Doctor of Philosophy (Agricultural Biotechnology)

DEGREE

Agricultural Biotechnology — **FIELD**

Interdisciplinary Graduate Program — **PROGRAM**

TITLE: Characterization, Genetic Variation and Diagnosis of *Sugarcane streak mosaic virus*, a New Poacevirus Infecting Sugarcane in Thailand

NAME: Miss Paweena Kasemsin

THIS THESIS HAS BEEN ACCEPTED BY

______________________ **THESIS ADVISOR**

(Assistant Professor Pissawan Chiemsombat, Dr.Agr.)

______________________ **THESIS CO-ADVISOR**

(Assistant Professor Ratchanee Hongprayoon, Ph.D.)

______________________ **GRADUATE COMMITTEE CHAIRMAN**

(Assistant Professor Sermsiri Chanprame, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON ______________________

______________________ **DEAN**

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

CHARACTERIZATION, GENETIC VARIATION AND DIAGNOSIS OF *Sugarcane streak mosaic virus*, A NEW POACEVIRUS INFECTING SUGARCANE IN THAILAND

PAWEENA KASEMSIN

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Agricultural Biotechnology)
Graduate School, Kasetsart University
2016

Paweena Kasemsin 2016: Characterization, Genetic Variation and Diagnosis of *Sugarcane streak mosaic virus*, a New Poacevirus Infecting Sugarcane in Thailand. Doctor of Philosophy (Agricultural Biotechnology), Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Assistant Professor Pissawan Chiemsombat, Dr.Agr. 87 pages.

Sugarcane leaves showing yellow streak mosaic symptoms were strikingly observed in farmers' fields in Kamphaeng Saen District, Nakhon Pathom Province, Thailand during disease surveys conducted in 2010. Diagnosis of symptomatic leaf samples by RT-PCR for *Sugarcane mosaic potyvirus* failed, but it revealed the presence of *Sugarcane streak mosaic virus* (SCSMV). In this study, SCSMV-infected sugarcane, designated as THA-NP3 isolate, was subjected to RNA extraction, followed by RT-PCR-based viral gene cloning and sequencing. The complete genome sequence of the isolate THA-NP3 contained 9,781 nucleotides, which encoded for a polyprotein of 3,130 amino acid residues. Protein sequence analysis indicated nine putative cleavage sites that yielded ten functional proteins namely P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb and CP, and an additional frameshifted PIPO protein. Analysis by multiple sequence alignment revealed that THA-NP3 shared 97.84% nucleotide identity with JP2 from China and 81.39-97.78% identities to other recorded SCSMV sequences. Electron microscopy of purified virions revealed them to be flexuous rod shaped, and with length of 700-890 nm, which is characteristic of viruses in family *Potyviridae*. Molecular weight of the coat protein subunits as estimated by SDS-PAGE was 31 kilodaltons. To develop virus diagnosis tools, rabbit polyclonal antisera against SCSMV were produced, and specific virus detection was achieved by using the direct antigen coating enzyme linked immunosorbent assay (DAC-ELISA). In addition, the immunochromatographic strip (ICS) was successfully created for rapid virus diagnosis from a diseased leaf sap within 5 min. Surveys for streak mosaic disease incidence were conducted in natural sugarcane fields from 2010 to 2014 in five provinces of major sugarcane growing areas in Thailand, including Nakhon Pathom, Kanchanaburi, Nakhon Ratchasima, Khon Kaen and Udon Thani, and in two germplasm collection fields. Virus infected percentages obtained from SCSMV-positively diagnosed by DAC-ELISA and RT-PCR were 43.48-90.91% and 54.17-100% in collected farmers and germplasm fields, respectively. Genetic diversity based on complete coat protein (CP) coding sequences of the collective 58 SCSMV isolates showed 86.17-100% nucleotide identities among Thai isolates, and 85.70-99.29% identities to isolates from other countries. Phylogenetic analysis of CP sequences indicated two major clusters of virus variants, one in cropping fields and another in germplasm fields. Genetic variations of SCSMV isolates were consistently indicated according to the potential recombination events detected in CP gene regions. These findings represent essential knowledge and should be utilized to improve the SCSMV resistance of sugarcane varieties.

____________________ ___________________________ ___ /___ /___

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

First of all, I would like to thank Asst. Prof. Dr. Pissawan Chiemsombat, my thesis advisor, and Asst. Prof. Dr. Ratchanee Hongprayoon, my thesis co-advisor, for their guidance and suggestions throughout my study.

I would also like to thank Assoc. Prof. Dr. Ratana Tongwongkit, Assoc. Prof. Dr. Prasert Chatwachirawong, and the Thailand Department of Agriculture for their provision of sugarcane germplasm collections. I would also like to thank Assoc. Prof. Dr. Sonthichai Chanprame and Dr. Nonglak Parinthawong for their comments and suggestions in this research.

I would also like to thank all of the members and graduate students at the Molecular Plant Pathology Laboratory, and Serology and Diagnostic Laboratory, Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus for their assistance in laboratory experiments, disease surveys and sample collections. Also, special thanks to Mr. Jack E. Scogin for his assistance in proofreading this thesis.

This project received financial support from the Center for Agricultural Biotechnology, Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education (AG-BIO/PERDO-CHE).

Finally, I would also like to extend my gratitude to my parents and my sisters for their support and encouragement.

Paweena Kasemsin

January 2016

TABLE OF CONTENTS

Page

TABLE OF CONTENTS	i
LIST OF TABLES	ii
LIST OF FIGURES	iv
LIST OF ABBREVIATIONS	vi
INTRODUCTION	1
OBJECTIVE	3
LITERATURE REVIEW	4
MATERIALS AND METHODS	19
RESULTS AND DISCUSSION	36
Results	36
Discussion	72
CONCLUSION AND RECOMMENDATIONS	76
Conclusion	76
Recommendations	78
LITERATURE CITED	79
CURRICULUM VITAE	87

LIST OF TABLES

Table **Page**

Table		Page
1	List of specific primers for amplification of *Sugarcane streak mosaic virus* full length genome and coat protein gene	29
2	Viruses in the family *Potyviridae* used for phylogenetic tree reconstruction in this study	33
3	Putative protease cleavage sites on the polyprotein of *Sugarcane streak mosaic virus* isolate THA-NP3	45
4	Identity percentages of complete genome sequence comparison between THA-NP3 and other SCSMV isolates, and potyviruses causing mosaic diseases in sugarcane	47
5	Enzyme-linked immunosorbent assay values (60 min reaction) from reciprocal of the cross absorbed IgG dilution against the infected plant when used to analyze 1:10 w/v the plant extracts	52
6	Enzyme-linked immunosorbent assay values (60 min reaction) from the reciprocal of sap dilutions	52
7	Detection of SCSMV by DAC-ELISA from sugarcane leaf samples obtained from collected farmers' fields in 5 provinces and germplasm collection fields	57
8	List of sugarcane varieties obtained from collected germplasm collection fields in Kanchanaburi (GKB), Nakhon Pathom (GNP) and Suphan Buri (GSB) provinces	58
9	The CP coding regions of Thai SCSMV isolates obtained from farmers' fields in 5 provinces	61
10	The CP coding regions of Thai SCSMV isolates obtained from germplasm collection fields	63

LIST OF TABLES (Continued)

Table **Page**

11 The CP coding regions of SCSMV isolates obtained from other countries 64

12 Recombination events detected in the CP coding region of SCSMV isolates using RDP4 (version 4.5) 70

13 Analysis of the recombination breakpoints in the CP coding region 71

LIST OF FIGURES

Figure **Page**

Figure		Page
1	Genome organization of the viruses in the family *Potyviridae*	6
2	The generally accepted model of RNA recombination presumes that RNA viruses recombine according to a copy-choice mechanism	18
3	Genome map of *Sugarcane streak mosaic virus* displaying positions of primers used for full length genome amplification	29
4	Yellow streak mosaic symptoms on sugarcane leaves observed at farmers' fields and germplasm collection fields	37
5	Symptoms of *Sugarcane streak mosaic virus* on the inoculated plants	38
6	Transmission electron micrograph of the purified virions of *Sugarcane streak mosaic virus*, isolate THA-NP3 from the infected sorghum leaves	40
7	SDS-Polyacrylamide gel electrophoresis of the purified virions of *Sugarcane streak mosaic virus*, isolate THA-NP3 from the infected sorghum leaves	41
8	Schematic representation of genome organization of *Sugarcane streak mosaic virus*, the isolate THA-NP3 (JN163911) and putative cleavage sites	44
9	Neighbor-joining phylogenetic tree reconstruction using the polyprotein amino acid sequences of the viruses within the family *Potyviridae*	48
10	The graph shows antiserum titer of the obtained antisera (AS1-AS7)	49
11	Enzyme-linked immunosorbent assay values (60 min reaction) of the cross-absorbed IgG at the dilution 1:200 against the positive samples of 12 other tested viruses	50
12	Detection of SCSMV by immunochromatographic strip (ICS)	54
13	Analysis of the coat protein (CP) in the infected plants and the healthy control by Western blotting	55

LIST OF FIGURES (Continued)

Figure **Page**

Figure		Page
14	The survey maps of streak mosaic disease incidence in major sugarcane growing areas in Central and Northeastern regions	59
15	Alignment of coat protein amino acid sequences of 85 isolates of *Sugarcane streak mosaic virus* (SCSMV) showing more variable residues at the positions 1-31	66
16	Maximum-likelihood tree based on the aligned CP nucleotide sequences of the 58 Thai SCSMV isolates and the 27 SCSMV isolates from other countries	67
17	Phylogenetic networks of SCSMV isolates based on analysis of the CP coding sequences	69
18	Prediction of the possible recombination in the CP coding region of the recombinant isolate GK76-4 by RDP4	70
19	Prediction of the possible recombination in the CP coding region of the recombinant isolate GROC7 by RDP4	71

LIST OF ABBREVIATIONS

aa	=	Amino acid
bp	=	Base pair
dNTP	=	Deoxyrionucleotide triphosphate
EDTA	=	Ethylene diamine tetraacetic acid
g	=	Gram
h	=	Hour
kb	=	Kilobase
kDa	=	Kilodalton
µg	=	Microgram
µl	=	Microlitre
LB	=	Luria bertani broth
M	=	Molar
mM	=	Millimolar
mg	=	Milligram
ml	=	Millilitre
ng	=	Nanogram
nt	=	Nucleotide
OD	=	Optical density
ORF	=	Open reading frame
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
RNA	=	Ribonucleic acid
RT-PCR	=	Reverse transcription-Polymerase chain reaction
rpm	=	Round per minute
SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel Electrophoresis
SCSMV	=	*Sugarcane streak mosaic virus*
SCMV	=	*Sugarcane mosaic virus*
SrMV	=	*Sorghum mosaic virus*

LIST OF ABBREVIATIONS (Continued)

JGMV	=	*Johnsongrass mosaic virus*
TBE	=	Tris borate EDTA
TEMED	=	N, N, N', N'-tetra methylethylene diamine
TriMV	=	*Triticum mosaic virus*
UV	=	Ultraviolet
V	=	Volts

CHARACTERIZATION, GENETIC VARIATION AND DIAGNOSIS OF *Sugarcane streak mosaic virus*, A NEW POACEVIRUS INFECTING SUGARCANE IN THAILAND

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a valuable crop for the sugar industry and is widely cultivated in Brazil, India, China, Thailand and Pakistan. In Thailand, it is largely cultivated in central, and northeastern areas such as Nakhon Sawan, Kanchanaburi, Nakon Ratchasima, Khon Kaen and Udon Thani. The major commercial sugarcane varieties in Thailand are LK92-11, K76-4, K84-200, K84-69, K88-87, K90-54, K92-60, UT1, UT3, UT8, SP50 and KK3. Sugarcane production in Thailand was affected by various biotic diseases. Major constraints of sugarcane production in Thailand are sugarcane white leaf disease caused by phytoplasma, sugarcane red rot disease caused by *Colletotrichum falcatum*, and mosaic disease caused by *Sugarcane mosaic virus* (SCMV). During surveys conducted from 2002-2004, the diagnosis of symptomatic sugarcane plants showing mosaic, yellow streak, chlorotic or necrotic streak, chlorotic mild mottle or mild mosaic revealed the presence of SCMV in many sugarcane production fields (Gemechu *et al*., 2004). In 2005, a distinct virus species, namely *Sugarcane streak mosaic virus* (SCSMV) was detected in sugarcane and sorghum plants which showed typical mosaic symptoms as those caused by SCMV but failed to react with the antisera against SCMV, SrMV and JGMV (Chatenet *et al*., 2005). SCSMV had been reported for its devastating effects in Asia, including India (Viswanathan *et al*., 2008), Pakistan, Sri Lanka, Vietnam, Thailand (Chatenet *et al*., 2005), China (Li *et al*., 2011) and Indonesia (Damayanti and Putra, 2011). The natural hosts of SCSMV are sugarcane, sorghum and Egypian crowfoot grass (*Dactylactonium aegypticum*) (Hema *et al*. 1999; Srinivas *et al*. 2010).

The virion of SCSMV is flexuous rod shaped, size of 890 ×15 nm, comprising of a positive sense single stranded RNA molecule of 9.8 kb characteristic of viruses in family *Potyviridae* (Hema *et al*., 1999). The viral genome contains a single open

reading frame (ORF) which encodes for a polyprotein of 3130 amino acid residues. The polyprotein is processed by protease cleavages to yield ten different proteins, namely P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb and CP. The first published complete genome sequence of the SCSMV which was isolated from sugarcane in Pakistan, SCSMV-PAK (GQ388116), contained 9782 nucleotides (nt), excluding 3′ Poly (A) tail (Xu *et al*., 2010). Sequence comparison and a phylogenetic tree of viral complete genome revealed that SCSMV was a distinct group from those of other members of the existing genera in the family *Potyviridae*, and has been recently named *Poacevirus* (Xu *et al*., 2010). More isolates of SCSMV and their complete genome sequences were reported from China, (Li *et al*., 2011) and India (Parameswari *et al*., 2013). Moreover, the study on genetic variability of SCSMV had been by using the analysis of CP (Viswanathan *et al*., 2008; He *et al*., 2013), P1 (He *et al*., 2013) and HC-Pro (Bagyalakshimi *et al*., 2012) coding regions. Genetic variations had been studied and results revealed evidences on genetic recombination among isolates of the virus.

In this study, sugarcane leaves showing yellow streak mosaic symptom in farmers' fields were diagnosed, and the causal virus was identified. A full length genome of Thai isolate of SCSMV was amplified and its nucleotide sequence was determined for the first time. Disease surveys were conducted, samples were collected for viral gene amplification, and genetic variations among Thai SCSMV isolates were analyzed based on coat protein (CP) coding region. Purified SCSMV virions were used for production of rabbit polyclonal antiserum. The diagnosis by using immunological methods including direct antigen coating enzyme linked immunosorbent assay (DAC-ELISA) and immunochromatographic strip (ICS) were successfully developed.

OBJECTIVES

1. To study the genome sequence and organization of the SCSMV isolate in Thailand.

2. To study the genetic variation of SCSMV isolates in Thailand based on coat protein gene analysis.

3. To produce polyclonal antiserum against SCSMV and develop immunological methods for virus detection.

LITERATURE REVIEW

1. Sugarcane planting in Thailand

Sugarcane (*Saccharum officinarum* L.) has a long period of vegetative growth and storage tissue is adapted for sucrose accumulation. It has been widely cultivated in tropical countries such as Brazil, India, China, Pakistan and Thailand. In addition, sugarcane biomass can be used for cellulose and bioethanol products. In Thailand, the major sugarcane planting areas are in northern, central and northeastern areas (Jaisil, 2014). The most cultivated sugarcane varieties as raw materials for the sugar industry include K84-200, K88-92, K95-84, LK92-11, UT84-13, K-2000-89, KK3 and UT3 (Jaisil, 2014). There are many sugarcane varieties which had been imported from abroad for using in the breeding program, such as Phil6607, Q130Triton, F154, ROC1, Q67, CO1148, CO62175, Phil63-17 and Phil58-260 (Office of The Cane and Sugar Board: www.ocsb.go.th).

The studies of genetic diversity and relationships among the commercial hybrid sugarcane varieties in Thailand revealed that they were derived from only 26 exotic varieties with higher closely relationship to OCSB3 (37.3%) than UT1 (20.4%). Among these hybrids, *S. officinarum* was the major genetic contributor (25.1%), whereas *S. barberi* (10.8%), *S. spontaneum* (5.9%) and *S. sinense* (2.4%) were minor ones. These results suggested that Thai improved sugarcane varieties have narrow genetic base (Thongpaiyai *et al*., 2012).

2. Viruses in the family *Potyviridae*

Plant virus species belong to family *Potyviridae* have one or two positive sense single stranded RNA molecules. Virus particles are flexuous rod shaped virion size of 11-15×650-900 nm long with a helical pitch of *ca*. 3.4 nm (Adam *et al*., 2005). The nucleocapsid was composed of approximate 2000 units of a single structural protein, surrounding one RNA molecule.

There are eight genera in this family which have been readily recognized, namely *Brambyvirus*, *Potyvirus*, *Macluravirus*, *Ipomovirus*, *Tritimovirus*, *Rymovirus*, *Bymovirus and* the newly established genus, *Poacevirus* (Xu *et al*., 2010)

The monopartite genome has been found in members of genera *Brambyvirus*, *Potyvirus*, *Macluravirus*, *Ipomovirus*, *Tritimovirus*, *Poacevirus* and *Rymovirus*. The bipartite genomes with RNA1 and RNA2 have been found in genus *Bymovirus* (Adam *et al*., 2005). The RNA genome is translated via a long ORF to obtain a polyprotein and subsequently cleavage into ten putative proteins by the viral proteinase, namely nuclear inclusion-a peptidase (NIa), helper component proteinase (HC-Pro) and P1 peptidase (Figure 1). Ten putative proteins are P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb and CP (Figure 1).

The viruses in family *Potyviridae* were transmitted by aphids (genera *Potyvirus* and *Macluravirus*), eriophyid mites (genera *Rymovirus, Tritimovirus* and *Poacevirus*), whiteflies (genus *Ipomovirus*) and plasmodiophorid fungi (genus *Bymovirus*) (Adam *et al*., 2005).

Replication of the RNA genome is started from uncoating which exposes nucleic acid as mRNA for translation. Translation produces structural and non-structural proteins such as the viral proteinases (P1, HC-Pro, NIa), RNA dependent RNA polymerase (RdRp) for genome replication and the CP for encapsidation. The viral genome is replicated using the negative RNA strand as a template yielding the positive RNA strand which is catalyzed by RdRp. Finally, the progeny genomic strands are encapsidated with the coat protein: CP (Adam *et al*., 2005).

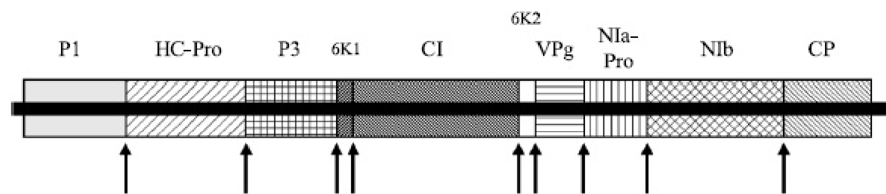

Figure 1 Genome organization of the viruses in the family *Potyviridae.*
The upper diagram shows 10 mature proteins produced by proteolytic cleavage sites of polyprotein (arrows) in most members of the family.

Source: Adam *et al*. (2005)

3. Genus *Poacevirus*

The genus *Poacevirus* is the newly established genus in the family *Potyviridae*. Viral genome is a positive sense single stranded RNA. The name, *Poacevirus* is related to the host plant generic name. The plants include wheat (*Triticum aestivum* L.), sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*) and Egypian grass (*Dactylactonium aegypticum*) (Tatineni *et al*., 2009). At present, there are two virus species in this genus, namely *Triticum mosaic virus* (TriMV) and *Sugarcane streak mosaic virus* (SCSMV). The type species of this genus is TriMV, which caused disease on wheat firstly reported in Kansas, and was transmitted by wheat curl mite (Seifers and Martin, 2008). Diseased plants showed light green or yellow streaking, spotting or mottling which were similar to those of wheat infected with WSMV (Seifers *et al*., 2009).

Sequence alignments of the CP gene have been demonstrated that TriMV has 45.9% identity to SCSMV strain AP (SCSMV-AP), but it shares only 23.2% identity to WSMV. TriMV is closely related to SCSMV and both are placed in the newly established genus *Poacevirus* (Fellers *et al*., 2009). Phylogenetic analysis of the polyprotein, NIa-Pro, NIb and CP sequences among the representative species of six genera and unassigned members of the family *Potyviridae* suggested that TriMV and

SCSMV are sister taxa and share a most recent common ancestor with tritimoviruses or ipomoviruses (Tatineni *et al*., 2009).

4. *Sugarcane streak mosaic virus* (SCSMV)

SCSMV is a distinct virus species with flexuous rod particle of 890 × 15 nm in the newly established genus, *Poacevirus* in the family *Potyviridae* (Hema *et al*., 1999). It was detected in sugarcane which imported from Pakistan to the USA and identified as SCMV strain F (SCMV-F) based on symptom expression on indicator hosts (Gillaspie *et al*., 1978). Jensen and Hall (1993) reported that there were no serological relationships between S CSMV and potyviruses such as SCMV-A, MDMV-A, (JGMV), SrMV, and other viruses in the genus *Rymovirus* such as *Hordeum mosaic virus* (HoMV) and *Agropyron mosaic virus* (AgMV). These data suggested that the Pakistani isolate was actually a distinct species. Hall *et al*. (1998) demonstrated that this virus isolate was most closely related to the viruses in genus *Tritimovirus*, namely *Wheat streak mosaic virus* (WSMV) and *Brome streak mosaic virus* (BrSMV).

Another isolate of SCSMV was found in sorghum showing mosaic symptoms in Andhra Pradesh state of India (Srinivas *et al*., 2010). This isolate is serologically related to SCSMV and *Sorghum mosaic parbhani virus* (SPMV). Sequence alignment of this isolate showed 95% nucleotide identity to the isolate of Andhra Pradesh (SCSMV-AP), and SCSMV-Coimbatore, and the CP gene showed 97.8% identity to the SCSMV-AP.

In 2010, the complete nucleotide sequence and genome organization of the Pakistani isolate (SCSMV-PAK) was reported by Xu *et al*. (2010). The complete genome of SCSMV-PAK composes of 9782 nucleotides, excluding 3′ Poly (A) tail. The genome contains an ORF encoding for the polyprotein of 3130 amino acid residues. The deduced polyprotein is cleaved at nine putative protease sites by three viral proteases (P1, NIa, HC-Pro) to obtain ten functional proteins, according to the virus in the family *Potyviridae* (Hema *et al*., 2003). The overall base composition of

SCSMV-PAK genome is 34.4% adenine, 20.7% cytosine, 22.5% guanine and 25.4% uracil, similar to that of other potyviruses (Xu *et al*., 2010). The 5′UTR (199 nts) has a 48% GC content and forms a stable secondary structure with a free energy of -49 kcal/mol, which is different from that of most potyviruses. The 3′UTR (191 nts) has a stable secondary structure with a free energy of -48 kcal/mol (Xu *et al*., 2010).

The genome organization of SCSMV-PAK is identical to the viruses in genera *Ipomovirus*, *Potyvirus*, *Rymovirus* and *Tritimovirus* in the family *Potyviridae*. However, phylogenetic relationships and sequence analysis indicate that the SCSMV-PAK is a distinct species separate from SCMV and SrMV. However, it caused mosaic disease in sugarcane with symptoms similar to those caused by SCMV and SrMV. SCSMV-PAK shares 52.7% identity with TriMV and 26.4 to 31.5% identities with the species of the existing genera and unassigned viruses in the family *Potyviridae*. Therefore, SCSMV and TriMV formed a distinct group in the new genus *Poacevirus* in the family *Potyviridae* (Xu *et al*., 2010).

More isolates of SCSMV were reported for their complete genome nucleotide sequences from China, i.e. JP1: JF488064, JP2: JF488065 and ID: JF488066 (Li *et al*., 2011), and India, i.e. TPT: GQ246187 and IND671: JN941985 (Parameswari *et al*., 2013). Phylogenetic relationship of the complete genome sequences revealed that isolates JP1, JP2 and ID clustered together but separated from isolate PAK and IND671 (Parameswari *et al*., 2013).

5. Potyviruses causing mosaic diseases in sugarcane

5.1 *Sugarcane mosaic virus* (SCMV)

SCMV is a member of SCMV subgroup in the genus *Potyvirus*, family *Potyviridae*. SCMV particle is a filamentous rod of 730-755 x 13 nm. It has been known as grass mosaic virus, sorghum red stripe virus or maize dwarf mosaic virus strain B (Shukla *et al.*, 1989). SCMV is transmitted by numerous species of aphids in a non-persistent manner, such as *Rhopalosiphum maidis* (Fitch), *Aphis gossypii*,

Lipaphis pseudobrassicae, *Longuinguis saccari*. Transmission through the infected seed cane such as SCMV strain J has been found at very low level in maize (Williams *et. al*., 1968). However, there are no more reports on seed transmission of SCMV in sugarcane, sorghum and other hosts. Natural infection of SCMV had been reported on various species of cultivated and wild grasses, including the genera *Arundinaria*, *Brachiaria*, *Cynodon*, *Dactyloctenium*, *Digitaria*, *Echinochloa*, *Eleusine*, *Eragrostis*, *Erianthus* (*Ripidium*), *Panicum*, *Paspalum*, *Paspalidium*, *Pennisetum*, *Rhynchelytrym*, *Rottboellia*, *Setaria*, *Sorghum*, *Stenotaphrum*, *Tripsacum* and *Zae* (Koike and Gillaspie, 1989). In general, *S. officinarum* L. is the most susceptible species of *Saccharum*.

In Thailand, the mosaic disease of sugarcane was first found in 1973 and had been previously characterized to be SCMV strain A, F and H according to the reactions on a set of sorghum assay hosts (Nateewatana, 1985). Gemechu *et al*. (2004) demonstrated that six isolates of SCMV obtained from 5 provinces of Thailand exhibited mosaic symptoms depending on host cultivars. Incubation period for symptom expression of SCMV in sugarcane, corn and sorghum was 4-15 days after mechanical inoculation depending on host cultivars. The inoculated sorghum exhibited lethal necrosis, chlorotic spot and severe mosaic associated with streak or stripe pattern, while whitish mosaic or streak, mottle were found in the inoculated corn. Virus infection rates varied from 15-92% in sorghum, 17-90% in corn and 0-88% in sugarcane. Infected sugarcane showed chlorotic or necrotic streak, chlorotic mild mottle or mild mosaic. This report suggested that UT-3 and Q-67 sugarcane varieties were the most susceptible cultivars to SCMV isolates as evaluated by infection rate.

Gemechu *et al*. (2005) studied sequence comparisons and phylogenetic relationship of SCMV-CP gene which cloned from SCMV infected sugarcane (UT6THH-sge, UD7TH-sge) and maize (SBC2TH-mz) in Thailand. The results revealed that Thai SCMV CP gene contained 942 nucleotides encoded for coat protein MW of 33.65 kDa. The nucleotide sequence identities among cloned CPs from three isolates were 98-99% to each other. The N-terminal of Thai SCMV-CP contained a

distinctive sequence, especially at nucleotide positions 28-46 of the N-terminal variable 70 amino acid residues. Phylogenetic relationship revealed that Thai SCMV were placed in a separate branch of SCMV-MDB cluster which is closely related to most of SCMV isolates from maize and distinct from the other cluster containing sugarcane-SCMV strains.

5.2 *Sorghum mosaic virus* (SrMV)

Sorghum mosaic virus (SrMV) was originally described by Abbott (1961) as a strain of SCMV strain H (SCMV-H). It caused a severe disease in sugarcane and sorghum in U.S.A, India, China, Japan and the Philippines (Grisham, 1994). Shukla *et al*. (1989) proposed and named as SrMV to be an independent member in the genus *Potyvirus*. The virus particles are flexuous filamentous of *ca*.750 nm long, containing a single stranded RNA genome. The host plants of SrMV were sorghum and sugarcane (Grisham, 2007). It was found the formation of cytoplasmic, cylindrical (pinwheel and scrolls) and amorphous inclusions in the host cells. The virus was transmitted by aphids in a non-persistent manner. The infected sorghum developed red-leaf and mosaic symptom whereas the infected sugarcane developed mild mottle symptom (Giorda *et al*., 1986). There are several strains of SrMV that have been found to infect sugarcane, namely SrMV strain H, I, M (in U.S.A) and HH, SrMV-HH in China (Li *et al*., 2013). These viruses had not been detected in Thailand neither in sugarcane or sorghum.

5.3 *Johnsongrass mosaic virus* (JGMV)

JGMV was originally described as Australian maize dwarf mosaic virus and Australian Johnsongrass strain of SCMV (Teakle and Grylls, 1973). Shukla *et al.* (1988) purposed that it should be an independent member of the *Potyvirus* group and named as *Johnsongrass mosaic virus* (JGMV). JGMV is serologically closely related to watermelon mosaic virus 2 and distantly related to bean yellow mosaic, maize dwarf mosaic, sorghum mosaic, sugarcane mosaic and clover yellow mosaic viruses (Shukla *et al*., 1988). The virus particle was flexuous rod of 750 × 12 nm, containing

positive sense single stranded RNA genome. It was transmitted by aphids in a non-persistent manner. The host plants of JGMV are sorghum, maize and sweet corn, grains and fodder. Recently, the identified host of JGMV was *Pennisetum purpureum* that was found in Brazil. The infected plants showed mosaic or necrotic foliage, stunting, chlorotic and yield reduction, especially in sorghum (Silva and Nicolini, 2013).

Other isolates of JGMV was named U.S. oat strain of maize dwarf mosaic virus (JGMV-MDO) that was found in the USA (Mc Deniel and Gordon, 1985). The Nigerian isolate of JGMV (JGMV-N) was found in Nigeria in 2004 (Seifers *et al*., 2005) and a virus isolate from *Brachiaria* spp. in Columbia, South America was identified as JGMV-Brac (Seifers *et al*., 2005). The Nigerian isolate (JGMV-N) differs from the JGMV-MDO because it reacts against only JGMV antiserum and infects sorghum and smooth brome but it could not infect oat or johnsongrass (Seifers *et al*., 2005). JGMV is serologically closely related to watermelon mosaic virus 2 and distantly related to bean yellow mosaic, maize dwarf mosaic, sorghum mosaic, sugarcane mosaic and clover yellow mosaic viruses (Shukla *et al*., 1988).

6. Incidence of SCSMV in Asia and yield loss

SCSMV is one of the major viral diseases caused an outbreak of streak mosaic disease in major sugarcane plantations in India (Rao *et al*., 2006; Viswanahan *et al*., 2008), China (He *et al*., 2013), Indonesia (Damayanti and Putra, 2011; Putra *et al*., 2014) and Thailand (Chatenet *et al*., 2005; Kasemsin *et al*., 2011).

In India, Hema *et al*. (1997) characterized the virus isolate causing mosaic disease of sugarcane in Andhra Pradesh state, South India and the virus isolates was named as *Sugarcane streak mosaic virus*-Andhra Pradesh isolate: SCSMV-AP (Hema *et al*., 1999). In 2006, Rao *et al*. (2006) reported that SCSMV was found more commonly associated with sugarcane mosaic samples in India (Uttar Pradesh, Maharashtra and Tamil Nadu) in comparison to SCMV. Interestingly, mixed infection of SCMV and SCSMV was detected in sugarcane infected leaf samples obtained from Maharashtra and Tamil Nadu. Six sugarcane varieties, UP 0090, CoLk 8102, CoSe

93232, VSI-9-20, CoM 9006 and Co 740 were positive to SCSMV infection. Among these positive sugarcane varieties, three varieties (VSI-9-20, CoM 9006 and Co 740) were found to be mixed infection of SCMV and SCSMV while rest of six varieties were infected only SCSMV. These results suggested that existence of SCSMV was more prevalent in India as compared to SCMV and mixed infections were detected in sugarcane mosaic infected leaf samples. Viswanathan *et al*. (2008) performed disease survey for the presence of SCSMV in 58 sugarcane varieties which collected during 2006-2007 at germplasm collections in India. These sugarcane varieties represented 11 major sugarcane growing states of India. The results suggested that high disease incidence of SCSMV was found in germplasm collections in India.

In China, sugarcane varieties including germplasms have been imported from abroad in order to improve the local cultivars. This approach might increase the risk of the spread of sugarcane diseases. In 2010, sugarcane germplasms maintained in National Nursery for Sugarcane Germplasm Resources in Kaiyuan, Yunnan province, China were found SCSMV infection and the positive samples were imported from Japan and Indonesia. In 2013, He *et al*. (2013) reported that SCSMV was widespread in Yuanjiang, Kaiyuan, Changning, Honghe, Mile and Xinping prefectures in Yunnan province. The highest infection rate (50%) for SCSMV was observed in Yuanjiang prefecture.

In Indonesia, SCSMV was first reported by Damayanti and Putra in 2011. Putra *et al*. (2014) reported that the disease survey in 2007 at sugarcane fields in Central and East Java revealed mosaic disease caused by SCSMV in 38 sugarcane fields with the disease incidence ranging from 0.28 to 62.18%. The sugarcane variety, PS 864 was predominantly affected with SCSMV, 1 to 62% of the plants having symptoms. This report suggested that the virus mostly infected commercial sugarcane varieties and predominantly infected PS 864 with more severe symptoms. In 2008-2009, the extensive survey was performed at commercial sugarcane plantations in Java again. The result revealed that the occurrence of SCSMV was more widespread and 32% of 931 observed fields were infected by SCSMV with the disease incidence ranged from 0.1 to 94.7%. The popular commercial varieties such as PS 864, BL, PS

862 and PSJT 941 were found severely infected by SCSMV. Yield loss assessment of SCSMV infected sugarcane was investigated on the variety PS864 by mechanical and vegetative transmissions. The results showed that cane tonnage and sugar yields reduced significantly at the infection level ≥50%. The reduction ranged from 16 to 17% and 19 to 21% for cane tonnage and sugar yield, respectively. In contrast, SCSMV infection did not influence sucrose content. Hot water treatment of cane cutting was not able to eliminate the virus in cane stalks but only postponed the appearance of the symptom (Putra *et al*., 2014).

In Thailand, SCSMV was first reported in 2005 by Chatenet *et al*. (2005). This research revealed that sugarcane leaf samples exhibiting mosaic symptoms originated from Bangladesh (BSRI), India (SBI-ICAR and SRS Kunraghat), Sri Lanka (SRS Uda Walawe), Thailand (DOA Bangkok) and Vietnam (QNSC) were positively diagnosed by RT-PCR using the designed primer for SCSMV. This is the first report on the occurrence of SCSMV and streak mosaic disease is widespread in several Asian countries including Thailand. In 2010, sugarcane disease survey was performed at farmers' fields in Kamphaeng Saen, Nakhon Pathom province. Random samples of the virus-like symptomatic leaves were collected and diagnosed for the presence of SCSMV by RT-PCR using the designed specific primer to the CP gene of SCSMV. The result revealed that SCSMV was the causal agent of streak mosaic disease according to the previous report by Chatenet *et al*. (2005). Yield loss by SCSMV on sugarcane production in Thailand needs further investigation.

7. Antibody against SCSMV and virus diagnosis

Immunological diagnosis of SCSMV had been successfully developed by using the antibodies against SCSMV. Hema *et al*. (2003) produced a rabbit polyclonal antibody against expressed CP protein of SCSMV isolate from India (SCSMV-AP). The purified recombinant CP was used as the antigen to produce rabbit antiserum and SCSMV had been readily diagnosed by DAC-ELISA and dot-blot immunobinding assay. In addition, the antiserum was used to develop the sensitive technique such as the immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR).

This method can be used for virus detection in oxidizing plant extracts and inhibitory plant constituents. The immobilized virions were used as template for RT-PCR reaction. This is the first report on the application of recombinant CP antibody based IC-RT-PCR for the detection of SCSMV-CP gene (Hema *et al*., 2003). This antiserum was also applied for SCSMV detection in sugarcane leaves collected in Indonesia (Prabowo *et al*., 2014).

8. Genetic variability and phylogenetic studies of SCSMV

The study on genetic variability of SCSMV have been investigated using analysis of CP (Viswanathan *et al*., 2008; He *et al*., 2013), P1 (He *et al*., 2013) and HC-Pro coding regions (Bagyalakshimi *et al*., 2012).

Viswanathan *et al*. (2008) investigated the genetic diversity of SCSMV isolates obtained from India, Australia, South Africa and USA base on analysis of the partial CP coding regions (690 bp). The results revealed that among Indian SCSMV isolates shared 85.7-100% (nt) and 89.9-100% (aa) identities and 82.2-97.5% (nt) and 89.7-98.6% (aa) identities with other database sequences. Phylogenetic relationships of Indian SCSMV isolates were distributed in 14 phylogenetic groups and the grouping pattern revealed that the virus isolates could not be grouped based on geographical origin of the host varieties or longevity of the host variety.

Bagyalakshmi *et al*. (2012) investigated the genetic variability and potential recombination events in HC-Pro gene of SCSMV. Comparison of 12 Indian SCSMV isolates and 4 other database sequences revealed high level of diversity in the HC-Pro gene, 72-97% (nt) and 83-99% (aa) identities. This result suggested that Indian isolates were found to be the most divergent, up to 12% variation at the amino acid level. Phylogenetic analysis revealed clustering of 16 SCSMV isolates into two groups. Group I included isolates from India and Pakistan and group II consisted of isolates from Japan and Indonesia. Recombination analysis revealed nine potentially significant recombination events and recombination sites were identified throughout HC-Pro gene such as the recombinant isolate, CBV2000V59 had recombination site at

the nucleotide position (nt 569-832) and recombinant isolate, CB 86010 had two recombination sites (nt 1-663 and nt 693-753). In addition, analysis of selection pressure indicated that HC-Pro gene of SCSMV was under strong negative selection.

He *et al*. (2013) investigated the molecular variability of SCSMV in China based on analysis of P1 and CP protein coding regions. In this report, P1 and CP coding region nucleotide sequences for 22 representative SCSMV isolates were analyzed. Sequence analysis of P1 protein revealed four motifs (H-x8-D/E, GxSG, RG and VELL) while CP protein revealed the motif, R-x_{44}-D involving long-distance movement. Recombination detection analysis revealed that CP coding sequences appeared to have recombination sites while P1 coding sequences were not found. Using RDP4 and SISCAN2 programs, recombination sites were detected in CP coding sequences of four isolates, CB671-1, CB740, CB9217-1 and S-8. Phylogenetic analysis of Chinese SCSMV isolates using recombination sites in CP coding regions (nt 59-483 and nt 484-831) revealed that Chinese SCSMV isolates were divided into three major lineages and the lineages seem to reflect geographical origin. In addition, Thai isolate of SCSMV (THA-NP3) was formed in the lineage III with Chinese SCSMV isolates and THA-NP3 was the major parental isolate of the recombinant, CB740, CB9217-1 and S-8.

9. Genetic recombination of plant RNA viruses

Recombination is one of the major driving forces for genetic variability in most plant viruses. This mechanism joined variants that arise independently within the same molecule, creating the new opportunities for viruses, to overcome the selective pressures, and to adapt to new environments and hosts. The recombination occurred when at least two viral genomes co-infected the same host cell and exchanged genetic segments. Different types of the viral recombination such as homologous and non-homologous recombinations were recognized based on the structure of the crossover site (Scheel *et al*., 2013). In addition, a particular type of recombination known as reassortment, occurred in viruses with segmented genomes such as *Tomato spotted wilt virus* (TSWV), a virus in genus *Tospovirus* (Lian *et al*., 2013). Recombination

occurred in both **(-)** ssRNA viruses and (+) ssRNA viruses. The recombination rates occurring in (+) ssRNA viruses revealed higher than (-) ssRNA viruses (McVean *et al*., 2002). The most widely accepted model, copy-choice, assumed that RNA recombination was mediated by a viral replicase as RNA-dependent RNA polymerases (RdRp), the enzymes mediating RNA recombination which were encoded by both viruses and plants. The model of RNA recombination was summarized in Figure 2. The first population of plant RNA viruses for recombination analysis was investigated by Haseloff *et al*. (1984). This research compared the genomic sequences of the (+) ssRNA viruses namely *Alfalfa mosaic virus* (AMV), *Brome mosaic virus* (BMV) and *Tobacco mosaic virus* (TMV). The genomic sequences of these viruses showed homology among them so that the reshuffling of viral genes during evolution was possible by recombination at the RNA level (Haseloff *et al*., 1984). The recombination and mutation created new genetic combinations which were the main source of genetic diversity. Mutations introduced changes in nucleotide levels and therefore new variants. The recombination allowed the movement of variants across genomes to produce new haplotypes. Therefore, recombination did not create new mutations (at the nucleotide level) but introduced new combinations of the existing ones (Reviewed by Perez-Losada *et al*., 2015).

Sequencing and phylogenetic relationship have been used for detection and characterizing recombination events among RNA viruses (He *et al*., 2013). Several detection methods for detecting recombination events and location breakpoints were involved in statistical programs such as Recombination Detection Program: RDP (Martin *et al*., 2015), a pairwise homoplasy index (PHI), implemented in the PhiPack package (Bruen *et al*., 2006) and Split Decomposition analysis (Huson, 2005). Several other graphic applications, including boot-scanning, PhylPro, TOPAL and DIVERT (Reviewed by Worobey and Holmes, 1999).

In this study, the authors would like to review about the recombination in plant (+) ssRNA viruses. The first evidence of genetic recombination in plant (+) RNA virus was provided by Bujarski and Kaesberg in the *Brome mosaic virus* (BMV) system (Reviewed by Sztuba-Solinska *et al*., 2011). The recombination of the viruses

in the family *Potyviridae* was also investigated. The recombination occurred at the CP ORF and 3′UTR sequences in *Potato virus Y* (PVY), *Bean common mosaic virus* (BCMV), *Sweet potato feathery mottle virus* (SPFMV), *Yam mosaic virus* (YMV), *Bean yellow mosaic virus* (BYMV), *Zucchini yellow mosaic virus* (ZYMV) and *Plum pox virus* (PPV). However, the recombination of non-structural proteins were also found in several viruses such as *Turnip mosaic virus* (TuMV), SPFMV, *Soybean mosaic virus* (SMV), PVY and *Watermelon mosaic virus* (WMV) (Reviewed by Sztuba-Solinska *et al*., 2011).

Umer Farooq *et al*. (2013) investigated the genetic diversity and recombination events *in Grapevine leafroll*-associated virus 3 (GLRaV-3) in China based on analysis of the CP gene. This research revealed genetic variation showing nucleotide sequence similarity ranging from 89.9-100% identities among 16 GLRaV-3 isolates in China. Phylogenetic analysis of the CP gene sequences revealed the existence of four well defined variants group, which corresponded to the previously reported phylogenetic groups (1, 2, 3, and 5). In addition, two new sub-groups designated as sub-group 1B and sub-group 3B in groups 1 and 3, respectively, were indentified in the Chinese GLRaV-3 population. Recombination analyses illustrated that those two new sub-groups (1B and 3B) were emerged as a result of recombination events between variants in groups 1 and 2, and variants in groups 1 and 3, respectively. These results further indicated that the variants in those new sub-groups are viable and evolutionary successful. In addition, these analyses provide evidence about CP gene as one of the recombination hotspots in GLRaV-3 genome.

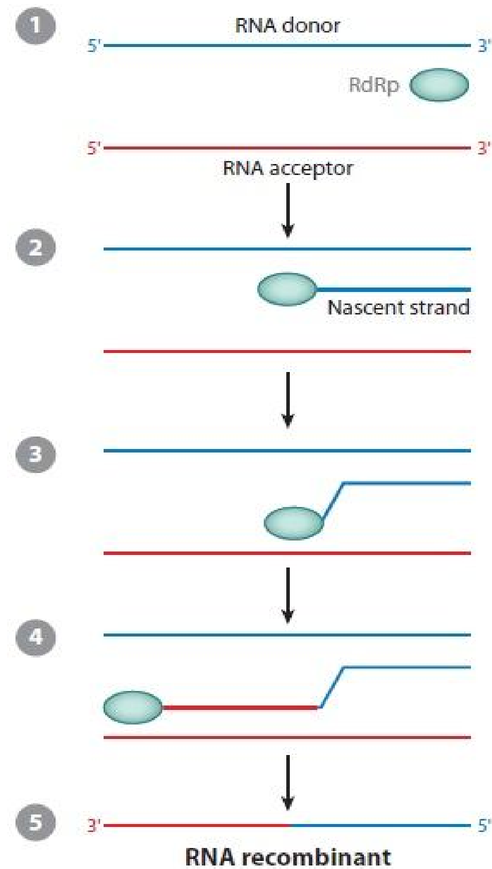

Figure 2 The generally accepted model of RNA recombination presumes that RNA viruses recombine according to a copy-choice mechanism. Recombinants are formed during the replication of a viral genome. (1) Virus-encoded RNA-dependent RNA polymerases (RdRp) initiate nascent strand synthesis at the 3′ end of the genomic RNA (RNA donor). (2) If RdRp is paused during the elongation, (3) a replicase nascent complex can dissociate from the donor template and reassociate with another template called an RNA acceptor. (4) Finally, the replicase resumes nascent strand synthesis on the acceptor template. (5) They are synthesized based on two different RNA templates.

Source: Sztuba-Solinska *et al*. (2011)

MATERIALS AND METHODS

1. Virus isolation and propagation

Sugarcane plants in farmers' fields in Kmaphaeng Saen District, Nakhon Pathom Province, Thailand were carefully inspected for SCSMV infection. Sugarcane leaves exhibiting yellow streak mosaic symptoms along the leaf vein were collected for SCSMV diagnosis by reverse-transcription polymerase chain reaction (RT-PCR). The collected samples were recorded for their locations by using Global Positioning System (GPS). Young leaves were excised from symptomatic plants, put in zip-lock plastic bag and kept on ice during transition back to the laboratory. Maize, sorghum and grasses growing around sugarcane fields were also randomly collected for SCSMV diagnosis.

Samples which were diagnosed for the presence of SCSMV by RT-PCR were subsequently diagnosed for SCMV infection by RT-PCR using specific primers for SCMV-CP gene amplification according to Gamechu *et al*. (2002). Only samples which were singly infected by SCSMV were processed for SCSMV propagation.

The selected isolate THA-NP3 from Nakhon Pathom (NP) was propagated in sorghum by mechanical inoculation. Inoculum was prepared by grinding 10 g of symptomatic SCSMV-infected sugarcane leaf tissues in a pre-chill blender added with 90 ml of 0.1 M sodium phosphate buffer (NaH_2PO_4, Na_2HPO_4, pH 7.0, 0.5% sodium sulphite). The homogenate was placed on ice and mixed with 0.1% 600 mesh-carborundum. Leaves of 15 days old-sorghum (*Sorghum bicolor* cv. UT325B) seedlings were rubbed with the prepared sugarcane leaf homogenate, and left for 5 min before rinsing with tap water. The inoculated plants were kept in a screenhouse for virus multiplication and symptom observation.

2. Virus purification

Sorghum fresh leaves showing severe symptom after 15 days post-inoculation (dpi) of SCSMV were used for virus purification following the protocol described by Hema *et al*. (1999). Briefly, 100 g of the infected fresh leaves were harvested, washed with water and incubated for 2 h at 4°C. The infected leaves were ground in a pre-chilled blender containing 500 ml of the extraction buffer (0.1 M sodium phosphate buffer, pH 7.2, 0.25% [v/v] 2-mercaptoethanol, 0.1% [w/v] sodiumdiethyldithiocarbamate, 1% [w/v] Polyvinylpyrolidone [PVP 40: mol wt. 40,000]. The homogenate was filtered by using miracloth. Virus suspensions were treated with an equal volume of chloroform and stirred gently for 20 min at room temperature. The supernatant was collected by centrifugation at 8,000×g for 20 min at 4°C. The virus preparation was concentrated by adding 6% polyethylene glycol (PEG, 6000 MW) and 0.2 M NaCl, followed by stirring gently for 1 h at 4°C. The pellet was collected by centrifugation at 8,000×g for 20 min at 4°C, then dissolved in 100 ml of 0.02 M phosphate buffer containing 1% Triton X-100, and stirred gently for 1 h at 4°C. The solution was centrifuged at 8,000×g for 20 min at 4°C. The virus suspension was precipitated in 20% sucrose by ultracentrifugation at 78,000×g for 3 h at 4°C. The pellet was dissolved in 10 ml of 0.01 M phosphate buffer, pH 7.2 containing 0.01 M EDTA and stirred gently for 2 h at 4°C. The solution was centrifuged at 8,000×g for 15 min and dialyzed (MW. Cut off: 12,000-14,000 Da) in PBS buffer (1x PBS, pH 7.4). The purified virus solution was diluted to 1:100 for measuring at O.D. 260 nm by using spectrophotometer.

3. Characterization of the purified virions

3.1 SDS-PAGE

The purified preparation was analyzed for viral coat protein M.W. by SDS-PAGE using 5% stacking and 12% separating polyacrylamide gels (Sambrook *et al*., 1989). A 10 µl of purified virions was mixed with an equal volume of 2x sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 0.02% bromophenol blue, 16% glycerol, 5%

2x mercaptoethanol). The mixture was denatured by boiling for 10 min. After electrophoresis, the gel was stained with Comassie Brilliant Blue-R250 at room temperature for 10 min and then de-stained in de-staining solution (25% methanol, 7% glacial acetic acid, 68% distilled water) until the gel turned to be visualized.

3.2 Electron microscopy

The purified virus particles were observed under a transmission electron microscope (TEM) by dip preparation method. A 1 mg/ml of purified virus solution was diluted to 1:100 in distilled water and dropped on a parafilm sheet. A grid (G-200-Cu, Ø 3.05 mm, 200 lines/inch square mesh) was placed on the virus suspension drop and was dried for two minutes at room temperature. The grid was stained with 2% uracil acetate and the virus particles were visualized by using TEM (TEM-1230, JEOL, 80KV).

4. Production of polyclonal antibody and immunoglobulin (IgG)

4.1 Injection

The polyclonal antibody was produced in a New Zealand White rabbit. Blood was collected as a normal serum before injection. One mg of the purified virus suspension in 500 µl was prepared as an antigen for each injection by mixing thoroughly with 500 µl of adjuvant (Freund’s complete adjuvant for the first injection and Freund’s incomplete adjuvant for the second injection). The rabbit was injected twice at 15 days interval by intradermal injection (ID) method for the first injection, and intramuscular (IM) method for the second injection. Blood was collected weekly after the second injection. The antisera were collected by centrifugation for 10 min at 8,000 rpm at 4°C, added with 0.02% sodium aside, and stored at 4°C.

4.2 Antiserum titer

Antiserum titer was determined against the purified SCSMV comparing with the normal serum by DAC-ELISA. The purified SCSMV was diluted to 5 µg/ml in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), loaded of 100 µl of the virus solution in each well of the microtiter plate and incubated overnight at 4°C. The plate was washed three times with PBST (1x PBST: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4, 0.05% Tween-20), each time for 2 min. Each well was then added with 100 µl of blocking solution (3% skim milk in 1x PBST), incubated at 37°C for 1 h and followed by washing as above. The antiserum was diluted by two-fold serial dilution starting from 200 to 409,600 in 1% skim milk solution (1% skim milk in PBST) and a 100 µl volume of each dilution was added to the well and incubated at 37°C for 1 h followed by plate washing. A 100 µl of the secondary antibody (Sigma-Aldrich Goat Anti-Rabbit IgG-AP conjugate, diluted at 1:10,000 in 1% skim milk solution) was added to each well, incubated at 37°C for 1 h and followed by plate washing. The plate was finally added with 100 µl of substrate, 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma, USA: 1 mg/ml in diethanolamine buffer, pH 9.6) and incubated for 60 min at 37°C. The developing color reaction of positive virus detection was measured at 405 nm absorbance (A_{405}) after incubation for 60 min.

4.3 Cross absorption of antisera

Sorghum plant proteins were used for cross absorption of non-specific antibodies in the obtained polyclonal antisera. Healthy sorghum was made homogenous by grinding in PBS buffer. Ratio of leaf to buffer was 1:10 (w/v). The SCSMV antiserum was add to the prepared healthy sorghum at the dillution 1:500 and followed by incubation at 37°C for 1 h. The supernatant was collected by centrifugation for 15 min at 12,000 rpm at 4°C. This cross-absorbed antiserum was used for SCSMV detection by DAC-ELISA.

4.4 Immunoglobulin (IgG) preparation

Healthy sorghum sap was prepared as above (4.3). A 9 ml of the healthy sorghum sap was mix with 1 ml of the antiserum and incubated at 37°C for 1 h. The supernatant was collected by centrifugation for 15 min at 12,000 rpm at 4°C. The supernatant was mix with an equal volume of Binding buffer (20 mM CH_3COONa, pH 7.4) and subjected to IgG purification using protein G plus agarose (Pierce: Thermo Scientific, USA). The purification protocol was performed according to the manufacturer's instruction. The IgG fractions were eluted using Elution buffer (0.1M CH_3COONa, pH 3.5) and each IgG fraction was neutralized by adding 100 µl of Neutralization buffer (1 M Tris, pH 9.0). The neutralized IgG fractions were dialyzed in 1xPBS, pH 7.4 and stored at -20°C. The IgG solution was measured by spectrophotometer at $O.D._{280}$ and the IgG yield (mg/ml) was calculated from the absorbance values (OD 280) and the extinction coefficient of rabbit IgG (1.4), mg/ml of IgG = O.D.280/1.4, according to Clark and Adam (1977). The M.W. of the purified IgG was analyzed by SDS-PAGE. This cross-absorbed IgG was subjected to develop immunochromatographic strips (ICS).

5. Immunological assays

5.1 Direct antigen coating ELISA (DAC-ELISA)

Leaf samples were made homogenous by grinding in plastic bags containing extraction buffer (1x PBS, pH 7.4, 0.1% sodiumdiethyldithiocarbamate) at ratio of leaf to buffer, 1:1 (w/v). The homogenate was diluted at 1:10 in coating buffer. A 100 µl of the diluted sap was loaded in each well of the microtiter plate, incubated overnight at 4°C and followed by washing and blocking as described above (4.2). The cross-absorbed antiserum was diluted to 1:500 in 1% skim milk solution, loaded in each well of the microtiter plate and incubated at 37°C for 1 h. The secondary antibody was added and incubated, followed by washing and adding substrate as previously described (4.2).

5.2 Dilution end-point (DEP) and sensitivity tests of the crossed-absorbed IgG

The plant extracts were prepared as described above (5.1) and used as working solutions for DEP and sensitivity tests by DAC-ELISA.

The working solutions of the plant extracts were diluted at 1:10 and used as antigen for virus detection by DAC-ELISA against different dilutions of the cross-absorbed IgG. The cross-absorbed IgG was diluted from the stock solution (1 mg/ml) by 2-fold serial dilution, starting from 1:200 to 1:102,400, and performed the DAC-ELISA protocol as described above (4.2). The DEP of the cross-absorbed IgG was evaluated from the highest dilution of the cross-absorbed IgG which was still showing the absorbance (A_{405}) values as positive diagnosis.

The DEP of the plant extracts from the SCSMV infected plants was also determined. The working solutions of the plant extracts were diluted by 2-fold serial dilution, starting from 1:10 to 10,240 and used as antigen for virus detection by DAC-ELISA against the cross-absorbed IgG. The cross-absorbed IgG was diluted to 1:400 in 1% skim milk solution and used for virus detection according to the DAC-ELISA protocol as described in 4.2. The DEP was evaluated from the highest dilution of the plant extracts from the virus-infected plants which was still showing the absorbance (A_{405}) values as positive diagnosis.

For specificity tests, the cross-absorbed antiserum including IgG was tested against the virus infected samples of twelve virus species known to infect *Poaceae* plants including sugarcane, maize and sorghum. Ten virus infected samples were bought from Agdia (Agdia, USA) and other virus infected samples (SCMV-MDB and MCMV) were isolated in Thailand. All positive virus samples were resuspended in extraction buffer (Agdia, USA) and diluted at 1:10 in coating buffer. A 100 μl of the diluted positive samples were loaded in each well of the microtiter plate, incubated at 4°C overnight and followed by washing and blocking. The cross-absorbed IgG (1 mg/ml) was diluted at 1:200 in 1% skim milk solution, and followed the DAC-ELISA protocol as described above (4.2).

5.3 Western blotting

Total protein was extracted from fresh leaves of healthy and diseased sugarcane and sorghum using the protocol described by Macintosh *et al*. (1992). One gram of fresh leaves were excised to small pieces and ground in 2.5 ml of protein extraction buffer (0.05 M Tris-HCl, 0.06 M sodium sulphite, pH 8.5) and transferred into a 1.5 ml microcentrifuge tube. The homogenate was centrifuged at 12,000 rpm for 5 min. The supernatant was transferred into a new microcentrifuge tube and kept at -20°C for further analysis. Total protein concentration was estimated by using Bradford method (Bradford MM, 1976). Approximately 5 µg of total protein was separated by SDS-PAGE using 5% stacking and 12% separating polyacrylamide gels. After electrophoresis, protein from gel was transferred to nitrocellulose membrane (Amersham, USA). The membrane was soaked in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4) at room temperature for 5 min. The membrane was incubated overnight at 4°C in blocking solution (3% BSA in TBST) and followed by washing with TBST 3 times, each time for 3 min with gently shaking. The membrane was added with the cross-absorbed IgG, diluted to 1:400 in 1% skim milk solution and incubated at room temperature for 1 h with gently shaking. The membrane was washed as described above, followed by incubation in the secondary antibody which was diluted to 1:10,000 in 1% skim milk solution at room temperature for 1 h. The membrane was washed and incubated in substrate, BCIP/NBT kit (Invitrogen, USA) for 30 min at room temperature. The reaction was stopped by adding distilled water and letting the membrane dry at room temperature.

6. Development of immunochromatographic strip (ICS)

6. 1 Preparation of colloidal gold conjugated SCSMV-IgG (CG-SCSMV-IgG)

Colloidal gold (CG) particles, 40 nm in diameter (Heron Diagnostic, distributed by Biogenomed, Thailand) were used for conjugation with the prepared IgG. A 10 ml of prepared colloidal gold (pH 7.3) was mixed with 100 µl of the cross-absorbed IgG (1 mg/ml). The mixture was stirred gently for 1 h at room temperature,

and 1 ml of 10% BSA solution (10 % BSA in distilled water) was added. The mixture was stirred gently for 30 min at room temperature. The pellet of the CG-SCSMV-IgG was collected by centrifugation at 12,000 rpm for 10 min at 4°C and resuspended in 500 µl of gold diluted buffer (0.02 M Na_2HPO_4, 0.1% NaN_3, 1% BSA, pH 7.4). The CG-SCSMV-IgG solution was mixed gently with 20% sucrose and stored at 4°C for future analysis.

6.2 Set up of immunochromatographic strip (ICS)

The ICS was 6 cm long, 0.5 cm wide, and was composed of sample application pad (SAP), conjugate releasing pad (CRP) glass fiber, nitrocellulose membrane (NCM) spread with the cross-absorbed IgG to make test line (T), and goat-anti rabbit IgG to make control line (C), respectively, and absorbent pad (AP), all components were placed on plastic backing. The CG-SCSMV-IgG was applied onto the glass fiber sheet (standard 17, Whatman, England) by hand with approximate amount of 5 µl/cm, and incubated for 2 h at 37°C. The cross-absorbed IgG (1 mg/ml in 1xPBS, pH 7.4) was applied twice as the test line (T) onto the nitrocellulose membrane (AE99, Whatman, England) by using a programmable automatic dispenser BioDot XY 2000 (BioDot Inc, USA) at the rate of 5 µl/cm. The goat anti-rabbit IgG (1 mg/ml in distilled water) was applied once as the control line (C), at the separate row approx. 0.5 cm distance from the test line, on the same nitrocellulose membrane with the same rate. The testing membrane was incubated for 2 h at 37°C. All components of the ICS were arranged overlapping one after another on 10 cm plastic backing, starting from SAP, CRP, nitrocellulose membrane and AP. A set of ICS was cut by a paper cutter (No. W-9001) to obtain a narrow strip pad of about 0.5 cm width for single use or assemble into a plastic holder for convenient use in the field.

7. Viral genome amplification and cloning

7.1 Primer design

Eleven specific primers for SCSMV whole genome amplification were

designed based on nucleotide alignment of the complete genome sequence of SCSMV-PAK: GQ388116 and the others previously recorded in GenBank: Y17738, EU650179, EF088799, EU650178, EU883391, EF088797, DQ421788, AM920686, AM920685, AB563503, GQ386845, GQ386843, GQ386844, Y17738, AY193783, AY189681. Two specific primers for amplification of the entire coat protein gene were also designed (Table 1 and Figure 3).

7.2 Total RNA extraction

Total RNA was extracted from fresh leaves of the collected plants and the healthy control using the extraction buffer described by Verwoerd *et al*. (1989). Approximately 1 g of leaf tissue was frozen in liquid nitrogen and ground to fine powder in a cold mortar and pestle. The powder was transferred into a 1.5 ml microcentrifuge tube containing 700 µl of TLES buffer (100 mM Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, pH 8.0, 1% SDS, 1% sodium sulphite) and mixed by inverting. The mixture was mixed with 500 µl of phenol (saturated in Tris-HCl, pH 8.0) and vortex for 2 min. Supernatant was collected by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was re-extracted with an equal volume of CI (chloroform: iso-amyl alcohol, 24:1) and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was mix gently with an equal volume of 4 M LiCl and incubated at 4°C overnight. The RNA pellet was collected by centrifugation at 13,000 rpm for 15 min at 4°C. The pellet was resuspended gently in 100 µl of DEPC treated water (0.1% DEPC in distilled water). RNA suspension was mix gently with 2.5 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate, pH 5.4. The RNA solution was incubated for 3 h at -20°C and centrifugated at 13,000 rpm for 15 min at 4°C. The RNA pellet was washed with 70% ethanol and resuspended gently in 30 µl of DEPC treated water. The concentration and purity of total RNA were measured by spectrophotometer at OD 260 nm and separated by electrophoresis on 0.8% agarose gel electrophoresis in 0.5X TBE buffer.

7.3 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR reaction was started to synthesize the full length cDNA from the viral mRNA template using an oligo d-T and a reverse transcriptase. The cDNA reaction consisted of 5 μg of total RNA, 2.5 μM of oligo $(dT)_{24}$, 0.5 mM dNTP mix and RNase-free water to adjust the total volume to 10 μl. The mixture was incubated for RNA denaturation at 65°C for 5 min and immediately placed on ice for 5 min. The denaturated RNA solution was mix with 10 μl of the solution containing 1X RT buffer, 5 mM $MgCl_2$, 10 mM DTT, 40 U of RNaseOUT, 200 U of SuperScript III RT (Invitrogen, USA) and RNase-free water. The mixture was started for cDNA synthesis at 50°C for 50 min, followed by termination at 85°C for 5 min and immediately placed on ice. This cDNA was used as a template for PCR reactions for synthesis the 11 overlapping sequences of SCSMV complete genome using our designed specific primers (Table 1).

PCR reaction consisted of 1X PCR buffer, 0.4 mM dNTP mix, 2mM $MgSO_4$, 10 pmol of each primer (Table 1), 1 U of Hi-Fidelity *Taq* with (Invitrogen, USA), 1 μl of cDNA and RNase-free water to adjust the total volume to 25 μl. The reaction was started with the initial step of denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing for 1 min at temperature according to the melting temperature (Tm) of each primer (Table 1), extension at 68°C for 1-2 min according to the length of the overlapping sequence (1 kb/1 min) and 1 cycle of final extension at 68°C for 7 min. PCR product was analyzed on 0.8% agarose gel electrophoresis in 0.5X TBE buffer.

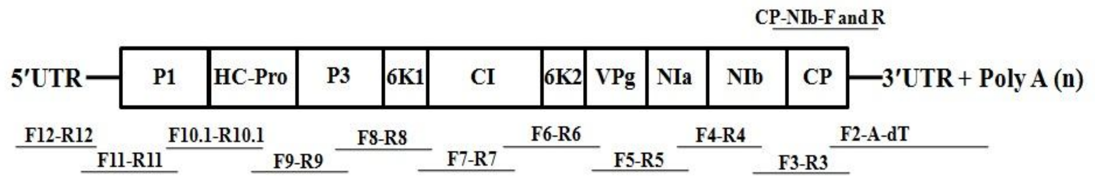

Figure 3 Genome map of *Sugarcane streak mosaic virus* displaying positions of primers used for full length genome amplification.
Ten functional protein names are shown in clear box. All of 11 primer designations are indicated below genome map and the specific primer pair for CP coding region (NIb-CP:F and NIb-CP:R) is above.

Table 1 List of specific primers for amplification of *Sugarcane streak mosaic virus* full length genome and coat protein gene.

Primer pair	Sequence 5′-3′	Length (bp)	Annealing Temp (°C)	Product size (bp)
CP-F	GATGAGGTACACACCACCACAAGC	24	59	588
CP-R	CGCAGGTCCGTGTCCTCATCTC	22		
A-d-T	TTTTTTTTTTTTTTTTTCCTCCN	23	55	881
F2	GCCAAGGCAAAGCAGATGATGAG	23		
F3	AGAGACAAGCTGGGTCACACTG	22	57	1,129
R3	CGTATTGATGCGGCCGATGAAG	22		
F4	CTGGAATGATGCAGTATGCGCTC	23	57	1,043
R4	GTCCGCGTCAATGAACTTCCAG	22		

Table 1 (Continued)

Primer pair	Sequence 5′-3′	Length (bp)	Annealing Temp (°C)	Product size (bp)
F5	AAGCGCCGAACACACGCT CGTG	22	58	1,096
R5	TCACTACCCGAGCTGCCG AATG	22		
F6	GCCACAACTTCCAATCCC ATTC	22	55	755
R6.1	TTCCGGCATTCTTCTTGGCG	20		
F7	AGCTACACCACCAGGAGC ACG	21	57	1,043
R7	GCGCTTCAGACGTTGACA ATCG	22		
F8	GACGACACAACAAGGTTA GCGC	22	55	1,067
R8	ATGAGTATACGCCCTCCG TTTG	22		
F9	GGAGGTGTGTTACCAGAT TTGCG	23	57	1,003
R9	CCGTCTTTCCTTGTGCGT GGTG	22		
F10.1	CAGATCCTGAACGAAATT GCACG	23	57	1,102
R10.1	CTGCAATATCGGGATGAT TCCTC	23		
F11	CGACAACACTGGTGAAGT TGAAG	23	57	1,183
R11	CGATAGTGGTTGGCTAGC GGTG	22		
F12	AAATGTAAT TTCAAATTG ACTAC	23	57	872
R12	CATTAGTTCGCATAATCA CACG	22		
CP-NIb:F	TTGGTGGAGCAAGCACACAG	20	61	1094
CP-NIb:R	CGGTCAGGCAACTACCATCA	20		

7.4 Cloning of PCR products

PCR product was purified by using the PCR purification kit (Favogen, Taiwan) and used for ligation with the cloning vector. Reaction of ligation consisted of 1X ligation buffer (Promega, USA), 2 µl of the purified PCR product, 1 unit of a T4 DNA ligase, 0.5 µl of the pGEM-T cloning vector (Promega, USA) and adding distilled water to adjust the total volume to 10 µl. The mixture was incubated at 16°C for 3 h.

The competent cells, *E. coli* strain DH5α were prepared according to Sambrook *et al*. (1989). The ligation mixture was mixed with the competent cells and

incubated on ice for 30 min. The mixture was incubated at 42°C for 1 min and immediately placed on ice for 5 min. The transformed cells were grown in 1 ml of LB liquid medium at 37°C with shaking for 45 min. Cell pellet was harvested by centrifugation at 8,000 rpm for 1 min and resuspended in 100 µl of LB liquid medium. The cell suspension was spread on LB plate containing 100 mg/l of ampicilin, 20 µl of X-GAL (20 mg/ml), 20 µl of IPTG (0.1 M) and followed by incubation at 37°C over night. The expected white colonies were screened for the presence of the inserted fragments by colony PCR. The positive recombinant clones from the 11 overlapping sequences were grown for plasmid isolation by using plasmid isolation kit (Favogen, Taiwan). The plasmid with the gene insert was sent to sequencing service at BioDesign, Pathum Thani, Thailand.

8. Nucleotide and protein sequence analyses

8.1 Genomic nucleotide and protein sequences

Nucleotide sequences were identified for virus gene by using Blast program (www.ncbi.nlm.nih.gov/Blast.cgi) and subjected for genome assembly by using CAP contig assembly program (BioEdit 7.2.5, http://www.mbio.ncsu.edu/bioedit/bioedit.html). The complete genome sequences were searched for an open reading frame (ORF) by using ORFinder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The putative cleavage sites of deduced proteins on the polyprotein were determined by comparing the potential cleavage sites with those of previously recorded SCSMV sequences (SCSMV-PAK: GQ388116, SCSMV-TPT: GQ246187, SCSMV-ID: JF488066, SCSMV-JP1: JF488064, SCSMV-JP2: JF488065 and SCSMV-IND671: JN941985). Viral genome with functional protein mapping was drawn according to the analyzed protein cleavage sites.

8.2 Multiple sequence alignment and phylogenetic analysis

Nucleotide sequences of the studied SCSMV isolates were used for multiple sequence alignment by using ClustalW in the CLC program package

(http://www.clcbio.com). Other recorded sequences in GenBank database were selected as the representative species from each genus in the family *Potyviridae* for multiple sequence alignment (Table 2). Pairwise comparisons were also created.

Phylogenetic trees were reconstructed by using MEGA6 (Tamura *et al.*, 2013) with the multiple aligned nucleotide and amino acid sequences of Thai SCSMV isolates and other representative species in the family *Potyviridae*. Neighbor-joining tree based on the aligned polyprotein was constructed using 1,000 bootstrap replicates.

Table 2 Viruses in the family *Potyviridae* used for phylogenetic tree reconstruction in this study.

No.	Genus	Species name	Acronym	Accession no.
1	*Potyvirus*	*Maize dwarf mosaic virus*	MDMV	NC_003377
2		*Sorghum mosaic virus*	SrMV	NC_004035
3		*Sugarcane mosaic virus*	SCMV	NC_003398
4		*Johnsongrass mosaic virus*	JGMV	NC_003606
5	*Rymovirus*	*Ryegrass mosaic virus*	RGMV	AF035818
6		*Agropyron mosaic virus*	AgMV	NC_005903
7		*Hordeum mosaic virus*	HoMV	NC_005904
8	*Ipomovirus*	*Cucumber vein yellow virus*	CVYV	NC_006941
9		*Squash vein yellowing virus*	SqVYV	EU259611
10		*Sweet potato mild mottle virus*	SPMMV	NC_003797
11	*Poacevirus*	*Sugarcane streak mosaic virus*	SCSMV-JP1	JF488064
12		*Sugarcane streak mosaic virus*	SCSMV-JP2	JF488065
13		*Sugarcane streak mosaic virus*	SCSMV-PAK	GQ388116
14		*Sugarcane streak mosaic virus*	SCSMV-TPT	GQ246187
15		*Sugarcane streak mosaic virus*	SCSMV-ID	JF488066
16		*Sugarcane streak mosaic virus*	SCSMV-IND671	JN941985
17		*Sugarcane streak mosaic virus*	SCSMV-THA-NP3	JN163911
18		*Triticum mosaic virus*	TriMV	NC_012799
19	*Tritimovirus*	*Wheat eqlid mosaic virus*	WEqMV	NC_009805
20		*Oat necrotic mottle virus*	ONMV	NC_005136
21		*Wheat streak mosaic virus*	WSMV	NC_001886
22	*Bymovirus*	*Oat mosaic virus*	OMV	NC_004016
23		*Barley yellow mosaic virus*	BaYMV	NC_002990
24		*Wheat yellow mosaic virus*	WYMV	NC_002350
25	*Brambyvirus*	*Blackberry virus Y*	BlVY	NC_008558
26	*Macluravirus*	*Chinese yam necrotic virus*	ChYNMV	NC_018455
27	unassigned genus	*Rose yellow mosaic virus*	RoYMV	JF280796

9. Disease surveys

Sugarcane field surveys for SCSMV infection were conducted from 2010 to 2014 in five provinces of major sugarcane growing areas including Nakhon Pathom, Kanchanaburi, Udon Thani, Khon Kaen and Nakhon Ratchasima. The virus-like symptomatic sugarcane leaf samples, particularly young mosaic leaves, were collected and kept in sealed plastic bags. Samples were divided into two groups (i) those from the farmers' fields and (ii) those from germplasm collection fields belonging to Kasetsart University, in Nakhon Pathom and Kanchanaburi provinces. Some samples were kindly provided by the Department of Agriculture, Ministry of Agriculture and Cooperatives. All collected sugarcane leaf tissues were diagnosed for the presence of SCSMV by RT-PCR, and/or by DAC-ELISA with the use of our produced antiserum against the purified virions of SCSMV. These country-wide collected samples were used for genetic variation study based on coat protein gene sequences analysis.

10. Genetic variation study based on coat protein (CP) gene sequences analysis

10.1 Cloning, sequencing and sequence analysis

The CP coding region of the selected isolates were amplified by RT-PCR using CP the specific primers (CP-NIb:F, CP-NIb:R) which were designed in this study (Table 1). The cDNA was synthesized as described above (7.3). PCR reaction consisted of 1X PCR buffer, 0.4 mM dNTP mix (TOYOBO, Japan), 2 mM $MgSO_4$, 10 pmol of each primer, 1 U of KOD-Plus-Neo (TOYOBO, Japan), 1 µl of cDNA and RNase-free water to adjust the total volume to 25 µl. The amplification cycle was the same as described above (7.3) except that the annealing temperature was 61°C for 1 min. PCR products were analyzed by 0.8% agarose gel electrophoresis. The products were subsequently purified and submitted to direct sequencing in both directions using DNA sequencing service (SolGent, South Korea). Some selected RT-PCR

products were cloned into pGEM-T cloning vector (Promega) and the plasmids containing the gene insert were sequenced in both directions.

Nucleotide sequences were edited by using Chromas, version 2.31 and assembled for a full length sequence using CAP contig assembly program in BioEdit, version 7.2.5 (http://www.mbio.ncsu.edu/). The assembled sequences were confirmed for the presence of the CP gene using blast (www.ncbi.nlm.nih.gov/BLAST). All corrected sequences obtained from each isolate were submitted to GenBank database. Sequence alignment, pairwise comparisons of nucleotide and amino acid sequences were created using CLC program package (http://www.clcbio.com). Phylogenetic tree was constructed by maximum-likelihood (ML) method using 1,000 bootstrap replicates which was implemented in MEGA6 (Tamura *et al*., 2013).

10.2 Genetic variation determination

Two types of analysis were conducted for possible recombination events of the viral genome. Firstly, the recombination events were investigated by using RDP4 program (version 4.5). The detection algorithms used in this study were automated RDP, GENECONV, Chimaera, MaxChi, BOOTSCAN, SISCAN and 3Seq with default setting (Martin *et al*., 2015).

Secondly, Phylogenetic network or Splits networks of the CP sequences among Thai SCSMV isolates were created by using SplitsTree4 program, version 4.13.1 (Huson and Bryant, 2006).

RESULTS AND DISCUSSION

Results

1. Virus isolation and symptomatology

In our primary survey experiments, totally 23 samples of symptomatic sugarcane leaves showing yellow streak mosaic symptoms (Figure 4) from Nakhon Pathom, Kanchanaburi, and Saraburi, were collected and diagnosis for SCSMV infection by RT-PCR using designed CP primers, CP-F and CP-R (Table 1). There were 11 samples from Nakhon Pathom, 6 samples from Kanchanaburi and 4 samples from Suraburi provinces gave expected 588 bp of partial CP gene. Sequence analysis of these fragments revealed that they were the partial sequences of SCSMV-CP coding regions.

The isolate THA-NP3 from Nakhon Pathom was selected for further investigation on viral full length genome sequence and organization. The virus isolate NP3 was propagated on sorghum cv. UT325B by mechanical inoculation. Symptoms on the inoculated sorghum leaves showed yellow steak mosaic, especially on young leaves at 15 days post-inoculation (dpi) (Figure 5A). On the commercial corn cv. Tender58, yellow streak mosaic symptoms were observed at 5 dpi (Figure 5B). Symptomatic leaves of sorghum and corn were confirmed for SCSMV infection by RT-PCR.

Figure 4 Yellow streak mosaic symptoms on sugarcane leaves observed at farmers' fields and germplasm collection fields.

Sugarcane leaf samples obtained from the farmers' fields in Kamphaeng Saen District, Nakhon Pathom Province (A) and Dan Makham Tia District, Kanchanaburi Province (B) in 2010. Sugarcane leaf samples obtained from germplasm collection fields in Kanchanaburi Province, UT13 in 2014 (C) and Suphan Buri Province, UT8 in 2012 (D).

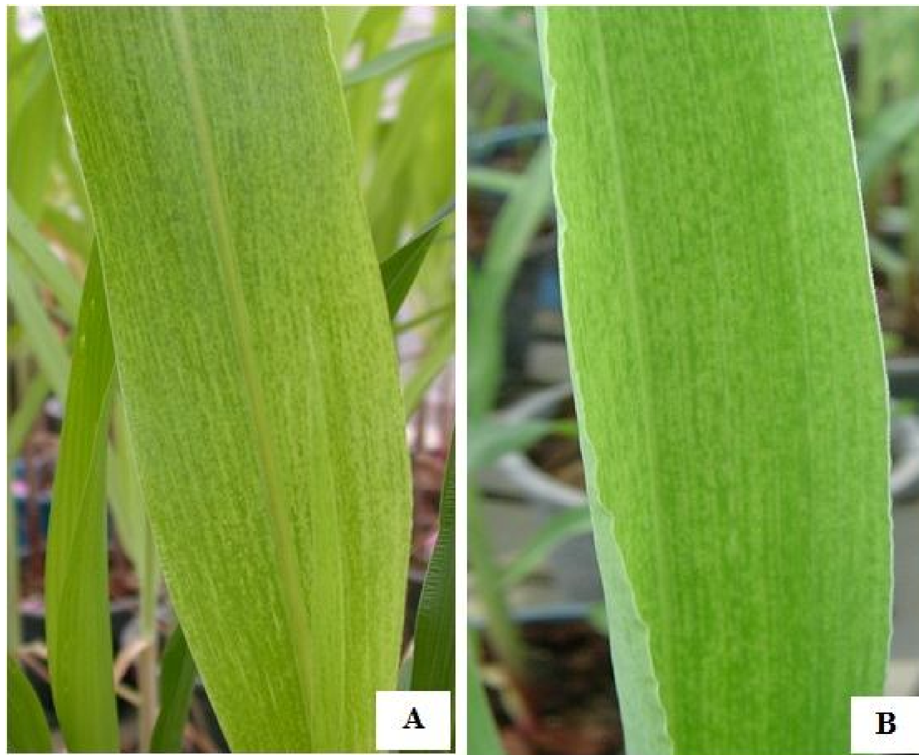

Figure 5 Symptoms of *Sugarcane streak mosaic virus* on the inoculated plants. Yellow streak mosaic symptoms express on the inoculated sorghum leaves, cv. UT325B at 15 dpi (A) and the commercial corn cv. Tender58 at 5 dpi (B).

2. Virus purification and properties

Electron microscopy of the purified virions revealed flexuous rod, ranged in length between 700-890 nm. However, several particles appeared as smaller size ranging from 500-600 nm were also found (Figure 6). The purified preparation showed 2.679 absorbance value of A_{260}, and spectral ratio of A260/280 was 2.04, giving 2.014 mg/ml concentration of the virus suspension which yielded 2.014 mg virus from 800 g of sorghum leaf tissues. This virus yield was calculated by using the sedimentation coefficient ($E260^{0.1\%}$) of the flexuous rod viruses mean of 1.33.

SDS-Polyacrylamide gel electrophoresis of the protein extracts from the purified preparation revealed subunits of the coat protein (CP) with a size of 31 kDa (Figure 7). In addition, the larger band of an unknown protein, approximately 40 kDa was also found in the same purified preparation (Figure 7).

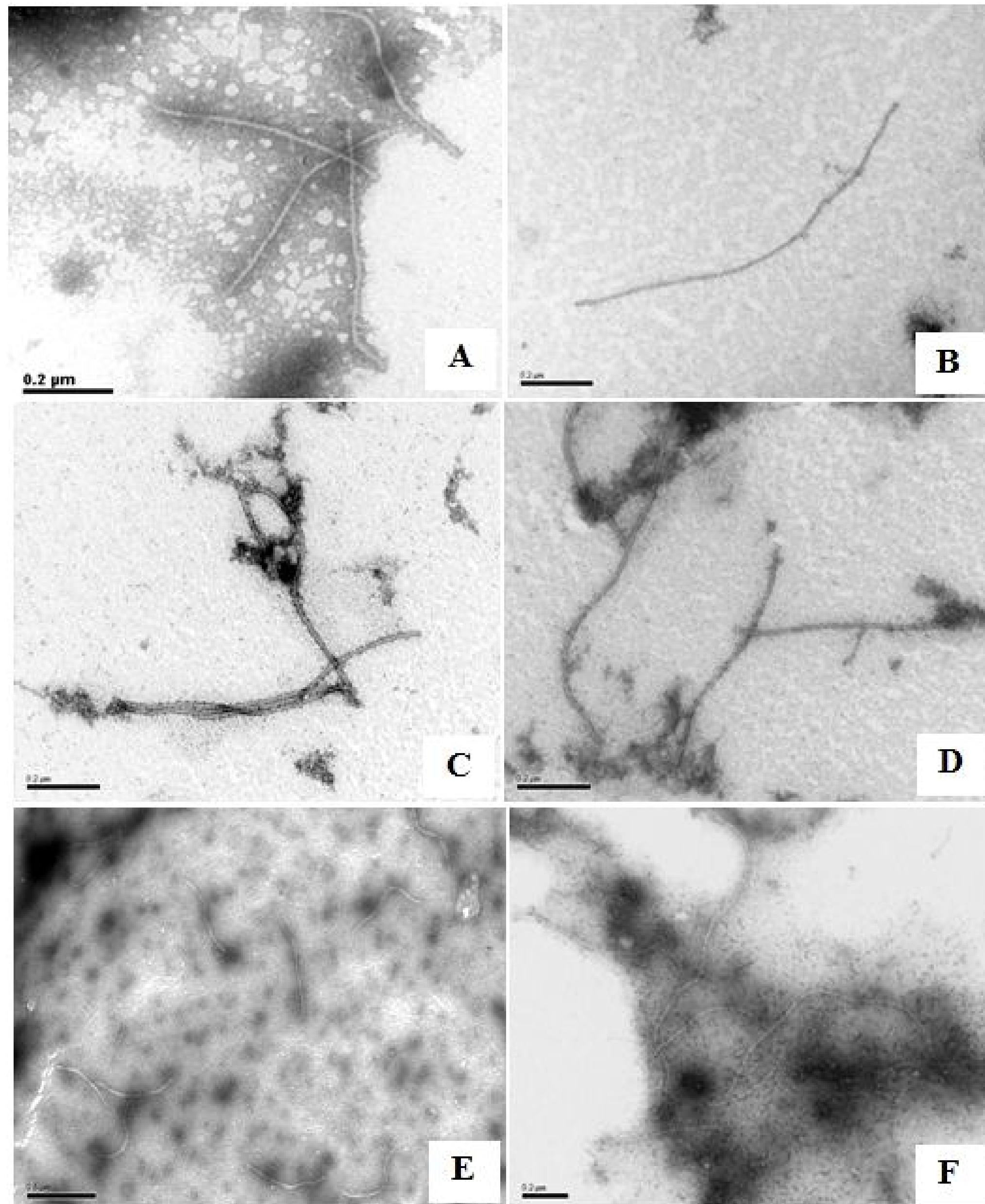

Figure 6 Transmission electron micrograph of the purified virions of *Sugarcane streak mosaic virus*, isolate THA-NP3 from the infected sorghum leaves. The flexuous rod particles of the purified preparation are visualized by TEM at 80,000x (A-D), 30,000x (E) and 50,000x (F).

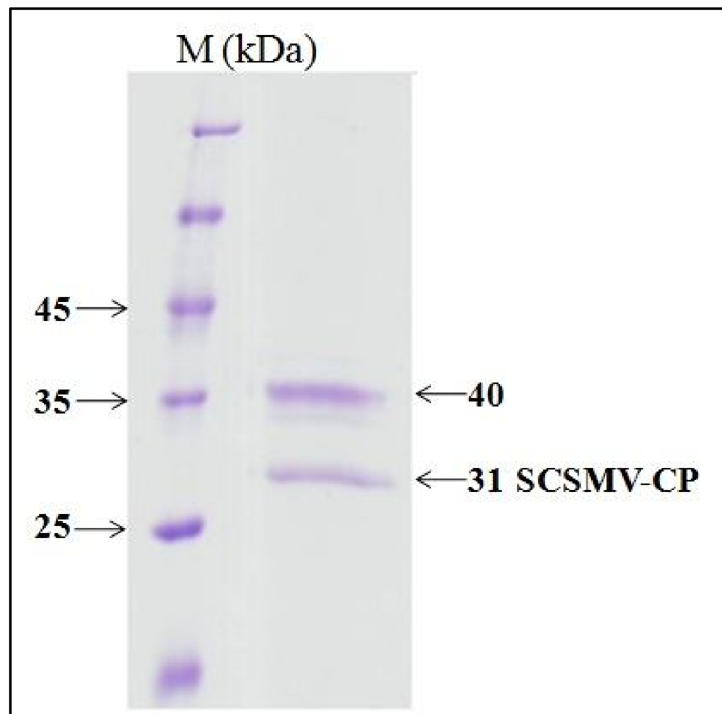

Figure 7 SDS-Polyacrylamide gel electrophoresis of the purified virions of *Sugarcane streak mosaic virus*, isolate THA-NP3 from the infected sorghum leaves.

3. Genome sequence and organization of isolate THA-NP3

The complete genome sequence of isolate THA-NP3 was obtained from eleven overlapping fragments assembly, starting from 5′ end of the genomic RNA strand downstream to 3′ end, excluding the 3′ poly (A) tail. The assembled THA-NP3 genome consisted of 9781 nucleotides and the sequence was deposited in the GenBank database (Acc. No. JN163911). Each of 11 fragment sequences as well as the assembled full length genome sequence showed the highest similarity to SCSMV isolates recorded in the database. According to the species demarcation criteria for members of family *Potyviridae*, the isolate THA-NP3 was identified as SCSMV which belonged to genus *Poacevirus*.

The genome of isolate THA-NP3 contained one long open reading frame (ORF), starting from nucleotide position 200 ending to position 9592. The 5′ untranslated region (UTR) consisted of 199 nucleotides while the 3′ UTR was 189 nucleotides long (Figure 8). The long ORF of 9393 nucleotides encoded a polyprotein of 3130 amino acid residues with a calculated *M*r of 356.53 kDa. Nine putative cleavage sites of the polyprotein were identified (Table 3). Ten functional proteins of THA-NP3 isolate, in ascending order of genome organization from 5′ to 3′ direction, were P1 (44.47 kDa), HC-Pro (54.41 kDa), P3 (37.39 kDa), 6K1 (5.43 kDa), CI (74.82 kDa), 6K2 (5.55 kDa), NIa-VPg (22.47 kDa), NIa-Pro (26.64 kDa), NIb (57.43 kDa) and CP (31.07 kDa).

According to the NCBI-CDD database, the motif scanning revealed 19 motifs on the THA-NP3 polyprotein. The **P1** protein contained a serine protease active site sequence, the catalytic triad H-8X-D-32X-S (aa 263-308) and the conserved motif F-V-V-L-G (aa 332-336) at the N-terminal region. The **HC-Pro** protein had the putative protease motif C-71-X-H (aa 715-787) at the C-terminal region. The **CI** protein, the largest protein among 10 functional proteins, contained an NTP-binding motif, G-T-G-K-S-3X-P at the amino acid positions 1302-1310. In addition, there were 6 motifs of superfamily 2 (SF2) RNA helicase at the following amino acid positions, 1318-

1331 (P-5X-P-S-R-4X-N), 1387-1390 (D-E-X-H), 1416-1420 (T-A-T-P-P), 1441-1442 (P-S), 1526-1532 (T-T-2X-Q-X-G) and 1566-1579 (N-5X-Q-R-X-G-R-X-G-R).

The **VPg** protein contained a conserved tyrosine for linking VPg to the 5′-terminus of viral RNA by the amino acid motif L-Y-D-L-D at the positions 1988-1992. The **NIa** protein contained the catalytic motif of H-3X-D-66X-G-X-C-G in cysteine protease at the positions 2152-2257. The viral replicase protein, **NIb**, contained the conserved motif, D-G-S-R-Y-D (aa 2589-2594) that represented the function for RNA dependent RNA polymerase. The **CP** protein contained conserved amino acid residues, i.e.Y-X-P-17X-W at the positions 2915-2935, A-X-P-2X-R-2X-M-6X-A at the positions 3012-3027, and D-F at the positions 3062-3063. There was no DAG-motif (characteristic of aphid transmitted potyvirus) in the SCSMV-CP sequence. The **6K1** protein contained a motif, A-22X-K (aa 1170-1193) and this motif was also found in the 6K1 proteins of all potyviruses. The **6K2** protein contained a stretch of predominantly hydrophobic residues at the position, 1882-1896, suggesting an association with membrane structures.

The genome sequence of THA-NP3 was analyzed for the presence of Pretty Interesting *Potyviridae* ORF (PIPO) in the P3 gene with a highly conserved motif, $G_{1-2}A_{6-7}$ similar to the previous report of potyviruses (Chung *et al.*, 2008). The result revealed that the conserved motif, GGAAAAAAA was found at the nucleotide position 3085-3093 which was similar to the SCSMV-PAK (Xu *et al.*, 2010). The deduced 139 aa of PIPO of THA-NP3 was derived from 420 bp in +1 frame at the nucleotide position 3091-3510.

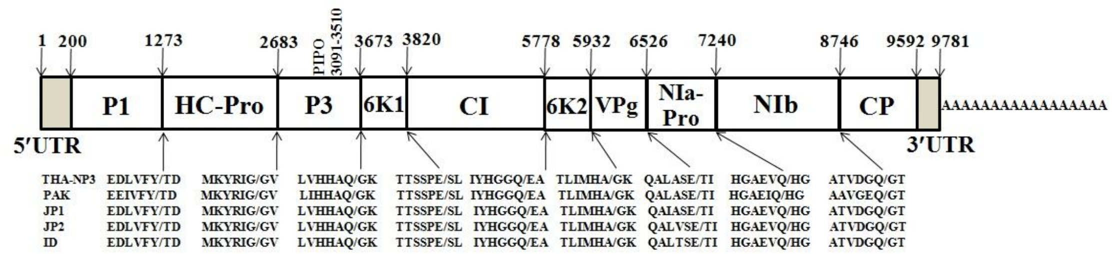

Figure 8 Schematic representation of genome organization of *Sugarcane streak mosaic virus*, the isolate THA-NP3 (JN163911) and putative cleavage sites. The 5′UTR (199 nts) and 3′UTR (189 nts) regions are shown as shaded boxes. The boxes show each of 10 functional proteins. Numbers above the boxes indicate nucleotide positions of each protein initial codon. The cleavage sites for viral proteinases are indicated below. The PIPO protein encoded in +1 frame at the nucleotide position, 3091-3511.

5′ UTR	: 5′ untranslated region consisted of 199 nucleotides
P1	: P1 Serine protease protein
HC-Pro	: Helper component protein
P3	: P3 protein
6K1 and 6K2	: Two small proteins
CI	: Cylindrical inclusions protein
VPg	: Viral genomic protein
NIa	: Nuclear inclusion a protein
NIb	: Nuclear inclusion b protein
CP	: Coat protein
3′ UTR	: 3′ untranslated region consisted of 189 nucleotides
PIPO	: Pretty Interesting *Potyviridae* ORF

Table 3 Putative protease cleavage sites on the polyprotein of *Sugarcane streak mosaic virus* isolate THA-NP3.

Protein cleavage	Cleavage sites on SCSMV isolates						
	THA-NP3	**PAK**	**JP1**	**JP2**	**ID**	**TPT**	**IND671**
P1/HC-Pro	EDLVFY/TD	EEIVFY/TD	EDLVFY/TD	EDLVFY/TD	EDLVFY/TD	EDLVFY/TD	EELVFY/TD
HC-Pro/P3	MKYRIG/GV	MKYRIG/GV	MKYRIG/GV	MKYRIG/GV	MKYRIG/GV	MKYRIG/GI	MKYRIG/GV
P3/6K1	LVHHAQ/GK	LIHHAQ/GK	LVHHAQ/GK	LVHHAQ/GK	LVHHAQ/GK	LVHHAQ/GK	LIHHAQ/GK
6K1/CI	TTSSPE/SL	TTSSPE/SL	TTSSPE/SL	TTSSPE/SL	TTSSPE/SL	TTSSPE/SL	TTSSPE/SL
CI/6K2	IYHGGQ/EA	IYHGGQ/EA	IYHGGQ/EA	IYHGGQ/EA	IYHGGQ/EA	IYHGGQ/EA	IYHGGQ/EA
6K2/VPg	TLIMHA/GK	TLIMHA/GK	TLIMHA/GK	TLIMHA/GK	TLIMHA/GK	TLIMHA/GK	TLIMHA/GK
VPg/NIb-Pro	QALASE/TI	QALASE/TI	QAIASE/TI	QALVSE/TI	QALTSE/TI	QAIASE/TI	QALASE/TI
NIa-Pro/NIb	HGAEVQ/HG	HGAEIQ/HG	HGAEVQ/HG	HGAEVQ/HG	HGAEVQ/HG	HGAEVQ/HG	HGAEIQ/HG
NIb/CP	ATVDGQ/GT	AAVGEQ/GT	ATVDGQ/GT	ATVDGQ/GT	ATVDGQ/GT	AAVDEQ/GT	AAVGEQ/GP

4. Virus relationship to other SCSMV isolates

The complete nucleotide sequence of THA-NP3 showed high similarity to those of JP2, JP1, ID and TPT isolates at 97.84%, 97.78%, 97.73% and 94.80% identities, respectively (Table 4). It was less similar to PAK and IND671 isolates, with 81.83% and 81.39% nucleotide identities, respectively (Table 4). The NIb protein of THA-NP3 showed 94.82-99.00% identities to other SCSMV isolates, but 64.62% identity to TriMV. The CP protein sequence was 94.72-98.93% identities to CP of other SCSMV isolates, but it shared 48.48% identity to TriMV-CP sequence.

Comparison of THA-NP3 nucleotide sequence to other potyviruses causing mosaic diseases in sugarcane such as SCMV, SrMV, MDMV and JGMV showed less similarity with 29.01-29.38% (nt) and 17.49-18.33% (aa) identities (Table 4). Thus, THA-NP3 is a distinct group apart from potyviruses causing the mosaic diseases in sugarcane. Neighbor-joining tree of the polyprotein among SCSMV isolates revealed that THA-NP3 was clustered in the same group with JP1, JP2, ID and TPT isolates while it was clustered separately from PAK and IND671 isolates (Figure 9). Moreover, THA-NP3 shared 52.40% nt and 50.31% aa identities with TriMV suggesting that TriMV is a type member in the same genus (Figure 9). Nucleotide sequence comparisons among seven complete genome isolates (THA-NP3, PAK, ID, JP1, JP2, IND671 and TPT) revealed more genetic variations in the P1, HC-Pro and CP genes.

Table 4 Identity percentages of complete genome sequence comparison between THA-NP3 and other SCSMV isolates, and potyviruses causing mosaic diseases in sugarcane.

Virus name / Gene name	SCSMV PAK	SCSMV JP1	SCSMV JP2	SCSMV ID	SCSMV TPT	SCSMV IND671	SCSMV AP	TriMV	SCMV	SrMV	MDMV	JGMV
whole genome (9781nt[a])	81.83	97.78	97.84	97.73	94.80	81.39	na[c]	52.40	29.24	29.01	29.38	29.17
Polyprotein (3130aa[b])	95.27	98.98	98.98	98.98	98.40	94.38	na	50.38	18.33	17.49	18.14	17.65
P1 (358 aa)	95.25	98.32	98.60	98.60	98.04	88.37	na	38.92	9.84	9.84	11.17	10.11
HC-Pro (470 aa)	90.85	98.51	98.51	98.72	98.30	90.64	na	45.11	14.95	15.15	15.76	14.34
P3 (330 aa)	95.15	99.09	99.09	99.39	98.18	96.06	na	34.94	9.81	9.81	9.26	10.90
6K1 (49 aa)	93.88	100	100	100	87.50	91.84	na	50.00	13.04	10.14	15.94	8.06
CI (656 aa)	96.95	99.24	99.09	99.24	97.71	96.95	na	59.15	24.26	23.96	24.70	21.88
6K2 (48 aa)	100	100	100	97.92	92.16	100	100	49.02	8.57	10.00	8.57	7.86
NIa-VPg (198 aa)	96.97	98.48	98.48	98.48	94.12	97.98	95.45	22.27	22.01	20.57	na	22.49
NIa-Pro (238 aa)	97.48	100	100	100	93.70	94.96	95.80	44.12	18.42	16.17	19.55	16.23
NIb (502 aa)	95.02	99.00	99.00	98.80	97.04	94.82	97.01	64.62	32.29	32.66	31.38	33.64
CP (281 aa)	95.73	98.58	98.93	98.58	95.47	94.72	96.80	48.48	14.91	12.86	12.99	14.41

[a/]nts: nucleotides; [b/]aa: amino acid residues; [c/] na : not available

GenBank accession no. of the virus isolates are these followings: SCSMV-PAK: GQ388116 (Pakistan), SCSMV-JP1: JF488064 (China), SCSMV-JP2: JF488065 (China), SCSMV-ID: JN163911 (China), SCSMV-TPT: GQ246187 (India), SCSMV-IND671: JN941985 (India), SCSMV-AP: Y17738 (India), TriMV: NC_012799 (USA), SCMV: NC_003398 (China), SrMV: NC_004035 (China), MDMV: NC_003377 (Bulgaria), JGMV: NC_003606 (unknown; direct submission)

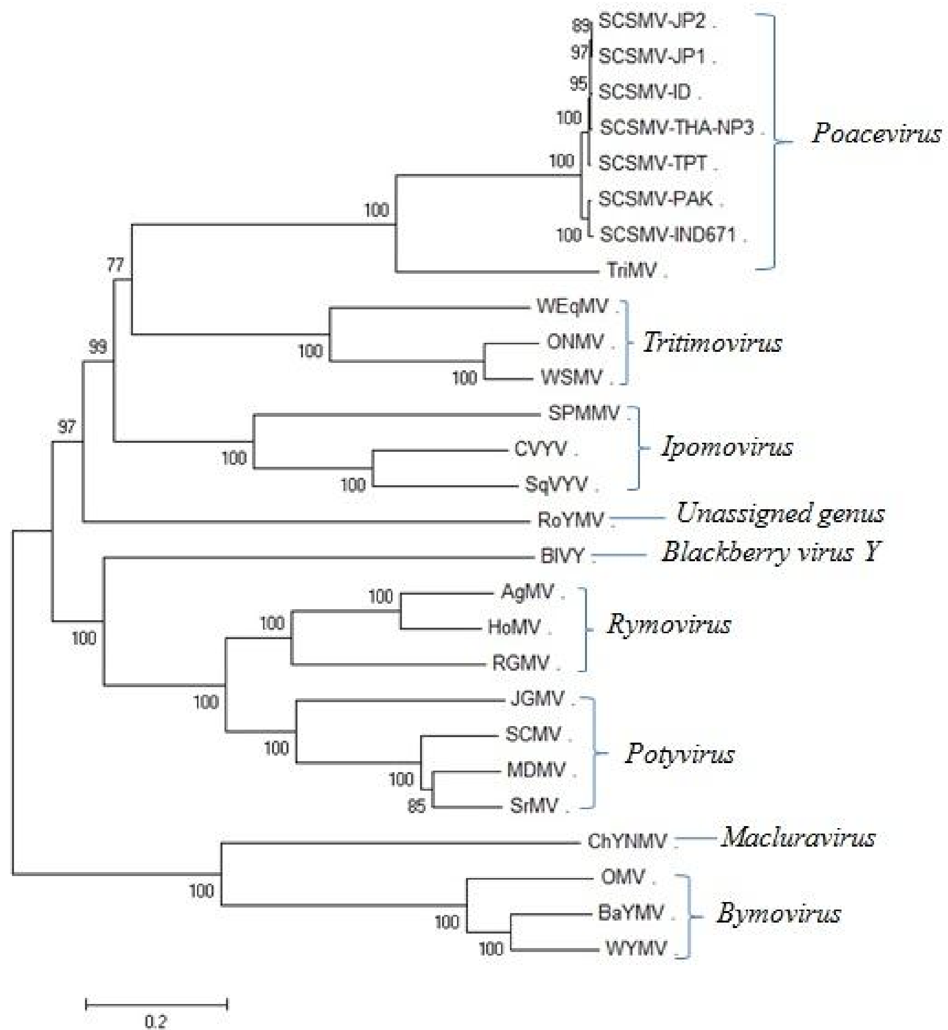

Figure 9 Neighbor-joining phylogenetic tree reconstruction using the polyprotein amino acid sequences of the viruses within the family *Potyviridae*.

5. Antiserum production

5.1 Titer

The antiserum, approximately 80 ml was obtained from seven bleeds. The highest antiserum titer was 409,600 which was obtained at the 3rd week (AS2-AS3) when compared with the normal serum (Figure 10). The absorbance values (A_{405}) in 60 min ranged from 0.223-0.346 at the dilution, 1:800-1:1600. Therefore, this antiserum was subjected to eliminate non-specific reactions by cross absorption with the healthy sorghum protein. After cross-absorption, the antiserum was diluted at 1:500 for DAC-ELISA, and the absorbance values of the healthy controls were lower than 0.2 in 60 min.

Figure 10 The graph shows antiserum titer of the obtained antisera (AS1-AS7). The antiserum titer was determined by DAC-ELISA by using the purified SCSMV (5 µg/ml) as the antigen raised against the antisera (AS1-AS7). The antiserum titers of AS1-AS7 were the following: 204800, 409600, 409600, 204800, 102400, 204800 and 204800, respectively.

5.2 Specificity

The specificity test of the cross-absorbed IgG against 12 other viruses revealed specific reactivity only to SCSMV infected plants (sugarcane and sorghum) at the dilution 1:200 by DAC-ELISA (Figure 11). The absorbance values (A_{405}) showed negative diagnosis in all tested viruses ranging between 0.098-1.432 (Figure 11). Therefore, this produced antiserum showed high specificity without cross reactions to other tested viruses including the virus causing mosaic diseases in sugarcane and sorghum such as JGMV, MDMV, SCMV-corn strain and SCMV-sugarcane strain (Figure 11).

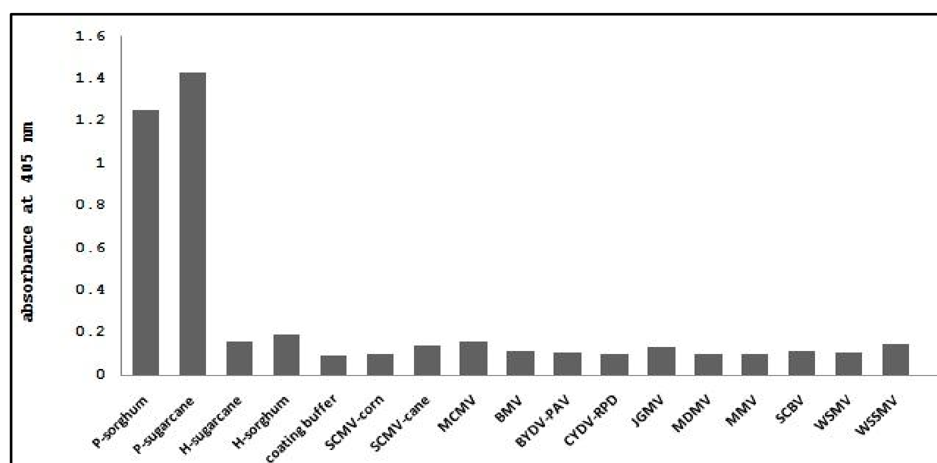

Figure 11 Enzyme-linked immunosorbent assay values (60 min reaction) of the cross-absorbed IgG at the dilution 1:200 against the positive samples of 12 other tested viruses. The exact absorbance (A_{405}) values of each sample are in brackets.

p-sorghum: SCSMV infected sorghum (1.249), p-sugarcane: SCSMV infected sugarcane (1.431), H-sugarcane: healthy sugarcane (0.161), H-sorghum: healthy sorghum (0.188), SCMV-corn: *Sugarcane mosaic virus* corn strain (0.098), SCMV-cane: *Sugarcane mosaic virus* sugarcane strain (0.137), MCMV: *Maize chlorotic mottle virus* (0.160), BMV: *Brome mosaic virus* (0.114), BYDV-PAV: *Barley yellow dwarf virus* (0.105), CYDV-RPD: *Cereal yellow dwarf virus* (0.102), JGMV: *Johnsongrass mosaic virus* (0.133), MDMV: *Maize dwarf mosaic* (0.102), MMV: *Maize mosaic virus* (0.102), SCBV: *Sugarcane bacilliform virus* (0.114), WSMV: *Wheat streak mosaic virus* (0.106), WSSMV: *Wheat spindle streak mosaic virus* (0.147)

5.3 Sensitivity

The cross-absorbed IgG at 1 mg/ml concentration was evaluated for virus detection by DAC-ELISA against 1:10 diluted plant extracts. The dilution end point (DEP) of the cross-absorbed IgG in positive reaction was 1:51,200, A_{405}=0.225 (Table 5). The cross-absorbed IgG reacted strongly at the dilution 1:400 and used this dilution for sensitivity tests. The results showed that the DEP of the diseased sugarcane revealed positive diagnosis in ELISA at the dilution 1:5,120, A_{405}=0.250 (Table 6). The negative controls (healthy sugarcane and buffer) showed negative diagnosis in all dilutions (Table 5 and Table 6).

Table 5 Enzyme-linked immunosorbent assay values (60 min reaction) from reciprocal of the cross absorbed IgG dilution against the infected plant when used to analyze 1:10 w/v the plant extracts.

	Reciprocal of IgG dilution									
Sample[a]	**200**	**400**	**800**	**1,600**	**3,200**	**6,400**	**12,800**	**25,600**	**51,200**	**102,400**
SCSMV Infected sugarcane	1.538	1.389	1.239	1.115	0.902	0.706	0.477	0.320	0.225	0.159
Healthy sugarcane	0.101	0.115	0.115	0.100	0.101	0.103	0.104	0.107	0.102	0.106
Coating buffer	0.076	0.077	0.079	0.079	0.076	0.076	0.081	0.079	0.079	0.075

[a] The plant samples were prepared at the dilution 1:10 in coating buffer and incubated at 4°C overnight for DAC-ELISA.

Table 6 Enzyme-linked immunosorbent assay values (60 min reaction) from the reciprocal of sap dilutions.

	Reciprocal of sap dilution										
Sample[b]	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**1280**	**2560**	**5120**	**10240**
SCSMV infected sugarcane	2.483	2.369	2.206	1.845	1.368	1.007	0.697	0.508	0.368	0.250	0.178
Healthy sugarcane	0.117	0.115	0.103	0.101	0.102	0.107	0.105	0.108	0.109	0.106	0.107
Coating buffer	0.078	0.077	0.081	0.077	0.077	0.086	0.083	0.078	0.075	0.077	0.089

[b] The plant samples were prepared by 2-fold serial dilution, starting from 1:10 to 1:10240 in coating buffer and incubated at 4°C overnight for DAC-ELISA.

6. Immunological diagnosis

6.1 DAC-ELISA

Detection of SCSMV in the plant extracts by DAC-ELISA was successfully developed. The plant extracts were diluted to 1:10 in coating buffer (pH 9.6) and then incubated at 4°C overnight. The specific conditions of the DAC-ELISA used in this study were the following: (i) 3% skim milk solution was used as blocking reagent and incubation for 1 h at 37°C, (ii) the cross-absorbed antiserum was diluted to 1:500 while the cross-absorbed IgG (1 mg/ml) was diluted to 1:400, and incubation at 37°C for 1 h, and (iii) the secondary antibody was diluted to 1:10,000 and incubation at 37°C for 1 h. SCSMV detection by using our DAC-ELISA protocol revealed that sugarcane leaf samples showing yellow streak mosaic symptoms, especially on young leaves revealed high absorbance values (A_{405}) ranging from 0.8-1.6 while the older leaf samples with mild symptoms revealed the absorbance values (A_{405}) ranging from 0.3-0.6 (Table 7). Some samples with the absorbance values (A_{405}) ranging from 0.2-0.3 were confirmed for the presence of SCSMV infection by RT-PCR using the SCSMV-CP specific primers (Table 1).

6.2 Immunochromatographic strip (ICS)

The ICS either dipped or dropped the plant extract into the sample pad. The extraction buffers for rapid virus detection by using ICS were investigated. The result revealed that Adgen extraction buffer showed only C line while other tested buffers including Bioreba (Switzerland), Na_2BO_3 and Agdia (USA) showed both T and C lines. Therefore, Adgen buffer was a suitable buffer that used as the extraction buffer for rapid virus detection by using ICS. The control line appeared within 2 min after the solution was migrated through the absorbent pad. The reaction was completed within 5 min. The positive reactions of the infected sugarcane plant (NR3/9; A_{405} in 60 min = 1.640) revealed both lines of test (T) and control (C) (Figure 12A). Sensitivity test of ICS was also investigated. The results revealed that strong reactions were found at the dilutions 1:5 to 1:40 while weak reactions were found at

the dilutions, 1:80, 1:160 and 1:320 (Figure 12B). The negative controls showed only the control lines (Figure 12C).

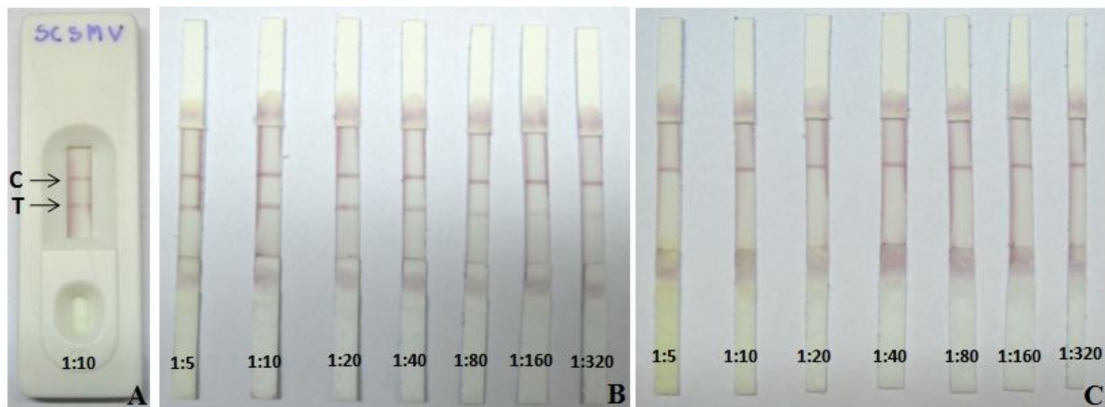

Figure 12 Detection of SCSMV by immunochromatographic strip (ICS).
The strip in a plastic holder was dipped with the diseased sugarcane sap at the dilution 1:10 (A), and individual strips were tested for sensitivity against different dilution, 1:5 to 1:320 of diseased sugarcane sap (B) and the healthy sugarcane (C).

6.3 Western blotting

SCSMV-CP was also detected in the plant extracts by Western blotting. The viral coat protein with a size of 31 kDa was appeared in SCSMV infected leaf samples (Figure 13, lane 2-5) and there were no viral proteins in non-infected samples and healthy control (Figure 13, lane 6-8). In addition, the larger band of an unknown protein with a size of 40 kDa was also found in all infected samples (Figure 13, lane 2-5). These results suggested that the specially produced antiserum showed high specificity only to SCSMV proteins in the infected plant extracts, except that the healthy control (Figure 13, lane 8).

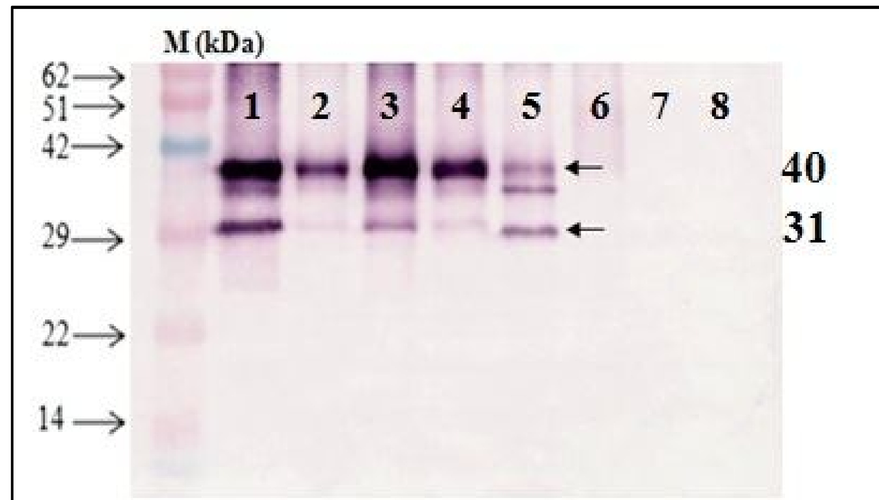

Figure 13 Analysis of the coat protein (CP) in the infected plants and the healthy control by Western blotting. The absorbance values (A_{405}) of each sample in 60 min are in brackets.

Lane M: Pre-stained marker (Vivantis, USA)

Lane 1: Purified SCSMV (50 ng)

Lane 2: SCSMV infected sorghum (1.251)

Lane 3: SCSMV infected sugarcane (1.574)

Lane 4: infected sugarcane leaf sample, UD12-7 (0.909)

Lane 5: infected sugarcane leaf sample, UD12-4 (0.278)

Lane 6: non-infected sugarcane leaf sample, KH4-6 (0.207)

Lane 7: non-infected sugarcane leaf sample, KH5 (0.108)

Lane 8: Healthy sorghum (0.097)

7. Incidence of streak mosaic disease in sugarcane crop in Thailand

In sugarcane fields, diseased sugarcane plants showed yellow streak mosaic, especially on young leaves, characteristic symptoms of SCSMV infection (Figure 4), while the older leaves showed mild symptoms. Two hundred and thirty-three sugarcane leaf samples were collected from 14 sugarcane fields of which 153 samples tested positive by DAC-ELISA. Some samples obtained from the older leaves had positive reactions by DAC-ELISA but the absorbance values (0.277-0.339) were lower than in younger leaves (Table 7). Therefore, selected samples with the absorbance values (A_{405}) ranging from 0.2-0.3 were confirmed for SCSMV infection by RT-PCR. Our disease surveys indicated that SCSMV was widespread in all collected sugarcane fields in Nakhon Pathom, Kanchanaburi, Udon Thani, Khon Kaen and Nakhon Ratchasima provinces (Table 7 and Figure 14). The percentage of the infected samples of all collected sugarcane fields ranged from 43.48-90.91% (Table 7). In three fields containing germplasm collections, 138 samples from 73 sugarcane varieties were collected. Of these, 91 samples obtained from 50 varieties indicated positive reactions by ELISA. The percentage of positive reactions within varieties ranged from 54.17-100% (Table 7). The sugarcane variety groups (UT, K and RT) revealed high infection rate of SCSMV. Other sugarcane variety groups such as ROC, Q and CP were also infected with SCSMV (Table 8). In addition, there were 21 sugarcane varieties revealed negative diagnosis by DAC-ELISA and RT-PCR (Table 8).

Table 7 Detection of SCSMV by DAC-ELISA from sugarcane leaf samples obtained from collected farmers' fields in 5 provinces and germplasm collection fields.

Collection fields	No. of collected sample	No. of positive sample	% of infected sample	Positive ELISA value in 60 min
Farmers' fields				
FSCPA1-2	21	12	57.14	0.375-0.998
FSCPA3	19	14	73.68	0.382-1.513
FSCBP1	23	10	43.48	0.509-1.108
FSCBP2-3	10	8	80.00	0.609-0.909
FSCMK1	14	10	71.43	0.394-0.806
FSCDT1	11	10	90.91	0.376-1.134
FSCKPS1	22	13	59.09	0.464-1.463
FSCKPS2	15	8	53.33	0.393-0.790
FSCKPS3	10	6	60.00	0.380-0.472
FNR1	10	8	80.00	0.392-1.640
FUD9-10	23	17	73.91	0.431-0.794
FUD11-12	18	11	61.11	0.387-0.909
FKH4-5	16	11	68.75	0.454-1.121
FKH6-10	21	15	71.43	0.381-1.347
Germplasm collection fields				
GKB	98	62	63.27	0.277-1.106
GNP	24	13	54.17	0.339-0.721
GSB	16	16	100.00	0.518-0.656

FSCPA1-2, FSCPA3, FSCBP1, FSCBP2-3, FSCMK1, FSCDT1 represent the sugarcane growing areas in Kanchanaburi (KB) province. FSCKPS1, FSCKPS2, FSCKPS3 represent the sugarcane growing areas in Nakhon Pathom (NP) province and FNR1, FUD9-10, FUD11-12, FKH4-5, FKH6-10 represent the sugarcane growing areas in Nakhon Ratchasima (NR), Udon Thani (UD) and Khon Kaen (KH) provinces, respectively. GKB, GNP and GSB represent the germplasm collection fields in Kanchanaburi (KB), Nakhon Pathom (NP) and Suphan Buri (SB) provinces, respectively.

Table 8 List of sugarcane varieties obtained from collected germplasm collection fields in Kanchanaburi (GKB), Nakhon Pathom (GNP) and Suphan Buri (GSB) provinces.

(+) positive diagnosis of SCSMV infection	94-2-105, 94-2-106, 99-2-294, 03-287, 03-208, 95-2-213, 194, K83-74, 294, K200, 135, K84-200, 03-2-395, 02-483, 395, UT3, 435, UT10, 477, UT11, 519, UT13, 352, RT2002-041, 527, RT2003-639, 1317, RT2007-027, RT2007-091, RT2001-1800, RT2003-619, RT2003-545, ROC1, ROC7, ROC16, Q85, CP29-166, CP63-588, CP385, CP48-103, LK92-11 and Ehaew
(-) negative diagnosis of SCSMV infection	94-128, K03-109, K06-23, KK61, UT (1,4), UT12, RT2004-077, RT07-068, RT01-1800, RT03-510, RT04-078, RT03-503, 254, 03-003, 85-2-352, Q229, CP29-221, CB1 (Brazil1), CB2 (Brazil2), CHB106-143 and CHB06-3-3575

These sugarcane varieties were diagnosed for the presence of SCSMV infection by DAC-ELISA and some selected samples were also confirmed SCSMV infection by RT-PCR.

Figure 14 The survey maps of streak mosaic disease incidence in major sugarcane growing areas in Central and Northeastern regions.

A-H: the collected areas in Kanchnaburi provinve

I-K: the collected areas Nakhon Pathom province

L-M: the collected areas in Nakhon Ratchasima province

O-R: the collected areas in Khon Kaen province

S-U: the collected areas in Udon Thani province

8. Genetic variation of SCSMV in Thailand based on coat protein

In a subsequent study, we selected 36 isolates from different farmers' fields in 5 provinces and 22 isolates from fields containing germplasm collections to examine the genetic variation based on sequence analysis of the viral CP gene. All selected isolates yielded the expected 1094 bp RT-PCR product. Sequence analysis of the RT-PCR product revealed the CP coding region containing 846 nucleotides which encoded for 281 amino acid residues. All of 58 SCSMV isolates obtained in this study (Table 9 and Table 10) were compared to 27 SCSMV isolates from other countries (Table 11). A nucleotide sequence comparison showed that the 36 isolates from different fields shared 86.17-100% identities while they shared 86.05-99.29% identities to SCSMV isolates from other countries. The 22 isolates from germplasm collections shared 86.52-100% identities among them and 85.70-99.29% identities to the isolates from other countries. The CP gene of Thai SCSMV isolates shared only 60.28-63.71% identities to the isolates of TriMV (NC_012799) which was used as an outgroup for construction of a phylogenetic tree.

The amino acid sequence comparison revealed 96.09-100% identities among the 36 isolates from collected farmers' fields, while they shared 94.31-100% identities to the isolates from other countries. The 22 isolates from collected germplasm collections shared 94.66-100% identities among each other, while they shared 93.24-100% identities to the isolates from other countries. Moreover, the CP protein of Thai SCSMV isolates shared 47.69-48.40% identities to the isolate of TriMV (NC_012799), a virus species in the same genus. Most variations of the amino acids were found at the amino acid positions 1-31 of N-terminal of the CP (Figure 15).

Phylogenetic relationships of the CP gene (846 nucleotides) obtained from 58 Thai SCSMV isolates and 27 SCSMV isolates from other countries (Table 9-11) were determined using a maximum-likelihood method. The CP sequences from Thai SCSMV isolates clustered in four well defined variant groups (Figure 16). Two sub-groups, which were designated as sub-groups 1A and 2A, contained 38 isolates from Thailand, 9 isolates from China, 2 isolates from Japan, 1 isolate from Indonesia, 2

isolates from India and the unique variant, GK76-4 (Figure 16). The second major group consisted of 2 sub-groups, 1B and 2B which represented most of the germplasm isolates from Thailand, India and China (Figure 16). The second sub-group, 2B contained only the isolates from collected germplasm collections (Figure 16). These results suggested that Thai SCSMV isolates obtained from farmers' fields were more closely related to the isolates from China while the isolates from collected germplasm collections were more closely related to the isolates from India and Pakistan (Figure 16).

Table 9 The CP coding regions of Thai SCSMV isolates obtained from farmers' fields in 5 provinces.

No.	SCSMV isolates	Location of isolation	Collection year	Sugarcane varieties	GenBank acc. no.
1	FDT1	Kanchanaburi	2012	Unknown	KP987806
2	FKB1	Kanchanaburi	2012	Unknown	KP987848
3	FKB13	Kanchanaburi	2012	Unknown	KP987807
4	FKB6	Kanchanaburi	2012	Unknown	KP987839
5	FSC1	Kanchanaburi	2012	Unknown	KP987820
6	FSC2	Kanchanaburi	2012	Unknown	KP987821
7	FSC7	Kanchanaburi	2012	Unknown	KR057207
8	FSC8	Kanchanaburi	2012	Unknown	KP987822
9	FKPS10	Nakhon Pathom	2012	Unknown	KR057206
10	FKPS19	Nakhon Pathom	2012	Unknown	KP987811
11	FKPS22	Nakhon Pathom	2012	Unknown	KP987812
12	FNP5	Nakhon Pathom	2012	Unknown	KP987813
13	FNP-KPS	Nakhon Pathom	2012	Unknown	KP987814
14	FTHA-NP3	Nakhon Pathom	2010	Unknown	JN163911
15	FNS4	Nakhon Pathom	2012	Unknown	KP987840
16	FNS5	Nakhon Pathom	2012	Unknown	KP987819

Table 9 (Continued)

No.	SCSMV isolates	Location of isolation	Collection year	Sugarcane varieties	GenBank acc. no.
17	FUD9-3	Udon Thani	2012	Unknown	KP987824
18	FUD9-5	Udon Thani	2012	Unknown	KP987825
19	FUD10-7	Udon Thani	2012	Unknown	KR057211
20	FUD10-4	Udon Thani	2012	Unknown	KP987826
21	FUD10-12	Udon Thani	2012	Unknown	KP987827
22	FUD11-1	Udon Thani	2012	Unknown	KR057212
23	FUD11-2	Udon Thani	2012	Unknown	KP987828
24	FUD12-3	Udon Thani	2012	Unknown	KP987829
25	FUD12-6	Udon Thani	2012	Unknown	KR057213
26	FUD12-8	Udon Thani	2012	Unknown	KP987830
27	FUD12-9	Udon Thani	2012	Unknown	KP987831
28	FUD12-10	Udon Thani	2012	Unknown	KP987804
29	FKH8	Khon Khan	2012	Unknown	KP987805
30	FKH4-8	Khon Kaen	2012	Unknown	KP987808
31	FKH5-1	Khon Kaen	2012	Unknown	KP987809
32	FKH5-2	Khon Kaen	2012	Unknown	KP987810
33	FNR3-1	Nakon Ratchasima	2012	Unknown	KP987815
34	FNR3-4	Nakon Ratchasima	2012	Unknown	KP987816
35	FNR3-6	Nakon Ratchasima	2012	Unknown	KP987817
36	FNR3-9	Nakon Ratchasima	2012	Unknown	KP987818

Table 10 The CP coding regions of Thai SCSMV isolates obtained from germplasm collection fields.

No.	SCSMV isolates	Location of isolation	Collection year	Sugarcane varieties	GenBank acc. no.
1	GK88-65	Nakhon Pathom	2014	K88-65	KP987836
2	GK88-87	Nakhon Pathom	2014	K88-87	KP987837
3	GUT6	Nakhon Pathom	2014	UT6	KP987842
4	GUT4	Nakhon Pathom	2014	UT4	KP987832
5	GUT5	Nakhon Pathom	2014	UT5	KP987841
6	GK76-4	Nakhon Pathom	2014	K76-4	KP987847
7	GRT2007-091	Kanchanaburi	2014	RT2007-091	KP987843
8	GRT2003-639	Kanchanaburi	2014	RT2003-639	KP987844
9	GEhaew	Kanchanaburi	2014	Ehaew	KP987802
10	G03208	Kanchanaburi	2014	03208	KP987803
11	GUT3	Kanchanaburi	2014	UT3	KP987834
12	G519	Kanchanaburi	2014	519	KP987835
13	GROC7	Kanchanaburi	2014	ROC7	KP987846
14	G99-2-294	Kanchanaburi	2014	99-2-294	KR057205
15	G02-483	Kanchanaburi	2014	02-483	KR057208
16	G94-2-106	Kanchanaburi	2014	94-2-106	KR057209
17	G95-2-213	Kanchanaburi	2014	95-2-213	KR057210
18	GUT10	Kanchanaburi	2014	UT10	KR057214
19	G03041	Suphan Buri	2012	03041	KP987845
20	GSP50-2	Suphan Buri	2012	SP50-2	KP987823
21	GUT8	Suphan Buri	2012	UT8	KP987833
22	GK92-80	Suphan Buri	2012	K92-80	KP987838

Table 11 The CP coding regions of SCSMV isolates obtained from other countries.

No.	SCSMV isolates	Location of isolation	Collection year	Sugarcane varieties	GenBank acc. no.
1	M16	unknown	2004	Unknown	JQ954718
2	M126	Yuanjiang	2011	Yun07-912	JQ954701
3	M119	Yuanjiang	2011	Yunyin58	JQ954719
4	M55	Changning	2008	Q170	JQ954717
5	M61	Yuanjiang	2008	Unknown	JQ954716
6	M71	Yuanjiang	2009	Badila	JQ954714
7	W14	France	2010	FR93-635	JQ954700
8	W17	Indonesia	2010	POJ2878	JQ954699
9	W18	Reunion	2010	R570	JQ954698
10	W4	Cuba	2010	MY55-14	JQ954720
11	ID	Indonesia	2010	Unknown	JF488066
12	JP1	Japan	2010	Unknown	JF488064
13	JP2	Japan	2010	Unknown	JF488065
14	Co419-24	Unknown	<2007	Co419	AM749404
15	Co7527-37	Unknown	Unknown	Co7527	AM749409
16	Co7527-39	Unknown	<2007	Co7527	AM749410
17	Co740-13	Unknown	<2007	Co740	AM749398
18	Co86032-17	Unknown	<2007	Co86032	AM920684
19	Co86032-19	Unknown	<2007	Co86032	AM920685
20	Co86032-4	Unknown	<2007	Co86032	AM920678
21	Co94012-4	Unknown	<2007	Co94012	AM920678

Table 11 (Continued)

No.	SCSMV isolates	Location of isolation	Collection year	Sugarcane varieties	GenBank acc. no.
22	Co95012-33	Unknown	<2007	Co95012	AM749407
23	CB671-1	Unknown	<2006	Unknown	DQ421788
24	IND671	Unknown	2011	IND671	JN941985
25	AP	Andhra Pradesh	Unknown	Unknown	Y17738
26	TPT	Unknown	2006	Unknown	GQ246187
27	PAK	Unknown	1983	Unknown	GQ388116

No. 14-26: SCSMV isolates from India with unknown location of isolation

No. 27: SCSMV isolate from Pakistan with unknown location of isolation

Figure 15 Alignment of coat protein amino acid sequences of 85 isolates of *Sugarcane streak mosaic virus* (SCSMV) showing more variable residues at the positions 1-31. Dot symbol represent conserved residues. Residue variations are indicated.

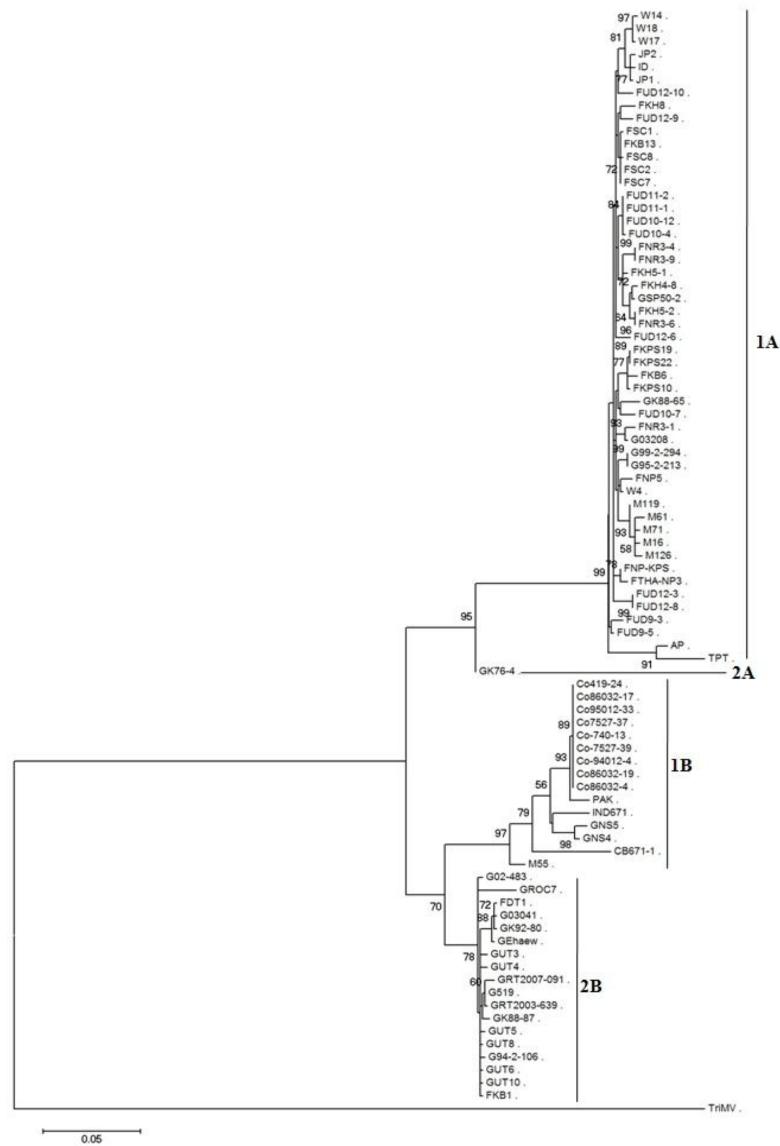

Figure 16 Maximum-likelihood tree based on the aligned CP nucleotide sequences of the 58 Thai SCSMV isolates and the 27 SCSMV isolates from other countries. The 13 SCSMV isolates from China are the followings: M16 (JQ954718), M126 (JQ954701), M119 (JQ954719), M55 (JQ954717), M61 (JQ954716), M71 (JQ954714), W14 (JQ954700), W17 (JQ954699), W18 (JQ954698), W4 (JQ954720), ID (JF488066), JP1 (JF488064) and JP2 (JF488065). The 13 SCSMV isolates from India are the followings: Co419-24 (AM749404), Co7527-37 (AM749409), Co7527-39 (AM749410), Co740-13 (AM749398), Co86032-17 (AM920684), Co86032-19 (AM920685), Co86032-4 (AM920678), Co94012-4 (AM920678), Co95012-33 (AM749407), CB671-1 (DQ421788), IND671 (JN941985), AP (Y17738), TPT (GQ246187), one SCSMV isolate from Pakistan (GQ388116) and an outgroup, TriMV (NC_012799).

9. **Phylogenetic network and genetic recombination of SCSMV in Thailand**

The splits networks based on the alignment of the CP gene of 58 Thai SCSMV isolates revealed that the recombination events occurred among Thai SCSMV isolates that divided Thai SCSMV isolates into two major network groups (Figure 17). The recombinant isolate GK76-4 shared with these two network groups suggested that recombination occurred between the virus isolates from the collected farmers and germplasm fields. Nine SCSMV isolates (AP, TPT, IND671, JP1, JP2, ID, M55, CB671-1 and PAK) with likely to be the recombinant were selected for splits network analysis with 58 Thai SCSMV isolates. The splitstree based on the selected 67 isolates exhibited two major network groups (Figure 17). JP1, JP2, ID, TPT and AP isolates shared the same network group with the collected farmer isolates. The second network group consisted of 4 isolates, CB671-1, M55, IND671 and PAK which shared the same network with the collected germplasm isolates (Figure 17).

Using RDP4 seven algorithms, four recombinant isolates, namely GK76-4, M55, CB671-1 and GROC7 were detected (Table 12). The recombinant isolate GK76-4 had two recombination breakpoints at the nucleotide position 1-196 and 694-846 (Table 12 and Figure 18). The recombinant isolate GK76-4 was distributed from the major parental isolate (FUD12-10) and the minor parental isolate (GRT2007-091) (Table 12). Another recombinant isolate, namely GROC7 had only one recombination breakpoint at the nucleotide position 562-798 which was contributed by the major parental isolate (GRT2007-091) and the minor parental isolate (FUD12-8) (Table 12 and Figure 19). The recombinant isolate M55 from China was distributed from the major parental isolate (GRT2007-091) and the minor parental isolate (PAK), while the recombinant isolate (CB671-1) from India was distributed from the major parental isolate from Thailand (FUD10-7) and the minor parental isolate from India (IND671). The recombination breakpoints of M55 were similar to positions of the recombinant isolate CB671-1 (Table 12). These results confirmed that the recombination occurred in the CP coding region among SCSMV isolates from different geographical regions, and sugarcane varieties in the presence of four recombinant isolates, GK76-4, GROC7, M55 and CB671-1 (Table 12).

The recombination breakpoints in the CP coding regions of all isolates were defined by examining the composition of nucleotide sequences such as nucleotide composition and restriction sites (Table 13). Only the restriction sites of *Xba*I, *Hae*III, *Hinc*II *and Taq*I were found in the recombination breakpoints of M55 and CB671-1 (Table 13).

Figure 17 Phylogenetic networks of SCSMV isolates based on analysis of the CP coding sequences.

The CP coding sequences obtained from the 67 SCSMV isolates (58 Thai isolates and 9 SCSMV isolates from other countries) were analyzed using the median network in SplitsTree4. The 9 SCSMV isolates are the following: ID (JF488066), JP1 (JF488064), JP2 (JF488065), PAK (GQ388116), IND671 (JN941985), AP (Y17738), TPT (GQ246187), M55 (JQ954717) and CB671-1 (DQ421788).

Table 12 Recombination events detected in the CP coding region of SCSMV isolates using RDP4 (version 4.5).

Event no.	Recombinant isolate	Recombination breakpoint	Major parent	Minor parent	Detection method*						
					R	G	B	M	C	S	T
1	K76-4	1-196, 694-846	UD12-10	RT2007-091	-	+	+	+	+	+	+
2	M55	99-831	RT2007-091	PAK	-	-	-	+	+	+	+
3	CB671-1	59-820	UD10-7	IND671	+	-	+	+	+	+	+
4	ROC7	562-798	RT2007-091	UD12-8	+	+	+	+	+	-	+

*The methods used for recombination detection are as the following, R: RDP, G: GENECONV, B: Bootscan, M: MaxChi, C: Chimaera, S: SiScan and T: 3Seq.

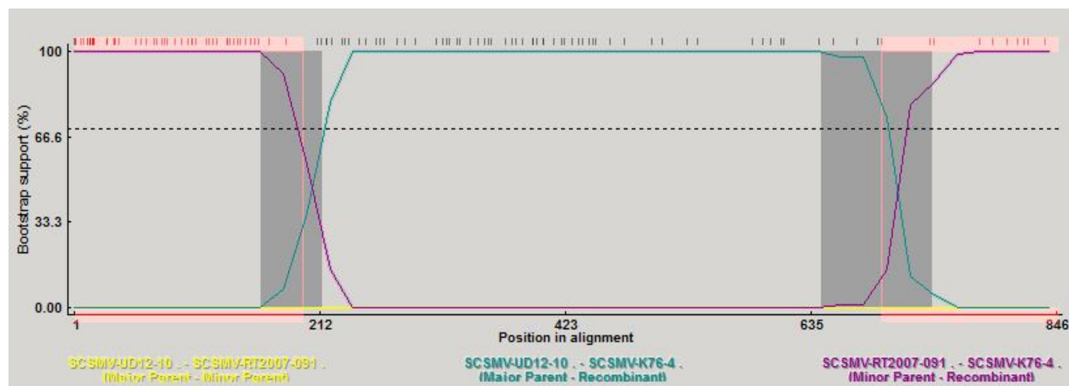

Figure 18 Prediction of the possible recombination in the CP coding region of the recombinant isolate GK76-4 by RDP4.

Pink areas represented recombination breakpoints at the positions, nt 1-196 and nt 694-846 of the isolate GK76-4 which was predicted by Bootscan method in RDP4.

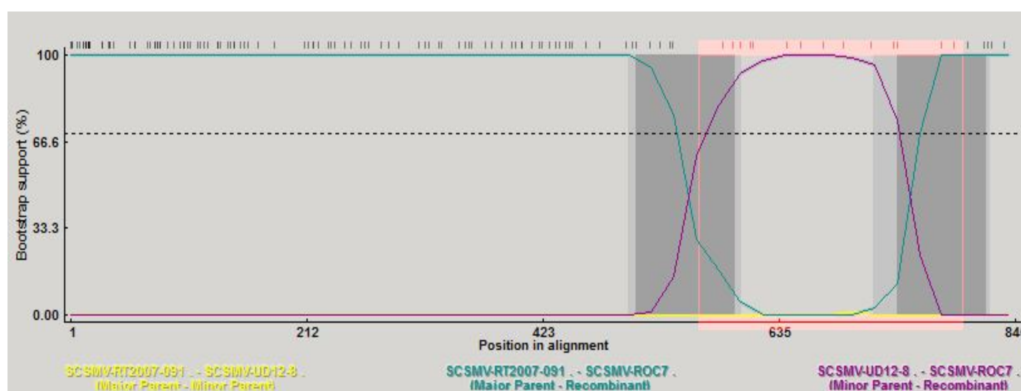

Figure 19 Prediction of the possible recombination in the CP coding region of the recombinant isolate GROC7 by RDP4.

Pink area represented recombination breakpoint at the positions (nt 562-798) of the isolate GROC7 which was predicted by Bootscan method in RDP4.

Table 13 Analysis of the recombination breakpoints in the CP coding region.

Recombination site	**Nucleotide composition**		**Restriction site***
	No. of A/U (%)	**No. of G/C (%)**	
1-196 (K76-4)	58.16	41.84	-
694-846 (K76-4)	43.79	56.21	-
59-820 (CB671-1)	51.18	48.82	*Xba*I, *Hae*III, *Hinc*II, *Taq*I
99-831 (M55)	51.57	48.43	*Xba*I, *Hae*III, *Hinc*II, *Taq*I
562-798 (ROC7)	52.74	47.26	-

* Restriction sites were obtained by nucleotide sequence analysis using CLC program.

Discussion

Yellow streak mosaic is a typical symptom of streak mosaic disease in sugarcane caused by *Sugarcane streak mosaic virus* (SCSMV). In addition, the mosaic symptoms in sugarcane are associated with several viruses such as *Sugarcane mild mosaic virus* (SCMMV), *Sugarcane striate mosaic associated virus* (SCSMaV), *Sugarcane mosaic virus* (SCMV) and *Sorghum mosaic virus* (SrMV) (Rott *et al.*, 2008). The typical symptoms and host ranges are similar among these viruses. In this study, SCSMV infected sugarcane from field surveys showed yellow streak mosaic symptoms, especially on young leaves while the older leaves showed mild symptoms. Host range tests on plant species in genus *Poaceae* including sorghum cv. UT325B and the commercial corn cv. Tender58 were investigated by mechanical inoculation. The typical symptoms of yellow streak mosaic were also exhibited on the inoculated sorghum and corn at 15 and 5 dpi, respectively (Figure 5A). These exhibited streak mosaic symptoms on the inoculated sorghum and corn were similar to those symptoms caused by SCMV and reported as potyvirus causing mosaic diseases in sugarcane, corn and sorghum in Thailand (Gemechu *et al.*, 2004).

Our results revealed the whole genome sequence of one isolate (THA-NP3). The complete genome sequence of THA-NP3 was successfully assembled from 11 overlapping sequences by using a set of primers (Table 1). All cleavage sites of THA-NP3 proteins as well as the position of amino acids were exactly identical to those of SCSMV isolates PAK, ID, JP1, JP2 and TPT (Table 3). The amino acid sequences of the conserved motifs were slightly different among seven complete genome isolates (Table 3). Nucleotide sequence comparisons among seven complete genome isolates (THA-NP3, PAK, ID, JP1, JP2, IND671 and TPT) revealed more genetic variations in the P1, HC-Pro and CP genes. Similar reports have concluded that these three proteins expressed genetic variability among SCSMV isolates from different sugarcane varieties (Bagyalakshmi *et al.*, 2012; He *et al.*, 2013). Amino acid sequence comparisons of the individual functional proteins among seven SCSMV isolates revealed that all proteins shared identity in the range of 90.64-100%, except the P1 of

IND671 and 6K1 of TPT, which shared 88.37% and 87.50% identities, respectively. However, the P1 of IND671 (361 aa) and 6K1 (56 aa) of TPT are larger than other isolates (Table 4).

Another approach focused on the production of SCSMV antiserum by using the purified virions as antigen. The purified virions of the isolate THA-NP3 revealed the flexuous rod ranging from 700-890 nm (Figure 6) and were characterized as the flexuous rod virus in genus *Poacevirus* in the family *Potyviridae* similar to those of *Triticum mosaic virus* (TriMV), a type member of the virus in genus *Poacevirus* (Seifers *et al*.,2008). The previous report revealed that SCSMV flexuous rod particles from India (Hema *et al*., 1999) and Indonesia (Damayanti and Putra 2011) were 890 nm in length, which were similar in size to our results. The virus yields, 0.25 mg per 100 g of infected sorghum leaf tissues were obtained in this study. The virus yields losses occurred due to virus aggregation that was found in some flexuous rod shaped viruses (Hammond and Lawson, 1988). SDS-PAGE of protein extract from purified preparation revealed the presence of viral coat protein subunits with a size of 31 kDa which were similar in size to the deduced coat protein, calculation from 846 nucleotides encoding 281 amino acid residues (Figure 7). In addition, the larger band of approximately 40 kDa of an unknown protein was also found (Figure 7) in the same purified preparation. However, the previous reports revealed that there were two sets of CP subunits, with sizes of 31 and 40 kDa in the purified preparation of SCSMV isolates from India, SCSMV-AP (Hema *et al*., 2002) and Indonesia, SCSMV-ID (Damayanti and Putra 2011).

The purified virions of the isolate THA-NP3 were successfully used as antigen to produce specific SCSMV antiserum. Specificity tests indicated that the SCSMV-THA antiserum was distinctive from an array of 12 other tested viruses that infected sugarcane, sorghum, corn and grasses (Figure 11). Previous studies reported that SCSMV-AP antiserum failed to react against U06-123, the isolate of TriMV (Hall *et al*., 1998; Seifers *et al*., 2008). In addition, the purified SCSMV-AP reacted positively to the antiserum against *Narcissus latent virus* (NLV), a member of the genus *Macluravirus* in the family *Potyviridae* while it could not react to *Sugarcane mosaic*

virus strains or potyvirus group-specific antiserum (Hema *et al*., 1999). The purified virions of THA-NP3 failed to react against the locally produced SCMV antiserum. Thus, our specially produced antiserum revealed high specificity only to SCSMV and was subjected to immunological diagnosis methods including DAC-ELISA and ICS for disease surveys of SCSMV infection in major sugarcane growing areas in Thailand.

Our results revealed that virus diagnosis by using DAC-ELISA and ICS could assist and facilitate the disease surveys. DAC-ELISA is a sensitive method for virus detection in plant extracts for large-scale testing while ICS provided simple field testing with results rapidly available within five minutes. Studies of SCSMV were performed from 2010 to 2014 at major sugarcane growing areas in Thailand including Nakhon Pathom, Kanchanaburi, Nakhon Ratchasima, Khon Kaen and Udon Thani provinces. The first disease survey in 2010 revealed sugarcane leaves showing yellow streak mosaic symptoms at the farmers' fields in the Kamphaeng Saen District of Nakhon Pathom Province. These sugarcane leaf samples failed to react with the locally produced antiserum against SCMV. In our subsequent study, we performed diagnosis by RT-PCR method using our designed primers, CP-F and CP-R (Table 1), then we cloned and sequenced the isolates. The results revealed that 588 bp (HQ171896) of the RT-PCR product matched SCSMV-CP in the databases. Thus, we strongly confirmed that the streak mosaic symptom in these sugarcane leaves was caused by SCSMV, as previously reported by Chatenet *et al*. (2005).

The second disease survey carried out from 2012 to 2014 revealed that the incidence of SCSMV was widespread across the major sugarcane growing areas in 5 provinces and germplasm collection fields in. Recently, there are eight sugarcane varieties that can be planted in all geographical areas such as K84-200, K88-92, K95-84, K92-11, K2000-89, KK3, UT3 and UT84-13 (Jaisil, 2014). Our surveys suggested that the commercial sugarcane varieties including germplasm collections were widely infected with SCSMV. The commercial sugarcane varieties such as UT3, UT4, UT5, UT6, UT8 and UT10 were more frequently infected with SCSMV. Other commercial sugarcane varieties such as K76-4, K88-65 and K88-87 were also found with SCSMV

infection. Additional widespread observation of SCSMV in many sugarcane fields occurred when these susceptible varieties became widely planted in Thailand sugarcane fields.

Our results revealed the genetic variability of 58 Thai SCSMV isolates based on analysis of the CP gene. By analyzing gene sequence variability, Thai SCSMV isolates were divided into two distinct groups (Figure 16), demarcating the virus obtained from farmers' fields and germplasm collections. However, some virus isolates from the farmers' fields were clustered in the same group with germplasm isolates. These results suggested that the variation of the CP gene occurred among various sugarcane varieties but was not associated with the geographical origin of the isolate. Network analysis of 58 Thai SCSMV isolates also confirmed that recombination events occurred in the CP gene among the virus isolates obtained from different fields and germplasm collections. Recombination detection by RDP4 also revealed two recombinant isolates, GK76-4 and GROC7 (Table 12). A previous studied revealed that the recombinant isolate from China, CB671-1 was distributed from the parents, W23×IND671 and three recombinant isolates (CB740, CB9217-1 and S-8) were distributed from the same parents, THA-NP3× CB671-1 (He *et al.*, 2013). In this study, we found that two recombinant isolates from China, CB671-1 and M55 were distributed from the parents, UD10-7×IND671 and RT2007-091×PAK, respectively. These results suggest that the recombination events occurred in the CP gene among the virus isolates from Thailand, China and India.

In conclusion, this is the first report on the incidence of SCSMV infection in the commercial sugarcane growing areas and germplasm collection fields of Thailand. These results will assist in improving the screening of sugarcane as well as the breeding of virus resistant sugarcane varieties.

CONCLUSION AND RECOMMENDATIONS

Conclusion

The complete genome of the virus isolate, THA-NP3 (JN163911) was first characterized in this study. Full length viral genome contained 9781 nucleotides, excluding 3′ Poly (A) tail. This complete genome encoded the polyprotein 3130 amino acid residues comprising 10 functional proteins, namely P1 (44.47 kDa), HC-Pro (54.41 kDa), P3 (38.01 kDa), 6K1 (5.43 kDa), CI (74.82 kDa), 6K2 (5.55 kDa), NIa-VPg (22.47 kDa), NIa-Pro (26.64 kDa), NIb (57.43 kDa) and CP (31.07 kDa). Phylogenetic analysis revealed that THA-NP3 formed the same cluster with JP1, JP2, ID and TPT isolates but separated from PAK and IND671 isolates.

The CP gene of Thai SCSMV isolates contained 846 nucleotides which encoded 281 amino acid residues. Sequence comparison revealed 86.17-100% nucleotide identities among 58 Thai SCSMV isolates and 85.70-99.29% nucleotide identities to SCSMV isolates from other countries. This gene showed high genetic variation which divided Thai SCSMV isolates into two distinct groups and involved in the potential recombination. SCSMV isolates from the farmers' fields formed in the first group while SCSMV isolates from germplasm collections formed in the second group.

The purified THA-NP3 showed the flexuous rods, ranged in length between 700-890 nm, indicating the characteristic of the virus in genus *Poacevirus* in the family *Potyviridae*. The viral coat protein with a size of 31 kDa was found in the purified preparation, and was successfully used for production of SCSMV antiserum. The antiserum and IgG were successfully used to develop the immunological methods such as DAC-ELISA and ICS for SCSMV detection.

The disease surveys from 2010 to 2014 revealed yellow streak mosaic symptoms in sugarcane and the causal agent was identified as SCSMV. The streak mosaic disease was widely distributed throughout the major sugarcane growing areas

in 5 provinces including Nakhon Pathom, Kanchanaburi, Udon Thani, Khon Kaen and Nakhon Ratchasima and germplasm collection fields of Thailand.

Recommendations

The immunological methods, DAC-ELISA and ICS revealed highly specific and sensitive detection of SCSMV in plant extracts. These two methods can be used for further study in screening for SCSMV resistant varieties. The commercial sugarcane, sorghum, and corn varieties including their germplasm collections should be evaluated for SCSMV resistance. The virus detection by using ICS provides convenient virus detection in field surveys and can be used as the first diagnosis.

In this study, sequence analysis of SCSMV genes revealed high variation in the CP, HC-Pro and P1 coding regions among SCSMV isolates from different locations. Thus, further studies for genetic variability among Thai SCSMV isolates should be investigated in the P1 and HC-Pro coding regions as well as analysis of the CP coding regions.

LITERATURE CITED

Abbott, K.V. 1961. A new strain of *Sugarcane mosaic virus*. **Phytopathology**. 51: 642.

Adam, M.J., J.F. Antoniw and C.M. Fauquet. 2005. Molecular criteria for genus and species discrimination within the family *Potyviridae*. **Arch. Virol**. 150: 459-479.

Bagyalakshimi, K., B. Parameswari, C. Chinnaraja, R. Karuppaiah, V. Ganesh Kumar and R. Viswanathan. 2012. Genetic variability and potential recombination events in the HC-Pro gene of *Sugarcane streak mosaic virus*. **Arch. Virol**. 157: 1371-1375.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. **Anal. Biochem**. 72: 248-254.

Bruen, T.C., H. Philippe and D. Bryant. 2006. A simple and robust statistical test to detect the presence of recombination. **Genetics**. 172: 2665-2681.

Byzova, N.A., I.V. Safenkova, S.N. Chirkov, A.V. Zherdev, A.N. Blintsov, B.B. Dzantiev and I.G. Atabekov. 2009. Development of Immunochromatographic Test Systems for Express Detection of Plant Viruses. **Appl**. **Bioch. Microbiol**. 45: 204-209.

Chatenet, M., C. Mazarin, J.C. Girard, E. Fermandez, D. Gargani, G.P. Rao, M. Royer, B. Lockhart and P. Rott. 2005. Detection of *Sugarcane streak mosaic virus* in sugarcane from several Asain countries. **Sugarcane Intl**. 23: 12-15.

Clark, M.F. and A.N. Adams. 1977. Characteristics of the microplate method of enzyme linked immuno-sorbent assay for the detection of plant viruses. **J. Gen. Virol**. 34: 475-483.

Chung, B.Y. W., W.A. Miller, J.F. Atkins and A.E. Firth. 2008. An overlapping essential gene in the *Potyviridae*. **Proc Natl Acad Sci USA**. 105: 5897-5902**.**

Damayanti, T.A. and K.L. Putra. 2011. First occurrence of *Sugarcane streak mosaic virus* infecting sugarcane in Indonesia. **J. Gen. Plant Pathol**. 77: 72-74.

Fellers, J.P., D. Seifers, M. Ryba-White and T.J. Martin. 2009. The complete genome sequence of *Triticum mosaic virus*, a new wheat-infecting virus of the High Plains. **Arch. Virol**. 154: 1511-1515.

Gemechu, A.L., P. Chiemsombat, S. Attathom, R. Lersrutaiyotin and K. Reanwarakorn. 2004. The variations among isolates of *Sugarcane mosaic virus* in Thailand as Determined by Virus-Host Interaction. **Kasetsart J. (Nat. Sci.).** 38: 369-379.

Gillaspie, A.G., R.G. Mock and F.F. Smith. 1978. Proc. Intl. Soc. **SugarCane Technol**. 16: 347-355.

Giorda, L.M., R.W. Toler and F.R. Miller. 1986. Identification of *Sugarcane mosaic virus* strain H isolate in commercial grain sorghum. **Plant Dis**. 70: 624-628.

Grisham, M.P. 1994. Strains of *Sorghum mosaic virus* causing sugarcane mosaic in Louisiana. **Plant Dis**. 78: 729-732.

Grisham, M.P. and Y.B. Pan. 2007. A genetic shift in the virus strains that cause mosaic in Louisiana. **Plant Dis**. 91: 453-458.

Hall, J.S., B. Adams, T.J. Parsons, R. French, L.C. Lane and S.G. Jensen. 1998. Molecular cloning, sequencing, and phylogenetic relationships of a new *Potyvirus*: *Sugarcane streak mosaic virus* and a reevaluation of the classification of the *Potyviridae*. **Mol. Phylogenet. Evol**. 10: 323-332.

Haseloff, J., P.T. Goelet, D. Zimmern, P. Ahlquist, R. Dasgupta and P. Kaesberg. 1984. Striking similarities in amino acid sequence among nonstructural proteins encoded by RNA viruses that have dissimilar genomic organization. **Proc**. **Natl**. **Acad**. **Sci**. **USA**. 81: 4358–62.

He, Z., W. Li, R. Yasaka, Y. Huang, Z. Zhang, K. Ohshima and S. Li. 2013. Molecular variability of *Sugarcane streak mosaic virus* in China based on an analysis of the P1 and CP protein coding regions. **Arch. Virol**. 13: 1854-1859.

Hema, M., M. Venkatranmana, H.S. Savithri and P. Sreenivasulu. 1999. Biological antigenic and genomic relationship among the virus isolates causing mosaic disease of sugarcane in South India. **Curr. Sci**. 77: 698-702.

Hema, M., N. Kirthi, P. Sreenivasulu, and H.S. Savithri. 2003. Development of recombinant coat protein antibody based IC-RT-PCR for detection and discrimination of *Sugarcane streak mosaic virus* isolates from Southern India. **Arch. Virol.** 148: 1185–1193.

Hema, M., P. Sreenivasulu and H.S. Savithri. 2002. Taxonomic position of *Sugarcane streak mosaic virus* in the family Potyviridae. **Arch**. **Virol**. 147: 1997-2007.

Huson D.H. and D. Bryant. 2005. Application of phylogenetic networks in evolutionary studies. **Mol. Biol. Evol**. 23: 254-267.

Juttupornpong, S., S. Srisink, W. Suchato, P. Thumthong, K. Lertprasertrat, U. Duanmeesuk and M. Sukhnimitre. 2012. Sugarcane varieties improvement of Suphan Buri agricultural research and development center. **Khonkaen Agr. J**. 3: 1-7.

Kasemsin, P., P. Chiemsombat and R. Hongprayoon. 2011. New virus disease of sugarcane caused by *Sugarcane streak mosaic virus*. **The proceeding of Thailand Research Symposium**, Bangkok, Thailand.

Koike, H. and A.G. Gillespie. 1989. In Ricaud, BT Egan, AG Gillespie, CG Hughes eds., "**Mosaic Disease of sugarcane-major disease**. pp 301-322.

Li, W., Z. He, S. Li, Y. Huang, Z. Zhang, D. Jiang X. Wang and Z. Luo. 2011. Molecular characterization of a new strain of sugarcane streak mosaic virus (SCSMV). **Arch. Virol**. 156: 2101-2104.

Lian, S., J-S. Lee, W.K. Cho, J. Yu, M-K. Ki, H-S. Choi and K-H. Kim. 2013. Phylogenetic and recombination analysis of *Tomato spotted wilt virus*. **PLOS ONE**. 8: 1-11.

Macintosh, S., D.J. Robinson and B.D. Harrison. 1992. Detection of three whitefly transmitted geminiviruses occurring in Europe by tests with heterologous monoclonal antibobies. **Ann**. **Appl**. **Biol**. 121: 297-303.

Martin D.P., B. Murrell, M. Golden, A. Khoosal and B. Muhire. 2015. Detection and analysis of recombination patterns in virus genome. **Virus Evol.** 1: 1-5.

McDaniel, L.L. and D.T. Gordon. 1985. Identification of a new strain of maize dwarf mosaic virus. **Plant Dis**. 69: 602-607.

McVean, G., P. Awadalla and P. Fearnhead. 2002. A coalescent-based method for detecting and estimating recombination from gene sequences. **Genetics**. 160: 1231-1241.

Nateewatana, S. 1985. A flexuous rod virus causing mosaic disease of sugarcane in Thailand. **M.S Thesis**. Kasetsart University, Bangkok.

Ohshima, K., Y. Yamaguchi, R. Hirota, T. Hamamoto, K. Tomimura, Z. Tan, T. Sano, F. Azuhata, J.A. Walsh, J. Fletcher, J. Chen, A. Gera and A. Gibbs. 2002. Molecular evolution of *Turnip mosaic virus*: evidence of host adaptation, genetic recombination and geographical spread. **J Gen Virol**. 83: 1511-1521.

Ohshima, K., Y. Tomitaka, J.T. Wood, Y. Minematsu, H. Kajiyama, K. Tomimura and A.J. Gibbs. 2007. Patterns of recombination in turnip mosaic virus genomic sequences indicate hotspots of recombination. **J Gen Virol.** 88: 298-315.

Parameswari, B., K. Bagyalakshmi, R. Viswanathan and C. Chinnaraja. 2013. Molecular characterization of Indian *sugarcane streak mosaic virus* isolate. **Virus Genes**. 46: 186-189.

Perez-Losada, M., M. Arenas, J.C. Galan, F. Palero and F. Gonzalez-Candelas. 2015. Recombination in viruses: Mechanisms, methods of study, and evolutionary consequences. **Infection**, **Genetics and Evolut**. 30: 296-307.

Prabowo, D.B., T. Hadiastono, T. Himawan and L.K. Putra. 2014. Detection disease of *Sugarcane streak mosaic virus* (SCSMV) via serological test on sugarcane (*Saccharum officinarum* L.), weed and insect vector. **Internat J of Sci and Res (IJSR)**. 3: 88-92.

Putra, L.K., A. Kristini, E.M. Achadian and T.A. Damayanti. 2014. *Sugarcane streak mosaic virus* in Indonesia: distribution, characterization, yield losses and management approaches. **Sugar Tech**. 16: 392-399.

Rao, G.P., M. Chatenet, J.G. Girard and P. Rott. 2006. Distribution of Sugarcane mosaic and sugarcane streak mosaic virus in India. **Sugar Tech**. 8: 79-81.

Rott, P., E. Fernandez and J.-C. Girard. 2008. "Mosaic diseases in sugarcane." In Characterization, diagnosis & management of plant viruses. **Industrial crops.** edited by G. P. Rao, S. M. P. Kanura and S. L. Lenardon, 99–110. Houston: Studium Press LCC.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. **Molecular Cloning: A Laboratory Manual**. 2nded. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA.

Scheel, T.K. H., A. Galli, Y.P. Li, L.S. Mikkelsen, J.M. Gottwein and J. Bukh. 2013. Productive homologous and non-homologous recombination of Hepatitis C virus in cell culture. **PLoS Pathog**. 9, e1003228.

Seifers, D.L., S. Haber,W. Ens, Y-M. She, K.G. Standing, and R. Salomon. 2005. Characterization of a distinct *Johnsongrass mosaic virus* strain isolated from sorghum in Nigeria. **Arch Virol**. 150: 557-576.

Seifers, D.L and T.J. Martin. 2008. *Triticum mosaic virus*: A new virus isolated from wheat in Kansas. **Plant Dis**. 92: 808-817.

Seifers, D. L., T. J. Martin, T. L. Harvey, J. P. Felleres and J. P. Michaud. 2009. Identification of the wheat curl mite as the vector of *Triticum mosaic virus*. **Plant Dis**. 94: 25-29.

Shukla D.D. and D.S. Teakle. 1989. *Johnsongrass mosaic virus*. AAB descriptions of plant viruses. **Aust. Applied Biol**. No. 340.

Silva K. N. and C. Nicolini. 2013. First Report of *Johnsongrass mosaic virus* (JGMV) Infecting *Pennisetum purpureum* in Brazil. **Plant Dis**. 97: 1003.

Srinivas, K.P., Ch.V. Subba Reddy, B. Ramesh, P. Lava Kumar and P. Sreenivasulu. 2010. Identification a virus naturally infecting sorghum in India as *Sugarcane streak mosaic virus*. **Eur.J.Plant Pathol**. 127: 13-19.

Susaimuthu, J., I.E. Tzanetakis, R.C. Gergerich and R.R. Martin. 2008. A member of a new genus in the *Potyviridae* infects Rubus. **Virus Res**. 131: 145-151.

Sztuba-Solinska, J., A. Urbanowicz, M. Figlerowicz and J.J. Bujarski. 2011. RNA-RNA recombination in plant virus replication and evolution. **Annu.Rev**. **Phytopathol**. 49: 415-443.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar. 2013. MEGA6: molecular evolution genetics analysis version 6.0. **Mol. Biol. Evolut**. 30: 2725-2729.

Tatineni, S., A.D. Ziems, N.S. Wegulo and R. French. 2009. *Triticum mosaic virus*: A distinct member of the family *Potyviridae* with an unusually long leader sequence. **Virol**. 99: 943-950.

Teakle, D.S. and N.E. Grylls. 1973. Four strains of sugarcane mosaic virus infecting cereals and other grasses in Australia. **Aust J Agric Res.** 24: 465-477.

Thongpaiyai, C., A. Wongpraneekul and P. Chatwachirawong. 2012. Genetic diversity and relationships among commercial sugarcane varieties in Thailand. **Khonkaen Agr. J**. 40-3: 60-67.

Tosic, M. and R.E. Ford. 1972. Grasses differentiating sugarcane mosaic and maize dwarf mosaic viruses. **Phytopathology**. 62: 1466-1470.

Umer Farooq, A.B., Y-X. Ma, Z. Wong, N. Zhuo and X. Wenxing. 2013. Genetic diversity analyses reveal novel recombination events in *Grapevine leafroll-associated virus* 3 in China. **Virus Reses**. 171: 15-21.

Verwoerd, T.C., B.M. Dekker and A. Hoekema. 1989. A small-scale procedure for the rapid isolation of plant RNAs. **Nucl. Acids Res**. 17: 2362.

Viswanathan, R., M. Balamuralikrishnan and R. Karuppaiah. 2008. Characterization and genetic diversity of *Sugarcane streak mosaic virus* causing mosaic in sugarcane. **Virus Genes**. 36: 553-564.

Worobey, M. and E.C. Holmes. 1999. Evolutionary aspects of recombination in RNA viruses. **J Gen Virol.** 80: 2535-2543.

Xu, D.L., G.H. Zhou, Y.J. Xie and R. Mock. 2010. Complete nucleotide sequence and taxonomy of *Sugarcane streak mosaic virus*, member of a novel genus in the family *Potyviridae*. **Virus Genes**. 40: 432-439.

CURRICULUM VITAE

NAME	**:** Miss Paweena Kasemsin
BIRTH DATE	**:** January 9, 1980
BIRTH PLACE	**:** Nakhon Phanom

EDUCATION **:**

YEAR	**INSTITUTE**	**DEGREE**
2003	Ubon Ratchathani Univ.	B.Sc. (Microbiology)
2007	Kasetsart Univ.	M.S. (Agricultural Biotechnology)

SCHORLARSHIP/AWARDS:

June 2004-May 2006:	National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathum Thani, Thailand for M.S. program in the field of Agricultural Biotechnology at Kasetsart University, Kamphaeng Saen Campus
June 2010-May 2013:	Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus and Center of Excellence on Agricultural Biotechnology, Science and Technology Office, Office of Higher Education Commission, Ministry of Education (AG-BIO/PERDO-CHE)
June 2012-May 2013:	Thailand Research Council
Feb 2012:	Praiseworthy Research Award from Thai Phytopathological Society