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THESIS

CHARACTERIZATION OF TOMATO NECROTIC RINGSPOT VIRUS, A NEW SPECIES OF TOSPOVIRUS INFECTING TOMATO IN THAILAND

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A Thesis Submitted in Partial Fulfillment of
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Tospoviruses cause severe damages on various economically important crops in Thailand such as tomato, pepper, peanut, watermelon, cantaloupe, cucumber and loofah. In this study, a new tospovirus isolated from naturally infected tomato plants grown in Nakhon Pathom province was characterized on the basis of particle morphology, serology, host range, nucleotide sequence of S and M RNA segments and thrips transmission. Infected tomato plants showed typical tospovirus symptoms consisting of necrotic spots, necrotic ringspots and stem necrosis. This virus was detected by using general antibodies that could recognize *Watermelon silver mottle virus* (WSMoV), Capsicum chlorosis virus (CaCV) and Melon yellow spot virus (MYSV). However, it did not react with specific monoclonal antibodies (MAbs) to WSMoV and CaCV or a specific MAb to MYSV. The complete nucleotide sequences of S and M RNAs of the virus were determined. They were 3,023 and 4,716 nucleotides in length, respectively, and contained two ORFs in an ambisense arrangement. Sequence analysis indicated that amino acid sequence of the nucleocapsid (N) protein shared 58.2%, 56.0% and 51.8% identity with those of CaCV, WSMoV and MYSV, respectively. The virus was experimentally transmitted by *Thrips palmi* and *Ceratothripoides claratris* with an efficiency of 83% and 12%, respectively. Based on our results, we conclude that this tospovirus isolate should be considered a member of a new species. The name Tomato necrotic ringspot virus (TNRV) is proposed for this tospovirus. Moreover, TNRV was also compared with Capsicum chlorosis virus (CaCV), an established member of tospovirus previously isolated from peanut. Results confirmed that TNRV and CaCV are different species of tospovirus and various isolates of CaCV could exist.

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This work is dedicated to my family, for their love, support and understanding.

Channarong Seepiban

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LIST OF ABBREVIATIONS

ANSV	=	Alstroemeria necrotic streak virus
BSA	=	bovine serum albumin
CaCV	=	Capsicum chlorosis virus
CCSV	=	Calla lily chlorotic spot virus
CSNV	=	Chrysanthemum stem necrosis virus
cm	=	centrimeter
cDNA	=	complementary DNA
°C	=	degree celsius
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
DIECA	=	diethydithiocarbamate
E. coli	=	*Escherichia coli*
EDTA	=	ethylene-diamine-tetra-acetic acid
GBNV	=	*Groundnut bud necrosis virus*
GRSV	=	*Groundnut ringspot virus*
h	=	hour
INSV	=	*Impatiens necrotic spot virus*
IYSV	=	Iris yellow spot virus
IR	=	intergenic region
kb	=	kilobase pairs
LB medium	=	Luria-Bertani medium
µl	=	microliter
MYSV	=	Melon yellow spot virus
MeSMV	=	Melon severe mosaic virus
MAb	=	monoclonal antibody
mg	=	milligram
mm	=	millimetre
mM	=	millimolar
MW	=	molecular weight
MgC_{l2}	=	magnesium chloride

LIST OF ABBREVIATIONS (Continued)

NaCl	=	sodium chloride
Na_2CO_3	=	disodium carbonate
$NaHCO_3$	=	sodium carbonate
NBT/BCIP	=	nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate
ng	=	nanogram
nt	=	nucleotide
PCFSV	=	Peanut chlorotic fan-spot virus
PYSV	=	*Peanut yellow spot virus*
PolRSV	=	Poligonum ringspot tospovirus
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate-buffered salts
PCR	=	polymerase chain reaction
PAb	=	polyclonal antibody
PTA-ELISA	=	plate trapped antigen enzyme-linked immunosorbent assay
RNA	=	ribonucleic acid
rpm	=	rotation per minute
RT	=	reverse transcription
SDS	=	sodium dodecyl sulfate
sec	=	second
TBST	=	tris buffered saline plus Tween 20
TCSV	=	*Tomato chlorotic spot virus*
TSWV	=	*Tomato spotted wilt virus*
TYRV	=	Tomato yellow ring virus
TZSV	=	Tomato zonate spot virus
TBE	=	Tris-borate-EDTA electrophoresis
WBNV	=	Watermelon bud necrosis virus
WSMoV	=	*Watermelon silver mottle virus*
ZLCV	=	*Zucchini lethal chlorosis virus*

CHARACTERIZATION OF TOMATO NECROTIC RINGSPOT VIRUS, A NEW SPECIES OF TOSPOVIRUS INFECTING TOMATO IN THAILAND

INTRODUCTION

The *Tospovirus* genus in the family *Bunyaviridae* includes plant viruses that infect a wide range of plant species including vegetable, fruit and ornamental crops. The virus is distributed in tropical, subtropical, and temperate regions throughout the Northern Hemisphere, Western Europe and Asia (German *et al.*, 1992). So far, at least twenty tospovirus species and tentative species have been reported. In Thailand, the incidence and severity of the diseases caused by tospoviruses have increased rapidly in many economically important crops, such as tomato, pepper, peanut, watermelon, loofah, cantaloupe and cucumber (Wongkaew, 1993; Gajanandana *et al*., 2006; Chiemsombat *et al*., 2008). Moreover, tospoviruses were found to infect various species of weeds (Wongkaew, 1993). The recent wide spread of tospoviruses in several economically crops in Thailand urges to understand the occurrence and distribution of the virus in Thailand.

Detection and identification of tospoviruses in Thailand were based on serological studies and amino acid sequence analysis of the nucleocapsid (N) protein. Plate-trapped antigen ELISA (PTA-ELISA) using antibodies against N protein of various tospoviruses and RT-PCR using N protein-specific primers were developed and used for identification of tospoviruses found in Thailand (Gajanandana *et al*., 2006; Chiemsombat *et al*., 2008). Up to now, three tospovirus species including *Watermelon silver mottle virus* (WSMoV), Melon yellow spot virus (MYSV) and Capsicum chlorosis virus (CaCV) have been identified in Thailand. WSMoV infected several plants of the families *Cucurbitaceae* and *Solanaceae*, such as snake loofah, melon, watermelon, pepper, tomato and *Physalis minima* in central, northern and northeastern regions of Thailand. MYSV was found to infect cucumber, watermelon, cantaloupe, loofah and *Physalis minima* in central, northern and northeastern regions

of Thailand. CaCV was found to infect peanut, tomato and pepper in central, northern and northeastern regions of Thailand. In addition, the whole genomic sequence, biological characteristics and thrips vector of CaCV isolate AIT (CaCV-AIT) isolated form tomato in central region of Thailand have been reported by Premachandra *et al* (2005) and Knierim *et al* (2006).

Tospoviruses are transmitted naturally by at least eleven species of thrips (*Thysanoptera*: *Thripidae*) in a persistent propagative manner (Ullman *et al*., 1993). Of eleven thrips species known as vectors of tospoviruses, at least four thrips species including *Thrips palmi, Ceratothripoides claratris, Thrips tabaci and Scirtothrips dorsalis* were recorded in agriculture fields in Thailand (Bansiddhi and poonchaisri, 1991; Bernardo, 1991; Murai *et al*., 2000; Rodmui, 2002). Thrips are polyphagous insect and considered as serious pest on several crop plants. *T. palmi* and *S. dorsalis* are the most common species of thrips found in peanut, pepper and tomato (Bansiddhi and poonchaisri, 1991; Bernardo, 1991). *C. claratris* was reported as the predominant thrips species found in tomato fields and greenhouses in central and northeastern regions of Thailand (Murai *et al*., 2000; Rodmui, 2002). However, little is known about the capability of these thrips species in transmitting tospoviruses found in Thailand. Premachandra *et al*. (2005) reported that *C. claratris* could transmit CaCV-AIT with a high efficiency (70%). The ability to elucidate the relationships between tospoviruses and thrips is essential for understanding epidemiology and spread of tospoviruses.

In this study, we have successfully characterized a member of a proposed new species of virus found on infected tomatoes in Thailand. Several approaches were performed for characterization of this tospovirus isolate, including particle morphology, biological indexing on different host plants, determination of the serological relationship of the N protein, genome sequence analysis and testing of thrips transmission.

In 2008, a tospovirus-like disease was observed in tomato plants grown in Nakhon Pathom province. Infected tomato plants showed symptoms consisting of necrotic spots, necrotic ringspots and stem necrosis. Preliminary identification showed that this virus isolate could be detected with general antibodies to tospoviruses. However, this tomato isolate gave a negative result in RT-PCR when specific primers for amplifying the N protein gene of MYSV, WSMoV or CaCV were used. Preliminary results suggested that this tospovirus isolate was different from previously identified tospoviruses found in Thailand. This tomato isolate was identified and characterized as a new member of *Tospovirus* genus within the *Bunyaviridae* family. The name Tomato necrotic ringspot virus (TNRV) was proposed for this tospovirus.

Moreover, TNRV was also compared with Capsicum chlorosis virus (CaCV), an established member of tospovirus. Several CaCV isolates were frequently observed on tomato, pepper and peanut. CaCV-AIT isolated form tomato in central region of Thailand was well characterized (Premachandra *et al*., 2005 and Knierim *et al*., 2006). However, only N protein sequences data are available for CaCV isolated from peanut. The whole genomic sequence, biological characteristics and thrips vector of CaCV isolated from peanut has not yet determined. In this study, Capsicum chlorosis virus isolate NRA (CaCV-NRA) isolated from peanut in Nakhorn Rachasrima was characterized and compared with TNRV. CaCV-NRA was determined based on host range, serology, the complete genomic sequence of S and M RNA segments and thrips vector. Our results confirmed that TNRV and CaCV are different species of tospovirus and various isolates of CaCV could exist.

OBJECTIVES

1. To characterize a new tospovirus species, tentatively named Tomato necrotic ringspot virus (TNRV), based on particle morphology, serology, host range, molecular characterization and thrips transmission.

2. To compare Tomato necrotic ringspot virus (TNRV) with Capsicum chlorosis virus (CaCV), an established member of tospovirus previously isolated from peanut.

LITERATURE REVIEW

1. Background and Spread of Tospoviruses

A tospovirus-like disease was first reported in Australia in 1915 (Brittlebank, 1919). The causal agent was identified as a virus named *Tomato spotted wilt virus* (TSWV) (Samuel *et al*., 1930). In 1991, Francki *et al* (1991) identified Impatiens necrotic spot virus (INSV) which led to the classification of viruses in the *Tospovirus* genus within the *Bunyaviridae* family. TSWV is considered as the type member of the genus.

The genus *Tospovirus* contains plant viruses that infect a wide range of plant species including vegetable, fruit and ornamental crops. The virus is distributed in tropical, subtropical, and temperate regions throughout the Northern Hemisphere, Western Europe and Asia (German *et al*., 1992). So far, twenty tospovirus species including Alstroemeria necrotic streak virus (ANSV) (Hassani-Mehraban *et al*., 2010), Capsicum chlorosis virus (CaCV) (McMichael *et al*., 2002), Calla lily chlorotic spot virus (CCSV) (Chen *et al*., 2005), Chrysanthemum stem necrosis virus (CSNV) (Resende *et al*., 1996), *Groundnut bud necrosis virus* (GBNV) (Reddy *et al*., 1992), *Groundnut ringspot virus* (GRSV) (De Ávila *et al*., 1993), *Impatiens necrotic spot virus* (INSV) (Law *et al*., 1991), Iris yellow spot virus (IYSV) (Cortez *et al*., 2002), Melon yellow spot virus (MYSV) (Kato and Hanada, 2000), Melon severe mosaic virus (MeSMV) (Ciuffo *et al*., 2009), Peanut chlorotic fan-spot virus (PCFSV) (Chen and Chiu, 1996), *Peanut yellow spot virus* (PYSV) (Reddy *et al*., 1991), Poligonum ringspot tospovirus (PolRSV) (Ciuffo *et al*., 2008), *Tomato chlorotic spot virus* (TCSV) (De Ávila *et al*., 1993), *Tomato spotted wilt virus* (TSWV) (Francki *et al*., 1991), Tomato yellow ring virus (TYRV) (Hassani-Mehraban *et al*., 2005), Tomato zonate spot virus (TZSV) (Dong *et al*., 2008), Watermelon bud necrosis virus (WBNV) (Jain *et al*., 1998), *Watermelon silver mottle virus* (WSMoV) (Yeh and Chang, 1995) and *Zucchini lethal chlorosis virus* (ZLCV) (Resende *et al*., 1996) have been reported (Table 1).

Table 1 Tospoviruses species and their geographical distribution.

Tospovirus	Abbreviation	Geographical distribution	Reference
Alstroemeria necrotic streak virus	ANSV	Columbia	Hassani-Mehraban *et al*. (2010)
Capsicum chlorosis virus	CaCV	Australia, Thailand, China, Taiwan, India	McMichael *et al*. (2002)
Calla Lily chlorotic spot virus	CCSV	Taiwan	Chen *et al*. (2005)
Chrysanthemum stem necrosis virus	CSNV	Brazil	Resende *et al*. (1996)
Groundnut bud necrosis virus	GBNV	India	Reddy *et al*. (1992)
Groundnut ringspot virus	GRSV	South America, South Africa	De Ávila *et al*. (1993)
Impatiens necrotic spot virus	INSV	USA, Europe	Law *et al*. (1991)
Iris yellow spot virus	IYSV	Brazil, Israel, USA, The Netherlands	Cortez *et al*. (2002)
Melon yellow spot virus	MYSV	Taiwan, Japan, Thailand	Kato and Hanada (2000)
Melon severe mosaic virus	MeSMV	Mexico	Ciuffo *et al*. (2009)
Peanut chlorotic fan virus	PCFV	Taiwan	Chen and Chiu (1996)
Peanut yellow spot virus	PYSV	India	Reddy *et al*. (1991)
Polygonum ringspot virus	PolRSV	Italy	Ciuffo *et al*. (2008)
Tomato chlorotic spot virus	TCSV	South America	De Ávila *et al*. (1993)
Tomato spotted wilt virus	TSWV	Worldwide	Francki *et al*. (1991)
Tomato yellow ring virus	TYRV	Iran	Hassani-Mehraban *et al*. (2005)
Tomato zonate spot virus	TZSV	China	Dong *et al*. (2008)
Watermelon bud necrosis virus	WBNV	India	Jain *et al*. (1998)
Watermelon silver mottle virus	WSMoV	Japan, Taiwan, Thailand	Yeh and Chang (1995)
Zucchini lethal chlorosis virus	ZLCV	Brazil	Resende *et al*. (1996)

2. Morphology and genome organization

The morphology of the tospovirus is typical of members of the family *Bunyaviridae* (Whitfield *et al*., 2005) (Figure 1). The virus has spherical particles of approximately 80-120 nm in size with an envelope containing glycoprotein (G_N and G_C) spikes (Tsompana *et al*., 2008). The particle contains a tripartite RNA genome which is tightly packaged by numerous copies of nucleoprotein (N) subunits, and few copies of a large (L) protein (Van Poelwijk *et al*., 1993) (Figure 1). Terminal sequences of each RNA segments are inverted complementary sequences. The eight-nucleotide sequences (5'-AGAGCAAU-3') are strictly conserved at the termini of each segment of all tospoviruses. Due to the complementary sequences at 5' and 3' ends, these RNA segments from a panhandle-like structure (De Haan *et al*., 1991; Law *et al*., 1991; Law *et al*., 1992). The intergenic region (IR) on the M and S segments is AU rich and is predicted to form a stable hairpin structure (De Haan *et al*., 1990; Kormelink *et al*., 1992).

Tospovirus genome consists of three linear single stranded RNA molecules denoted small (S) RNA, medium (M) RNA and large (L) RNA (Whitfield *et al*., 2005; Goldbach *et al*., 1996; Elliott *et al*., 1990) (Figure 2). Both S and M RNA have an ambisense coding strategy (De Haan *et al*., 1990; Kormelink *et al*., 1992; Law *et al*., 1992). The S RNA encodes the nucleocapsid protein (N) and non-structural proteins (NSs) (Kormelink *et al*., 1991; De Haan *et al*., 1990). The N protein involves in particle structure and transcription/replication regulation (Snippe *et al*., 2007). NSs protein involves in the suppression of gene-silencing (Bucher *et al*., 2003). The M RNA encodes the two envelope glycoproteins (G_N and G_C) and another non-structural protein (NSm) (Kormelink *et al*., 1992). NSm protein is responsible for cell to cell movement and involves in stimulation of tubule formation in protoplasts (Kormelink *et al*., 1994; Storms *et al*., 1995). The G_NG_C glycoproteins are major determinants for thrips transmission (Wijkamp *et al*., 1995, Sin *et al*., 2005, Whitfield *et al*., 2005) The L RNA encoding the viral polymerase has a negative polarity (De Haan *et al*., 1991).

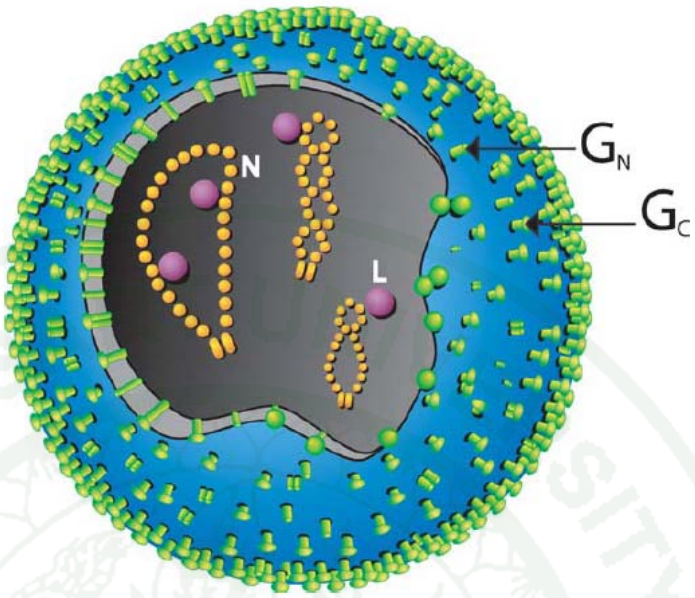

Figure 1 Particle morphology of *Tomato spotted wilt virus* (TSWV), the type species of the genus *Tospovirus*. The virus has spherical particle with an envelope containing glycoprotein (G_N and G_C) spikes. The virus particle contains a tripartite RNA genome which is tightly packaged by numerous copies of nucleoprotein (N) subunits, and few copies of a viral polymerase (L).

Source: Whitfield *et al*. (2005)

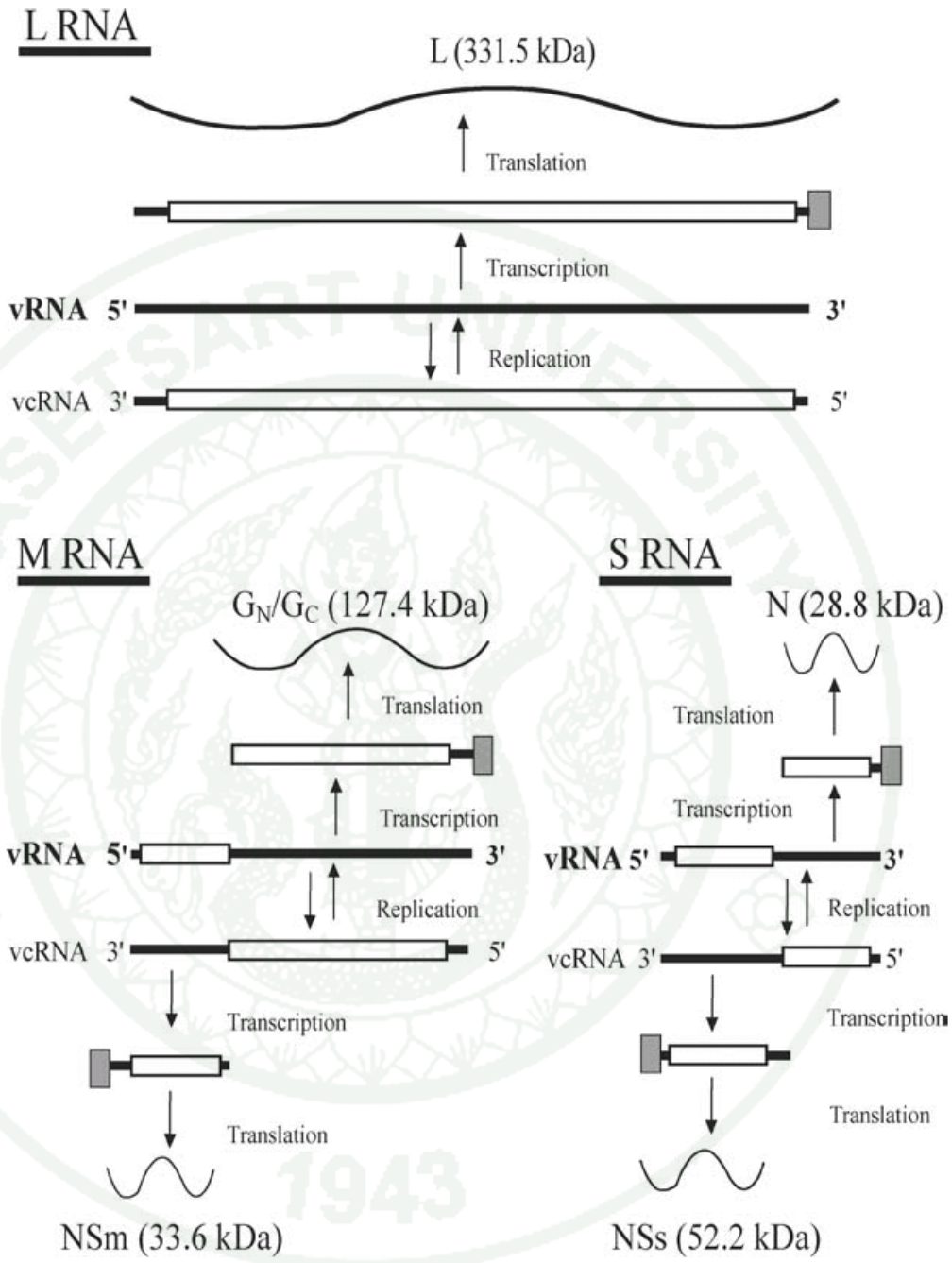

Figure 2 Genome organization and expression strategy of *Tomato spotted wilt virus* (TSWV), the type species in the genus *Tospovirus*.

Source: Whitfield *et al*. (2005)

3. Host Range

The host range of tospoviruses varies greatly with the virus species. For instant, TSWV has a very broad host range which includes more than 800 different plant species within 82 families (Moyer, 1999). Economically important plants which are susceptible to TSWV include tomato, potato, tobacco, peanut, pepper, lettuce, papaya, and the ornamentals such as chrysanthemum, begonia, agetarium and impatiens (German *et al*., 1992). Other tospoviruses have a narrower host range. For instance, INSV is predominant in greenhouse of ornamental crops, including cineraria, ranunculus, impatiens, New Guinea impatiens and others. PolRSV was found to infect two Polygonum species (Ciuffo *et al*., 2008).

General tospoviral symptoms include necrosis on several plant parts, chlorosis, local lesions, ring patterns, mottling, silvering, stunting, leaf spots, necrotic areas (Mumford *et al*., 1994; German *et al*., 1992). Young plants are especially vulnerable to infection. However, it is difficult to identify tospovirus infection using visual symptoms alone. Symptoms of tospovirus vary depending on plant species, cultivar, developmental stage of the plant, and environmental conditions (German *et al*., 1992). Moreover, tospovirus symptoms often mimic symptoms caused by nutrient deficiencies or other biotic and abiotic stresses (Moyer, 1999).

4. Viral Transmission

Tospoviruses are transmitted naturally by thrips (*Thysanoptera: Thripidae*) in persistent propagative manner (Ullman *et al*., 1993; Whitfield *et al*., 2005). Until now, at least eleven thrips species of the genera *Frankliniella, Thrips, Scirtothrips* and *Ceratothripoides* have been reported as vectors of tospoviruses (Ullman *et al*., 1997; Premachandra *et al*., 2005; Whitfield *et al*., 2005; De Borbon *et al*., 2006; Ohnishi *et al*., 2006). These thrips species included *Frankliniella occidentalis, F. schultzei, F. intonsa, F. fusca, F. bispinosa, F. zucchini, Thrips palmi, T. Setosus, T. tabaci, Scirtothrips dorsalis* and *Ceratothripoides claratris* (Table 2). These thrips species feed exclusively on plants and most of them are polyphagous (Ullman *et al*., 1997).

Transmission of tospovirus is characterized by a unique relationship between the virus and the vector. The thrips life cycle from egg to adult involves two larval and two pupal stages (Ullman *et al*., 1993) (Figure 3). The females lay eggs in plant leaves and the first larval stage hatchs within 3-6 days dependent on the temperature. The first and second instar larvae take 2 and 4 days, respectively. The pupal stage occurs in litter or soil, while the larval and adult stages can be found on the top parts of a plant (Figure 3). Thus, initiation of the infection cycle occurs only when female adult thrips lay eggs on tospovirus-infected leaves. Only the first and second instar larvae can acquire the virus (Ullman *et al*., 1997). After acquisition, the virus replicates and circulates in its thrips vector, and is transmitted by second instar larvae and adults (Ullman *et al*., 1993). The virus replication was found first in the epithelial cells of the midgut and then in the muscle cells surrounding the midgut (Ullman *et al*., 1997; Whitfield *et al*., 2005). The virus was then translocated to salivary glands (Ullman *et al*., 1993; Ullman *et al*., 1995; Nagata *et al*., 2004). The infection of the midgut and salivary glands is a critical event for thrips to become transmitters. The adult may remain viruliferous over lifetime. Adult thrips that feed on infected plants do not become viruliferous since a midgut barrier prevents viral acquisition by adult thrips (Ullman *et al*., 1992; Ullman *et al*., 1997).

The competence and specificity of each thrip species in transmission of different tospoviruses have been demonstrated (Nagata *et al*., 2004; Wijkamp *et al*., 1993; Wijkamp *et al*., 1995). Wijkamp *et al*. (1995) showed that *F. occidentalis* transmitted TSWV, INSV, GRSV, and TCSV with variable efficiency. INSV and TSWV was efficiently transmitted by *F. occidentalis* (85% and 66%, respectively), whereas GRSV and TCSV were poorly transmitted by this thrip species (10% and 27%, respectively). Nagata *et al*. (2004) also showed that each thrip species transmitted tospoviruses with varying efficiency according to the virus species. In Brazil, *F. occidentalis* transmitted TSWV (32%), TCSV (32%), GRSV (18%) and CSNV (72%). *F. schultzei* transmitted TCSV (70%), GRSV (93%) and CSNV (66%), but did not transmit the TSWV isolate. *T. tabaci* and *T. palmi* did not transmit any of these four tospoviruses TSWV, TCSV, GRSV and CSNV (Nagata *et al*., 2004). In Asia, *T. palmi* was reported to be a vector of Asian tospoviruses such as WSMoV,

CaCV, WBNV, CCSV, GBNV and MYSV (Chen and Chiu, 1996; Kato and Hanada, 2000; Lakshmi et al., 1995; Palmer et al., 1990; Persley et al., 2006; Yeh et al., 1992). *C. claratris* transmits CaCV isolate AIT (CaCV-AIT) isolated from tomato in Central Thailand at an efficiency rate of 70% (Premachandra *et al*., 2005).

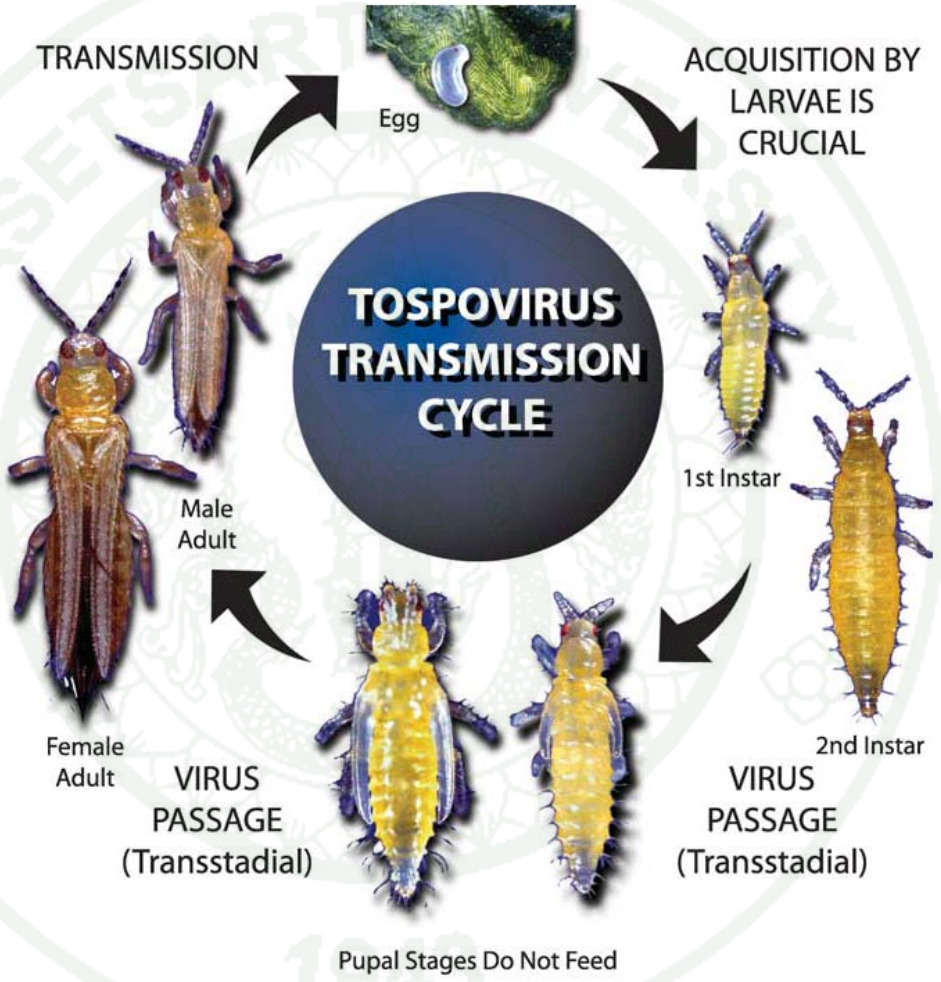

Figure 3 Life-cycle of the western flower thrips (*Frankliniella occidentalis*) and transmission cycle of tospovirus.

Source: Whitfield *et al*. (2005)

Table 2 Geographical distribution of known thrips vectors of tospoviruses.

Thrips vector	Tospovirus	Geographical distribution of thrips vector
Frankliniella fusca	TSWV, INSV	North America, Mexico
Frankliniella intonsa	TSWV, TCSV	Europe, Asia, U.S.A., India
Frankliniella occidentalis	TSWV, INSV, GRSV, TCSV, CSNV	America, Europe, Hawaii, New Zealand, Asia, South-Africa, Australia, Argentina
Frankliniella schultzei	TSWV, GRSV, TCSV, CSNV	South America, Caribbean, Africa, Australia, Pacific, Asia, Florida, The Netherlands, Italy, Britain
Frankliniella bispinosa	TSWV	Bahamas, Bermuda, Puerto Rico
Frankliniella zucchini	ZLCV	Brazil
Thrips palmi	WSMoV, CaCV, CCSV, GBNV, WBNV, MYSV	Asia, Australia, Pacific, Caribbean, Florida, Guyana, Venezuela, Sudan,Nigeria, The Netherlands, Finland
Thrips setosus	TSWV	Japan, Korea
Thrips tabaci	TSWV, IYSV	Worldwide, widespread on all Continents
Scirtothrips dorsalis	GBNV, PCFSV, PYSV	Asia, Papua New Guinea, Solomon Islands, Australia, South Africa
Ceratothripoides claratris	CaCV	India, Thailand

Source: Whitfield *et al*. (2005); Jones (2005); Premachandra *et al*. (2005)

5. Serology

Tospoviruses have been classified into serogroups based on serological relationships of the N protein. Numerical classification of serogroups, such as serogroup I, II, III and IV has been used (Table 3) (Adam *et al*., 1993; De Avila *et al*., 1990; Bezerra *et al*., 1999; Kato and Hanada, 2000; Moyer, 1999). However, this system is apparently not appropriate because of an increase in the number of tospoviruses and N protein cross-reactions that occur between members of different tospovirus species (Moyer, 1999; Chu *et al*., 2001; Jones *et al*., 2005). Moyer (1999) and Chu *et al*. (2001) have called for replacement of original system with a type species in a serogroup. Two major serogroups, TSWV and WSMoV, have been proposed (Table 3). GRSV, TCSV, CSNV and ZLCV, which show serological relationships to TSWV, are grouped in the TSWV serogroup. By these criteria, GBNV, CaCV, WBNV, CCSV and TZSV are grouped in the WSMoV serogroup. The recently reported PolRSV showed a serological relationship with TYRV, and a slight serological cross-reaction with IYSV (Ciuffo *et al*., 2008). Other tospoviruses have not yet been assigned to groups, since they are not serologically related to members of other species (Chu *et al*., 2001) (Table 3).

6. Virus taxonomy

The plant-infecting members of the *Bunyaviridae*, which are propagatively transmitted by thrips species, are classified into a genus *Tospovirus*, (Francki *et al*., 1991). The family *Bunyaviridae* compasses 5 genera including *Bunyavirus*, *Phlebovirus*, *Hantavirus*, *Nairovirus* and *Tospovirus* (Elliot *et al*., 1990). The *Tospovirus* genus is the only plant-infecting virus and the four other genera are animal-infecting virus. Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus* (Fauquet, 2005).

Viruses within the genus *Tospovirus* are classified on the basis of virion morphology, genome structure and organization, and thrips transmission. Species classification is based on vector specificity, host range, serological relationships

among their N proteins and molecular relationships of their N protein genes (Fauquet *et al*., 2005; Goldbach *et al*., 1996). Amino acid sequence similarity with previously reported sequences is the major descriptor for the identification of new species. Criteria for species of tospovirus based on N protein sequence similarity are as follows: strains of the same virus (>90%), strains or members of distinct species (80-90%) depending on additional criteria, and members of different species (<80%) (Fauquet *et al*., 2005; Jones, 2005; Tsompana *et al*., 2008). To date, twenty *Tospovirus* species have been reported (Table 1). Of these, eight definitive species and eight tentative species have been recognized by the International Committee on Taxonomy of Viruses (ICTV) (Fauquet *et al*., 2005; Jones *et al*., 2005). Tospoviruses have also been grouped according to their geographical origins. As a result, Eurasian, American, Asian and European-Middle Eastern groups have been proposed (Ciuffo *et al*., 2008; Hassani-Mehraban *et al*., 2005; Jones *et al*., 2005).

7. Tospoviruses in Thailand

At least 10 tospovirus species have been recorded in Asia (Table 1). *Groundnut bud necrosis virus* (GBNV) is an extremely destructive disease of peanut in South and Southeast Asia (Reddy *et al*., 1983). Distribution of the disease seems to be limited to peanut producing countries including India, Nepal, Pakistan, China, Vietnam and Thailand (Reddy *et al*., 1983). WSMoV and MYSV have been reported to systemically infect members of the family *Cucurbitaceae* (Yeh and Chang, 1995; Kato *et al*., 2000). WSMoV caused severe damages on watermelon, wax gourd, melon, cucumber and bitter gourd productions in Japan and Taiwan (Yeh and Chang, 1996). MYSV cause severe damages on melon and cucumber productions in Japan (Kato *et al*., 2000). CaCV was found to infect tomato and pepper in India (Kunkalikar *et al*., 2010), peanut in China (Chen *et al*., 2007), tomato and pepper in Australia (McMichael *et al*., 2002) and *Phalaenopsis* orchid in Taiwan (Zheng *et al*., 2011).

Table 3 Serogroups of tospoviruses.

Serogroup	Numerical serogroup	Tospovirus
Tomato spotted wilt (TSWV) serogroup	I	*Tomato spotted wilt virus*
	II	*Groundnut ringspot virus*
	II	*Tomato chlorotic spot virus*
	VIII	Chrysanthemum stem necrosis virus
	IX	*Zucchini lethal chlorosis tospovirus*
Watermelon silver mottle (WSMoV) serogroup	IV	*Watermelon silver mottle virus*
	IV	*Groundnut bud necrosis virus*
	IV	Watermelon bud necrosis virus
	IV	Capsicum chlorosis virus
	IV	Calla lily chlorotic spot virus
	-	Tomato zonate spot virus
Serologically unrelated	III	*Impatiens necrotic spot virus*
	VI	Iris yellow spot virus
	V	*Peanut yellow spot virus*
	VII	Melon yellow spot virus
	-	Tomato yellow ring virus
	-	Melon severe mosaic virus
	X	Peanut chlorotic fan virus
	-	Polygonum ringspot virus
	-	Alstroemeria necrotic streak virus

Source: Adam *et al*. (1993); De Avila *et al*. (1990); Bezerra *et al*. (1999); Kato and Hanada (2000); Moyer (1999); Dong *et al*. (2008)

The presence of a tospovirus-like disease was first reported in peanut crops in Northeastern part of Thailand (Wongkaew, 1993). The symptoms of the disease included chlorotic and necrotic ringspot on leaf, bud necrosis and stunting. The disease was the major threat for groundnut production in Northeastern and Eastern part of Thailand (Wongkaew, 1993). Tospovirus-like disease found in peanut was identified as Capsicum chlorosis virus (CaCV) (Gajanandana *et al*., 2006; Chiemsombat *et al*., 2008). In 2000, tospovirus infected tomatoes with necrotic ringspot lesions on the leaves were collected from fields in Kalasin; northeastern part of Thailand (Pongsapich *et al*., 2002). This tospovirus isolate was further characterized and identified as a strain of Capsicum chlorosis virus-tomato necrosis strain (Chiemsombat *et al*., 2008). Tospoviruses were also found to infect many plants including pepper, tomato, watermelon, cantaloupe, tobacco, eggplant, cowpea and various species of weeds including *Physalis minima*, *Spilanthes paniculata*, *Vincia rosea*, *Cleome viscosa*, *Synedrella nodiflora* and *Richardia sp.* (Wongkaew, 1993).

Detection and identification of tospoviruses in Thailand were based on serological studies and nucleotide and amino acid sequence analysis of viral nucleocapsid (N) protein gene. Plate-trapped antigen ELISA using antibodies against N protein combined with RT-PCR using N protein-specific primers are useful techniques for detection and characterization of tospoviruses (Gajanandana *et al*., 2006, Chiemsombat *et al*., 2008). Monoclonal and polyclonal antibodies to N protein of CaCV, WSMoV and MYSV were established (Gajanandana *et al*., 2006). Specific primers for amplifying the N protein gene of CaCV, WSMoV or MYSV have been developed (Gajanandana *et al*., 2006; Bhunchoth *et al*., 2005). So far, three tospovirus species have been identified in Thailand including *Watermelon silver mottle virus* (WSMoV), Capsicum chlorosis virus (CaCV) and Melon yellow spot virus (MYSV) (Gajanandana *et al*., 2006, Chiemsombat *et al*., 2008, Bhunchoth *et al*., 2005).

7.1 *Watermelon silver mottle virus* (WSMoV)

WSMoV infected several plants of the families *Cucurbitaceae* and *Solanaceae*, such as snake loofah, melon, watermelon, pepper, tomato and *Physalis minima* in central, northern and northeastern regions of Thailand. Six isolates of WSMoV in Thailand were identified on snake loofah (WSMoV-SL, accession number AY514624), pepper (WSMoV- SP29, accession number AY514625), tomato (WSMoV-T40, accession number AY514626), melon (WSMoV-SN858, accession number AY514627) and *Physalis minima* (WSMoV- PhNR, accession number AY626761) (Chiemsombat *et al*., 2008). Sequence analysis showed that the N gene of those WSMoV isolates revealed extremely high homology with each other at 98-100% nucleotide identity. The N protein of WSMoV found in Thailand showed high homology (97-98% amino acid identity) with those of WSMoV (Yeh *et al*., 1995) reported in Taiwan.

7.2 Melon yellow spot virus (MYSV)

MYSV was found to infect cucumber, watermelon, cantaloupe, loofah and *Physalis minima* in central, northern and northeastern regions of Thailand. Five isolates of MYSV were identified on cucumber (MYSV-JTC, accession number AY673635), watermelon (MYSV-W3, accession number AY67363), cantaloupe (MYSV-M7, accession number AY574574), loofah (MYSV-Luffah13, accession number AM087020) and *Physalis minima* (MYSV-Ph122, accession number AM113769) (Chiemsombat *et al*., 2008). Sequence analysis showed that the N gene of those MYSV isolates revealed extremely high homology with Melon yellow spot virus from Japan (Kato *et al*., 2000) at 98.5-100% nucleotide identity. Moreover, Physalis severe mottle virus (PSMV) found in *Physalis minima* was also reported in Thailand (Cortez *et al*., 2001). PSMV was considered the same species of MYSV because of the extremely high homology of amino acid sequence of N protein gene between both viruses (99.6% identity).

7.3 Capsicum chlorosis virus (CaCV)

CaCV was found to infect peanut, tomato and pepper in central, northern and northeastern regions of Thailand (Gajanandana *et al*., 2006; Chiemsombat *et al*., 2008). Based on amino acid sequence of N protein, two groups of CaCV were identified in Thailand (Chiemsombat *et al*., 2008). The first group was comprised of CaCV found on peanut (CaCV-Pkk, accession number DQ022745) and pepper (CaCV- Psk55, accession number AM113770). These two CaCV isolates revealed extremely high homology with each other (98-100% nucleotide identity) and shared extremely high homology with CaCV-Aus from Australia (McMichael *et al*., 2002) at 99.3% amino acid identity. The second group was comprised of two CaCV isolates from tomato (CaCV-TD8, accession number AY647437; CaCV-ToK, accession number AY626762) in northeastern region of Thailand and one isolate of CaCV, designated CaCV-AIT (Asian Institute of Technology), from tomato in central region of Thailand. Sequence analysis of the N protein indicated that those three CaCV isolates from tomato (CaCV-TD8, CaCV-ToK and CaCV-AIT) shared 92-93% identity with those of two CaCV isolates from peanut and pepper (CaCV- Pkk and CaCV- Psk55). Moreover, the complete nucleotide sequence of S, M and L RNAs of CaCV-AIT was fully characterized (Knierim *et al*., 2006). CaCV-AIT could be transmitted by *C. claratris* with an efficiency of 70% (Premachandra *et al*., 2005).

MATERIALS AND METHODS

1. Plant materials

Plants showing tospovirus-like symptoms were collected from fields in several regions of Thailand during 2006-2009 (Table 4). Peanut showing necrotic spot, necrotic ringspot and bud necrosis were collected from field in Khon Kaen, Nong Khai, Udon Thani and Nakhorn Rachasrima. Tomato showing necrotic spot, necrotic ringspot and stem necrosis were collected from fields in Nakhon Pathom. Pepper showing necrotic spot on leaves and fruits were collected from fields in Nakhon Pathom, Khon Kaen and Kanjanaburi. Watermelon plants showing chlorotic spot and necrotic spot were collected from fields in Khon Kaen and Suphan Buri (Table 4).

Table 4 Plants and locations where tospovirus infected samples were collected.

Plant	Region	Location	Designated isolate	Collection date
Peanut	Northeast	Khon Kaen	KK	28 April 2006
Peanut	Northeast	Nong Khai	NK	28 April 2006
Peanut	Northeast	Udon Thani	UT	28 April 2006
Peanut	Northeast	Nakhorn Rachasrima	NRA	20 January 2008
Tomato	Central	Nakhon Pathom	TT1	18 February 2008
Pepper	Central	Nakhon Pathom	PP1	16 December 2008
Pepper	Western	Kanjanaburi	PP2	20 January 2009
Pepper	Northeast	Khon Kaen	PP3	26 January 2009
Watermelon	Central	Suphan Buri	WS1	18 August 2008
Watermelon	Central	Suphan Buri	WS2	18 August 2008
Watermelon	Northeast	Khon Kaen	WKA	31 March 2008
Watermelon	Northeast	Khon Kaen	WKB	31 March 2008

2. Local lesion isolation and maintenance of virus

Original samples were kept at -80°C. Single local lesion isolation was performed by mechanical inoculating *Chenopodium quinoa*. Briefly, samples of tospovirus-infected plant were ground in 0.05 M phosphate buffer (pH 7.0) containing 0.2% 2-mercaptoethanol and 1% celite. Extracts from infected plants were inoculated into *C. quinoa* (two-month-old plants). Single local lesion developed at 2-3 days post-inoculation was then cut and mechanically inoculated to systemic hosts. Virus isolates were maintained in *Datura stamonium* or *Physalis minima* by mechanical inoculation every 2-3 weeks.

3. Electron microscopic study

Leaves of tospovirus infected plant were cut into small pieces (1 × 1 × 2 mm). Samples were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 h. They were then washed 3 times in phosphate-buffered salts (PBS, pH 7.0) and post-fixed with 1% Osmium tetroxide for 3 h at 4 ^{o}C. The samples were dehydrated in a graded series of ethanol and embedded in Spurr's resin. Ultrathin sections were stained with 2% uranyl acetate and 0.5% lead citrate and examined using transmission electron microscope (JOEL, Ltd, Tokyo, Japan).

4. Host range studies

In order to determine the host range, the virus was mechanically inoculated to various plant species, at least five plants of each. Test plant species were *Chenopodium quinoa, Chenopodium amaranticolor*, *Arachis hypogea, Vigna unguiculata, Capsicum annum*, *Datura stramonium, Solanum lycopersicum, Nicotiana benthamiana, Nicotiana glutinosa, Nicotiana tabacum, Physalis minima, Cucumis sativus, Luffa acutangula* and *Citrullus lanatus*. Leaves of tospovirus infected plant giving a strong positive reactivity in plate-trapped antigen ELISA (PTA-ELISA) using general antibodies to tospoviruses (PAb MYSV6) were ground and mechanically inoculated onto seedlings at 3-5 true leaf stage. Inoculated plants

were kept in an insect-free greenhouse with temperature-controlled at 25-30 ^{0}C for 4 weeks to observe symptom development. Infections of leaves from tested plants were confirmed by PTA-ELISA. Mock-inoculated plant was used as a negative control.

5. Serological characterization

Mouse monoclonal (MAb) and rabbit polyclonal (PAb) antibodies were previously developed by using recombinant N protein of tospoviruses expressed in *E. coli* as immunogens (Gajanandana *et al.*, 2006). Binding activities of these antibodies to tospoviruses could be categorized into 3 groups (Table 5): 1) antibodies that reacted with WSMoV, CaCV and MYSV [PAb MYSV6 (prepared to N protein of MYSV) and MAb 2B2 (prepared to N protein of WSMoV)] 2) antibodies that reacted with WSMoV and CaCV [PAb A3 (prepared to N protein of CaCV), MAb 2D6 (prepared to N protein of WSMoV) and MAb L4E8 (prepared to N protein of CaCV)] and 3) antibodies that specifically reacted with MYSV [MAb 5E7 (prepared to N protein of MYSV)]. These antibodies were used to determine the serological relationship of the N protein of the new tospovirus compared to those of three tospoviruses previously found in Thailand (WSMoV, CaCV and MYSV) using PTA-ELISA and Western blot analysis.

5.1 Plate-trapped antigen ELISA (PTA-ELISA)

Plant extracts were prepared by grinding leaves from tospovirus-infected or healthy plants (1 in 5 w/v) in extraction buffer (14 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6 plus 0.2% sodium DIECA). Plates were coated with sap extracts and incubated for 24 h at 4 ^{o}C. After washing with PBS-Tween 20, plates were blocked by adding 2% BSA in PBS-Tween 20. Plates were then again washed with PBS-Tween 20. Polyclonal antibodies (1/5,000 dilution) and monoclonal antibodies (1/200 dilution) were applied to each plate. Plates were incubated for 1 h at 37 ^{o}C and rewashed. Alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse immunoglobulins (Sigma, USA) diluted in 0.5% BSA in PBS-Tween 20 was added. Plates were incubated for 1 h at 37 ºC and rewashed. Substrate solution (*p*-nitrophenyl phosphate)

was added. The absorbance at 405 nm was measured using automated microplate reader.

Table 5 Characteristic of available rabit polyclonal (PAb A3 and MYSV6) and mouse monoclonal (MAb 2B2, L4E8, 2D6 and 5E7) antibodies against tospoviruses found in Thailand including *Watermelon silver mottle virus* (WSMoV), Capsicum chlorosis virus (CaCV) and Melon yellow spot virus (MYSV).

Antibody	Antigen	WSMoV	CaCV	MYSV
PAb A3	CaCV-N protein	+	+	-
PAb MYSV6	MYSV-N protein	+	+	+
MAb 2B2	WSMoV-N protein	+	+	+
MAb L4E8	CaCV-N protein	+	+	-
MAb 2D6	WSMoV-N protein	+	+	-
MAb 5E7	MYSV-N protein	-	-	+

Source: Gajanandana *et al*. (2006)

5.2 Western blot analysis

Sap extracts were subjected to the discontinuous SDS-polyacrylamide gel electrophoresis using 5% stacking gel and 10% separating gel. Proteins were transferred to nitrocellulose membrane (Protran™ Nitrocellulose Transfer Membrane, Germany). The blotted membranes were air-dried. The membranes were soaked in 5 ml of Tris buffered saline plus Tween 20 (TBST; 10 mM Tris pH 8, 150 mM NaCl, 0.05% Tween 20) for 10 min and blocked with 5 ml of 4% (w/v) skim milk (Difco, USA) in TBST for 1 h. The nitrocellulose membranes were then incubated with 5 ml of polyclonal or monoclonal antibodies diluted in 1% (w/v) skim milk in TBST for 1 h. After washing with TBST 3 times for 10 min each, membranes were incubated in alkaline phosphatase conjugated goat anti-rabbit or anti-mouse immunoglobulins (Sigma, USA) for 1 h. The membranes were washed and incubated for 5-10 min in

the substrate solution NBT/BCIP (nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate) (ZYMED, USA). The color development was stopped by immersing the membrane in distilled water.

6. Molecular characterization

6.1 RNA extraction

Total RNA was extracted from infected plants using RNeasy Plant Mini kit (QIAGEN, Germany) according to manufacturer's instructions. Plant tissue (100 mg) was ground in liquid nitrogen with a mortar and pestle. Sample powder was immediately added to a new microcentrifuge tube containing 450 µl of lysis buffer (RLT buffer plus 1% β-mercaptoethanol). The tube was vortexed for 30 sec and incubated for 3 min at 56ºC in water bath. Lysate was then transferred through QIAshredder spin column and centrifuged at 13,000 rpm for 2 min. The flow through was added to a new tube and absolute ethanol (225 µl) was added to the clear lysate and mixed by pipetting. Lysate was then transferred through an RNeasy spin column and centrifuged at 13,000 g for 30 sec. Flow through was discarded and 700 µl of first wash buffer (RW1 buffer) was added to the same column and centrifuged for 30 sec. Flow through was discarded and 500 µl of second wash buffer (RPE buffer) was then added to the same column and centrifuged for 30 sec. The same step was repeated and centrifuged for 2 min. Finally, Total RNA was eluted with 50 µl RNase free water by centrifuging at 13,000 rpm for 1 min and was collected in a new tube. Total RNA was used as a template in two-step reverse transcription-polymerase chain reaction (RT-PCR).

6.2 Reverse transcription (RT)

The RT reaction was carried out using Superscript III (Invitrogen, USA). Briefly, 5 µg of total RNA and 100 ng of primer were incubated with Superscript III (200 units) at 50 ˚C for 1 h in a reaction mixture of 20 µl. The reaction was heated at

70 ˚C for 15 min and then treated with 2 units of *E. coli* RNase H (Invitrogen, USA) at 37 ˚C for 20 min to remove template RNA. The cDNA that was obtained was used as a template for PCR.

The first-strand cDNA of S RNA was synthesized from purified RNA using a primer J13 (Table 7) based on an 8-nt conserved sequence that is present at the 3' end of all tospoviral S RNA segments (Cortez *et al*., 2001). The cDNA of M RNA was synthesized using the primer 3'MRNA (Table 7), which contains a 14-nt conserved sequence that is present in all Asian tospoviral M RNA segments.

6.3 Polymerase chain reaction (PCR)

The PCR was carried out in a volume of 50 µl as following: 2 µl of cDNA template, 5 µl of 10X PCR buffer, 1 µl of 10 mM dNTP, 1.5 µl of 50 mM $MgCl_2$, 100 ng of primers, 5 units of Platinum Tag DNA Polymerase (Invitrogen, USA). Reactions were carried out in a thermocycler (PTC-200, MJ Research) with the following conditions: a denaturation temperature at 94˚C for 2 min, 30 cycles of 94˚C for 1 min, 50˚C or 55˚C for 1 min, 72˚C for 1 min per kb of template and a final extension temperature of 72 ˚C for 7 min. The PCR products were analyzed by electrophoresis on 0.8% agarose gels in 0.5X TBE buffer (1X TBE = 89 mM Tris-borate, 2 mM EDTA, pH 8.0).

6.4 Primers used for amplifying the N protein gene of *Watermelon silver mottle virus* (WSMoV), Capsicum chlorosis virus (CaCV) and Melon yellow spot virus (MYSV)

Specific primers used for amplifying the N protein gene of *Watermelon silver mottle virus* (WSMoV), Capsicum chlorosis virus (CaCV) and Melon yellow spot virus (MYSV) were developed (Gajanandana *et al*., 2006; Bhunchoth *et al*., 2005). The nucleotide sequence of each primer and the site of amplicon were described in Table 6.

Table 6 Specific primers used for amplification of N protein gene of Capsicum chlorosis virus (CaCV), *Watermelon silver mottle virus* (WSMoV) and Melon yellow spot virus (MYSV).

Primer	sequence (5' to 3')	site of the amplicon
Primer for CaCV		
CaCV-ExFB	GGATCC ATGTCTAACGTCAGG	0.8 kb
CaCV-ExRS	GTCGACTTACACCTCTATAGA	
Primer for WSMoV		
WSMV-C	TTACACTTCCAAAGAAGTGCTGGG	0.8 kb
WSMV-V	ATGTCTAACGTTAAGCAGC TCACA	
Primer for MYSV		
MN3	ATTCAACA TCAGCAAGTCAA	1 kb
MN5	TATTTCATTCAACTAGTTAA	

Source: Gajanandana *et al*. (2006); Bhunchoth *et al*. (2005)

6.5 Cloning strategies and primers used for amplifying S and M RNAs of Tomato necrotic ringspot virus (TNRV)

Cloning strategies and primers used for amplifying S and M RNA segments of TNRV were shown in Figure 4 and Table 7. Fragment 1 of S RNA was amplified with primer J13 which contains the eight nucleotides highly conserved in all tospoviral S RNA segments (Cortez *et al.,* 2001) and primer FST-2276 obtained from sequence within NSs gene. Fragment 2 of S RNA was amplified with primer RST-555 obtained from conserved sequence within NSs gene and primer FST-750 obtained from sequence within N protein gene. Fragment 3 of S RNA was amplified with primer UHP obtained from conserved sequence within intergenic region (IR) of Asian Tospoviral S RNA segments (Cortez *et al.,* 2001) and primer 3'G4S containing 13 nt conserved sequence at 3' terminus of all Asian tospoviral S RNA segments.

To amplify the entire M RNA segment of TNRV, fragments 4 was amplified with primer F-3199 obtained from conserved sequence within GnGc glycoprotein gene and primer 5'MRNA obtained from 13 nt conserved sequence at the 5' terminal of known tospoviral M RNA segments. Fragment 5 was amplified with primer RM-1628 obtained from sequence within GnGc glycoprotein gene and primer 3'MRNA containing 13 nt conserved sequence at 3' terminus of of all Asian tospoviral M RNA segments (Figure 4 and Table 7).

6.6 Cloning strategies and primers used for amplifying S and M RNA of Capsicum chlorosis virus (CaCV)

Cloning strategies used for amplifying S and M RNA segments of CaCV-NRA were shown in Figure 5 and Table 8. Primers were designed based on conserved regions within S and M RNA segments of CaCV-AIT (NC_008301 for S RNA; NC_008303 for M RNA) (Knierim *et al*., 2006) and Gloxinia-TH1 (AF059578 for N gene; AF059577 for NSs gene; AF023172 for M RNA). Fragments 1, 2, 3 and 4 of S RNA segments were amplified by using CaCV-ExRS /3'G4S primers, RS-2100/ FS-743 primers, RS-2729/ FS-1230 primers and J13/ FS-2481 primers, respectively. To amplify the entire M RNA segment of CaCV-NRA, fragments 5 and 6 were amplified by using F-3199/5'MRNA primers and R-3302/3'MRNA primers, respectively (Figure 5 and Table 8).

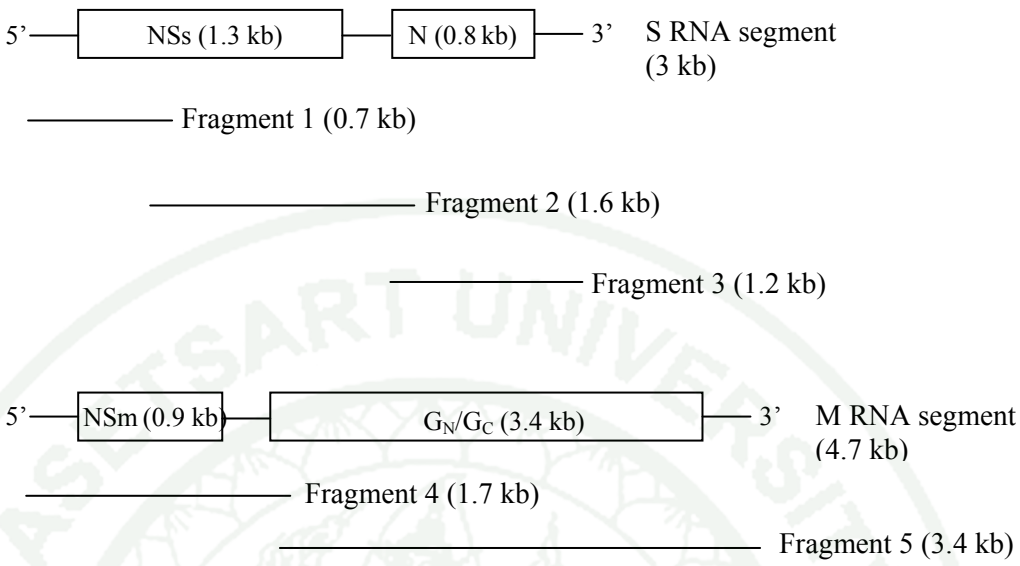

Figure 4 Cloning strategies used for amplification of S and M RNA segments of Tomato necrotic ringspot virus (TNRV).

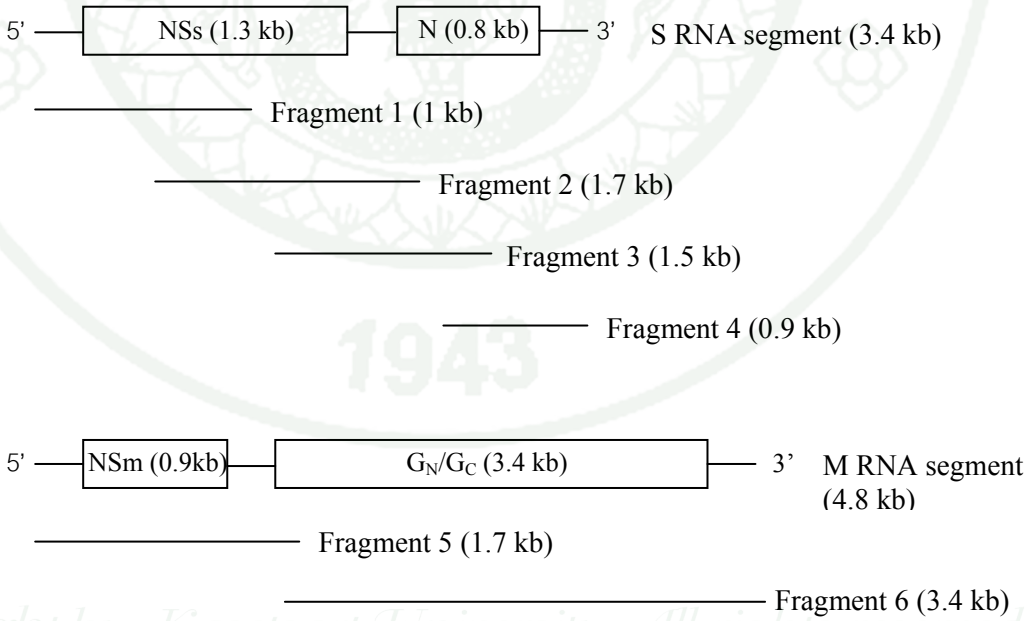

Figure 5 Cloning strategies used for amplification of S and M RNA segments of Capsicum chlorosis virus (CaCV).

Table 7 Primers used for amplifying of S and M RNA segments of Tomato necrotic ringspot virus (TNRV).

Primer sequence (5' to 3')		Fragment	Site of the amplicon
Primer used for S RNA			
J13*	CCCGGATCCAGAGCAAT	1	0.7 kb
FST-2276	GCTCTGATGCTATCTGAATGGC		
FST-750	GAAGACTAGCTCACCTGGAACA	2	1.6 kb
RST-555	ACAACATCTTACTGGAGAGA		
UHP*	CACTGGATCCTTTTGTTTTTGTTTTTTG	3	1.2 kb
3'G4S	AGAGCAATCGAGG		
Primer used for M RNA			
5'MRNA	AGAGCAATCGGTG	4	1.7 kb
F-3199	ACCTTTCAACTGGGTAGCTTCCTT		
RM-1628	CTAACAATGTCAAAGAAG	5	3.4 kb
3'MRNA	AGAGCAATCAGTGC		

* Conserved primer reported by Cortez *et al.* (2001)

Table 8 Primers used for amplifying of S and M RNA segments of Capsicum chlorosis virus (CaCV).

Primer sequence (5' to 3')		Fragment	site of the amplicon
Primer used for S RNA			
J13*	CCCGGATCCAGAGCAAT	1	1 kb
FS-2481	GCAGTTGATATGATTTCTGTTAGG		
RS-2729	AGGCATTCAGTTGGGTTCTGAGCT	2	1.7 kb
FS-1230	ACCTCTTCGGAGGCAAACTATTGG		
RS-2100	AGCATCAACATTTCACTGTGGAGC	3	1.5 kb
FS-743	AGGCTGTCAAGTTGCTTAGTGAGA		
CaCV-ExRS	GTCGACTTACACCTCTATAGA	4	0.9 kb
3'G4S	CCTCGATTGCTCT		
Primer used for M RNA			
5'MRNA	AGAGCAATCGGTG	5	1.7 kb
F-3199	ACCTTTCAACTGGGTAGCTTCCTT		
R-3302	GCATCTTCTAGTTTTTGTCTCCTC	6	3.4 kb
3'MRNA	AGAGCAATCAGTGC		

* Conserved primer reported by Cortez *et al.* (2001)

6.7 Cloning and sequencing

PCR reaction mixture was fractionated by electrophoresis using 0.8% agarose gel in 0.5X TBE (1X TBE= 89 mM Tris-borate, 2 mM EDTA, pH 8.0). Virus specific bands were excised from ethidium bromide stained gels, and PCR products were purified using QIAquick gel extraction kit (QIAGEN, Germany) following manufacturer's instructions. The gel containing desired DNA band was excised and placed in a 1.5 ml microcentrifuge tube. The gel was weighed by using an analytical

balance (Mettler-Toledo, USA). Then 100 µl of QG Buffer was added for every 100 mg agarose gel slice. The tube was incubated for 10 min at 50ºC and briefly vortexed every 2-3 min during incubation. After the gel completely dissolved, 100 µl of isopropanol was added for every 100 mg agarose gel slice and mixed thoroughly. The suspension was transferred through QIAquick column and centrifuged at 13,000 rpm for 1 min. Flow through was discarded and 500 µl of QG buffer was added to the same column and centrifuged for 1 min. Flow through was discarded and 750 µl of PE buffer was then added to the same column and centrifuged at 13,000 rpm for 1 min. To complete removal of PE buffer, the QIAquick column was centrifuged at 13,000 rpm for 1 min. The column was placed in the new tube. Finally, purified PCR product was eluted with 30 µl sterile water by centrifuging at 13,000 rpm for 1 min.

The eluted PCR products were ligated with pGEM-T easy Vector (Promega, USA) according to manufacturer's procedures. The reaction was carried out in a volume of 10 µl as following: 1 µl of 50 ng/µl of vector, 5 µl of 2X Rapid Ligation Buffer, 3 µl of purified DNA and 1 µl of 100 units of T4 DNA Ligase. The ligation mixture was incubated at 4ºC for overnight.

Recombinant plasmids were transformed into *E. coli*-DH5α cells by heat shock method (Sambrook *et al*., 1989). The tube of frozen *E. coli*-DH5α competent cells was removed from -80 ºC and placed in an ice bath until it was thawed. The ligation mixture was added to the tube and placed on ice for 15 min. The tube was incubated for 1 min at exactly 42 ºC in a water bath and the tube was returned to ice for 5 min. SOC medium (1 ml) was added to the tube and incubated for 1 hour at 37ºC with shaking. The cells were precipitated by centrifugation at 10,000 rpm for 1 min, and then resuspended with 100 µl of LB medium. The transformed culture was grown in LB agar plates containing ampicillin (100 mg/L), IPTG (50 µM) and X-gal (80 mg/L) at 37 ºC overnight. White colonies were then selected and grown in 5 ml LB medium plus 100 µg/ml ampicillin at 37 ºC overnight on a rotary shaker.

Recombinant plasmids were extracted from *E. coli* cells using QIAprep Spin Miniprep kit (QIAGEN, Germany) following manufacturer's instructions. Bacterial culture was transferred to a 1.5 ml microcentrifuge tube and harvested by centrifugation at 10,000 rpm for 5 min. Cell pellet was resuspended in P1 buffer (250 µl) and mixed vigorously by vortexing. The mixture was added with P2 buffer (250 µl) and mixed by inverting the tube for 4-6 times. The mixture was then added with N3 buffer (350 µl), mixed gently by inverting and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred through the QIAprep spin column and centrifuged at 13,000 rpm for 1 min. Flow through was discarded and 500 µl of PB buffer was added to the same column and centrifuged for 1 min. Flow through was discarded and 750 µl of PE buffer was then added to the same column and centrifuged at 13,000 rpm for 1 min. To complete removal of wash buffer, the column was centrifuged at 13,000 rpm for 1 min and placed in the new tube. Finally, purified plasmid was eluted with 30 µl sterile water by centrifuging at 13,000 rpm for 1 min.

Recombinant clones were confirmed to have DNA inserts by digestion with *Eco*RI restriction enzyme. The digestion reaction was electrophoresed through 0.8% agarose gel in 0.5X TBE buffer. After screening, at least three independent clones were sequenced. Purified plasmid DNA was sequenced by First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia).

6.8 Sequence analysis

Nucleotide sequences were assembled and analyzed using DNASTAR program (DNAStar Inc, USA). Multiple alignments of amino acid sequences were performed by ClustalX2 program (Larkin *et al*., 2007). Data obtained from ClustalX2 program were used for phylogenetic analysis using MEGA version 4.0 program (Tamura *et al*., 2007). The phylogenetic trees were constructed using neighbor-joining method with 500 bootstrap replications. Tospovirus sequences available on GenBank were used for comparative analysis (Table 9).

Table 9 Tospovirus sequences available on GenBank used in this study.

Virus	Abbreviation	S RNA	M RNA
Alstroemeria necrotic streak virus	ANSV	GQ478668	-
Capsicum chlorosis virus	CaCV-AIT	NC_008301	NC_008303
Capsicum chlorosis virus	CaCV-Aus	AY036058	-
Capsicum chlorosis virus	CaCV-CP	DQ355974	-
Capsicum chlorosis virus	CaCV-Ch-Pan	FJ011449	FJ011450
Capsicum chlorosis virus	CaCV-TwTom1	HM021139	-
Capsicum chlorosis virus	CaCV-Ph	HM021141	-
Capsicum chlorosis virus	CaCV-TD8	AY647437	-
Calla lily chlorotic spot	CCSV	AY867502	-
Chrysanthemum stem necrosis virus	CSNV	AF067068	AB274026, AF213675
Groundnut bud necrosis virus	GBNV	AY871098	NC_003620
Groundnut ringspot virus	GRSV	L12048	AY574055
Impatiens necrotic spot virus	INSV	NC_003624	NC 003616
Iris yellow spot virus	IYSV	AF001387	AF214014
Melon yellow spot virus	MYSV	NC_008300	AB061773
Melon severe mosaic virus	MeSMV	EU275149	-
Peanut chlorotic fan-spot virus	PCFSV	AF080526	-
Peanut yellow spot virus	PYSV	AF013994	-
Poligonum ringspot tospovirus	PolRSV	EF445397	EU271753
Tomato chlorotic spot virus	TCSV	AF282982	AF213674, AY574054
Tomato spotted wilt virus	TSWV	AY870391	NC_002050
Tomato yellow ring virus	TYRV	DQ462163	-
Tomato zonate spot virus	TZSV	NC_010489	NC_010490
Watermelon bud necrosis virus	WBNV	EU216028	ACN53946, ACN53945
Watermelon silver mottle virus	WSMoV	NC_003843	NC003841
Zucchini lethal chlorosis virus	ZLCV	AF067069	AB274027

7. Insect transmission

7.1 Thrips rearing

Adult *Thrips palmi* were collected from orchid farm in Nakhon Pathom province. Healthy colonies of *T. palmi* were continuously reared on okra pods (*Abelmoschus esculentus*) in glass bottles. Fresh okra pods were used as feeding and oviposition materials. New okra pods were replaced every two days. Adult *Ceratothripoides claratris* were collected from tomatoes growning in greenhouse in Nakhon Pathom province. Healthy colonies of *C. claratris* were reared on tomatoes in thrips-proof cages. Both thrips cultures were maintained under the laboratory conditions at 25±2°C with a photo period of 12-h.

7.2 Thrips identification

Thrips adults (50 individuals per sample) were identified to species based on morphological characteristics. Thrips was inspected under a compound microscope (40X objective yielding 400X total magnification). Morphological charactersistics used to distinguish *Thrips palmi* from other species were described in Table 10 (Nakahara, 1994; Murai, 2002; Mound and Masumoto, 2005; Draft diagnostic protocol for *Thrips palmi*, 2009). Morphological charactersistics used to distinguish *Ceratothripoides claratris* from other species were described in Table 11 (Palmer *et al.,* 1989; http://keys.lucidcentral.org/keys/v3/thrips_of_california/data/key/thysanoptera/media/html/browse_species/ceratothripoides_claratris.htm

7.3 Thrips transmission

A leaf disc assay combined with PTA-ELISA was used to study the vector capability of *T. palmi* and *C. claratris* in transmitting TNRV and CaCV (Figure 6). Because feeding preference or behavior of different thrips species may vary, it is very important to use appropriate host for virus acquisition. In this study, peanut was selected as the acquisition host for *T. palmi* and tomato for *C. claratris*. Moreover,

both peanut and tomato are susceptible to TNRV and CaCV infections. To compare transmission efficiencies of thrips species, the host plant should display high virus titers and be suitable for thrips feeding. In this study, *Physalis minima* was selected for virus transmission tests since it is a systemic host of TNRV and CaCV with high virus titers. Both *T. palmi* and *C. claratris* could feed well on *P. minima*.

7.3.1 Virus acquisition

Host plants were mechanically inoculation 2 weeks prior to the acquisition feeding. Systemically infected leaves showed high virus titers in a dilution series in PTA-ELISA were selected for virus acquisition. New born larvae (up to 4 h old) were given an acquisition access period (AAP) of 48 h on infected leaves which were floated on 2 ml water in plastic petri dishes (Figure 6a). Larvae of *T. palmi* were acquired on virus-infected peanut leaves while larvae of *C. claratris* were fed on virus-infected tomato leaves, respectively. The thrips were then transferred to 50-ml conical polystyrene tubes containing healthy leaves (peanut leaves for *T. palmi* and tomato leaves for *C. claratris*) and reared until they become adult (Figure 6b). Fresh leaves were provided every 2 days.

7.3.2 Virus transmission assay

Transmission efficiency was evaluated by leaf discs assay (Wijkamp *et al.*, 1993). Adult thrips were placed individually on a healthy *Physalis minima* leaf disc (0.8 cm in diameter) in a microcentrifuge tube for 2 days (Figure 6c). After 2 days, the old leaf disc was replaced with a new healthy leaf disc to begin the second inoculation access period (IAP). After another 2 days, the third IAP was performed. After each IAP, the discs were floated for 4 days on 2 ml water in a 24-well plate (Figure 6d). The infectivity of virus on the leaf discs were determined by PTA-ELISA using antibodies to tospoviruses (PAb MYSV6) as described above. Transmission efficiency was calculated from the percentage of infected leaf discs.

Table 10 Morphological characteristics of *Thrips palmi*.

Specimens	Characters
Specimens can be recognized as *T. palmi* by the following combination of characters	
Antenna	with seven or eight distinct segments; segments III and IV with forked sense cones
Head	with two pairs of ocellar setae (II and III); pair I missing
Pronotum	with two pairs of major posteroangular setae
Forewing	1st vein – setal row on the first vein interrupted (in most species)
Abdominal tergites V toVIII	with paired ctenidia
Abdominal tergite VIII	with ctenidia posteromesad to the spiracles
Specimens can be identified as *Thrips palmi* by the presence of the following characters	
Body colour	clear yellow body with no dark areas on the head, thorax or abdomen; antennal segments I and II are pale
Antennal segment V	usually yellowish in basal ⅓ to ½
Antennal segment VI	length = 42–48 μm
Head: ocellar setae pair III	with their bases sited outside of the ocellar triangle or touching the tangent lines connecting the anterior ocellus to each of the posterior ocelli
Forewing: 1st vein	with three (occasionally two) distal setae
Metascutum	with a pair of campaniform sensilla; with striate sculpture converging posteriorly
Abdominal pleurotergites	discal setae absent; lines of sculpture without numerous microtrichia
Abdominal tergite II	with four lateral marginal setae
Abdominal tergites III and IV	S2 almost equal to S3
Abdominal tergite VIII	with complete posteromarginal comb
Abdominal tergite IX	with two pairs of pores (anterior and posterior)
Male: sternites	transverse glandular areas on sternites III to VII

Source: Nakahara (1994); Murai (2002); Mound and Masumoto, (2005); Draft diagnostic protocol for *Thrips palmi*, (2009)

Table 11 Morphological characteristics of *Ceratothripoides claratris*.

Specimens	Characters
Specimens can be identified as *C. claratris* by the presence of the following characters	
Antenna	with eight segments
Body colour	brown body, antennal segment III is yellow, antennal segment IV–V are variably yellow and brown
Leg	yellow legs with extensive brown shadings particularly on femora
Head	with three pairs of ocellar setae, pairs III is almost twice as long as lateral margin of ocellar triangle, arising close to anterolateral margin
Forewing: 1st vein	with only two setae on distal half, second vein with 14–16 setae
Pronotum	with two pairs of posteroangular setae
Metanotum	metanotum irregularly reticulate, posterior reticles smallest, median setae at anterior margin and more than two-thirds as long as metanotum,without paired campaniform sensilla
Abdominal tergites	with sculpture lines scarcely extending mesad to campaniform sensilla, without ctenidia
Abdominal tergite VIII	with long regular marginal comb, sternites without discal setae, marginal setae as long as sternites; sternite VII setae S1 and S2 arising well in front of margin
Male sternites III–VII	with about 12 small glandular areas in two irregular transverse rows

Source: Palmer *et al*. (1989); http://keys.lucidcentral.org/keys/v3/thrips_of_california/data/key/thysanoptera/media/html/browse_species/ceratothripoides_claratris.htm

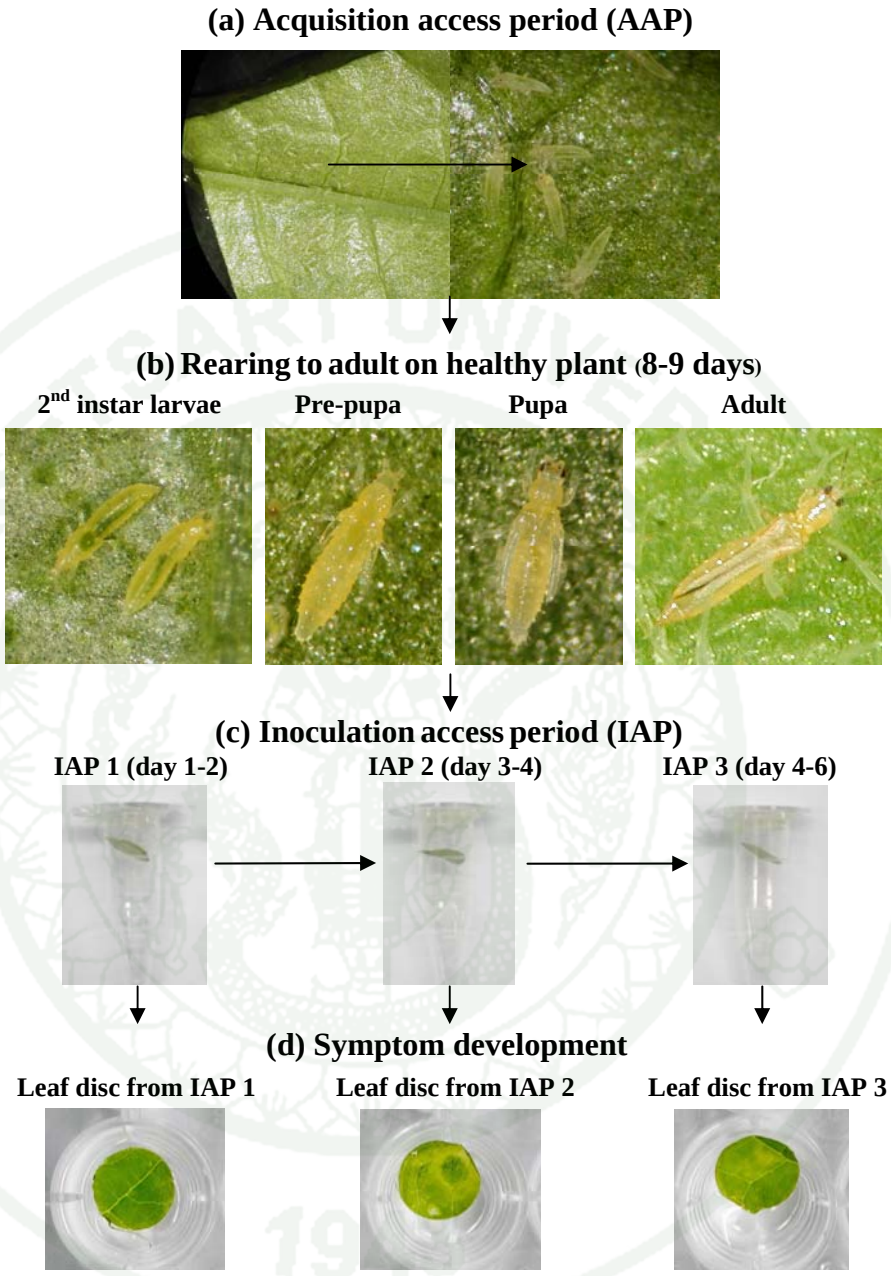

Figure 6 Method for transmission of tospovirus by thrips. First instar larvae were given an acquisition access period (AAP) for 48 h on tospovirus infected plant (a). Thrips were reared to adult on healthy plant (8-9 days) (b). For transmission, adult thrips were placed individually on *P. minima* leaf disc for 48 h. Inoculation access period (IAP) were performed 3 times (c). Leaf discs from each IAP were incubated on tap water for 4 days for symptom development (d).

RESULTS

1. Detection and identification of tospovirus in infected plant samples

Samples of peanut, watermelon, tomato and pepper showing typical tospovirus symptoms; such as chlorotic spots, necrotic spots, severe chlorosis, leaf necrosis, stem necrosis and bud necrosis; were collected from fields in seven provinces in Thailand (Table 12). Samples were proven for tospovirus infections by PTA-ELISA using general antibodies for detection of CaCV, WSMoV and MYSV (PAb MYSV6). The ELISA reactivities are shown in Table 12. All samples gave positive results for tospovirus infection (A_{405} nm range from 0.833 to 3.655). This result indicated the presence of tospovirus in all sample tested.

Six isolates including tospovirus-infected tomato (TT1), pepper (PP1), peanut (KK and NRA) and watermelon (WS1 and WS2) were subjected to further characterization. To identify the tospovirus species, RT-PCR with specific primers follwing with the complete nucleotide sequence of N protein gene were determined. Total RNAs were extracted from tospovirus-infected plants and used as a template for cDNA synthesis using 3'G4S primer, containing 13 nt conserved in all Asian tospoviral S RNA segments (Cortez *et al.,* 2001). PCR was then performed using specific primers to the N protein gene of MYSV (MN3/MN5 primers), WSMoV (WSMV-C/WSMV-V primers) and CaCV (CaCV-ExFB/CaCV-ExRS primers) (Table 6).

Approximately 1 kb fragment was detected with MN3/MN5 primers when tospovirus-infected watermelon isolate WS1 was examined (Figure 7 lane 1). Approximately 0.9 kb fragment was detected with WSMV-C/WSMV-V primers when tospovirus-infected watermelon isolate WS2 was examined (Figure 7 lane 7). Approximately 0.9 kb fragment was detected with CaCV-ExFB/CaCV-ExRS primers when tospovirus-infected peanut isolate NRA and KK were examined (Figure 7 lane 13 and 14). However, no amplification product was detected with specific primers for amplifying MYSV, WSMoV and CaCV when tospovirus-infected tomato isolate TT1

(Figure 7 lane 5, 10 and 15, respectively) and tospovirus-infected pepper isolate PP1 were examined.

Table 12 Detection of tospoviruses in naturally infected plant species by plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) using general antibodies to tospoviruses (PAb MYSV6).

Plant	Location	Designated virus isolate	A_{405}
Peanut	Khon Kaen	KK	1.781
Peanut	Nong Khai	NK	1.768
Peanut	Udon Thani	UT	2.031
Peanut	Nakhorn Rachasrima	NRA	2.268
Tomato	Nakhon Pathom	TT1	2.517
Pepper	Nakhon Pathom	PP1	0.833
Pepper	Kanjanaburi	PP2	3.655
Pepper	Khon Kaen	PP3	1.888
Watermelon	Suphan Buri	WS1	2.809
Watermelon	Suphan Buri	WS2	1.144
Watermelon	Khon Kaen	WKA	1.460
Watermelon	Khon Kaen	WKB	2.674
Healthy peanut	-	-	0.135
Healthy tomato	-	-	0.181
Healthy pepper	-	-	0.138
Healthy Watermelon	-	-	0.156

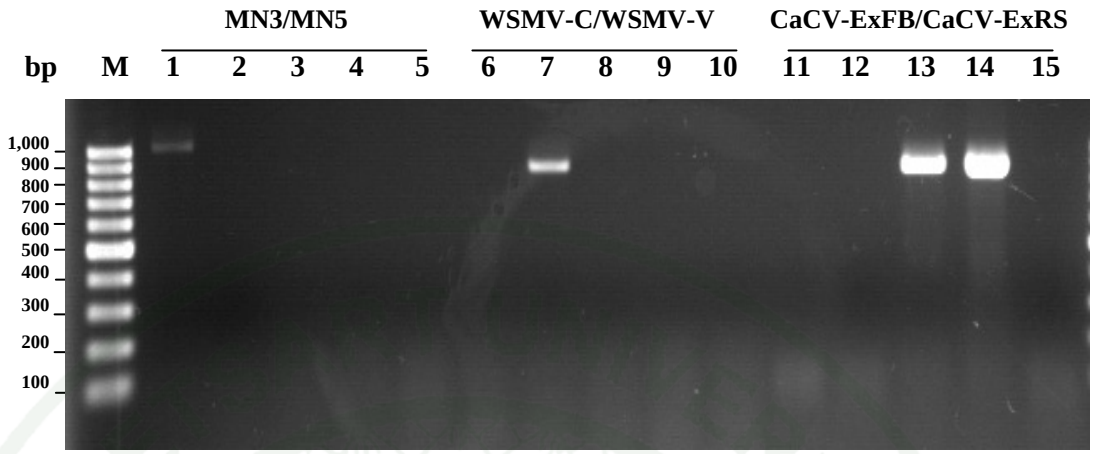

Figure 7 Agarose gel electrophoresis of RT-PCR products of N protein gene amplified with MN3/MN5 primers (lane 1-5), WSMV-C/WSMV-V primers (lane 6-10), and CaCV-ExFB/CaCV-ExRS primers (lane 11-15). Total RNA was extracted from tospovirus-infected watermelon isolate WS1 (lane 1, 6 and 11) and WS2 (lane 2, 7 and 12), tospovirus-infected peanut isolate NRA (lane 3, 8 and 13) and KK (lane 4, 9 and 14), and tospovirus-infected tomato isolate TT1 (Lane 5, 10 and 15). GeneRuler™ 100 bp DNA Ladder was loaded as molecular weight marker (M).

The obtained PCR products of four isolates (WS1, WS2, NRA and KK) were cloned, sequenced and compared with sequences deposited in NCBI database using BLAST program. Comparison of amino acid sequences revealed that amino acid sequence of N protein of tospovirus-infected watermelon isolate WS1 shared 98% amino acid identity with those of MYSV from Japan (Kato *et al*., 2000). Amino acid sequence of N protein of tospovirus-infected watermelon isolate WS2 showed very high identity (98%) with those of WSMoV from Taiwan (Yeh *et al*., 1995). Amino acid sequences of N protein of tospovirus infected peanut isolate KK and NRA revealed high homology with those of CaCV-Aus from Australia (McMichael *et al*., 2002) at 98% identity while shared high homology with CaCV-AIT isolated from tomato in central Thailand (Knierim *et al*., 2006) at 92% identity.

Based on criteria for species of tospoviruses, the isolates in the *Tospovirus* genus with >90% N protein sequence identity are classified as strains of the same virus species (Fauquet *et al*., 2005; Jones, 2005; Tsompana *et al*., 2008). Hence tospovirus-infected watermelon isolate WS1 should be regarded as a strain of MYSV (designated MYSV-WS1). Tospovirus-infected watermelon isolate WS2 should be regarded as a strain of WSMoV (designated WSMoV-WS2). Tospovirus-infected peanut isolate KK and NRA should be regarded as a strain of CaCV (designated CaCV-KK and CaCV-NRA). The complete nucleotide sequences of N protein gene of MYSV-WS1 from watermelon in Suphan Buri (accession no. FJ947154), WSMoV-WS2 from watermelon in Suphan Buri (accession no. FJ947155) and CaCV-KK from peanut in Khon Kaen (accession no. FJ947157) were submitted to GenBank.

To identify the N protein gene of tospovirus-infected tomato (TT1) and pepper (PP1) that could not be detected with specific primers for amplifying three tospoviruses found in Thailand, RCR with two conserved primers (UHP and 3'G4S) for amplifying 3' sequence of tospoviral S RNA segment were performed. The primer UHP obtained from conserved sequence within intergenic region (IR) of Asian Tospoviral S RNA segments (Cortez *et al.,* 2001) while the primer 3'G4S obtained from 13 nt conserved sequence at 3' terminus of all Asian tospoviral S RNA segments. The PCR product of approximately 1.2 kb was amplified with UHP/3'G4S primers when tospovirus-infected tomato isolate TT1 and pepper isolate PP1 were examined (Figure 8 lane 1 and 2, respectively). The obtained PCR products were cloned, sequenced and compared with sequences deposited in NCBI database using BLAST program.

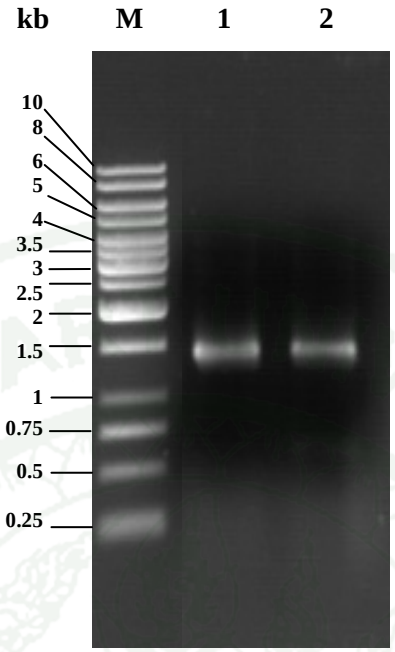

Figure 8 Agarose gel electrophoresis of RT-PCR products of N protein gene amplified with 3'G4S/UHP primers. Total RNA was extracted from tospovirus infected tomato isolate TT1 (lane 1) and pepper isolate PP1 (lane 2). GeneRuler™ 1 kb DNA Ladder was loaded as molecular weight marker (M).

Comparison of amino acid sequences revealed that amino acid sequence of N protein of tospovirus-infected tomato (TT1) and pepper (PP1) revealed extremely high homology at 99.6% identity. Results suggested that these two tospovirus isolates were the same species. Sequence comparison showed the amino acid sequence of N protein of tospovirus-infected tomato (TT1) and pepper (PP1) shared 58.2%, 56.0% and 51.8% identity with those of CaCV, WSMoV and MYSV reported in Thailand, respectively. This result indicated that these tospovirus isolates may different from other tospoviruses which have been reported in Thailand. Since this tospovirus isolate was first observed in tomato in Nakhon Pathom province with symptoms consisting of necrotic spot and necrotic ringspot on tomato leaves, the virus was tentatively designated Tomato necrotic ringspot virus (TNRV) for further studies. The complete nucleotide sequence of N protein gene of TNRV-PP1 from pepper in Nakhon Pathom (accession no. FJ947153) was submitted to GenBank.

In this study, Tomato necrotic ringspot virus isolated from tomato (TNRV-TT1) was further characterized based on particle morphology, host range, serology, the complete nucleotide sequence of S and M RNA segments and vector transmission. Capsicum chlorosis virus isolated from peanut (CaCV-NRA) was also characterized and compared with TNRV. Moreover, tospovirus isolates WSMoV-WS2 and MYSV-WS1 were also used for serological characterization.

2. Characterization of Tomato necrotic ringspot virus (TNRV)

2.1 Single local lesion isolation of Tomato necrotic ringspot virus (TNRV)

Tomato necrotic ringspot virus isolate TT1 was subjected to single local lesion isolation on *Chenopodium quinoa*. Three days after inoculation, chlorotic spots followed by necrotic local lesions were observed on inoculated leaves. Single local lesion was excised from *Chenopodium quinoa* and inoculated in systemic hosts such as *Datura stramonium* and *Physalis minima* to establish pure culture.

2.2 Symptoms of Tomato necrotic ringspot virus (TNRV) and electron microscopic study

Infected tomato plants showed typical tospovirus symptoms consisting of necrotic spots, necrotic ringspots and necrosis on leaves, stem necrosis and necrotic spots on tomato fruit (Figure 9). Back-inoculation on tomato induced symptoms similar to those observed in naturally infected plants. Electron microscopic observation of an ultra-thin section of infected tomato leaves showed a cluster of spherical particles of approximately 80-90 nm in diameter (Figure 10). These particles have morphological features resembling those of tospoviruses.

2.3 Host range studies

Symptoms induced by TNRV on various tested plants are shown in Table 13. Several characteristics of local and systemic symptoms were observed on TNRV-infected plants. Of 14 plant species inoculated with this virus isolate, eight developed a systemic viral infection, three showed only local lesions on the inoculated leaves and the other three showed no symptoms. TNRV systemically infected *Solanum lycopersicum*, *Capsicum annum*, *Arachis hypogea*, *D. stramonium*, *Nicotiana benthamiana*, *N. glutinosa*, *N. tabacum* and *P. minima* (Table 13). Necrotic local lesions were found on inoculated leaves of *C. quinoa* and *C. amaranticolor*, while chlorotic local lesions were found on inoculated leaves of *Vigna unguiculata*. No symptoms were observed on inoculated *Cucumis sativus*, *Luffa acutangula* and *Citrullus lanatus*. Tospovirus infection of leaves of inoculated plants was confirmed by serological tests. Leaves exhibiting tospovirus symptoms gave positive results with general antibodies to tospoviruses (PAb MYSV6) in PTA-ELISA (Appendix Table 1). Mock-inoculated control plants gave negative results.

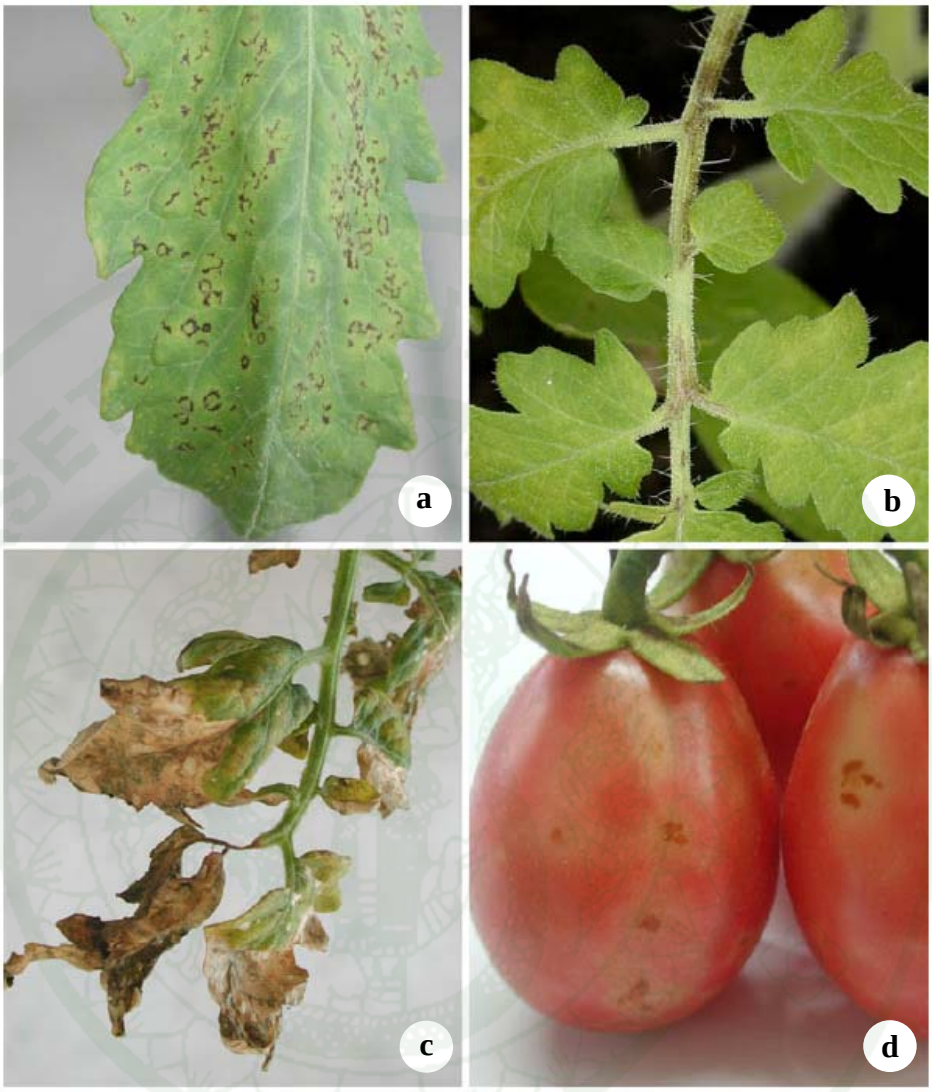

Figure 9 Symptoms induced by Tomato necrotic ringspot virus (TNRV) on tomato. (a) necrotic spots and necrotic ringspots on leave. (b) stem necrosis. (c) necrosis on leaves. (d) necrotic spots on fruits.

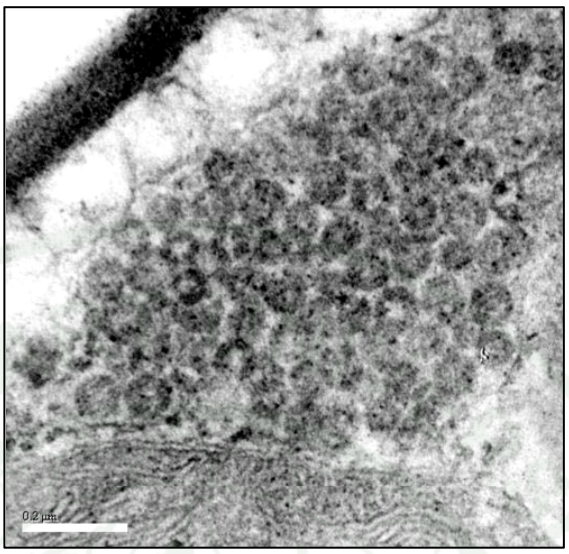

Figure 10 Electron micrograph of a cluster of virus particles in an ultra-thin section of leaf tissues of Tomato necrotic ringspot virus (TNRV) infected tomato. Bar represents 200 nm.

Table 13 Response of several host plants to Tomato necrotic ringspot virus (TNRV).

Tested plant	Local reaction	Systemic reaction	N
Chenopodiaceae			
Chenopodium quinoa	NL	-	8/10
C. amaranticolor	NL	-	8/10
Leguminosae			
Arachis hypogea	CS	CS, MO	4/10
Fabaceae			
Vigna unguiculata	CS	-	3/5
Solanaceae			
Capsicum annum	CS	CS, NS, MO, LD	9/12
Datura stramonium	CS	CS, NS, MO, LD	8/13
Solanum lycopersicum	NS, NRS	NS, NRS, SN, LN	7/10
Nicotiana benthamiana	CS	CS, NS	7/10
Nicotiana glutinosa	NR, NRS	NR, NRS	7/10
Nicotiana tabacum	CS	CS	8/10
Physalis minima	NS, NRS	NS, NRS	11/12
Cuberbitaceae			
Cucumis sativus	-	-	0/8
Luffa acutangula	-	-	0/8
Citrullus lanatus	-	-	0/8

CS, chlorotic spots; NL, necrotic lesion; NS, necrotic spots; NR, necrotic rings NRS, necrotic ringspots; LN, leaf necrosis; SN, stem necrosis; LD, leaf deformation; MO, mottling; -, no symptoms; N, number of symptomatic plants/number of inoculated plants

2.4 Serological characterization

Gajanandana *et al*. (2006) have generated the panel of antibodies against various N proteins of tospoviruses found in Thailand. In this study, these antibodies were used to characterize TNRV by PTA-ELISA (Figure 11) and Western blot analysis (Figure 12). The reactivity of antibodies to the other three tospoviruses previously reported in Thailand (CaCV, WSMoV and MYSV) was also compared. PAb MYSV6 and MAb 2B2 were shown to cross-react with all tospoviruses found in Thailand, including CaCV, WSMoV, MYSV and TNRV (Figure 11). Rabbit polyclonal antibodies A3 could react with CaCV, WSMoV and TNRV but not MYSV. However, TNRV did not react with MAb 2D6 and MAb L4E8, which were previously found to recognize CaCV and WSMoV, and MAb 5E7, which specifically reacted with MYSV.

Similar results were also obtained in Western blot analysis (Figure 12). TNRV was detected by general antibodies that could recognize WSMoV, CaCV and MYSV (PAb MYSV6 and MAb 2B2) and a polyclonal antibody (PAb A3) that could bind WSMoV and CaCV, yielding the N protein band at 32 kDa. However, the other two MAbs (MAb 2D6 and MAb L4E8), which detected WSMoV and CaCV, did not react with TNRV. A specific antibody to MYSV (MAb 5E7) failed to react with TNRV in Western blot analysis (Figure 12). This result suggests that TNRV is serologically related but not identical to those of WSMoV and CaCV found in Thailand.

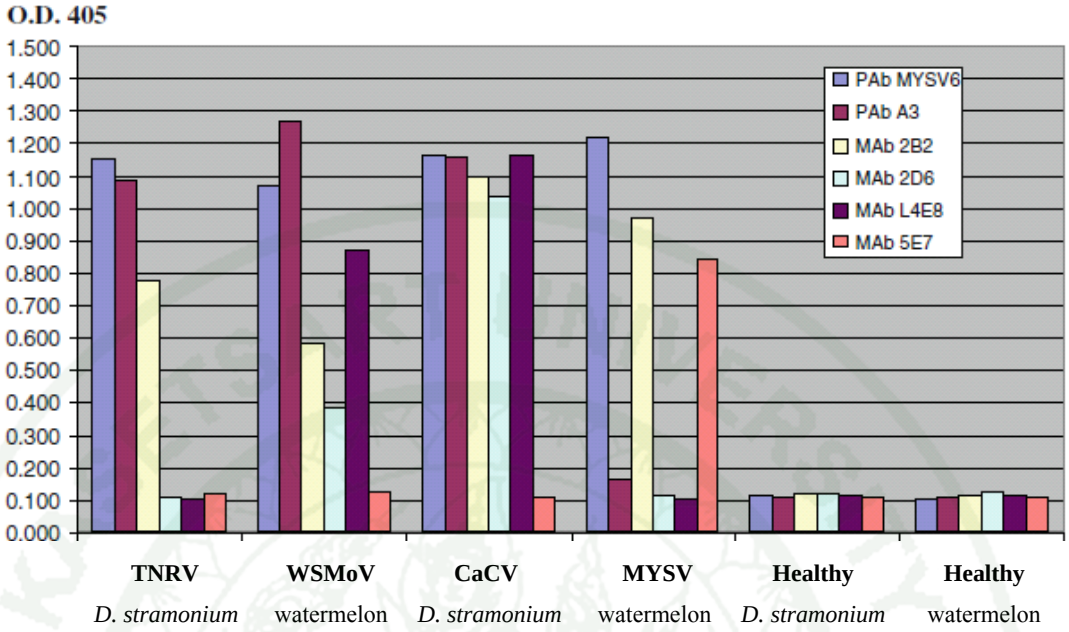

Figure 11 Serological reactions in plate-trapped antigen ELISA (PTA-ELISA) among four tospoviruses found in Thailand using various antibodies to N proteins of tospoviruses, using extracts from infected plants as antigen source.

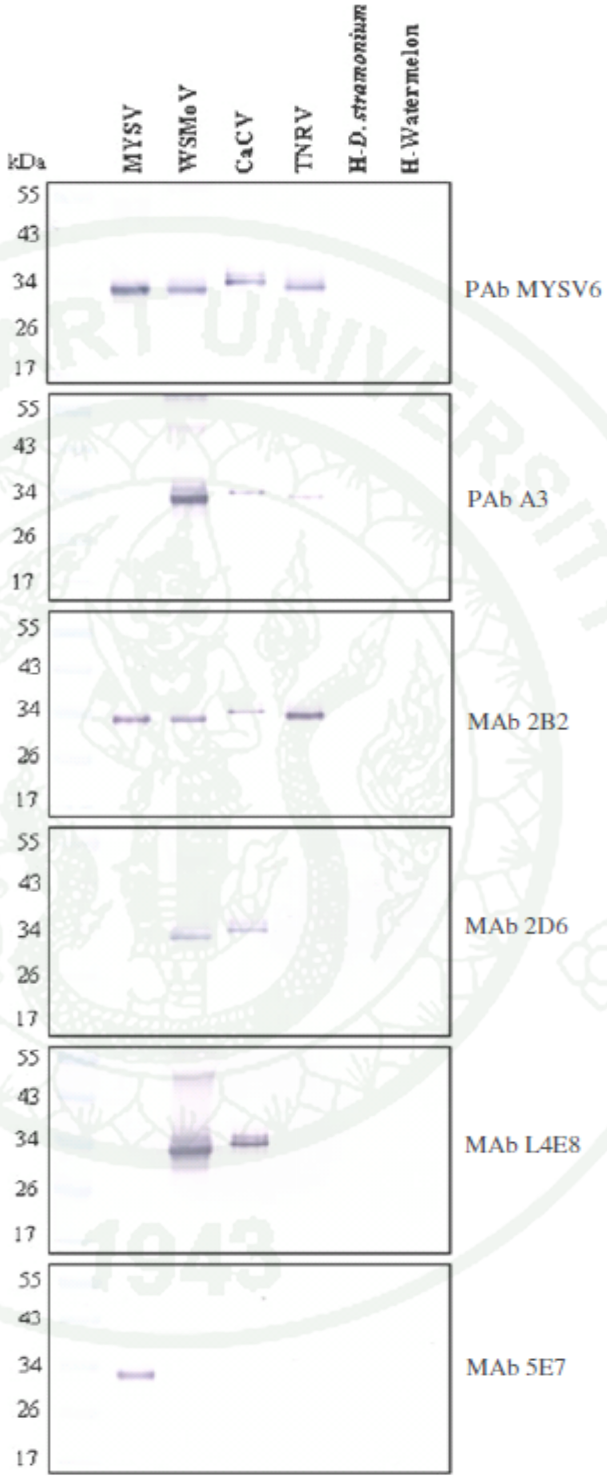

Figure 12 Western blot analysis of leaf extracts from plants infected with Melon yellow spot virus (MYSV), *Watermelon yellow spot virus* (WSMoV), Capsicum chlorosis virus (CaCV) and Tomato necrotic ringspot virus (TNRV) using various antibodies against N proteins of tospoviruses.

2.5 Cloning of S and M RNA fragments of Tomato necrotic ringspot virus (TNRV)

Three overlapping primers were designed to amplify a full-length S RNA segment. The PCR products of approximately 0.7, 1.2 and 1.6 kb were amplified from RNA extract of TNRV-infected tomato using J13/FST-2250, 3'G4S/UHP and FST-750/RST-555 primers, respectively (Figure 13 lane 1, 2, 3). Two overlapping primers were designed to amplify a full-length M RNA segment. The PCR products of approximately 1.7 and 3.4 kb were amplified using F-3199/5'MRNA and RM-1628/3'MRNA, respectively (Figure 13 lane 4, 5). The obtained PCR products were cloned, sequenced and assembled using SeqMan software (DNAStar Inc, USA). The complete nucleotide sequences of S and M RNA segments of TNRV were showed in Figure 14 and 15, respectively.

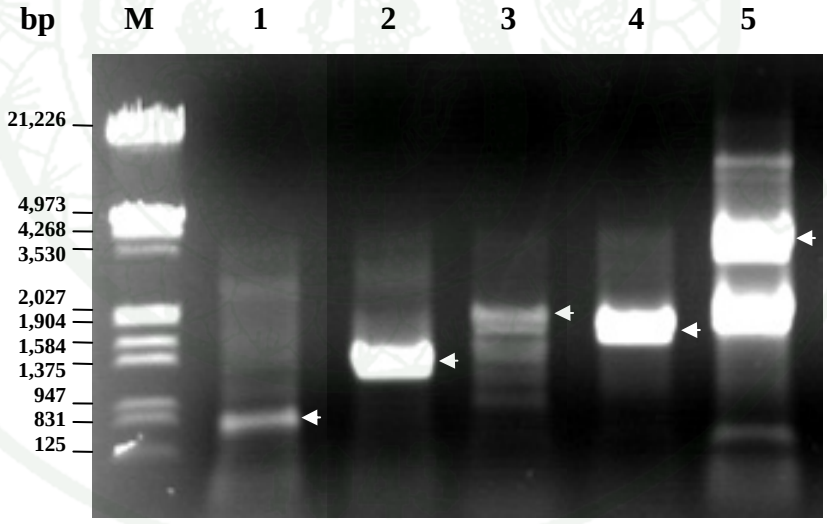

Figure 13 Agarose gel electrophoresis of RT-PCR products of S RNA fragments amplified with J13/FST-2250 primers (lane 1), 3'G4S/UHP primers (lane 2), FST-750/RST-555 primers (lane 3), F-3199/5'MRNA primers (lane 4) and RM-1628/3'MRNA primers (lane 5). The arrows indicate virus specific band. Total RNA was extracted from Tomato necrotic ringspot virus (TNRV) infected tomato. Lambda DNA/EcoRI/HindIII marker was used as molecular weight marker (M).

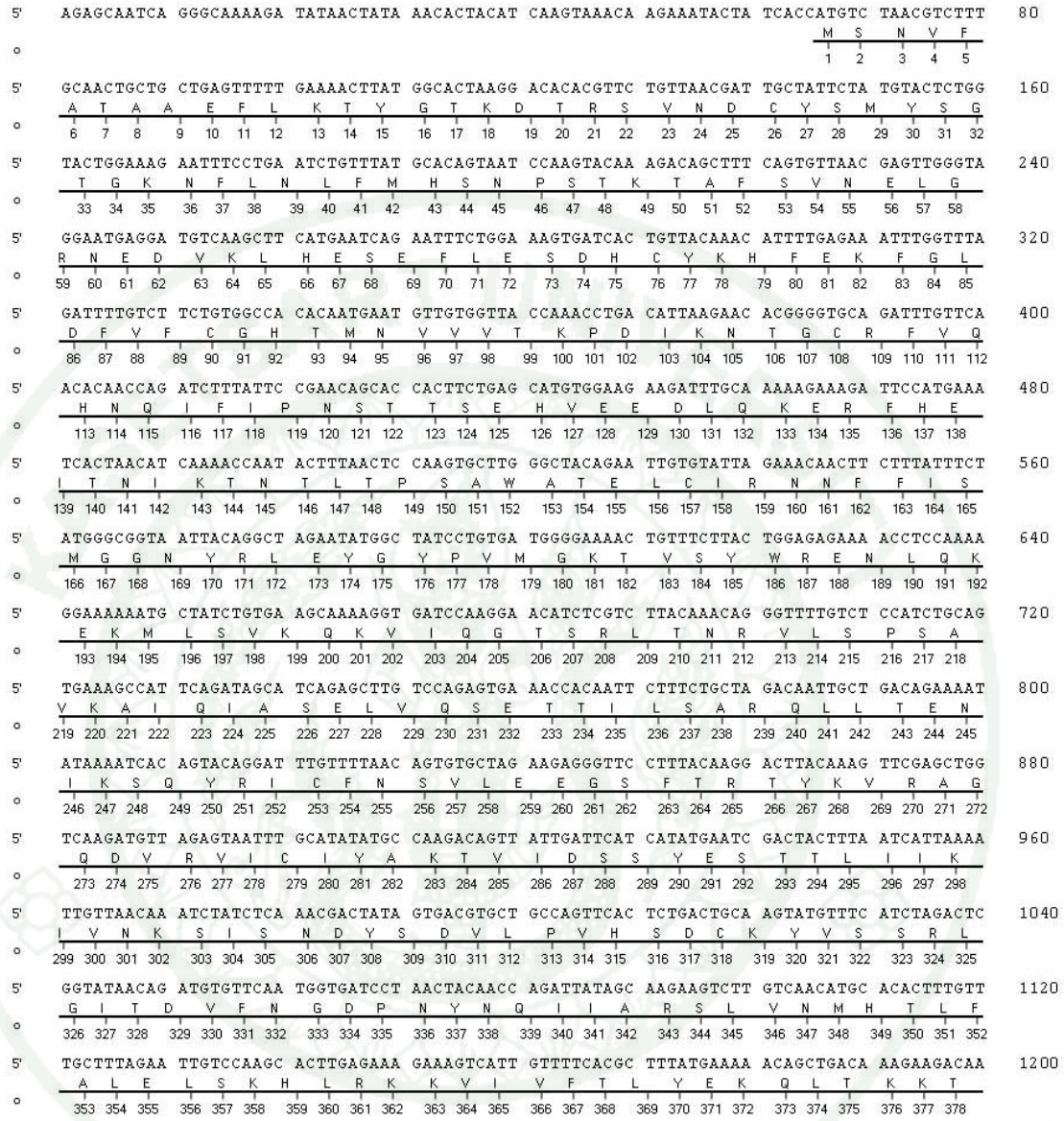

Figure 14 Nucleotide sequence of S RNA segment of TNRV. The sequence is displayed in the virion DNA sense (5' to 3').

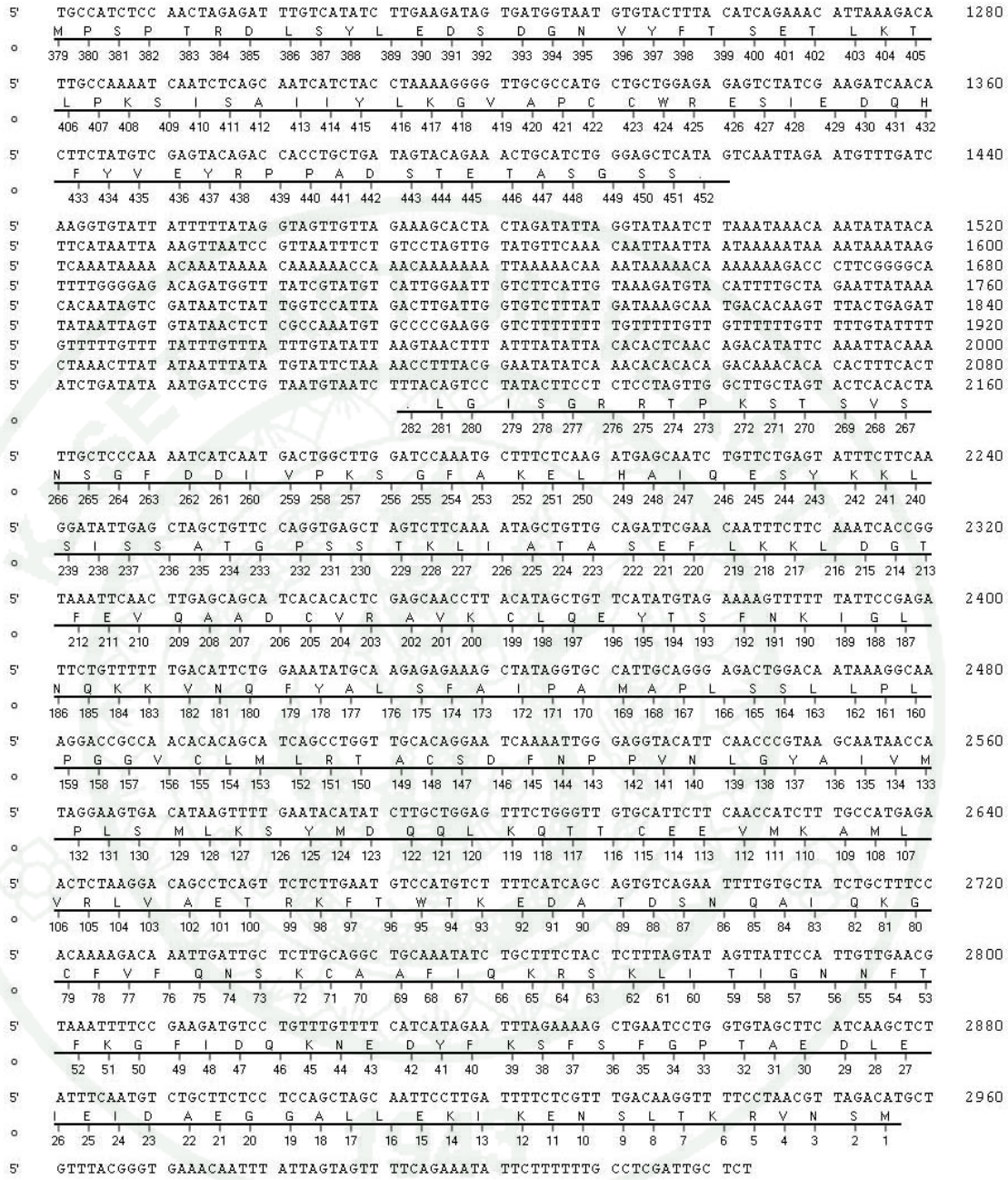

Figure 14 (Continued)

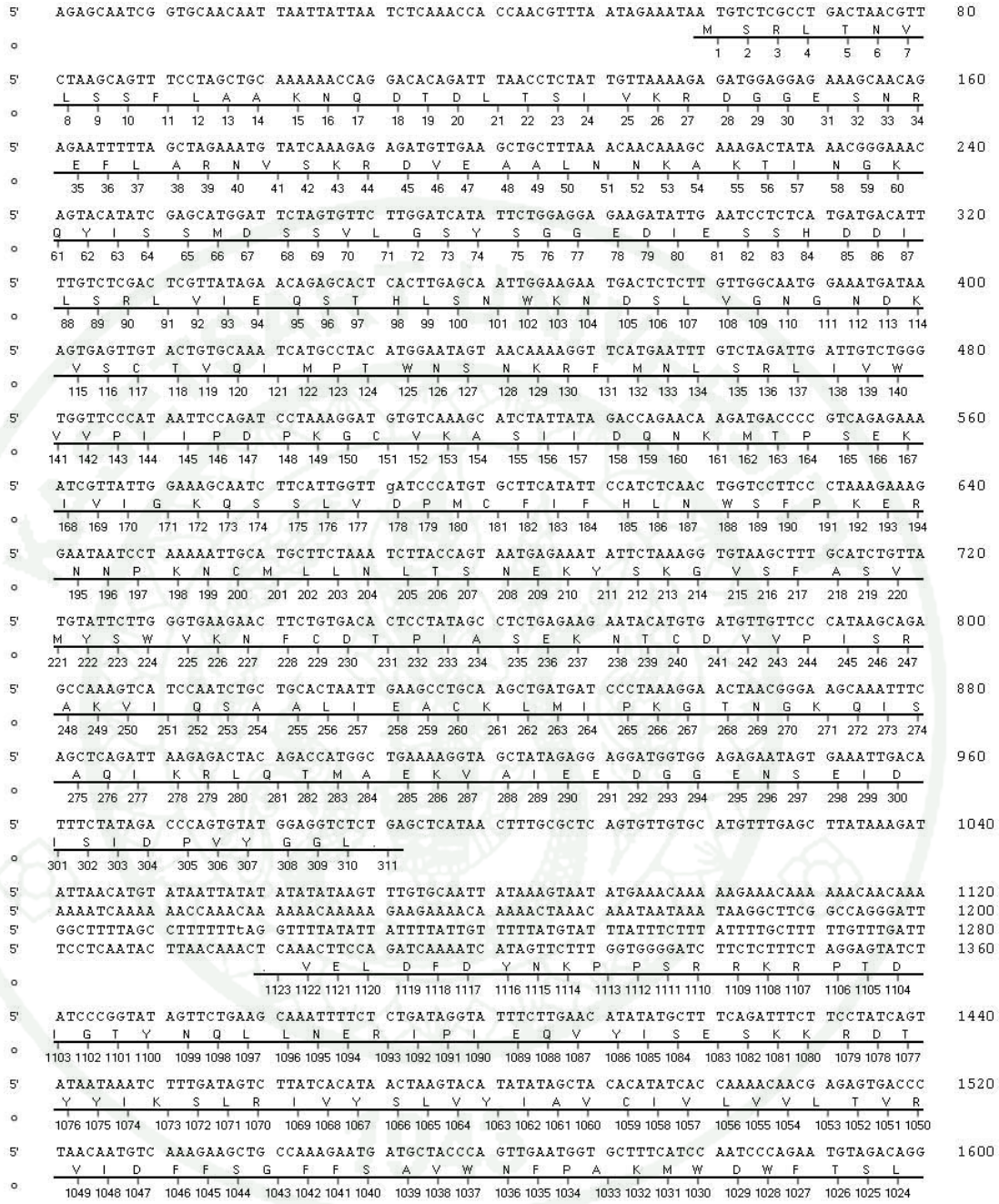

Figure 15 Nucleotide sequence of M RNA segment of TNRV. The sequence is displayed in the virion DNA sense (5' to 3').

Figure 15 (Continued)

Figure 15 (Continued)

2.6 Sequence analysis of S RNA of Tomato necrotic ringspot virus (TNRV)

Complete nucleotide sequences of S RNA segment of TNRV was determined and submitted to GenBank (accession no. FJ489600) (Figure 14). The S RNA was 3,023 nt in length and comprised of two ORFs in an ambisense arrangement. The first ORF on the viral strand was 1,356 nt in length, coding for the NSs protein (451 aa), with a total predicted molecular mass of 51 kDa. The second ORF on the viral complementary strand was 846 nt in length, encoding the N protein (281 aa), with a total predicted molecular mass of 31 kDa. The 5' and 3' untranslated regions (UTR), which have the potential to form a stable hairpin structure, were 65 and 66 nt long, respectively. The intergenic region (IR) between the two ORFs was 690 nt long.

Sequence identities of the N protein of TNRV compared with those of other tospoviruses are summarized in Figure 16. Amino acid sequence of the N protein of TNRV shared 51.8-58.2% identity with those of CaCV, GBNV, WSMoV, CCSV, WBNV, TZSV and MYSV, which were all found in Asia (Figure 16). The N protein of TNRV shared 44.6-47.8% identity with those of IYSV, TYRV and PolRSV, which were found in Europe and the Middle East, and shared 25.3-31.2% identity with those of America tospoviruses (MeSMV, INSV, ZLCV, CSNV, TSWV, ANSV, GRSV and TCSV) (Figure 16). Sequence analysis of the N protein revealed that TNRV was most similar to CaCV (58.2%). The amino acid sequence of the NSs protein shared 42.1-53.4% identity with those of CCSV, TZSV, CaCV, GBNV, WSMoV, MYSV, IYSV, TYRV and PolRSV and shared 14.6-18.9% with those of other tospoviruses (Table 14).

The phylogenetic tree derived from amino acid sequences of the N protein of tospoviruses grouped the viruses into four clusters (Figure 17). TNRV was in the same cluster with the Asian tospovirus group (CaCV, GBNV, WSMoV, CCSV, WBNV, TZSV and MYSV). Phylogenetic analysis of the NSs protein also showed similar clustering patterns (Figure 18a). Based on the low degree of homology of the

N and NSs proteins of TNRV compared with other tospoviruses, we concluded that TNRV should be considered a member of a distinct species in the genus *Tospovirus*.

2.7 Sequence analysis of M RNA of Tomato necrotic ringspot virus (TNRV)

The complete nucleotide sequence of M the RNA segment of TNRV was determined (GenBank accession no. FJ947152) (Figure 15). The M RNA was 4,716 nt in length and contained two ORFs in an ambisense arrangement. The first ORF on the viral strand was 933 nt in length, coding for the NSm protein (310 aa), with a total predicted molecular mass of 34 kDa. The second ORF on the viral complementary strand was 3,369 nt in length, coding for the GnGc glycoprotein (1,122 aa), with a total predicted molecular mass of 128 kDa. The 5' and 3' untranslated regions (UTR) were 59 and 48 nt long, respectively, and had the potential to form a stable hairpin structure. The intergenic region (IR) between two ORFs was 307 nt long.

Sequence identities of the GnGc glycoprotein and NSm protein of TNRV compared with those of other tospoviruses are summarized in Table 14. The amino acid sequence of the GnGc glycoprotein revealed 51.3-65.6% identity with those of Asian (CaCV, GBNV, WSMoV, WBNV, TZSV and MYSV) and European-Middle Eastern tosposviruses (PolRSV and IYSV) and shared 33.2-34.2 % identity with those of American tospoviruses (TSWV, TCSV, GRSV, INSV, ZLCV and CSNV) (Table 14). The NSm protein of TNRV shared 62.5-71.7% sequence identity with those of Asian and Eropean-Middle Eastern tosposviruses and shared 36.1-39.4% with those of other American tospoviruses. Sequence analysis of the NSm protein and the GnGc glycoprotein reveled that TNRV was most similar to CaCV (71.7% and 65.6%).

Phylogenetic analysis of the amino acid sequences of the GnGc glycoprotein and NSm protein of tospoviruses showed that TNRV clusters with Asian tospoviruses (CaCV, GBNV, WSMoV, WBNV, TZSV and MYSV) (Figure 18b and 18c).

	CaCV	GBNV	WSMoV	WBNV	CCSV	TZSV	MYSV	IYSV	TYRV	PolRSV	MeSMV	INSV	ZLCV	CSNV	TSWV	GRSV	ANSV	TCSV	PCFSV	PYSV	TNRV
CaCV	***	84.7	85.5	80.0	63.5	62.9	58.2	46.8	45.6	44.4	32.2	29.4	31.2	30.5	33.9	32.7	29.9	31.9	21.0	24.2	58.2
GBNV		***	86.2	86.2	65.7	62.9	58.3	45.0	44.8	43.3	33.3	28.6	30.9	29.3	31.1	31.5	30.3	29.9	21.3	20.1	57.8
WSMoV			***	83.3	63.9	62.2	57.1	42.8	44.1	43.3	33.3	29.8	31.2	30.1	32.3	33.1	30.3	30.3	20.6	20.5	56.0
WBNV				***	65.7	63.6	57.1	42.4	42.2	41.9	32.2	28.6	29.3	28.9	30.7	31.1	29.1	29.5	19.9	20.1	55.6
CCSV					***	80.9	57.0	48.1	47.6	45.0	29.7	24.2	27.2	27.2	27.5	28.2	28.2	26.7	18.8	17.6	56.7
TZSV						***	57.6	46.5	43.8	43.0	28.6	23.4	25.7	27.2	28.6	28.6	26.7	27.1	18.0	18.0	55.4
MYSV							***	49.4	47.4	48.5	30.5	27.0	29.6	31.5	28.2	29.4	27.8	29.0	19.4	21.3	51.8
IYSV								***	72.5	70.0	33.2	28.1	31.5	31.9	34.9	33.3	32.4	32.5	17.2	20.7	44.6
TYRV									***	82.8	32.7	29.5	32.9	31.8	33.2	35.6	35.5	33.2	16.3	19.0	47.8
PolRSV										***	32.7	28.7	34.1	32.9	33.2	35.2	35.9	34.4	18.3	20.2	47.1
MeSMV											***	51.9	59.6	60.8	56.6	59.3	59.7	59.3	20.0	22.2	29.2
INSV												***	52.3	54.7	53.9	54.3	53.9	52.7	19.4	20.0	25.3
ZLCV													***	79.6	74.4	76.7	76.4	75.6	18.6	18.4	27.1
CSNV														***	76.4	73.6	74.8	74.4	19.4	20.5	27.5
TSWV															***	79.1	79.5	79.5	17.5	18.4	31.2
GRSV																***	82.2	87.6	19.5	19.7	28.9
ANSV																	***	82.2	19.5	19.2	27.7
TCSV																		***	19.5	19.2	28.9
PCFSV																			***	67.5	19.0
PYSV																				***	18.4
TNRV																					***

Figure 16 Sequence identities (%) of N protein of Tomato necrotic ringspot virus (TNRV) compared with those of other tospoviruses.

Table 14 Sequence identities (%) of NSs protein, NSm protein and GnGc glycoprotein of Tomato necrotic ringspot virus (TNRV) compared with those of other tospoviruses.

	NSs			NSm			GnGc		
Virus	nt	%nt	%aa	nt	%nt	%aa	nt	%nt	%aa
TNRV	1,356			933			3,369		
CaCV	1,320	56.0	49.5	927	69.8	71.7	3,366	66.1	65.6
WSMoV	1,320	54.9	49.1	939	68.5	66.0	3,366	65.4	64.2
GBNV	1,320	55.5	49.3	924	70.4	70.9	3,366	66.4	65.6
WBNV	-	-	-	921	70.2	68.2	3,363	63.1	51.3
CCSV	1,383	57.8	53.4	-	-	-	-	-	-
TZSV	1,380	56.4	51.8	930	70.9	68.6	3,369	65.4	64.3
MYSV	1,410	57.0	44.1	927	63.4	62.5	3,384	61.8	59.6
IYSV	1,332	52.9	44.4	936	67.0	66.4	3,411	60.4	56.5
PolRSV	1,332	52.4	42.1	924	66.0	63.7	3,408	61.7	57.6
TYRV	1,332	54.2	44.9	-	-	-	-	-	-
MeSMV	1,368	36.2	18.9	-	-	-	-	-	-
TSWV	1,404	38.1	18.4	909	49.2	36.6	3,408	47.4	33.2
TCSV	-	-	-	912	48.9	37.7	3,405	47.6	33.5
GRSV	1,404	35.4	17.2	912	50.2	39.4	3,417	47.6	33.6
INSV	1,350	37.4	18.4	912	48.0	36.4	3,333	47.5	34.2
ZLCV	-	-	-	912	50.5	36.1	3,408	47.4	33.4
CSNV	-	-	-	912	50.1	38.4	3,408	48.2	33.6
PYSV	1,443	36.1	14.6	-	-	-	-	-	-
PCFSV	1,419	38.2	15.9	-	-	-	-	-	-

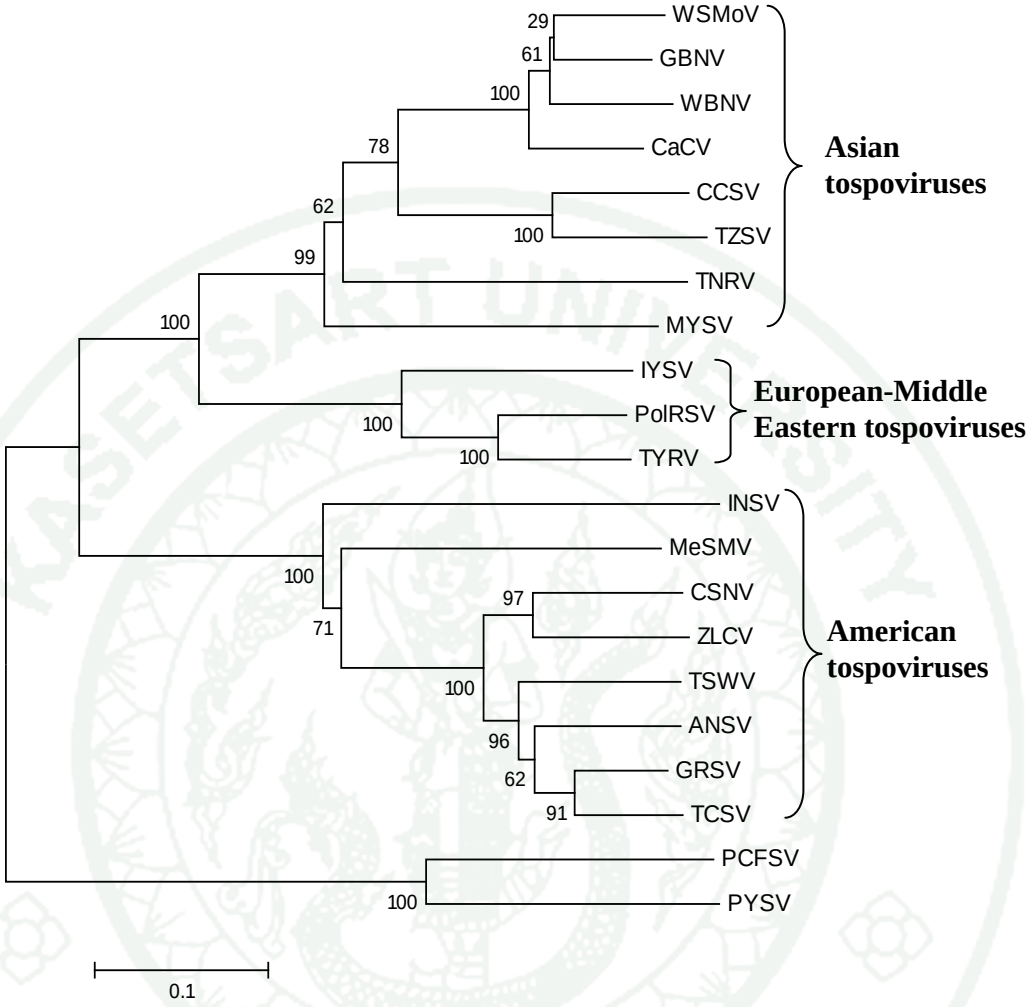

Figure 17 Phylogenetic analysis of tospovirus species based on amino acid sequence of N protein. The trees were constructed by MEGA 4.0 program using the neighbour-joining method options with 500 bootstrap replications.

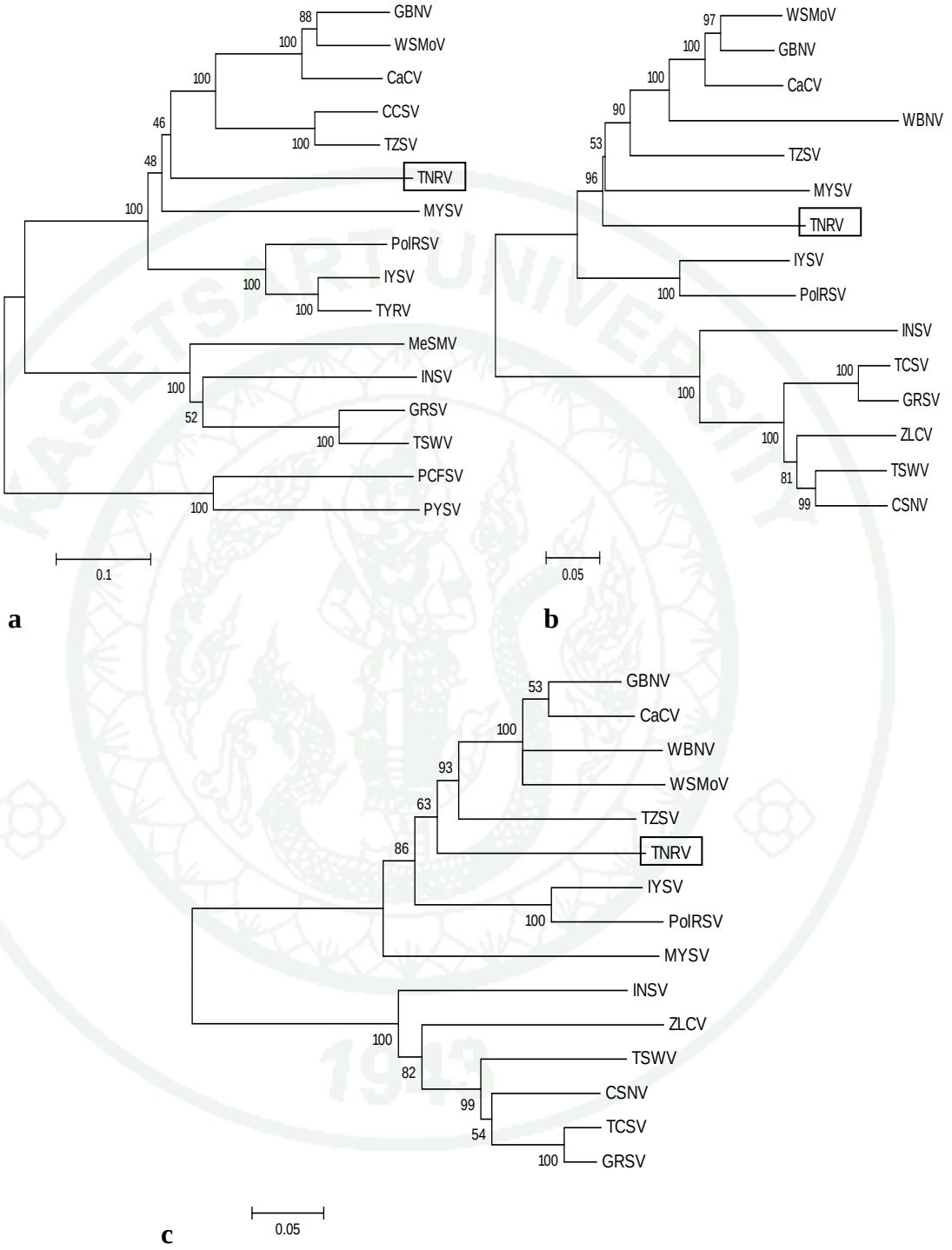

Figure 18 Phylogenetic analysis of tospovirus species based on amino acid sequence of NSs protein (a), GnGc glycoprotein (b) and NSm protein (c). The trees were constructed by MEGA 4.0 program using the neighbour-joining method options with 500 bootstrap replications.

2.8 Vector capability of *T. palmi* and *C. claratris* in transmitting Tomato necrotic ringspot virus (TNRV)

Two thrips species, *T. palmi* and *C. claratris*, which are known as vectors of tospoviruses, were studied for their capability to transmit TNRV using a leaf disc assay combined with ELISA. Morphological characteristics of *T. palmi* and *C. claratris* used for transmission assays in this study were determined (Figure 19 and 20, respectively).

This capacity was tested with adults that were given an acquisition access period of 48 h on TNRV-infected plants as newborn larvae, up to 4 h old. An adult was individually placed on healthy *P. minima* leaf disc for 48 h. Four days after inoculation, chlorotic spot symptoms were found on the infected leaf discs. Leaf discs were tested for tospovirus infection using PTA-ELISA (Appendix Table 3 and 4). *T. palmi* transmitted TNRV with an efficiency of 83 % while *C. claratris* transmitted TNRV with an efficiency of 12% (Table 15). There was no significant difference in transmission efficiency between adult males and females of both thrips species. Results suggested that TNRV could be experimentally transmitted by *T. palmi* and *C. claratris.*

Table 15 Transmission efficiency of Tomato necrotic ringspot virus (TNRV) by adults of *T. palmi* and *C. claratris* determined by leaf discs assay.

Thrip	Number of tested thrips	Transmission efficiency (%)		
		Male	Female	Total
T. palmi	97	88% (40 of 45)	78% (41 of 52)	83% (81 of 97)
C. Claratris	75	10% (3 of 30)	13% (6 of 45)	12% (9 of 75)

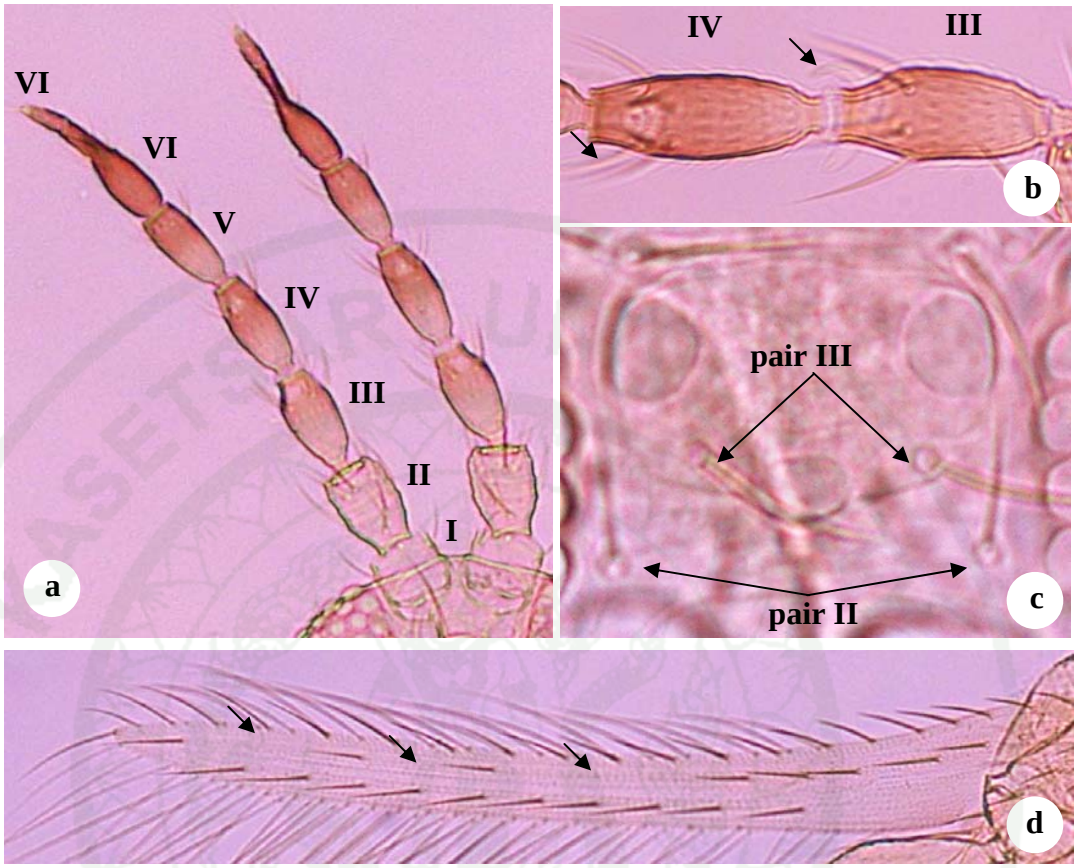

Figure 19 Characteristics of *Thrips palmi* identified in this study. Antenna with seven segments (a). Antenna: segments III and IV with forked sense cones (arrow) (b). Head: with two pairs of ocellar setae and ocellar setae pair III situated outside of ocellar triangle (c). Forewing: first vein-three setae with gaps in distal half (arrow) (d). Abdominal tergite II: with four lateral marginal setae (arrow) (e). Metascutum: with campaniform sensilla (arrow) (f). Abdominal tergite VIII: ctenidia posteromesad to the spiracle; posteromarginal comb complete (g). Thrips was inspected under a compound microscope (40X objective yielding 400X total magnification).

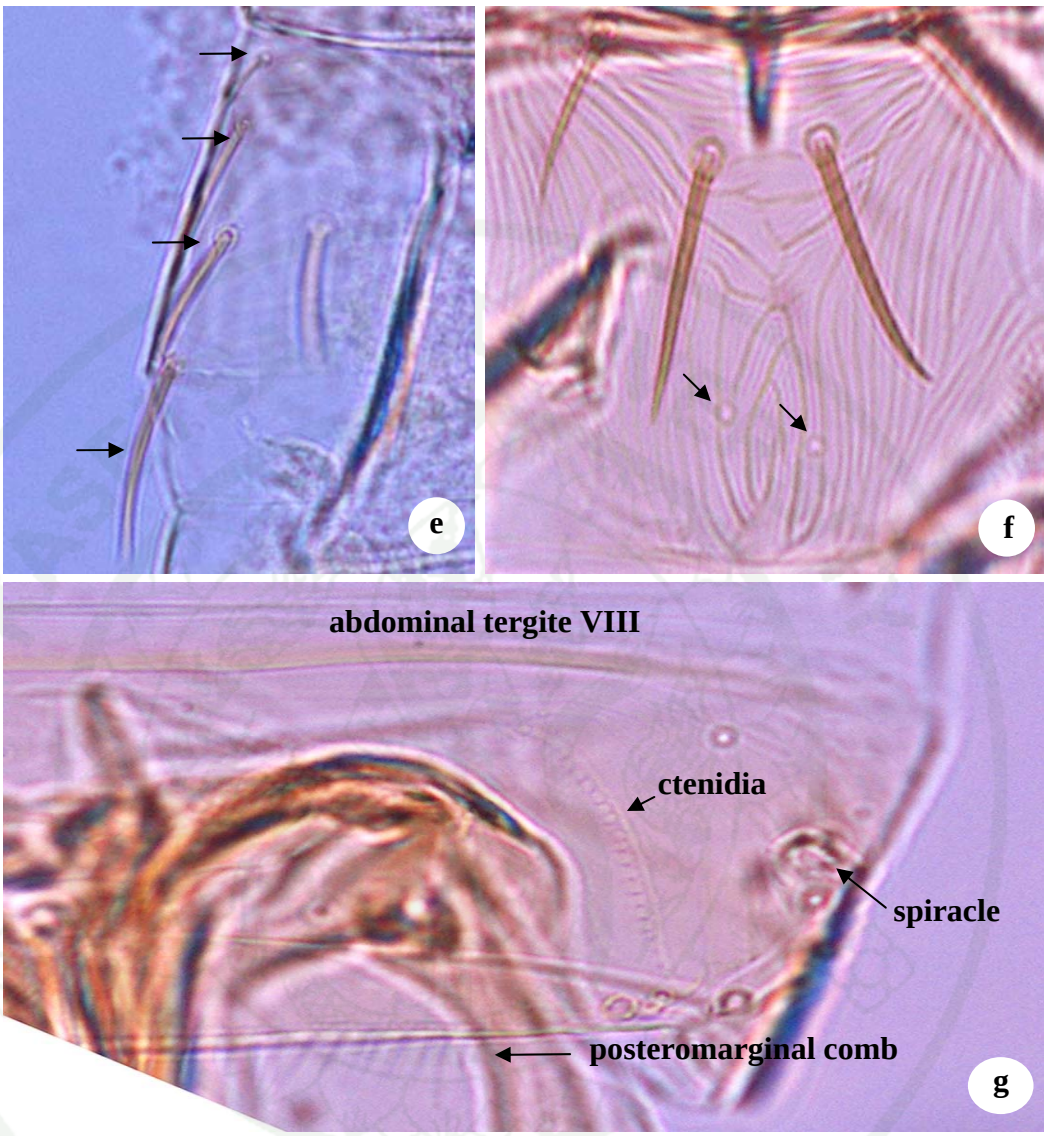

Figure 19 (continued)

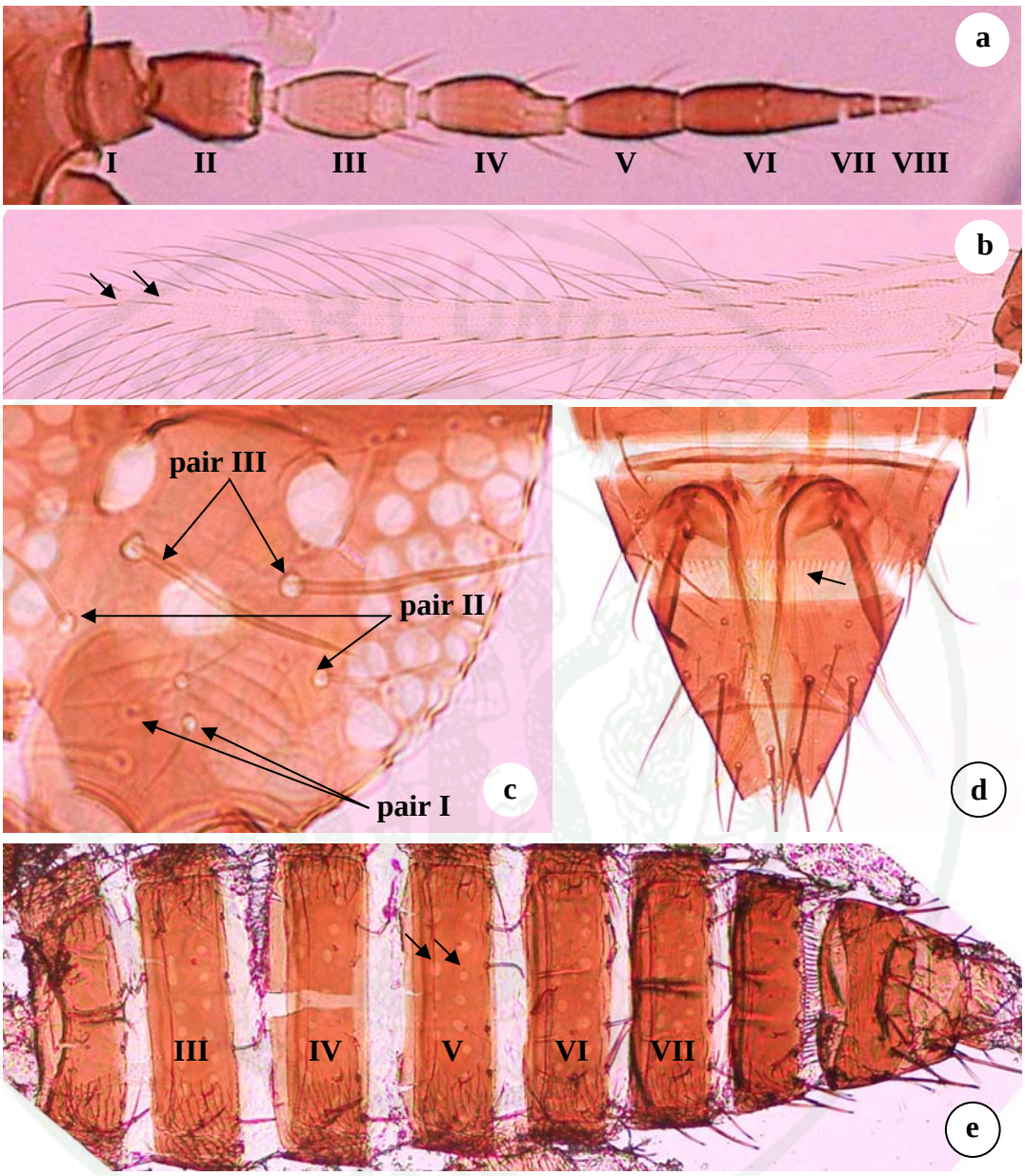

Figure 20 Characteristics of *Ceratothripoides claratris* identified in this study. Antenna: with eight segments (a). Forewing: first vein with only two setae on distal half (arrow), second vein with 18 setae (b). Head: with three pairs of ocellar setae present (c). Abdominal tergite VIII: with long regular marginal comb (arrow) (d). Male sternites III-VII: with about 12 small glandular areas in two irregular transverse rows (arrow) (e). Thrips was inspected under a compound microscope (40X objective yielding 400X total magnification).

3. Characterization of Capsicum chlorosis virus isolated from NRA (CaCV-NRA) from peanut

3.1 Single local lesion isolation of Capsicum chlorosis virus isolate NRA (CaCV-NRA)

Capsicum chlorosis virus isolate NRA (CaCV-NRA) isolated from peanut in Nakhorn Rachasrima was subjected to single local lesion isolation on *Chenopodium quinoa*. Three days after inoculation, chlorotic spots followed by necrotic local lesion were observed on inoculated leaves. Single local lesion was excised from *Chenopodium quinoa* and inoculated in systemic hosts such as *Datura stramonium* and *Physalis minima* to establish pure culture of virus.

3.2 Symptoms of Capsicum chlorosis virus isolate NRA (CaCV-NRA)

Typical tospovirus symptoms consisting of chlorotic spots, necrotic spots, necrotic ringspots and bud necrosis were observed in infected peanut plants (Figure 21). Back-inoculation on peanut induced similar symptoms of what we oserved in naturally infected plants.

3.3 Host range studies

Symptoms induced by CaCV-NRA on various plants were shown in Table 16. From the total of 14 plant species inoculated with this virus isolate, eight developed a systemic viral infection, three showed only local lesions on the inoculated leaves and the other three showed no symptoms. (Table 16). CaCV-NRA systemically infected *Arachis hypogea*, *Capsicum annum, Datura stramonium, Solanum lycopersicum, Nicotiana benthamiana, Nicotiana glutinosa, Nicotiana tabacum* and *Physalis minima* (Table 16). CaCV-NRA infected *C. quinoa* and *C. amaranticolor* showing only necrotic lesion on inoculated leaves. CaCV-NRA induced chlorotic local lesions and necrotic ringspots on inoculated leaves of *Vigna unguiculata*. No local or systemic symptoms were observed on *Cucumis sativus*, *Luffa*

acutangula and *Citrullus lanatus*. Leaves of test plants were collected and analyzed for viral infection by PTA-ELISA. All leaf samples with typical symptoms of tospovirus showed positive results by PTA-ELISA using general antibodies to tospovirus (PAb MYSV6) (Appendix Table 2). Mock inoculated control plants gave negative detection results.

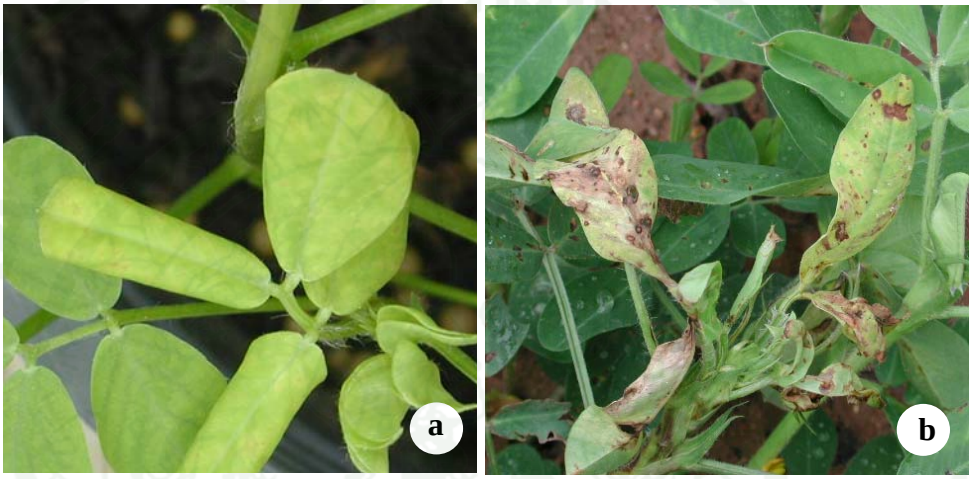

Figure 21 Symptoms induced by Capsicum chlorosis virus isolate NRA (CaCV-NRA) on peanut plants. (a) chlorotic spots on leaves. (b) necrotic ringspots and bud necrosis on shoot.

Table 16 Response of several host plants to Capsicum chlorosis virus isolate NRA (CaCV-NRA).

Tested plants	Local reaction	Systemic reaction	N
Chenopodiaceae			
Chenopodium quinoa	NL	-	6/10
C. amaranticolor	NL	-	7/10
Leguminosae			
Arachis hypogea	CS	CS, NS, NRS, SN	6/10
Fabaceae			
Vigna unguiculata	CS, NRS	-	3/5
Solanaceae			
Capsicum annum	CS	CS, MO	8/12
Datura stramonium	CS	CS, NS	8/12
Solanum lycopersicum	CS, NS	CS, NS, NRS, SN	6/10
Nicotiana benthamiana	CS	CS, NS	7/10
Nicotiana glutinosa	CS, NS	CS, NS	7/10
Nicotiana tabacum	CS	CS, MO	8/10
Physalis minima	CS, NS	CS, NS, MO, LD	12/12
Cuberbitaceae			
Cucumis sativus	-	-	0/8
Luffa acutangula	-	-	0/8
Citrullus lanatus	-	-	0/8

CS, chlorotic spots; NL, necrotic lesion; NS, necrotic spots; NRS, necrotic ringspots; SN, stem necrosis; LD, leaf deformation; MO, mottling; -, no symptoms;
N, Number of symptomatic plants/number of inoculated plants

3.4 Serological characterization

Serological relationships of the N protein of CaCV-NRA and other tospoviruses in Thailand (MYSV, WSMoV and TNRV) were analyzed by PTA-ELISA (Figure 11) and Western blot analysis (Figure 12). In both PTA-ELISA and Western blot analysis, CaCV-NRA could react with PAb MYSV6, PAb A3, MAb 2B2, MAb 2D6 and MAb L4E8. However, a specific antibody to MYSV (MAb 5E7) failed to react with CaCV-NRA in PTA-ELISA and Western blot analysis. All antibodies that could react to CaCV-NRA also reacted to WSMoV. This result suggested that the N protein of CaCV-NRA is closely related to WSMoV. Moreover, CaCV-NRA is serologically related but not identical to those of TNRV found in Thailand.

3.5 Cloning of S and M RNA fragments of Capsicum chlorosis virus isolate NRA (CaCV-NRA)

Four overlapping primers were designed to amplify a full-length S RNA segment. PCR products of approximately 1, 0.9, 1.7 and 1.5 kb were amplified from RNA extract of CaCV-NRA infected peanut using J13/FS-2481, CaCV-ExRS/3'G4S, RS-2729/FS-1230 and RS-2100/FS-743 primers, respectively (Figure 22 lane 1, 2, 3 and 4, respectively). Two overlapping primers were designed to amplify a full-length M RNA segment. PCR products of approximately 3.4 and 1.7 kb were amplified using R-3302/3'MRNA and F-3199/5'MRNA primers, respectively (Figure 22 lane 5 and 6, respectively). The obtained PCR products were cloned, sequenced and assembled using SeqMan software (DNAStar Inc, USA). The complete nucleotide sequence of S and M RNA of CaCV-NRA were shown in Figure 23 and 24, respectively.

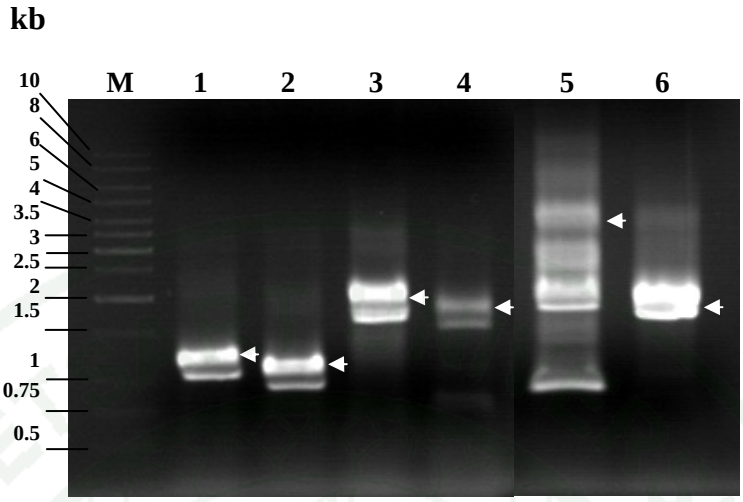

Figure 22 Agarose gel electrophoresis of RT-PCR products of S and M RNA fragments amplified with J13/ FS-2481 primers (lane 1), CaCV-ExRS /3'G4S primers (lane 2), RS-2729/ FS-1230 primers (lane 3), RS-2100/ FS-743 primers (lane 4), R-3302/3'MRNA primers (lane 5) and 5'MRNA /F-3199 primers (lane 6). The arrows indicate virus specific band. Total RNA was extracted from CaCV-NRA infected peanut. GeneRuler™ 1 kb DNA Ladder was used as molecular weight marker (M).

3.6 Sequence analysis of S RNA of Capsicum chlorosis virus isolate NRA (CaCV-NRA)

Complete nucleotide sequence of S RNA segment of CaCV-NRA from peanut was determined (Figure 23). The S RNA was 3,612 nt in length and comprised of two ORFs in an ambisense arrangement. The first ORF on the viral strand was 1,320 nt in length, coding for the NSs protein (439 aa) with a total predicted molecular mass of 48 kDa. The second ORF on the viral complementary strand was 828 nt in length, coding for the N protein (275 aa) with a total predicted molecular mass of 31 kDa. The 5' and 3' untranslated regions (UTR) were 66 and 67 nt long, respectively. The intergenic region (IR) between two ORFs was 1,331 nt long (Figure 23).

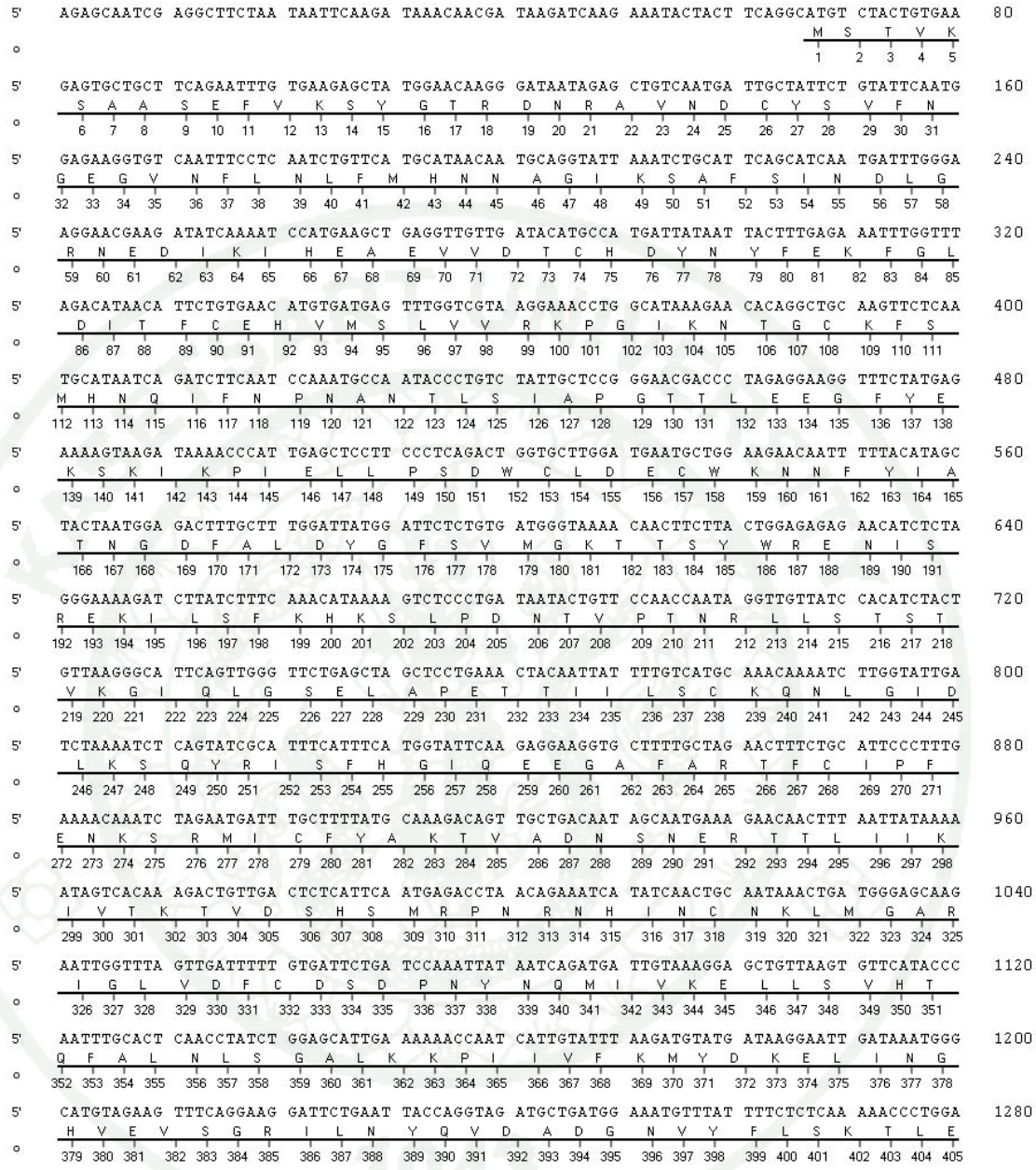

Figure 23 Nucleotide sequence of S RNA segment of CaCV-NRA. The sequence is displayed in the virion DNA sense (5' to 3').

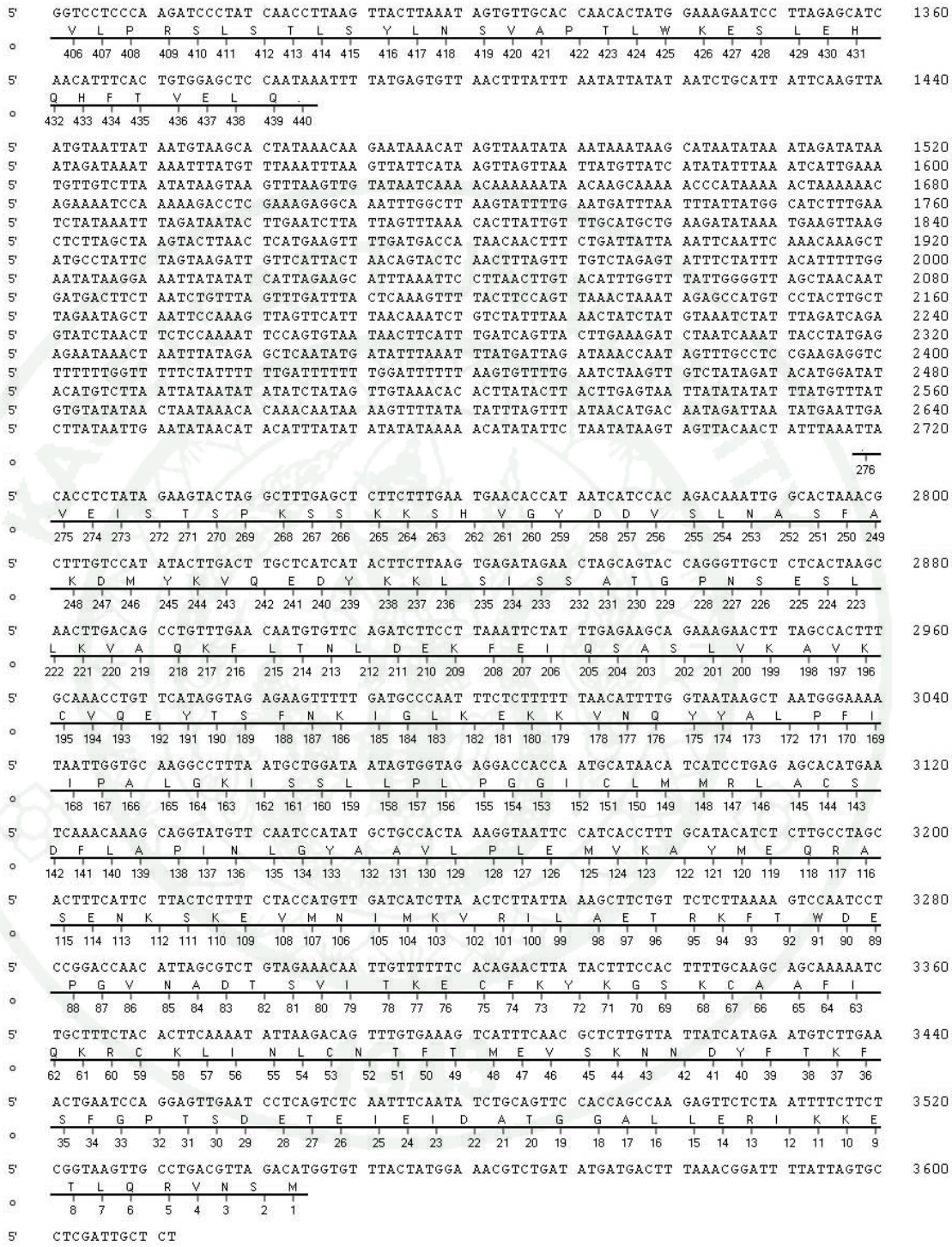

Figure 23 (Continued)

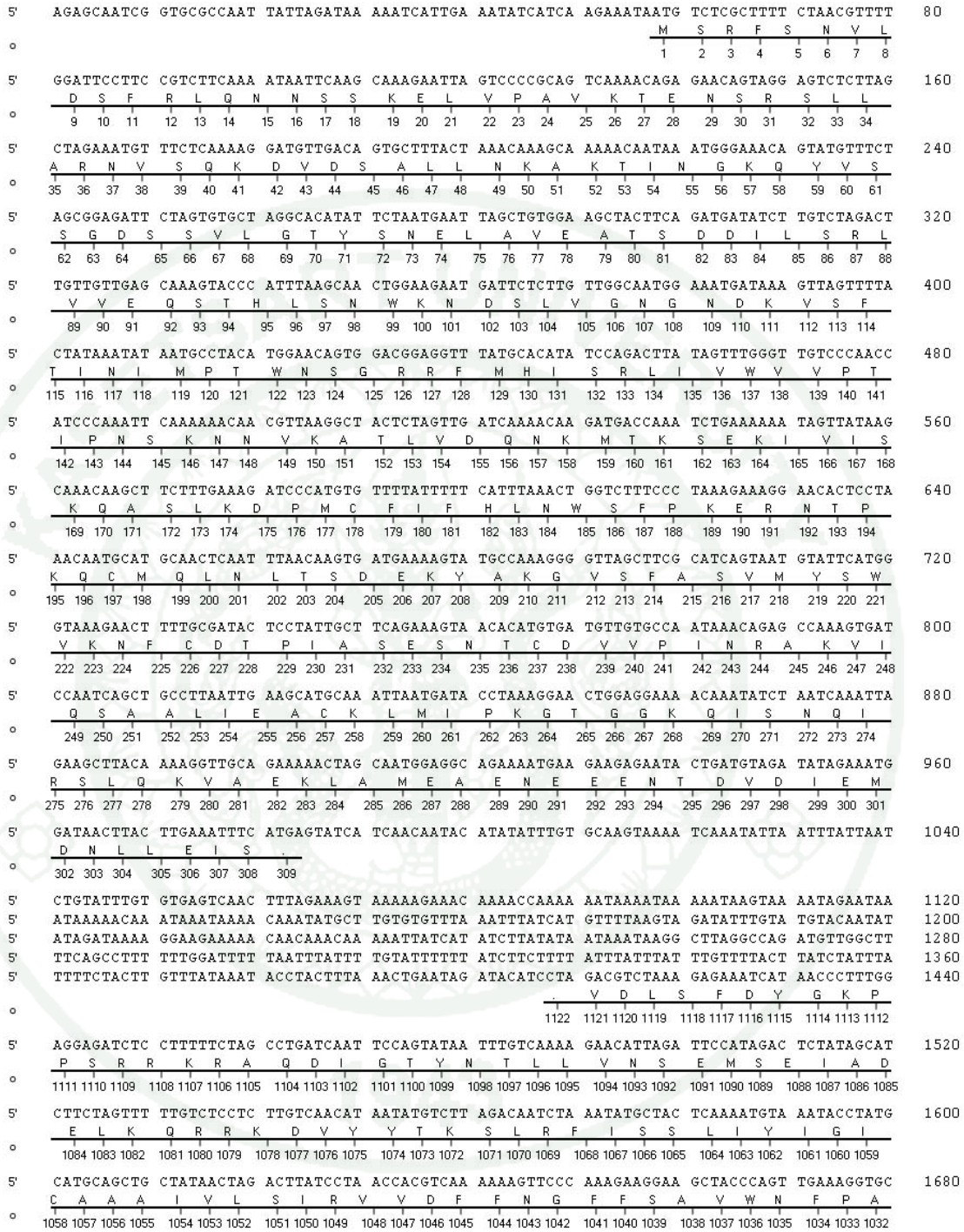

Figure 24 Nucleotide sequence of M RNA segment of CaCV-NRA. The sequence is displayed in the virion DNA sense (5' to 3').

Figure 24 (Continued)

Figure 24 (Continued)

Sequence identities of S RNA segment of CaCV-NRA as compared with those of other tospoviruses are summarized in Table 17. Amino acid sequence of N protein of CaCV-NRA revealed extremely high homology with CaCV-Ch-Pan isolated from pepper in India, CaCV-Aus isolated from pepper in Australia and CaCV-Ph isolated from *Phalaenopsis* orchid in Taiwan (97.1%, 98.9% and 98.5% identity, respectively) and shared high homology with CaCV-AIT isolated from tomato in Thailand, CaCV-TD8 isolated from tomato in Thailand, CaCV-CP isolated from peanut in China and CaCV-TwTom1 isolated from tomato in Taiwan (92.7%, 92.0%, 92.7 and 93.8% identity, respectively) (Table 17).

The N protein of CaCV-NRA shared 58.2-85.1% identity with those of Asia tospoviruses (WSMoV, GBNV, WBNV, CCSV, TZSV, TNRV and MYSV), and shared 43.3-45.0% identity with those of Europe and the Middle East tospoviruses (IYSV, TYRV and PolRSV), and shared 29.3-32.7% identity with those of America tospoviruses (MeSMV, INSV, ZLCV, CSNV, TSWV, ANSV, GRSV and TCSV) (Table 17). Similarly, comparison of NSs protein of CaCV-NRA revealed extremely high homology with CaCV-Ch-Pan (96.8% identity) and shared high homology with CaCV-AIT and CaCV-CP (87.9 and 90.0% identity, respectively) (Table 17). The amino acid sequence of the NSs protein shared 49.0-81.1% identity with those of CCSV, TZSV, GBNV, WSMoV, TNRV, MYSV, IYSV, TYRV and PolRSV and shared 13.2-19.8% with those of other tospoviruses (Table 17).

The phylogenetic tree derived from amino acid sequences of the N protein of tospoviruses grouped the viruses into four clusters (Figure 25). CaCV-NRA was in the same cluster with the Asian tospovirus group (CaCV isolates, GBNV, WSMoV, CCSV, WBNV, TZSV, TNRV and MYSV). Moreover, CaCV isolates were grouped into three clades. In the first clade, CaCV-NRA, CaCV-Ch-Pan, CaCV-Ph and CaCV-Aus clustered together. CaCV-CP and CaCV-TwTom1 clustered a second cluster. CaCV-TD8 and CaCV-AIT clustered the third cluster. Phylogenetic analysis of NSs protein showed that CaCV-NRA clusters with CaCV isolates in Asian tospoviruses cluster (Figure 26a).

Table 17 Untranslated regions (5'-UTR, IR and 3'-UTR) of S RNA of Capsicum chlorosis virus isolate NRA (CaCV-NRA) and sequence identities of NSs and N protein compared with those of other tospoviruses.

Virus	length	5'-UTR	IR	3'-UTR	NSs			N		
	nt	nt	nt	nt	nt	%nt	%aa	nt	%nt	%aa
CaCV-NRA	3,612	66	1,331	67	1,320			828		
CaCV-Ch-Pan	3,105	66	824	67	1,320	98.0	96.8	828	95.4	97.1
CaCV-Aus	-	-	-	-	-	-	-	828	96.7	98.9
CaCV-Ph	-	-	-	-	-	-	-	828	98.4	98.5
CaCV-AIT	3,477	66	1,196	67	1,320	82.8	87.9	828	85.1	92.7
CaCV-TD8	-	-	-	-	-	-	-	828	84.8	92.0
CaCV-CP	3,399	66	1,119	66	1,320	83.3	90.0	828	85.7	92.7
CaCV-TwTom1	-	-	-	-	-	-	-	828	85.6	93.8
WSMoV	3,534	66	1,255	65	1,320	77.7	81.1	828	76.9	85.1
GBNV	3,057	66	773	67	1,320	76.6	80.6	831	78.7	84.0
WBNV	-	-	-	-	-	-	-	828	78.0	81.1
CCSV	3,172	66	825	64	1,383	64.2	63.8	834	65.3	63.5
TZSV	3,279	64	934	64	1,380	65.5	62.9	837	63.9	61.8
TNRV	3,023	65	690	66	1,356	57.3	50.9	846	61.8	58.2
MYSV	3,232	68	847	67	1,410	59.7	49.0	840	62.3	59.3

Table 17 (continued)

Virus	length	5'-UTR	IR	3'-UTR	NSs			N		
	nt	nt	nt	nt	nt	%nt	%aa	nt	%nt	%aa
IYSV	3,105	70	811	70	1,332	56.2	52.2	822	55.9	45.0
PolRSV	2,484	72	183	72	1,332	56.0	50.3	825	55.5	43.3
TYRV	3,006	71	706	72	1,332	56.9	52.6	825	55.7	44.4
MeSMV	-	-	-	-	1,369	40.9	19.7	789	44.7	32.7
TSWV	3,047	88	625	153	1,404	40.3	19.8	777	45.4	32.7
TCSV	-	-	-	-	-	-	-	777	45.8	31.5
ANSV	-	-	-	-	-	-	-	777	45.8	30.3
GRSV	3,049	87	630	151	1,404	39.2	18.8	777	47.6	32.3
INSV	2,992	62	642	149	1,350	39.4	19.8	789	42.9	30.6
ZLCV	-	-	-	-	-	-	-	783	46.9	30.1
CSNV	-	-	-	-	-	-	-	783	46.0	29.3
PYSV	2,970	57	653	76	1,443	38.6	13.2	741	42.8	22.5
PCFSV	2,833	67	455	79	1,419	38.6	16.1	813	41.0	22.6

3.7 Sequence analysis of M RNA of Capsicum chlorosis virus isolate NRA (CaCV-NRA)

Complete nucleotide sequence of M RNA segment of CaCV-NRA from peanut was determined (Figure 24). The M RNA was 4,820 nucleotides in length and comprised of two ORFs in an ambisense arrangement. The first ORF on the viral strand was 927 nt in length, coding for the NSm protein (308 aa) with a total predicted molecular mass of 34 kDa. The second ORF on the viral complementary strand was 3,366 nt in length, coding for the GnGc glycoprotein (1,121 aa) with a total predicted molecular mass of 128 kDa. The 5' and 3' untranslated regions (UTR) were 57 and 47 nt long. The intergenic region (IR) between two ORFs was 423 nt long (Figure 24).

Sequence identities of M RNA segment of CaCV-NRA as compared with those of other tospoviruses were summarized in Table 18. Amino acid sequence of GnGc glycoprotein of CaCV-NRA revealed extremely high homology with CaCV-Ch-Pan isolated from pepper in India (96.2% identity) and shared high homology with CaCV-AIT isolated from tomato in Thailand (91.3% identity) (Table 18). The GnGc glycoprotein revealed 60.9-87.9% identity with those of Asian (GBNV, WSMoV, WBNV, TZSV, TNRV and MYSV) and European-Middle Eastern tosposviruses (PolRSV and IYSV) and shared 34.5-36.2 % identity with those of American tospoviruses (TSWV, TCSV, GRSV, INSV, ZLCV and CSNV) (Table 18). Amino acid sequence of NSm protein of CaCV-NRA revealed extremely high homology with CaCV-Ch-Pan (98.7% identity) and shared high homology with CaCV-AIT (91.2% identity). The NSm protein of CaCV-NRA shared 63.9-88.6% sequence identity with those of Asian (GBNV, WSMoV, WBNV, TZSV, TNRV and MYSV) and Eropean-Middle Eastern tosposviruses (PolRSV and IYSV) and shared 35.5-38.6% with those of other American tospoviruses (TSWV, TCSV, GRSV, INSV, ZLCV and CSNV) (Table 18). Phylogenetic analysis of the amino acid sequences of the GnGc glycoprotein and NSm protein revealed clustering of CaCV-NRA with CaCV-Ch-Pan, CaCV-AIT, GBNV, WBNV and WSMoV (Figure 26b and 26c, respectively).

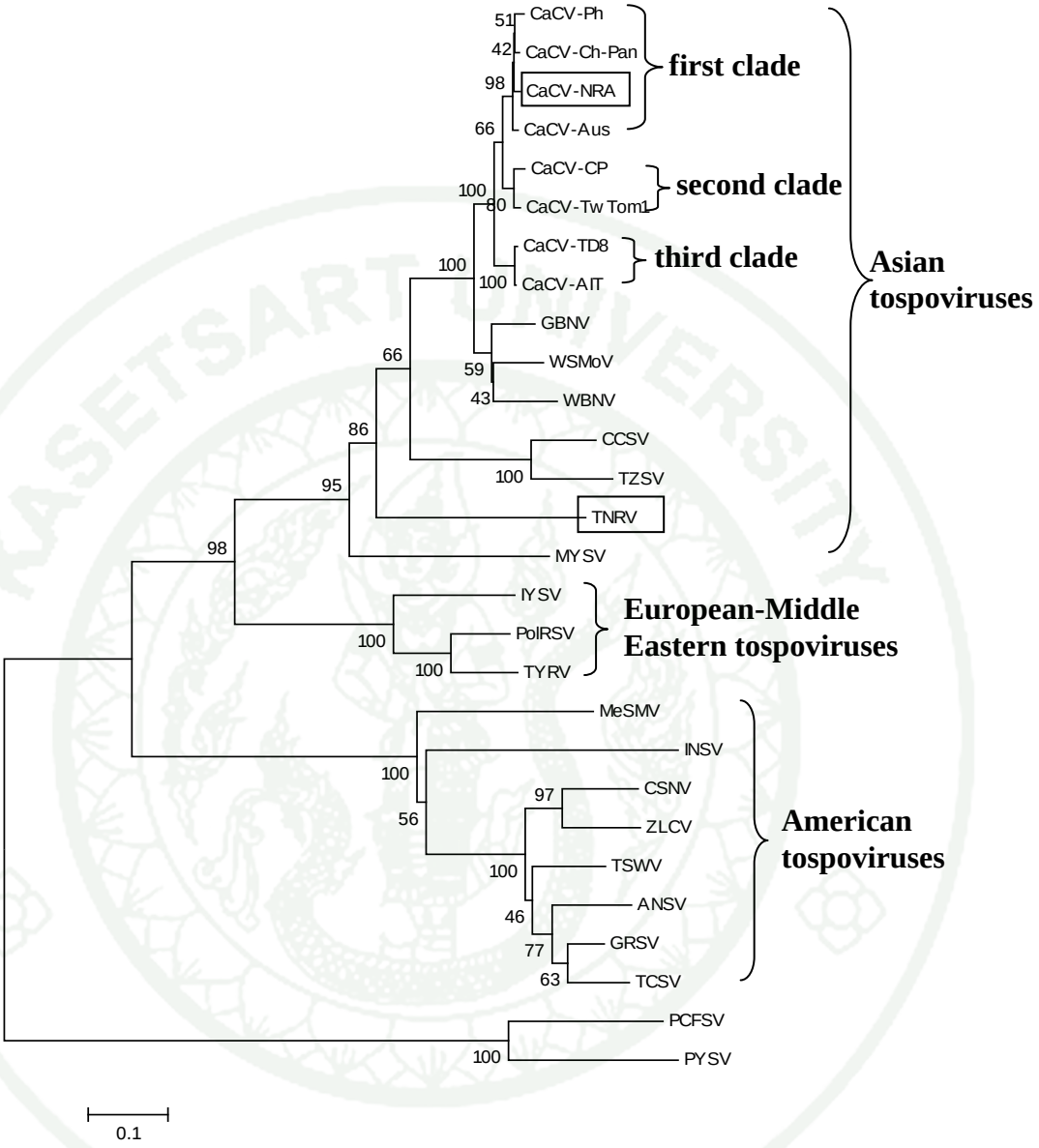

Figure 25 Phylogenetic analysis of CaCV isolates compared with other tospovirus species based on amino acid sequence of N protein. The trees were constructed by MEGA 4.0 program using the neighbour-joining method options with 500 bootstrap replications.

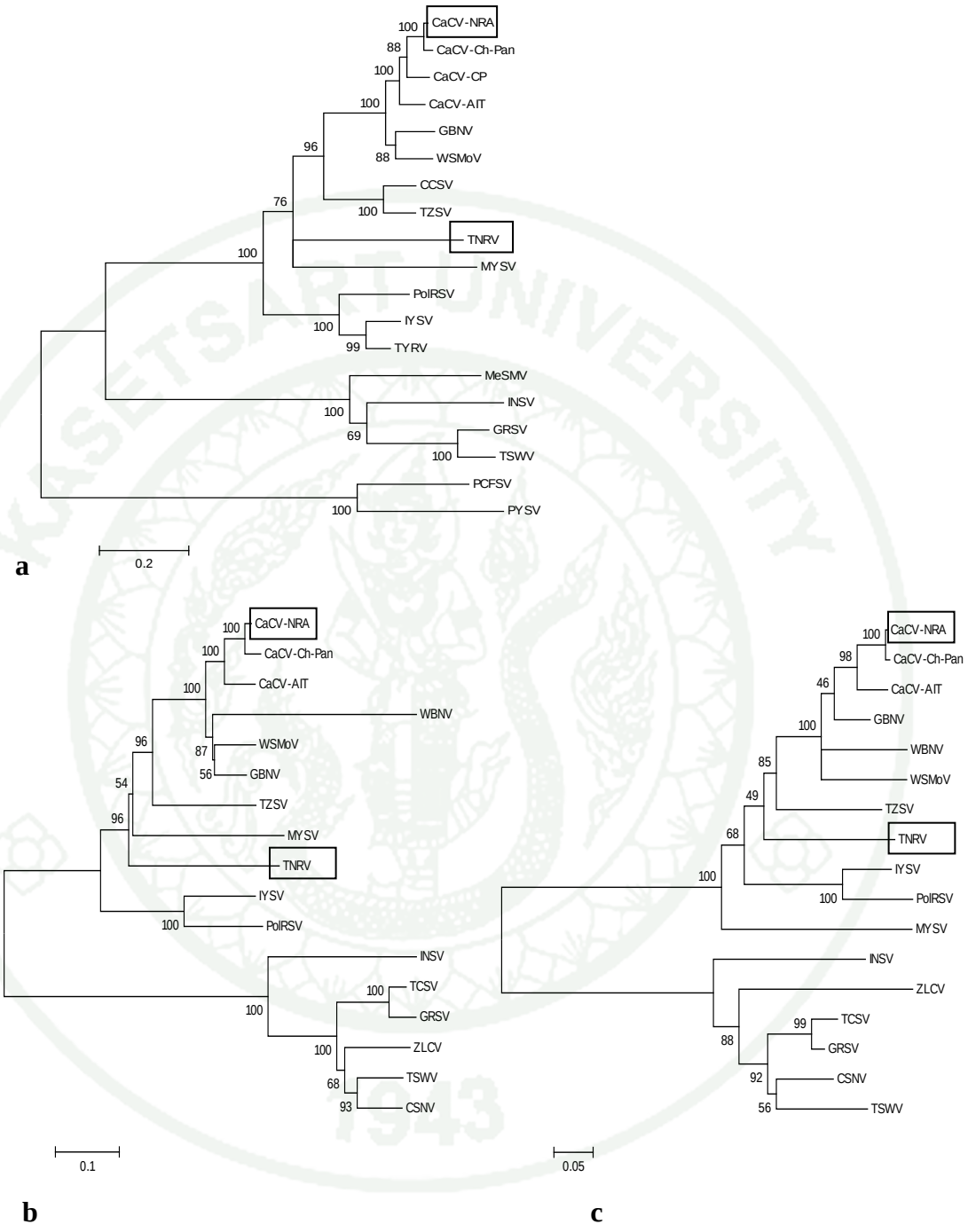

Figure 26 Phylogenetic analysis of CaCV isolates and other tospovirus species based on amino acid sequence of NSs protein (a), GnGc glycoprotein (b) and NSm protein (c). The trees were constructed by MEGA 4.0 program using the neighbour-joining method options with 500 bootstrap replications.

Table 18 Untranslated regions (5'-UTR, IR and 3'-UTR) of M RNA of Capsicum chlorosis virus isolate NRA (CaCV-NRA) and sequence identities of NSm and GnGc glycoprotein compared with those of other tospoviruses.

Virus	Length	5'-UTR	IR	3'-UTR		NSm			GnGc	
	nt	nt	nt	nt	nt	%nt	%aa	nt	%nt	%aa
CaCV-NRA	4,820	57	423	47	927			3,366		
CaCV-Ch-Pan	4,821	56	425	47	927	98.5	98.7	3,366	96.7	96.2
CaCV-AIT	4,823	56	427	47	927	84.5	91.2	3,366	83.2	91.3
WSMoV	4,880	55	473	47	939	78.8	82.1	3,366	78.5	85.1
GBNV	4,801	56	408	47	924	81.2	88.6	3,366	79.8	87.9
WBNV	4,795	55	412	44	921	78.5	82.4	3,363	75.1	67.4
TZSV	4,945	54	546	46	930	72.0	75.6	3,369	70.7	72.5
MYSV	4,815	58	398	48	927	65.6	63.9	3,384	66.0	64.9
TNRV	4,716	59	307	48	933	71.1	72.0	3,369	66.9	65.3
IYSV	4,838	63	379	49	936	68.2	69.0	3,411	64.0	61.5
PolRSV	-	62	267	-	924	65.7	65.9	3,408	64.5	60.9
TSWV	4,821	100	320	84	909	48.7	37.5	3,408	50.2	36.0
TCSV	-	-	-	-	912	50.9	37.3	3,405	50.1	35.9
GRSV	-	-	-	-	912	52.2	38.0	3,417	49.8	35.3
INSV	4,972	85	473	169	912	49.6	37.3	3,333	50.0	34.5
ZLCV	-	-	-	-	912	49.8	35.5	3,408	49.0	35.4
CSNV	-	-	-	-	912	52.1	38.6	3,408	49.9	36.2

3.8 Vector capability of *T. palmi* and *C. claratris* in transmitting Capsicum chlorosis virus isolate NRA (CaCV-NRA) from peanut

Two thrips species, *T. palmi* and *C. claratris*, which are known as vectors of tospoviruses, were studied for their capability to transmit CaCV-NRA using a leaf disc assay combined with ELISA. This capacity was tested with adults that were given an acquisition access period of 48 h on CaCV-NRA-infected plants as newborn larvae, up to 4 h old. An adult was individually placed on healthy *P. minima* leaf disc for 48 h. Four days after inoculation, chlorotic spot symptoms were found on the infected leaf discs. Leaf discs were tested for tospovirus infection using PTA-ELISA with general antibodies to tospoviruses (PAb MYSV6) (Appendix Table 5 and 6). *T. palmi* transmitted CaCV-NRA with an efficiency of 37 % while *C. claratris* transmitted CaCV-NRA with an efficiency of 70% (Table 19). This result suggested that CaCV-NRA could be experimentally transmitted by *T. palmi* and *C. claratris.*

Table 19 Transmission efficiency of Capsicum chlorosis virus isolate NRA (CaCV-NRA) by adults of *T. palmi* and *C. claratris* determined by leaf discs assay.

Thrip	Number of tested thrips	Transmission efficiency (%)		
		Male	Female	Total
T. palmi	145	46% (23 of 49)	32% (31 of 96)	37% (54 of 145)
C. Claratris	99	60% (21 of 35)	76% (49 of 64)	70% (70 of 99)

DISCUSSION

1. Detection and identification of tospoviruses in infected plant samples

In this thesis, the occurrence of tospoviruses in several crop plants in different regions of Thailand has been investigated during 2006-2009. Samples of peanut, tomato, pepper and watermelon showing typical symptoms of tospovirus were collected from many fields in seven provinces. The viruses were characterized based on serology (using general antibodies to tospoviruses) and nucleotide sequence of N protein gene. One isolate of Melon yellow spot virus (MYSV-WS1) was isolated from watermelon in Suphan Buri province. One isolate of *Watermelon silver mottle virus* (WSMoV-WS2) was isolated from watermelon in Suphan Buri province. Two isolates of Capsicum chlorosis virus (CaCV-NRA and CaCV-KK) were identified from peanut in Nakhorn Rachasrima and Khon Kaen province, respectively. Moreover, a new tospovirus species, named Tomato necrotic ringspot virus (TNRV-TT1), isolated from tomato was also identified in this study. TNRV was also detected in naturally infected pepper plants (TNRV-PP1) as confirmed by PTA-ELISA and nucleotide sequence analysis of the N protein gene. TNRV isolated from tomato was selected for comprehensive characterization and proposed as a new member of *Tospovirus* genus. Moreover, Capsicum chlorosis virus isolated from peanut (CaCV-NRA) was also fully characterized and compared with TNRV. CaCV is an established member of tospovirus and frequently observed on tomato, pepper and peanut in Thailand.

The present study combined with earlier reports (Wongkaew, 1993; Gajanandana *et al*., 2006; Chiemsombat *et al*., 2008) clearly revealed the predominance of four distinct tospovirus species in Thailand (WSMoV, CaCV, MYSV and TNRV). It is possible that unidentified new tospoviruses and various strains of previously identified tospovirus species may emerge as a potential threat to crop production in Thailand. The occurrence of tospoviruses in other agricultural crops such as onion, potato and ornamental crops such as orchids has not been studied. Moreover, the economical impact of tospoviral diseases in Thailand needs to be evaluated.

2. Characterization of Tomato necrotic ringspot virus, a new tospovirus isolated in Thailand

In this study, we isolated and identified a new tospovirus, namely Tomato necrotic ringspot virus (TNRV), from naturally infected tomato plants in Thailand. TNRV infected tomato showed symptoms consisting of necrotic spots, necrotic ringspots, stem necrosis, leaf necrosis and necrotic spots on tomato fruit. Several approaches were performed for characterization of this new tospovirus, including host range assay, determination of the serological relationship of the N protein, genome sequence analysis and testing of thrips transmission.

So far, three tospoviruses have been previously reported in Thailand, including CaCV, WSMoV and MYSV. CaCV was found to infect peanut, tomato and pepper (Chiemsombat *et al*., 2008). WSMoV and MYSV infected several plants of the families *Solanaceae* and *Cucurbitaceae*, such as tomato, pepper, cucumber, watermelon, cantaloupe and loofah (Gajanandana *et al*., 2006; Chiemsombat *et al*., 2008). In this study, it was shown that TNRV has different host range characteristics when compared with those three tospoviruses. TNRV was able to infect peanut (*Leguminosae*) and many members of the family *Solanaceae* but failed to infect three species of the family *Cucurbitaceae*. Unlike WSMoV and MYSV, which could systemically infect several cucurbit species, TNRV failed to mechanically infect three cucurbit species including *C. sativus*, *L. acutangula* and *C. lanatus*. Moreover, we have observed that symptoms induced by TNRV were different from those of CaCV. For instance, TNRV induced necrotic rings and necrotic ringspots on *N. glutinosa*, while chlorotic and necrotic spots were observed in CaCV infection.

For the serology approach, many antibodies to N proteins of various tospoviruses were used to characterize TNRV by ELISA and Western blot analysis. PAb MYSV6 and MAb 2B2, which could detect CaCV, WSMoV and MYSV, also reacted positively with TNRV. The virus was also detected by PAb A3, which could react with CaCV and WSMoV but not MYSV. However, TNRV was not detected by MAbs 2D6 and L4E8, which could detect CaCV and WSMoV, and also MAb 5E7,

which could specifically react with MYSV. Results suggested that TNRV was serologically related but not identical to WSMoV and CaCV found in Thailand. Monoclonal antibodies are known to be a powerful tool for identification of biomolecules. In general, we were able to generate MAbs that could bind to common epitopes as well as MAbs that could distinguish distinct epitopes. Considering the different degrees of similarity among the N proteins of different tospoviruses, it is not surprising to obtain various antibodies with different epitope-binding characteristics. Amino acid sequences of N proteins of TNRV shared 58.2%, 56.0% and 51.8% identity with those of CaCV, WSMoV and MYSV, respectively. A highly conserved region among these four tospoviruses was detected by MAb 2B2 and PAb MYSV6. PAb A3 detected different epitopes found on CaCV, WSMoV and TNRV but not MYSV. TNRV was not recognized by the other three MAbs (MAbs 2D6, L4E8 and 5E7). MAbs 2D6 and L4E8 should bind to other epitopes that are shared by CaCV and WSMoV but not presented on TNRV, whereas MAb 5E7 reacted with a distinct epitope on MYSV. Results show that all of the antibodies that reacted with CaCV also detected WSMoV, since both tospoviruses share 85.5% amino acid sequence identity in the N protein. Chen *et al*. (2010) also reported that WSMoV and CaCV were indistinguishable using their currently available MAbs. Similar to this study, they also found that a rabbit antiserum prepared against the N proteins of WSMoV or MYSV could recognize common epitopes present on MYSV, WSMoV, CaCV, GBNV, WBNV and CCSV, while specific MAbs to N proteins of WSMoV or MYSV were able to distinguish between these two tospoviruses (Chen *et al*., 2010).

TNRV has a virion morphology and a genome organization of S and M RNAs similar to those of other viruses in the genus *Tospovirus*. Amino acid sequence of the N protein of TNRV shared similarity of only 18.4-58.2% with those of other tospoviruses. Since the N protein similarity of TNRV with those of other tospoviruses was below the threshold level (80% amino acid identity) for distinguishing tospovirus species (Fauquet *et al*., 2005; Jones, 2005; Tsompana *et al*., 2008), we concluded that TNRV should be considered a member of a distinct species. Moreover, sequence analysis of proteins from N, NSm and GnGc of TNRV revealed that they were most similar to those of CaCV (58.2%, 71.7% and 65.6% identity, respectively).

Phylogenetic analysis of N, NSs, GnGc and NSm proteins also showed that TNRV clustered with Asian tospoviruses (CaCV, WSMoV, GBNV, WBNV, CCSV, TZSV and MYSV). These studies also suggested that TNRV was more closely related to Asian tospoviruses than other tospoviruses.

Transmission experiments revealed that TNRV can be transmitted by *T. palmi* and *C. claratris*. Both thrips species were found abundantly in agriculture fields in Thailand. *T. palmi* transmitted TNRV with an efficiency of 83%, while *C. claratris* transmitted TNRV with an efficiency of 12%. However, *C. claratris* has been reported to transmit CaCV-AIT at an efficiency rate of 70% (Premachandra *et al*., 2005). Transmission studies also confirmed species difference between TNRV and CaCV. *T. palmi* was reported to be a vector of Asian tospoviruses such as WSMoV, CaCV, WBNV, CCSV, GBNV and MYSV (Chen and Chiu, 1996; Kato and Hanada, 2000; Lakshmi et al., 1995; Palmer et al., 1990; Persley et al., 2006; Yeh et al., 1992). This high transmission competence of *T. palmi* suggested that it may contribute to the wide spread of TNRV in Thailand. With an abundance of this virus in the central, western and northern regions of Thailand, where most of tomatoes and peppers are grown, TNRV could be a serious threat to vegetable production in this country.

3. Characterization of Capsicum chlorosis virus isolate NRA (CaCV-NRA) from peanut

In this study, the tospovirus infected peanut from Nakhorn Rachasrima province was described as an isolate of CaCV, designated CaCV-NRA. CaCV-NRA showed symptoms on peanut consisting of chlorotic spot, necrotic spot, necrotic ringspot and bud necrosis. CaCV-NRA was characterized as a distinct isolate of CaCV based on serology, host range, the complete genomic sequence of S and M RNA segments and thrips vector.

For the serology approach, CaCV-NRA could be reacted with PAb MYSV6, PAb A3, MAb 2B2, MAb 2D6 and MAb L4E8 but not with MAb 5E7. Serological analysis revealed that CaCV-NRA is closely related to WSMoV. Moreover, CaCV-NRA is serologically related but not identical to those of TNRV found in Thailand. Comparative host ranges analysis revealed that there are biological differences between two CaCV isolates found in Thailand. Although both CaCV-NRA isolated from peanut and CaCV-AIT isolated from tomato in Thailand systemically infected *Capsicum annum*, *Solanum lycopersicum*, *Nicotiana benthamiana, Nicotiana glutinosa* and *Nicotiana tabacum*, the symptom expressions revealed some clear differences. Moreover, the host range of CaCV-NRA was different from those of CaCV-Aus isolated from pepper in Australia (Persley *et al*., 2006) and CaCV-Ph isolated from *Phalaenopsis* orchid in Taiwan (Zheng *et al*., 2007). CaCV-NRA systemically infected *Arachis hypogea*, *S. lycopersicum*, and *N. glutinosa* while CaCV-Aus did not systemically infect these three plant species (Persley *et al*., 2006). CaCV-Ph systemically infected *S. lycopersicum* but not *N. glutinosa* (Zheng *et al*., 2007).

Comparison of S and M RNAs of CaCV isolates from different counties was performed to understand variation in gene and intergenic regions (IR). Sequence analysis of N proteins of CaCV isolates showed that the N protein of CaCV-NRA revealed extremely high homology with CaCV-Ch-Pan isolated from pepper in India (Kunkalikar *et al*., 2010), CaCV-Aus isolated from pepper in Australia (McMichael *et*

al., 2002) and CaCV-Ph isolated from *Phalaenopsis* orchid in Taiwan (Zheng *et al*., 2011) at 97.1-98.9% identity. The N protein of CaCV-NRA shared high homology with CaCV-CP isolated from peanut in China (Chen *et al*., 2007), CaCV-TwTom1 isolated from tomato in Taiwan, CaCV-AIT isolated from tomato in Thailand (Knierim *et al*., 2006) and CaCV-TD8 isolated from tomato in Thailand (Chiemsombat *et al*., 2008) at 92.0-93.8% identity. Phylogenetic analysis of N protein also showed that CaCV isolates were grouped into three clades. In the first clades, CaCV-NRA, CaCV-Ch-Pan, CaCV-Ph and CaCV-Aus clustered together. CaCV-CP and CaCV-TwTom1 clustered a second cluster. CaCV-TD8 and CaCV-AIT clustered the third cluster.

Further comparison between CaCV-NRA isolated from peanut and CaCV-AIT isolated from tomato revealed significant molecular divergence. Amino acid sequence of N protein of CaCV-NRA shared 92.7% identity with those of CaCV-AIT. Similarly, two other tospovirus species (IYSV and TYRV) differ on the same extent in their N protein sequence. For instance, IYSV isolates from Brazil (IYSV-BR; Pozzer *etal*. 1999) and the Netherlands (IYSV-NL; Cortês *et al*., 1998) shared 91 % amino acid sequence identity in their N protein (Pappu *et al*., 2006; Pozzer *et al*., 1999). Also for two TYRV strains from Iran, the N protein of TYRV-t from tomato and TYRV-s from soybean shared 92 % amino acid sequence identity in their N protein (Hassani-Mehraban *et al*., 2007). Further comparative analysis of the S RNA sequences between CaCV-NRA and CaCV-AIT revealed clear differences in the intergenic region (IR). The IR of CaCV-NRA was 1,331 nt long while the IR of CaCV-AIT was 1,196 nt long. The length of IR varied greatly and shared lowest sequence identity among tospovirus species (Hassani-Mehraban *et al*., 2007). Moreover, amino acid sequence of GnGc and NSm proteins of CaCV-NRA revealed extremely high homology with CaCV-Ch-Pan (96.2% and 98.7% identity, respectively) and shared high homology with CaCV-AIT (91.3% and 91.2% identity, respectively).

Due to the presence of *T. palmi* and *C. claratris* in several crop plants in Thailand, these two thrips species were tested as vector of CaCV-NRA. Several transmission experiments have been performed by leaf disc assay combined with PTA-ELISA. *T. palmi* transmitted CaCV-NRA with an efficiency of 37 % while *C. claratris* transmitted CaCV-NRA with an efficiency of 70%. This result indicates that both *T. palmi* and *C. claratris* represent as efficient vector of CaCV-NRA. *C. claratris* has been reported to transmit CaCV-AIT at an efficiency rate of 70% (Premachandra *et al*., 2005). *T. palmi* has been reported to transmit CaCV-Aus from Australia (Persley *et al*., 2006). However, it has been shown that thrips species transmitting CaCV isolates in Thailand and Taiwan are different. CaCV-NRA from peanut in Thailand could be transmitted by *T. palmi*, however, CaCV-Ph from *Phalaenopsis* orchid in Taiwan could not be transmitted by this thrips species (Zheng *et al*., 2007).

In conclusion, data indicated that CaCV-NRA from peanut showed clear differences in biological characters, specifically host range and symptomatology, and the molecular divergence. This result suggested that CaCV-NRA isolated from peanut and CaCV-AIT isolated from tomato in Thailand may be considered as different isolates of CaCV.

CONCLUSION

1. A new tospovirus species, Tomato necrotic ringspot virus (TNRV), was isolated from tomato in Thailand. Infected plants showed symptoms consisting of necrotic spots, necrotic ringspots and stem necrosis. TNRV systemically infected *Solanum lycopersicum*, *Capsicum annum*, *Arachis hypogea*, *Datura stramonium*, *Nicotiana benthamiana*, *N. glutinosa*, *N. tabacum* and *Physalis minima.* TNRV was serologically related but not identical to WSMoV and CaCV found in Thailand. The complete nucleotide sequences of S and M RNAs of TNRV were 3,023 and 4,716 nucleotides in length, respectively, and contained two ORFs in an ambisense arrangement. Sequence analysis indicated that amino acid sequence of the N protein shared 58.2%, 56.0% and 51.8% identity with those of CaCV, WSMoV and MYSV, respectively. The virus was experimentally transmitted by *Thrips palmi* and *Ceratothripoides claratris* with an efficiency of 83 % and 12%, respectively.

2. The tospovirus infected peanut from Nakhorn Rachasrima province was described as an isolate of CaCV, designated CaCV-NRA. CaCV-NRA showed symptoms on peanut consisting of chlorotic spots, necrotic spots, necrotic ringspots and bud necrosis. CaCV-NRA systemically infected *Arachis hypogea*, *Capsicum annum, Datura stramonium, Solanum lycopersicum, Nicotiana benthamiana, Nicotiana glutinosa, Nicotiana tabacum* and *Physalis minima*. Serological analysis revealed that CaCV-NRA is closely related to WSMoV. The complete nucleotide sequence of S and M RNAs of CaCV-NRA were determined to be 3,612 and 4,820 nucleotides, respectively. Amino acid sequence of the N protein of CaCV-NRA shared 92.7%, 85.1%, 58.2% and 59.3% with those of CaCV-AIT, WSMoV, TNRV and MYSV, respectively. CaCV-NRA was experimentally transmitted by *T. palmi* and *C. claratris* with an efficiency of 37 % and 70%, respectively.

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APPENDIX

Appendix Table 1 Detection of Tomato necrotic ringspot virus (TNRV) in inoculated plant samples using plate-trapped antigen ELISA (PTA-ELISA) with general antibodies to tospoviruses (PAb MYSV6).

Inoculated plant	O.D. $_{405}$	Inoculated plant	O.D. $_{405}$
C. quinoa 1	0.329	*A. hypogea* 8	0.146
C. quinoa 2	0.430	*A. hypogea* 9	0.134
C. quinoa 3	0.689	*A. hypogea* 10	0.142
C. quinoa 4	0.386	*S. lycopersicum* 1	1.139
C. quinoa 5	0.812	*S. lycopersicum* 2	0.330
C. quinoa 6	0.871	*S. lycopersicum* 3	1.771
C. quinoa 7	0.292	*S. lycopersicum* 4	1.186
C. quinoa 8	0.479	*S. lycopersicum* 5	1.365
C. quinoa 9	0.172	*S. lycopersicum* 6	1.582
C. quinoa 10	0.154	*S. lycopersicum* 7	0.709
C. amaranticolor 1	0.522	*S. lycopersicum* 8	0.144
C. amaranticolor 2	0.530	*S. lycopersicum* 9	0.171
C. amaranticolor 3	0.417	*S. lycopersicum* 10	0.129
C. amaranticolor 4	0.912	*N. benthamiana* 1	1.669
C. amaranticolor 5	0.417	*N. benthamiana* 2	0.111
C. amaranticolor 6	0.817	*N. benthamiana* 3	0.106
C. amaranticolor 7	0.423	*N. benthamiana* 4	1.140
C. amaranticolor 8	0.313	*N. benthamiana* 5	0.540
C. amaranticolor 9	0.129	*N. benthamiana* 6	1.032
C. amaranticolor 10	0.150	*N. benthamiana* 7	0.806
A. hypogea 1	0.771	*N. benthamiana* 8	0.119
A. hypogea 2	1.409	*N. benthamiana* 9	1.031
A. hypogea 3	1.598	*N. benthamiana* 10	0.331
A. hypogea 4	0.153	*N. glutinosa* 1	1.592
A. hypogea 5	1.402	*N. glutinosa* 2	0.650
A. hypogea 6	1.245	*N. glutinosa* 3	0.159
A. hypogea 7	1.157	*N. glutinosa* 4	1.558

Appendix Table 1 (Continued)

Inoculated plant	O.D. $_{405}$	Inoculated plant	O.D. $_{405}$
N. glutinosa 5	1.596	*D. stramonium* 12	0.091
N. glutinosa 6	1.688	*D. stramonium* 13	0.120
N. glutinosa 7	0.384	*N. tabacum* 1	1.255
N. glutinosa 8	1.862	*N. tabacum* 2	1.174
N. glutinosa 9	0.131	*N. tabacum* 3	0.979
N. glutinosa 10	0.139	*N. tabacum* 4	1.759
C. annum 1	1.535	*N. tabacum* 5	0.860
C. annum 2	0.686	*N. tabacum* 6	0.131
C. annum 3	1.695	*N. tabacum* 7	0.121
C. annum 4	0.999	*N. tabacum* 8	2.101
C. annum 5	1.464	*N. tabacum* 9	0.479
C. annum 6	0.731	*N. tabacum* 10	1.148
C. annum 7	1.197	*P. minima* 1	1.833
C. annum 8	1.386	*P. minima* 2	1.785
C. annum 9	0.356	*P. minima* 3	0.160
C. annum 10	0.123	*P. minima* 4	0.353
C. annum 11	0.122	*P. minima* 5	1.589
C. annum 12	0.107	*P. minima* 6	1.593
D. stramonium 1	1.612	*P. minima* 7	1.560
D. stramonium 2	1.066	*P. minima* 8	1.741
D. stramonium 3	0.855	*P. minima* 9	2.407
D. stramonium 4	1.154	*P. minima* 10	1.725
D. stramonium 5	0.887	*P. minima* 11	1.425
D. stramonium 6	0.997	*P. minima* 12	1.989
D. stramonium 7	0.184	*V. unguiculata* 1	0.540
D. stramonium 8	0.149	*V. unguiculata* 2	0.712
D. stramonium 9	1.032	*V. unguiculata* 3	0.967
D. stramonium 10	0.806	*V. unguiculata* 4	0.123
D. stramonium 11	0.123	*V. unguiculata* 5	0.134

Appendix Table 2 Detection of Capsicum chlorosis virus isolate NRA (CaCV-NRA) in inoculated plant samples using plate-trapped antigen ELISA (PTA-ELISA) with general antibodies to tospoviruses (PAb MYSV6).

Inoculated plants	O.D. $_{405}$	Inoculated plants	O.D. $_{405}$
C. quinoa 1	0.611	*A. hypogea* 8	0.104
C. quinoa 2	0.212	*A. hypogea* 9	0.104
C. quinoa 3	1.034	*A. hypogea* 10	0.104
C. quinoa 4	0.216	*V. unguiculata* 1	0.867
C. quinoa 5	0.900	*V. unguiculata* 2	1.034
C. quinoa 6	0.145	*V. unguiculata* 3	1.184
C. quinoa 7	0.599	*V. unguiculata* 4	0.106
C. quinoa 8	0.722	*V. unguiculata* 5	0.097
C. quinoa 9	0.314	*C. annum* 1	2.616
C. quinoa 10	0.121	*C. annum* 2	2.693
C. amaranticolor 1	0.386	*C. annum* 3	2.662
C. amaranticolor 2	0.375	*C. annum* 4	2.558
C. amaranticolor 3	0.392	*C. annum* 5	2.318
C. amaranticolor 4	0.381	*C. annum* 6	2.469
C. amaranticolor 5	0.361	*C. annum* 7	1.144
C. amaranticolor 6	0.896	*C. annum* 8	1.101
C. amaranticolor 7	0.578	*C. annum* 9	0.096
C. amaranticolor 8	0.107	*C. annum* 10	0.104
C. amaranticolor 9	0.115	*D. stramonium* 1	2.956
C. amaranticolor 10	0.127	*D. stramonium* 2	2.831
A. hypogea 1	1.144	*D. stramonium* 3	2.765
A. hypogea 2	1.047	*D. stramonium* 4	2.752
A. hypogea 3	1.153	*D. stramonium* 5	2.790
A. hypogea 4	1.170	*D. stramonium* 6	2.812
A. hypogea 5	1.089	*D. stramonium* 7	0.128
A. hypogea 6	1.262	*D. stramonium* 8	0.122
A. hypogea 7	0.118	*D. stramonium* 9	1.101

Appendix Table 2 (Continued)

Inoculated plants	O.D. $_{405}$	Inoculated plants	O.D. $_{405}$
D. stramonium 10	1.276	*N. glutinosa* 6	2.716
D. stramonium 11	0.152	*N. glutinosa* 7	0.227
D. stramonium 12	0.123	*N. glutinosa* 8	2.867
S. lycopersicum 1	1.702	*N. glutinosa* 9	0.226
S. lycopersicum 2	2.159	*N. glutinosa* 10	0.221
S. lycopersicum 3	2.173	*N. tabacum* 1	2.562
S. lycopersicum 4	2.591	*N. tabacum* 2	2.677
S. lycopersicum 5	0.200	*N. tabacum* 3	1.016
S. lycopersicum 6	0.209	*N. tabacum* 4	1.187
S. lycopersicum 7	0.090	*N. tabacum* 5	1.356
S. lycopersicum 8	0.934	*N. tabacum* 6	1.264
S. lycopersicum 9	0.949	*N. tabacum* 7	1.556
S. lycopersicum 10	1.304	*N. tabacum* 8	1.234
N. benthamiana 1	1.422	*N. tabacum* 9	0.124
N. benthamiana 2	1.531	*N. tabacum* 10	0.115
N. benthamiana 3	1.294	*P. minima* 1	2.526
N. benthamiana 4	1.440	*P. minima* 2	2.718
N. benthamiana 5	1.607	*P. minima* 3	2.824
N. benthamiana 6	1.456	*P. minima* 4	2.400
N. benthamiana 7	1.683	*P. minima* 5	1.363
N. benthamiana 8	0.111	*P. minima* 6	1.912
N. benthamiana 9	0.179	*P. minima* 7	1.448
N. benthamiana 10	0.136	*P. minima* 8	1.458
N. glutinosa 1	1.483	*P. minima* 9	1.728
N. glutinosa 2	0.658	*P. minima* 10	1.587
N. glutinosa 3	0.206	*P. minima* 11	1.529
N. glutinosa 4	0.157	*P. minima* 12	1.339
N. glutinosa 5	0.139		

Appendix Table 3 Detection of Tomato necrotic ringspot virus (TNRV) in *Thrips palmi*-inoculated leaf discs using PTA-ELISA with general antibodies to tospoviruses (PAb MYSV6). Each leaf disc was given an inoculation access period (IAP) of 48 h by adult *T. palmi* that was given an acquisition access period (AAP) of 48 h on TNRV infected peanut as first stage larvae.

thrips	sex	O.D. $_{405}$			thrips	sex	O.D. $_{405}$		
		IAP1	IAP2	IAP3			IAP1	IAP2	IAP3
TP1	female	0.352	2.593	1.235	TP25	female	0.205	0.272	0.244
TP2	male	1.214	2.354	0.255	TP26	male	1.493	2.532	1.456
TP3	female	1.003	2.548	1.526	TP27	male	0.317	2.748	0.992
TP4	female	0.950	2.582	1.507	TP28	female	0.202	2.883	1.356
TP5	female	1.928	2.815	1.560	TP29	male	0.485	2.839	1.511
TP6	female	0.628	2.068	1.489	TP30	female	0.142	0.813	1.557
TP7	male	0.149	0.211	0.336	TP31	female	0.909	2.830	1.513
TP8	male	0.115	0.108	-	TP32	male	1.435	2.847	1.480
TP9	male	1.131	2.224	1.544	TP33	male	1.508	2.533	1.470
TP10	female	0.892	2.471	1.437	TP34	male	1.942	2.740	1.539
TP11	male	1.267	2.374	1.511	TP35	female	0.171	2.479	1.403
TP12	male	1.327	2.094	1.444	TP36	female	0.224	2.563	1.571
TP13	female	0.108	0.103	-	TP37	female	0.135	2.557	1.446
TP14	male	1.265	2.505	1.450	TP38	male	1.414	2.769	1.520
TP15	female	0.120	0.203	0.303	TP39	male	1.401	1.978	1.461
TP16	male	1.443	2.469	1.449	TP40	female	0.114	0.264	0.290
TP17	male	0.685	2.864	1.447	TP41	male	1.231	-	-
TP18	male	1.719	2.768	1.517	TP42	male	1.263	2.395	1.188
TP19	male	0.166	0.218	0.271	TP43	male	1.475	2.494	1.418
TP20	female	1.286	2.240	1.444	TP44	male	0.643	2.248	1.418
TP21	male	1.574	2.597	1.525	TP45	female	1.551	2.407	1.469
TP22	male	0.778	0.103	-	TP46	male	1.498	2.778	1.472
TP23	female	0.552	0.095	-	TP47	female	0.357	2.553	1.557
TP24	female	0.781	-	-	TP48	female	1.091	2.535	1.550

Appendix Table 3 (Continued)

thrips	sex	O.D. 405			thrips	sex	O.D. 405		
		IAP1	IAP2	IAP3			IAP1	IAP2	IAP3
TP49	female	0.241	2.674	1.346	TP74	male	1.733	2.580	1.514
TP50	male	1.268	2.586	1.412	TP75	female	1.547	-	-
TP51	female	1.410	2.586	1.434	TP76	male	2.033	2.492	1.513
TP52	female	0.108	-	-	TP77	male	1.638	2.487	1.315
TP53	female	1.021	2.588	1.349	TP78	female	1.959	2.490	1.310
TP54	female	1.135	2.710	1.365	TP79	female	0.994	2.483	0.772
TP55	male	0.946	-	-	TP80	male	0.144	0.228	0.247
TP56	male	1.004	2.611	1.477	TP81	female	0.756	0.107	-
TP57	female	1.250	2.573	1.481	TP82	male	1.864	2.702	0.255
TP58	female	1.399	2.457	0.845	TP83	female	1.935	2.750	1.363
TP59	male	1.152	2.544	1.438	TP84	female	2.056	2.697	1.454
TP60	female	1.396	2.552	1.450	TP85	female	1.643	0.104	-
TP61	female	0.166	0.221	0.276	TP86	female	0.871	0.110	-
TP62	female	0.731	2.515	1.349	TP87	male	1.814	2.800	1.495
TP63	male	1.302	2.942	1.360	TP88	female	0.196	0.250	0.225
TP64	male	1.223	2.664	1.412	TP89	male	1.193	2.918	1.470
TP65	female	1.085	2.588	1.420	TP90	female	0.172	0.248	-
TP66	female	0.637	1.463	0.288	TP91	female	0.155	0.587	0.273
TP67	male	1.922	2.630	1.416	TP92	female	0.143	0.143	-
TP68	female	0.181	0.193	0.266	TP93	female	0.211	2.717	1.433
TP69	female	1.328	1.412	0.295	TP94	female	1.923	2.710	1.266
TP70	male	0.170	0.108	-	TP95	male	1.541	2.788	1.280
TP71	male	1.719	2.723	1.380	TP96	male	0.371	2.741	1.078
TP72	female	0.167	0.204	0.339	TP97	female	1.260	0.889	-
TP73	male	1.548	2.318	1.431					

Appendix Table 4 Detection of Tomato necrotic ringspot virus (TNRV) in *Ceratothripoides claratris*-inoculated leaf discs using PTA-ELISA with general antibodies to tospoviruses (PAb MYSV6). Each leaf disc was given an inoculation access period (IAP) of 48 h by adult *C. claratris* that was given an acquisition access period (AAP) of 48 h on TNRV infected tomato as first stage larvae.

thrips	sex	O.D. $_{405}$			thrips	sex	O.D. $_{405}$		
		IAP1	IAP2	IAP3			IAP1	IAP2	IAP3
CC1	female	0.097	0.091	0.119	CC25	female	0.102	0.115	0.114
CC2	female	0.100	0.109	0.109	CC26	female	0.106	0.104	0.262
CC3	female	0.145	0.100	0.111	CC27	female	0.112	0.112	0.256
CC4	male	0.100	0.106	0.123	CC28	male	0.140	0.149	0.126
CC5	male	0.109	0.102	0.111	CC29	female	0.126	0.144	0.144
CC6	female	0.118	0.117	0.107	CC30	female	0.103	0.142	-
CC7	male	0.114	0.105	0.111	CC31	female	0.196	0.146	0.249
CC8	male	0.117	0.117	0.109	CC32	male	0.108	0.115	0.111
CC9	female	0.119	0.109	0.112	CC33	male	0.141	0.134	0.177
CC10	male	0.105	0.097	0.117	CC34	male	0.103	0.101	-
CC11	male	0.140	0.088	0.103	CC35	female	0.095	0.097	0.219
CC12	male	0.105	0.095	0.144	CC36	male	0.101	0.096	0.105
CC13	female	0.106	0.102	0.116	CC37	male	0.100	0.099	0.116
CC14	male	0.108	0.094	0.174	CC38	female	0.129	0.120	0.855
CC15	female	0.112	0.120	0.190	CC39	male	0.109	0.094	0.235
CC16	male	0.122	0.107	0.120	CC40	female	0.107	0.106	0.318
CC17	female	0.104	0.100	0.152	CC41	female	0.101	0.097	0.315
CC18	female	0.114	0.102	0.190	CC42	female	0.112	0.098	0.156
CC19	male	0.121	0.082	1.434	CC43	female	0.107	0.105	0.334
CC20	female	0.108	0.090	0.140	CC44	female	0.101	0.103	0.238
CC21	male	0.102	0.090	0.824	CC45	female	0.126	0.120	0.709
CC22	female	0.100	0.101	-	CC46	female	0.148	0.109	0.214
CC23	male	0.100	0.108	0.111	CC47	male	0.105	0.097	0.163
CC24	female	0.099	0.093	0.203	CC48	male	0.115	0.103	0.216

Appendix Table 4 (Continued)

thrips	sex	O.D. 405			thrips	sex	O.D. 405		
		IAP1	IAP2	IAP3			IAP1	IAP2	IAP3
CC49	female	0.106	0.101	1.228	CC63	female	0.108	0.132	2.304
CC50	male	0.111	0.103	-	CC64	female	0.101	0.116	1.174
CC51	female	0.142	0.093	0.285	CC65	female	0.098	0.094	0.260
CC52	female	0.123	0.096	1.690	CC66	female	0.109	0.136	0.276
CC53	male	0.116	0.099	0.166	CC67	female	0.129	0.135	-
CC54	female	0.115	0.102	0.125	CC68	female	0.108	0.121	-
CC55	male	0.095	0.092	0.122	CC69	male	0.099	0.103	0.279
CC56	female	0.098	0.098	0.136	CC70	female	0.124	0.115	-
CC57	male	0.099	0.137	0.876	CC71	male	0.119	0.112	0.101
CC58	male	0.105	0.160	-	CC72	female	0.237	0.127	0.295
CC59	female	0.114	0.125	-	CC73	female	0.122	0.129	0.154
CC60	female	0.110	0.094	0.277	CC74	female	0.107	0.123	0.188
CC61	male	0.096	0.105	-	CC75	male	0.149	0.121	0.139
CC62	female	0.104	0.107	0.281					

Appendix Table 5 Detection of Capsicum chlorosis virus isolate NRA (CaCV-NRA) in *Thrips palmi*-inoculated leaf discs using PTA-ELISA with general antibodies to tospoviruses (PAb MYSV6). Each leaf disc was given an inoculation access period (IAP) of 48 h by adult *T. palmi* that was given an acquisition access period (AAP) of 48 h on CaCV-NRA infected peanut as first stage larvae.

thrips	sex	O.D. $_{405}$			thrips	sex	O.D. $_{405}$		
		IAP1	IAP2	IAP3			IAP1	IAP2	IAP3
TP1	female	0.137	0.101	0.100	TP25	female	0.140	0.098	-
TP2	female	0.168	0.101	0.103	TP26	female	0.157	0.093	0.090
TP3	male	0.158	0.098	0.097	TP27	male	0.151	1.448	0.977
TP4	female	0.150	0.101	0.096	TP28	male	0.155	1.458	1.378
TP5	male	0.150	0.104	0.099	TP29	male	0.152	0.097	0.099
TP6	female	0.146	0.102	0.098	TP30	male	0.149	1.388	1.424
TP7	female	0.133	0.097	0.100	TP31	female	0.143	0.090	0.100
TP8	female	0.143	0.933	1.288	TP32	female	1.751	0.089	-
TP9	male	0.144	0.099	0.096	TP33	female	0.133	0.098	0.093
TP10	female	0.165	1.749	-	TP34	female	0.182	0.100	0.100
TP11	female	0.152	0.095	-	TP35	female	0.151	0.093	0.094
TP12	male	2.166	1.728	0.102	TP36	female	0.151	0.091	0.094
TP13	female	0.908	1.587	0.713	TP37	male	0.150	1.456	1.355
TP14	female	0.138	0.089	-	TP38	female	0.147	0.103	-
TP15	female	1.894	1.529	0.091	TP39	female	0.146	0.103	-
TP16	female	1.994	1.339	1.119	TP40	male	0.146	1.190	1.312
TP17	female	0.132	0.102	0.105	TP41	female	0.143	0.095	0.101
TP18	male	0.161	0.101	0.105	TP42	male	1.902	0.935	0.096
TP19	female	0.157	1.557	1.430	TP43	female	1.675	0.095	-
TP20	male	0.920	1.662	0.110	TP44	female	0.143	0.091	0.109
TP21	female	0.146	0.092	0.096	TP45	female	0.370	1.493	0.090
TP22	female	0.143	0.096	0.098	TP46	female	0.149	0.100	0.093
TP23	male	2.138	0.855	0.111	TP47	female	0.143		-
TP24	female	0.142	0.095	-	TP48	female	0.153	1.515	1.249

Appendix Table 5 (Continued)

thrips	sex	O.D. 405			thrips	sex	O.D. 405		
		IAP1	IAP2	IAP3			IAP1	IAP2	IAP3
TP49	female	0.140	0.097	0.102	TP79	female	0.138	0.090	0.099
TP50	female	2.031	0.100	0.704	TP80	female	0.137	0.089	0.096
TP51	female	0.150	0.094	0.098	TP81	female	0.163	-	-
TP52	female	0.149	0.098	0.100	TP82	female	0.166	1.444	1.322
TP53	male	0.151	0.099	0.097	TP83	female	0.170	1.492	1.344
TP54	female	0.145	1.449	1.130	TP84	female	0.194	0.099	0.095
TP55	female	0.152	0.096	0.104	TP85	female	0.147	1.440	1.365
TP56	male	0.152	0.092	0.104	TP86	male	0.146	0.087	0.106
TP57	female	0.146	0.095	0.100	TP87	male	0.149	1.294	1.021
TP58	female	0.168	0.093	0.110	TP88	male	0.148	0.092	0.101
TP59	female	0.173	0.094	0.100	TP89	female	0.155	1.607	0.113
TP60	female	0.159	0.097	0.108	TP90	female	0.156	0.092	-
TP61	female	0.139	0.095	0.097	TP91	female	2.166	1.683	0.095
TP62	female	0.130	0.096	1.180	TP92	male	0.143	0.091	0.099
TP63	female	0.154	0.097	0.101	TP93	male	0.129	0.090	0.110
TP64	female	0.143	0.102	0.101	TP94	female	1.604	1.332	0.102
TP65	female	0.153	0.096	-	TP95	male	0.136	0.112	0.104
TP66	female	0.151	0.099	-	TP96	female	0.163	0.103	0.109
TP67	female	0.147	0.092	0.101	TP97	male	2.245	1.594	1.425
TP68	female	1.226	1.456	0.520	TP98	female	2.374	0.101	0.106
TP69	female	0.159	0.096	0.105	TP99	male	2.260	1.512	0.103
TP70	male	0.130	0.098	0.099	TP100	female	0.147	-	-
TP71	female	0.140	1.623	1.483	TP101	male	0.133	0.097	0.098
TP72	female	0.134	0.095	0.093	TP102	female	0.154	0.101	-
TP73	male	1.449	1.483	1.482	TP103	male	0.150	0.107	0.102
TP74	female	0.173	0.090	0.092	TP104	male	0.156	0.099	0.097
TP75	male	1.649	1.356	0.251	TP105	female	0.152	0.127	-
TP76	male	0.150	1.264	1.256	TP106	female	0.160	0.102	-
TP77	male	1.942	1.556	0.853	TP107	female	0.149	0.088	0.106
TP78	female	0.132	0.095	0.095	TP108	female	0.147	0.093	0.093

Appendix Table 5 (Continued)

thrips	sex	O.D. 405			thrips	sex	O.D. 405		
		IAP1	IAP2	IAP3			IAP1	IAP2	IAP3
TP109	female	0.133	1.200	-	TP128	male	0.141	0.107	0.101
TP110	female	0.160	0.098	-	TP129	female	0.146	0.095	-
TP111	female	0.134	0.103	-	TP130	female	0.145	1.204	-
TP112	male	0.155	0.092	0.090	TP131	male	0.163	0.092	0.102
TP113	female	2.229	1.773	1.240	TP132	female	0.145	0.098	1.471
TP114	male	2.107	0.754	0.105	TP133	male	0.136	0.085	0.097
TP115	male	1.311	0.095	0.104	TP134	female	1.408	0.088	0.106
TP116	male	1.817	1.460	0.518	TP135	female	0.133	0.086	0.096
TP117	female	0.136	0.113	0.098	TP136	male	0.153	0.095	0.096
TP118	female	0.139	0.091	0.100	TP137	male	0.141	0.091	0.101
TP119	female	0.135	0.095	0.101	TP138	female	0.168	1.314	1.184
TP120	male	0.151	0.100	0.089	TP139	female	0.129	0.090	0.097
TP121	male	0.142	0.101	0.104	TP140	female	1.665	1.428	1.227
TP122	male	0.141	0.088	-	TP141	female	0.128	0.085	0.098
TP123	male	0.138	0.099	0.092	TP142	male	0.204	1.648	1.584
TP124	male	0.133	1.633	1.295	TP143	female	0.128	0.091	0.089
TP125	male	1.311	1.466	1.138	TP144	female	0.141	0.090	0.094
TP126	female	2.103	1.154	0.105	TP145	male	1.285	1.211	0.732
TP127	female	0.132	0.351	0.098					

Appendix Table 6 Detection of Capsicum chlorosis virus isolate NRA (CaCV-NRA) in *Ceratothripoides claratris*-inoculated leaf discs using PTA-ELISA with general antibodies to tospoviruses (PAb MYSV6). Each leaf disc was given an inoculation access period (IAP) of 48 h by adult *C. claratris* that was given an acquisition access period (AAP) of 48 h on CaCV-NRA infected tomato as first stage larvae.

thrips	sex	O.D. $_{405}$			thrips	sex	O.D. $_{405}$		
		IAP1	IAP2	IAP3			IAP1	IAP2	IAP3
CC1	female	0.324	1.670	0.312	CC24	female	1.124	0.080	0.272
CC2	female	0.125	0.106	0.113	CC25	male	0.121	0.096	1.122
CC3	male	1.325	1.812	0.188	CC26	female	0.105	0.135	0.263
CC4	female	1.391	1.663	0.168	CC27	male	1.813	0.105	1.014
CC5	female	0.501	1.587	0.157	CC28	male	1.724	1.390	0.454
CC6	female	0.142	1.900	0.547	CC29	female	1.439	0.108	0.329
CC7	male	0.111	0.098	0.117	CC30	female	0.909	0.544	0.245
CC8	female	0.855	0.803	0.680	CC31	female	1.318	0.239	0.902
CC9	male	0.304	0.130	0.117	CC32	female	1.104	0.812	0.281
CC10	male	0.098	0.111	0.108	CC33	male	0.098	0.101	0.107
CC11	male	0.108	0.100	1.464	CC34	female	0.102	0.109	0.360
CC12	male	0.116	0.108	0.638	CC35	female	1.673	0.124	0.311
CC13	male	0.105	0.419	0.761	CC36	female	0.105	0.155	1.261
CC14	male	0.304	1.187	1.124	CC37	female	0.105	0.095	0.195
CC15	female	0.106	0.099	0.102	CC38	male	1.228	1.331	0.134
CC16	female	0.129	0.107	-	CC39	female	0.106	0.127	0.130
CC17	female	0.115	0.797	1.635	CC40	female	1.204	0.100	0.113
CC18	female	1.598	0.921	0.110	CC41	female	0.089	1.799	1.521
CC19	female	0.114	1.123	0.143	CC42	female	0.180	1.810	0.605
CC20	male	1.332	0.917	0.430	CC43	female	0.137	1.935	0.118
CC21	male	0.116	-	-	CC44	female	0.163	0.092	0.108
CC22	female	0.106	0.151	1.421	CC45	male	0.107	0.097	0.150
CC23	female	0.551	1.136	0.860	CC46	female	0.119	2.099	0.516

Appendix Table 6 (Continued)

thrips	sex	O.D. 405			thrips	sex	O.D. 405		
		IAP1	IAP2	IAP3			IAP1	IAP2	IAP3
CC47	female	0.191	1.923	0.597	CC74	male	0.096	1.761	0.102
CC48	male	0.098	1.853	0.946	CC75	female	1.640	1.968	1.050
CC49	male	0.080	1.278	0.107	CC76	female	0.131	1.982	0.759
CC50	female	0.164	1.752	0.325	CC77	male	1.216	1.908	0.248
CC51	female	0.125	0.112	0.130	CC78	female	0.094	1.951	0.239
CC52	female	0.156	1.857	0.569	CC79	male	0.096	0.106	0.316
CC53	female	0.085	1.700	0.122	CC80	male	0.099	0.109	0.219
CC54	male	0.148	1.987	0.141	CC81	female	0.094	1.908	1.038
CC55	female	0.112	1.891	0.121	CC82	male	0.669	1.748	0.128
CC56	female	0.175	1.643	0.232	CC83	female	1.422	1.718	0.655
CC57	male	0.160	1.892	0.120	CC84	female	0.107	1.972	0.281
CC58	female	0.125	1.779	1.980	CC85	male	0.093	0.114	0.114
CC59	male	0.095	1.713	0.188	CC86	female	0.381	1.678	0.111
CC60	female	0.086	1.704	0.130	CC87	female	0.845	1.907	0.340
CC61	female	0.172	1.973	1.035	CC88	male	1.318	2.092	0.241
CC62	female	0.086	0.187	1.417	CC89	female	0.880	1.741	1.687
CC63	female	0.099	0.176	0.258	CC90	male	0.100	0.098	0.111
CC64	female	0.110	0.110	0.387	CC91	female	1.165	1.714	0.208
CC65	female	0.125	0.954	0.232	CC92	male	1.402	1.896	0.165
CC66	female	0.152	0.159	0.169	CC93	male	0.650	1.904	1.276
CC67	female	0.142	0.110	0.306	CC94	male	0.115	0.105	0.120
CC68	female	0.118	0.100	1.603	CC95	female	0.922	1.934	0.204
CC69	female	1.886	1.799	0.211	CC96	female	0.119	1.877	1.235
CC70	female	0.521	1.039	-	CC97	male	0.101	0.112	0.269
CC71	female	0.106	0.722	0.204	CC98	female	0.132	1.965	0.754
CC72	female	0.108	1.849	0.408	CC99	male	0.127	1.275	0.589
CC73	male	0.103	0.096	0.152					

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