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Molecular characterization of *Tuberose mild mosaic virus* and preparation of its antiserum to the coat protein expressed in bacteria

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Abstract. A 2-kb DNA product was amplified from purified *Tuberose mild mosaic virus* (TMMV) virions as well as from infected tissues of tuberose by the use of degenerate primers for potyvirus. The PCR product was subsequently cloned and its sequence analyzed. It was found comprised of 1947 nucleotides (nts) corresponding to the 3´-terminal region of potyviruses. The deduced amino acid sequence contained 598 residues encoding part of the 3´-terminal region of NIb gene (319 residues) and the complete sequence of coat protein (CP) gene (279 residues). A 136 nts of non-coding region (NCR) was found located at the 3´-terminal region of the DNA. A genetic code for aphid transmissibility of potyviruses, DAG triplet, was found at the 19-21 residues from the N-terminus of CP gene. Compared to the known sequences of potyviruses, the percent of nucleotide identities of the CP gene and the NCR were less than 62% and 39%, respectively. Similarly, percent identities of TMMV's CP amino acid sequence to those of other known potyviruses were all below 58%, confirming our previous finding that TMMV is a new species of *Potyvirus*. Using directional cloning technology, a 39-kDa fusion protein containing a complete CP sequence of TMMV and a partial sequence encoded by the expression vector plasmid (pET-30b, Novagene) was highly expressed and purified from *E. coli* cell cultures. The antigenicity of the fusion protein was determined to be indistinguishable from the viral CP. Antiserum prepared against this fusion protein showed comparable reactivities in the serological detection of TMMV with the conventional antibodies against purified virus particles.

Keywords: 3´-terminal region; Antiserum; Expression vector; Fusion protein; *Potyvirus*; Sequences; *Tuberose mild mosaic virus*.

Introduction

Tuberose (*Polianthes tuberosa* L.) has been cultivated in Taiwan for over 300 years and has become an economically important ornamental bulb crop (Shen et al., 1987, 1993). *Tuberose mild mosaic virus* (TMMV), a newly recognized potyvirus, based on its serological and biological distinction to other known potyviruses, was recorded in 1998 (Chen and Chang, 1998). However, the molecular characteristics of this tuberose virus such as the coat protein (CP) gene sequence, the key taxonomic factor, have not been described (Chen and Chang, 1998). One of the major objectives of this study is to clone and analyze the sequences of the CP gene and the 3´-non coding region (3´-NCR) of TMMV. This sequence information is provided as molecular evidence for finalizing its actual taxonomic status. Secondly, due to generations of vegetative propagation, all of the major tuberose varieties in Taiwan have been found infected by TMMV. Preparation of specific antiserum was hampered by the difficulties in obtaining sufficient quantities of purified TMMV (Horner and Person, 1988). Recently an antiserum against TMMV was successfully prepared and applied in virus identification and indexing in Taiwan (Chen and Chang, 1998). Using the antiserum, a complete virus indexing and bulb certification program for tuberose has been developed (Chen et al., 1998; Chen and Chang, 1999). In addition, the clone of a virus-free multi-petal tuberose variety was rescued by meristem-tip tissue culture and identified by indexing with this antiserum (Chen et al., 1999). As a result, an industrialized virus-free tuberose bulb propagation program is becoming established. However, its success will depend upon a consistent supply of antiserum to TMMV. The efficient production of good quality antiserum to TMMV is not an easy task under conventional methods due to their instability and extremely low yield in tuberose tissue. In this study, we took the approach of cloning the complete coat protein (CP) gene and expressing it in a bacterial culture system. The bacteria-expressed viral CP was then efficiently purified in sufficient quantity for antiserum preparation. Serological experiments showed that antiserum prepared by this approach was equivalent to traditional antiserum against purified virions in the detection of TMMV in tuberose tissue.

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Materials and Methods

Virus Isolate and Maintenance

Tbr1 isolate, the original culture for identification, of TMMV was used in this study (Chen and Chang, 1998). Tbr1 infected tuberose bulbs were routinely stored at 5°C and planted in a 25°C growth chamber to propagate infected tissue for virion purification (Chen and Chang, 1998).

Viral RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA template for RT-PCR was isolated from TMMV virions purified as described previously (Chen and Chang, 1998). The purified virion (1 mg/ml) was treated with 1/10 volume of disruption buffer (40 mM Tris-HCl, pH 7.8, containing 20 mM sodium acetate, 1 mM EDTA and 1% [w/v] SDS), extracted with phenol/chloroform (1:1), precipitated with ethanol, and resuspended in 20 µl of sterilized water. The first strand of cDNA was synthesized from the viral RNA by a cDNA synthesis kit (Strategene, La Jolla, CA) with the addition of an oligo dT primer. Subsequently, a set of potyvirus degenerate primers (Zerbini et al., 1995) was used to PCR-amplify the 3´-terminal region of TMMV genome. The exTaq polymerase (TaKaRa, Shuzo Co., Shiga, Japan) was used in the amplification reaction for 30 cycles (Perkin-Elmer GeneAmp model 2400, Norwalk, CT) as follows: denaturing at 94°C for 1 min, annealing at 60°C for 30 s, and DNA synthesis at 72°C for 2 min. An elongation step at 72°C for 8 min was conducted during the last cycle. The amplification products were analyzed by electrophoresis in a 1% agarose gel.

Cloning and Sequencing

The amplified DNA product in a total of 200 µl of PCR reaction mixture was precipitated with ethanol, resuspended in 20 µl of sterilized water, and cloned into pCRII-TOPO vector (Invitrogen, California, USA) according to manufacturer's instruction. Plasmid clones with expected size DNA inserts were identified and used for sequence analyses (Sambrook et al., 1989). Sequencing of the target insert DNA was done by an automatic DNA sequencer (ABI PRISM 377, Perkin-Elmer, CA, USA) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, CA, USA). Sequence data was analyzed by the ScanDNASIS program (Hitachi Software Engineering America, Ltd., California, USA).

Expression of Viral Coat Protein Gene

Based on the sequence data obtained from the previous experiment, a primer pair was designed for the site-specific amplification of the putative complete CP gene of TMMV. The restriction enzymes *Nco*I and *Not*I digestion sites (underlined) were created at the 5´-end of the upstream (CP1: 5´- CATCAT<u>CCATGG</u>CTAGCGGGAATCC ACCACCA-3´) and downstream (CP2: 5´-TAATGT <u>GCGGCCG</u>CGAGGGAACACTA-3´) primer, respectively, to facilitate subsequent directional cloning of the CP gene into expression vector plasmid. Using this primer pair to react with purified TMMV RNA in PCR, DNA fragment corresponding to the estimated size of TMMV CP gene was amplified, cloned into pET-30b vector (Novagen, Inc., Madison, WI, USA) by a site-specific directional cloning procedure, and subsequently transformed into the *E. coli* strain DH5α . Bacterial clones containing inserted pET DNA were identified by PCR using the same primer pair (CP1/CP2) followed by subcloning into the *E. coli* strain BL21(DE3)pLysS (Novagen) for protein expression. The inducer, isopropyl β-D-thiogalactopyranoside (IPTG), was added to a bacterial culture in LB broth to make a final concentration of 1 mM for extra protein expression (Li et al., 1998).

Analysis of the Bacteria-Expressed Coat Protein of TMMV

About 4 h after adding IPTG, an aliquot of 5 ml of bacterial culture was sampled from the main culture, centrifuged at 8,000 rpm for 5 min, resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA) and treated with an equal volume of protein denaturing solution (0.25 M Tris-HCl, pH 6.8, containing 2% [w/v] of SDS, 4% [w/v] of 2-mercaptoethanol, and 10% [w/v] of sucrose). The sample was then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The size and expression level of viral CP was determined by western blotting analysis using an antiserum (#060) against traditionally purified TMMV virions (Chen and Chang, 1998). Antigenecity of the expressed viral CP relative to the native protein was determined by the SDS-immunodiffusion test (Chang et al., 1988).

Purification of Expressed Viral Coat Protein

Since the complete CP gene of TMMV was inserted into the pET-30b, the N-terminus of the protein translation cassette of which contained a histidine-tag (Figure 3), we were enabled to efficiently purify the expressed protein by histidine affinity column chromatography. One of the bacterial clones expressing a fusion protein that positively reacted to TMMV antiserum was selected and grown in modified M9 medium (Li et al., 1998). In order to purify the expressed TMMV CP, a 1,000 ml of bacterial culture was pooled 4 h after IPTG induction and centrifuged at 8,000 rpm for 10 min to collect the bacterial cells. The cell pellet was resuspended in 100 ml of cell-lysis buffer (50 mM NaH_2PO_4, 10 mM Tris-HCl, 100 mM NaCl, 8 M Urea, pH 8.0) and was frozen at -20°C overnight. After thawing in a 37°C water bath, the bacterial cells were further disrupted by a sonicater (VCX 600, Sonics & Materials Inc., CT) followed by centrifuging at 10,000 rpm for 15 min to remove the cell debris. The supernatant was then mixed with histidine resin (Talon Resin, Clontech, Inc., CA) and subjected to affinity chromatography purification according to the manufacturer's instruction. The eluted protein solution was adjusted to a concentration of 1.0 OD_{280} per ml for immunological studies.

Antiserum Preparation and Serological Test

An antiserum (#079) against the TMMV CP expressed in bacteria was prepared by immunizing New Zealand white rabbit intramuscularly. The immunization protocol was the same as in those described previously for preparing antisera against native viral antigens (Chang et al., 1988; Chen and Chang, 1998). SDS-immunodiffusion, ELISA, and direct tissue blotting tests (Chen and Chang, 1998, 1999) were carried out to compare the reactivity of this antiserum (#079) with the antiserum (#060) prepared against TMMV virion purified by traditional protocols.

Results

Cloning the 3´-Terminal Region of TMMV Genome and Analysis of its Sequence

A DNA fragment 2-kb in size was consistently amplified from TMMV RNA or from total RNA of TMMV-infected tissue by running a potyvirus-specific RT-PCR (Figure 1, lane 1, 2). The PCR product was cloned into pCRII-TOPO plasmid and subsequently sequenced. The DNA fragment was 1947 nucleotides (nts) long, corresponding to the 3´-terminal region of potyvirus genome

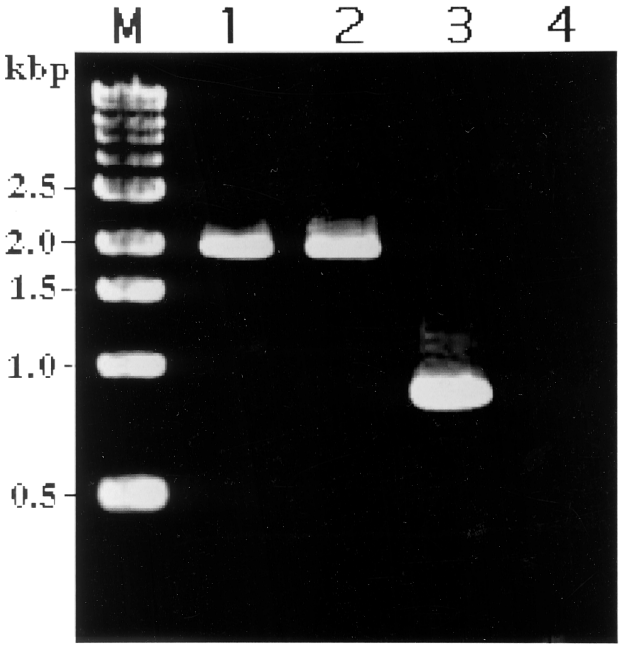

Figure 1. Agarose gel electrophoresis of DNA products derived by amplification of *Tuberose mild mosaic virus* (TMMV) RNA with potyvirus specific degenerate and TMMV coat protein specific primers by reverse-transcription polymerase chain reaction (RT-PCR). Lane M, molecular markers; lanes 1 and 2, DNA product amplified with potyvirus degenerate primers (Zerbini et al., 1995) from the RNA templates purified from TMMV virion and TMMV-infected tissue, respectively; lane 3, DNA product amplified from purified TMMV RNA with TMMV coat protein specific primers (CP1/CP2) as described in the text; lane 4, no product could be amplified from the RNA extracted from healthy tuberose tissue with CP1/CP2 primers.

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5´gtgtggatgactttcaattaccagttttatgatctacacatagcgggtccctggacagtt   60
ggcatgacaaaattcaacaggggatgggacgcgcttcttagcgagttaccagaaggctgg  120
ctttactgtgatgctgacgggtctcaattcgacagctctttatctccatacctcatcaac  180
tctgcgattcaaataagacaccatttttatggaggattgggctattggcgaaactatgttg  240
aggaatctgtataccgagatcgtttacacaccaatcgccacaccagatgggacagtcgtc  300
aagaagttcaagggcaacaacagcggacaaccatccacagttgtcgacaactcgctgatg  360
gtttgtgtcacaatgtttacgcgatggacaaggctggtgtcaacacacaaaaagtatatg  420
gatgtgcttcggttctttgtcacggagacgattggatcattgcactacggccagatatgt  480
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actcacaggacattcgaaaagagcgagctgtggttcatgtcgcaccaaggaatcaacaaa  600
gacggaatttatttttccgaagctagaaatggagagagtggtctccatactagtgtgggat  660
cgaagtagcgaaccggagcatagactggaggctatctgcgctgcaatgatcgaagcatgg  720
 R  S  S  E  P  E  H  R  L  E  A  I  C  A  A  M  I  E  A  W   20
ggtcatgaacaactgttgtaccaaatacgactgttctatgcatgggtgcttgagatggag  780
 G  H  E  Q  L  L  Y  Q  I  R  L  F  Y  A  W  V  L  E  M  E   40
ccatataaatcactagctacgattggcaaagcaccgtacatatctgagattgcactccga  840
 P  Y  K  S  L  A  T  I  G  K  A  P  Y  I  S  E  I  A  L  R   60
aagctgtatttggacgtcaagcattccgagcctgaactcgaagtttatttgcaatacatg  900
 K  L  Y  L  D  V  K  H  S  E  P  E  L  E  V  Y  L  Q  Y  M   80
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gtcccaaatgttgatggaattgccacacttagtcccgaacacttgctatcctacctacct 1200
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 R  D  M  H  T  L  L  G  V  R  N  L  K  G  P  I  I  N  *      378
tccctcgcggacacattaatgcattaatgttatgttgtttcatatttgtttcttatgtgc 1860
ctcaaactttcatgtttagtctcagtagcgaggttttacctccaagagcctttgcatggg 1920
agggcgaaacaaaaaaaaaaaaaaaa                                   1947
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Figure 2. The nucleotide and deduced amino acid sequences of the cloned 3´-terminal region of *Tuberose mild mosaic virus* (TMMV) genome. The putative protease cleavage site (Q/S) between nuclear inclusion protein b (NIb) and coat protein (CP) and the DAG triplet, a genetic indication for aphid transmissibility, are underlined. The cloned 1947 nucleotides of TMMV are shown starting from the first nt. The termination codon is indicated by an asterisk followed by 136 nts of non-translated region and 17 nts of poly-A tail. The sequence has been submitted to GenBank with the accession no. AF062926.

including a part of the NIb gene (957 nts), the CP gene (837 nts) and a 3´ non-coding region (NCR) of 136 nts followed by a poly-A tail of 17 nts (Figure 2). The deduced amino acid sequences of the partial NIb and the complete CP gene contained 319 and 279 residues, respectively (Figure 2). As with other potyviruses, the predicted protease recognition site for cleaving NIb and CP was between Q/S (Shukla et al., 1991). The DAG triplet needed for aphid transmissibility (Atreya et al., 1991) was found 19-21 amino acid residues from the N-terminus of CP (Figure 2). With the help of the ScanDNASIS program, the percent identities between nucleotide sequences of the CP gene and the 3´-NCR of TMMV to those of other known potyviral se-

quences documented in the GenBank were less than 62% and 39%, respectively (Table 1). In addition, the percent identities of the amino acid sequence of TMMV CP to other known potyviruses were below 58% (Table 1). These results provided strong evidence that TMMV was a unique member of the genus *Potyvirus*.

Expression of the CP Gene of TMMV in E. coli

Based on the derived 3´-terminal sequence of TMMV (Figure 2), two site-specific primers (CP1 and 2) were designed, and a 837 bp DNA product equivalent to the estimated size of the full length CP gene of TMMV was amplified by PCR (Figure 1, lane 3). This 837 bp DNA was subsequently constructed in expression vector pET-30b by directional cloning (Figure 3) and cloned in *E. coli* strain DH5α. After confirmation by PCR using CP1 and CP2 primers, six clones with correct DNA insert sizes were obtained, and the plasmids were then transformed into *E. coli* strain BL21 for protein expression. A protein about 39 kDa in size was detected by electrophoresis in bacterial lysates of transformed BL21 clones induced with IPTG, but not in lysates of control clones (Figure 4, A). Western blotting analyses showed that the 39-kDa protein as well as the TMMV CP purified from infected tissue both strongly reacted with TMMV antiserum #060 (Figure 4, B), indicating the expressed protein was antigenically related to the native TMMV coat protein. Furthermore, when compared in an SDS-immunodiffusion test they were antigenically indistinguishable as no spur reaction formed between their precipitation lines (Figure 5). These serological tests clearly showed that the cloned TMMV CP gene in pET-30b expressed a fusion protein antigenically indistinguishable from the native viral TMMV CP. In order to mass-produce the fusion protein for immunization, a high expressing clone, TM29, was selected for large-scale culture and the protein was purified by affinity chromatography. Routinely, about 4 mg of expressed protein was purified from 1,000 ml of bacterial culture.

Table 1. Percent identities of the nucleotide and amino acid sequence of the coat protein gene and the 3´-non coding region of *Tuberose mild mosaic virus* (TMMV) to those of known potyvirus species.

Potyvirus[b]	Percent identity[a]		
	CP		3´-NCR
	nt	aa	nt
BYMV	54.5	49.1	33.3
LMV	61.3	52.4	3.6
LYSV	58.5	55.1	12.3
PMtV	57.2	55.0	2.8
PPV	51.2	48.5	33.3
PRSV	55.4	49.2	0.9
PSbMV	30.8	51.0	0.0
PSMV	59.3	55.6	0.0
PVY	60.4	57.7	21.6
SMV	53.5	48.1	3.1
TEV	59.6	55.6	38.6
TuMV	57.3	55.4	36.9
TVBMV	25.5	52.6	0.0
WMV2	59.1	55.7	28.2
YaMV	53.7	49.4	36.3
ZYMV	60.8	57.0	3.3

[a]Percent identities of the respective nucleotide and amino acid sequences were analyzed by the ScanDNASIS programs (Hitach Software Engineering America, Ltd., California, USA).

[b]Sixteen potyviral sequences with highest percent identities are selected from the GenBank for comparison. The GenBank accession numbers: BYMV (*Bean yellow mosaic virus*, D28819), LMV (*Lettuce mosaic virus*, X97704), LYSV (*Leek yellow stripe virus*, D11118), PMtV (*Pepper mottle virus*, M96425), PPV (*Plum pox virus*, X81082), PRSV (*Papaya ringspot virus*, X78557), PSbMV (*Pea seedborne mosaic virus*, X89997), PSMV (*Pepper severe mosaic virus*), PVY (*Potato virus Y*, U09509), SMV (*Sugarcane mosaic virus*, U57354), TEV (*Tobacco etch virus*, M11458), TuMV (*Turnip mosaic virus*, L12396), TVBMV (*Tobacco vein banding mosaic virus*, L28816), WMV 2 (*Watermelon mosaic virus 2*, D13913), YaMV (*Yam mosaic virus*, U42596), ZYMV (*Zucchini yellow mosaic virus* , D13914).

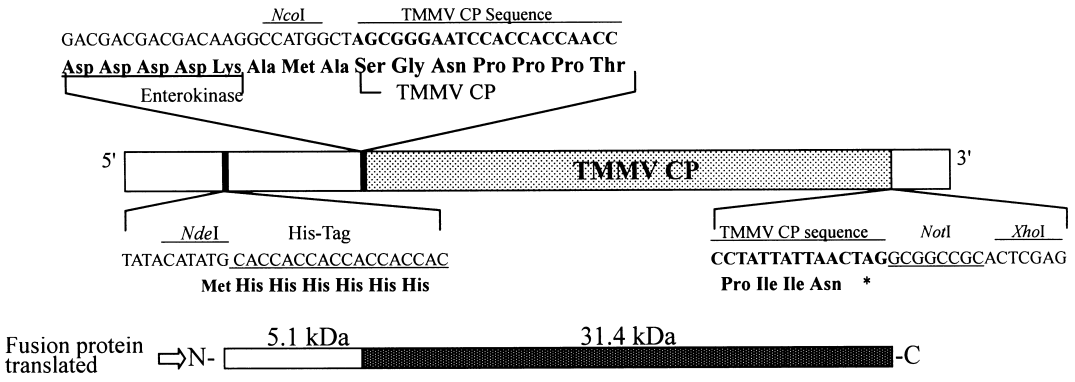

Figure 3. Schematic representation of the construction of full-length coat protein gene of *Tuberose mild mosaic virus* (TMMV-CP gene) in the expression vector pET-30b. The upper map shows the detail sequence composition in the linkage between TMMV-CP (▨), and the pET-30b (□) by *Nco*I and *Not*I restriction enzyme sites. Protein translation is done starting from the ATG codon of the *Nde*I site from the expression vector. After reading through a sequence encoded by the vector, translation is terminated at the TAG codon provided by the viral CP sequence. The lower map indicates the relative size of the translated protein with a 5.1 kDa vector-encoded fragment from the N-terminus and followed by the expressed 31.4 kDa of TMMV CP. The molecular size of the expressed fusion protein is calculated by ScanDNASIS program based on the amino acid sequence.

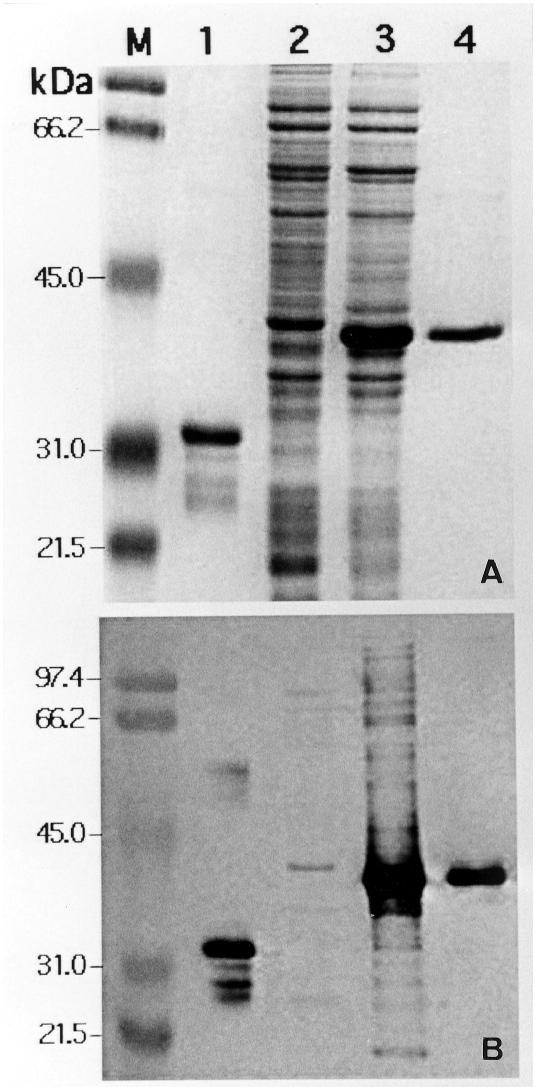

Figure 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the coat protein of *Tuberose mild mosaic virus* (TMMV) expressed in bacteria (A) and western blotting (B) with the antiserum against purified TMMV virion. Lane M, protein marker; lane 1, purified TMMV virion; lane 2, IPTG-induced bacteria (*E. coli* BL21(DE3)pLysS) lysate containing expression vector pET-30b without TMMV CP gene insert; lane 3, IPTG-induced bacteria lysate of clone TM29 containing TMMV CP gene inserted pET-30b; lane 4, bacteria-expressed TMMV CP purified by His-Tag affinity chromatography.

Serological Reactivity of the Antiserum Prepared Against Viral CP Expressed in Bacteria

An antiserum (#079) against the TMMV CP expressed in bacteria was successfully prepared, and its reactivities in ELISA, SDS-immunodiffusion, and direct tissue blotting tests as compared to the antiserum #060 against purified virions (Chen and Chang, 1998) were analyzed. In indirect ELISA, both antisera reacted with TMMV-infected tuberose tissue and the bacteria lysates containing expressed fusion protein, but did not react with healthy tuberose and the bacteria lysates without an insert of the TMMV CP gene (Table 2). Although ELISA tests implicated slight differences in the optimum reaction titers of these two antisera, both were successful in the detection of TMMV from tuberose tissue. On the other hand, the reactivities of antiserum #079 and #060 against the fusion protein expressed in bacteria were significantly different (Table 2). The dilution end point of the fusion protein in reacting with antiserum #060 was only 1/125, while that

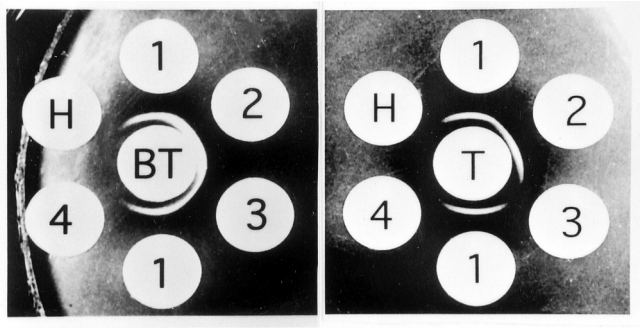

Figure 5. Reactivity of the antiserum prepared against coat protein (CP) of *Tuberose mild mosaic virus* (TMMV) expressed in bacteria as compared to the antiserum against purified TMMV virion in sodium dodecyl sulfate (SDS) immunodiffusion tests. The central wells BT and T were charged with antiserum #079 against bacteria expressed TMMV CP and antiserum #060 against purified TMMV virions, respectively. The peripheral wells were filled with SDS-treated antigens of TMMV-infected tuberose tissue (1), IPTG-induced lysate of bacteria (*E. coli* BL21(DE3)pLysS) clone TM29 containing TMMV CP gene inserted pET-30b (2), IPTG-induced bacterial lysate with pET-30b without TMMV CP gene insert (3), the same bacterial lysate as (3) but non-induced by IPTG (4), and healthy tuberose tissue (H), respectively.

Table 2. Reactivities in indirect ELISA of the antiserum #079 against 39-kDa *Tuberose mild mosaic virus* (TMMV) fusion coat protein expressed in bacteria compared to the antiserum #060 against TMMV virions.[a]

Antiserum	Five-fold dilution of antigens											
	Infected tuberose				CK-tuberose		Expressed lysates				CK-lysates	
	5^{-3}	5^{-4}	5^{-5}	5^{-6}	5^{-1}	5^{-3}	5^{-1}	5^{-3}	5^{-5}	5^{-7}	5^{-1}	5^{-3}
As #079	1.99[b]	0.88	0.20	0.01	0.01	0.07	3.45	3.35	3.14	2.88	0.09	0.04
As #060	1.42	0.43	0.08	0.02	0.01	0.01	0.55	0.30	0.06	0.05	0.04	0.01

[a]Antigen-coating type of indirect ELISA as described previously (Chen and Chang, 1999) was conducted to compare the reactivities of antiserum #079 with that of antiserum #060. Immunoglobulins of both antisera were diluted 1/1000 and alkaline phosphatase conjugated goat anti-rabbit immunoglobulin was used as the second antibody.

[b]Reactivities of the antisera are shown as the absorbance readings (A_{405nm}) taken 40 min after the addition of enzyme substrate solution.

against antiserum #079 was higher than $1/5^7$. This result indicated that antiserum #079 contained antibodies specific to the antigenic determinants residing in the vector-encoded protein sequence of the fusion protein. These antibodies however were not present in antiserum #060. Nevertheless, apparently these antibodies in antiserum #079 did not interfere with the detection of TMMV.

In SDS-immunodiffusion tests, antiserum #079 reacted strongly with IPTG induced bacterial lysates of clone TM29 and TMMV-infected tuberose tissues, and no discernible spur formed between them. It did not react with non-infected tuberose antigen. Unlike antiserum #060, #079 reacted with IPTG-induced control bacterial lysate (well 3, Figure 5) but not with the non-IPTG-induced culture (well 4, Figure 5). This result further confirmed the previous ELISA finding that antiserum #079 did contain antibodies specific to the vector-encoded fragment of the expressed fusion protein so that it reacted with the control bacterial lysate only after induction by IPTG. Without IPTG induction, the control lysate contained no vector protein and thus did not react with antiserum #079. Based on the intensity of the precipitation lines against TMMV-infected tuberose tissue, the reactions of antiserum #079 and #060 under the same dilution factor were indistinguishable (Figure 5).

In direct tissue blotting tests, both antisera #079 and #060 readily detected TMMV in infected tuberose bulbs or leaves (Figure 6). As in the results of SDS-immunodiffusion tests, no differences in the color intensity of positive reactions were observed between the two antisera.

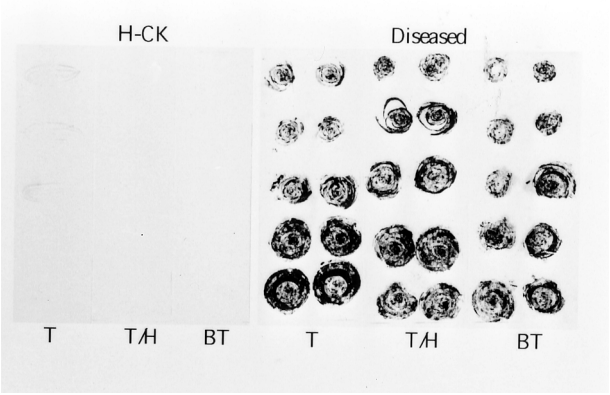

Figure 6. Reactivity of the antiserum prepared against coat protein (CP) of *Tuberose mild mosaic virus* (TMMV) expressed in bacteria as compared to the antiserum against purified TMMV virion in direct tissue blotting tests. Cross-sectioned TMMV-infected tuberose bulbs (Diseased) and healthy tuberose leaves (H-CK) were blotted on separated Nylon membrane (Duralon-UV membranes, Stratagene, La Jolla, CA) and reacted with the antiserum #079 against TMMV CP expressed in bacteria (BT), with antiserum #060 against purified TMMV virion (T), and with antiserum #060 but pre-absorbed with healthy tuberose tissue (T/H). The treated membranes were further reacted with alkaline phosphatase conjugated goat anti-rabbit immunoglobulin followed by treating with (BCIP/NBT) substrate solution as previously described (Chen and Chang, 1999). Positive reactions were shown by the dark bluish color on the blots.

However, antiserum #060 reacted slightly with control blots containing proteins from non-infected tissues, indicating a low background reaction still associated with it. Although this background reaction could easily be removed by cross absorption with non-infected tuberose antigen as shown in Figure 6, this procedure was unnecessary for antiserum #079.

Discussion

Based on our previous studies of serological and biological distinction (Chen and Chang, 1998), TMMV was proposed as a new species of the genus *Potyvirus*. It is currently the only documented potyvirus known to infect tuberose. In this study, we presented molecular evidence showing that the CP gene and 3´-NCR of TMMV has less than 62% identity with other known potyviruses at the nucleotide level. Similarly, the amino acid sequence identity of CP gene of TMMV with other known potyviruses was less than 58%. It is now widely accepted that amino acid sequence identities of CP among species of potyviruses ranged from 38 to 71% (average 54%) while those among strains of the same species ranged from 90 to 99% (average 95%) (Shukla and Ward, 1988, 1989; Ward et al., 1992). Therefore, our results of sequence comparison provide conclusive evidence that TMMV is a new species of *Potyvirus*.

TMMV was found in nearly every tuberose plant examined in Taiwan but it did not cause evident damage on cut flower production during the summer season. However, during other growing seasons when the temperature is lower than 25°C infected tuberoses tend to express pronounced foliar symptoms and usually their growth vigor is significantly reduced (unpublished data). Therefore, implementation with TMMV-indexed bulbs as growing materials is proposed as a solution to this problem. In order to eliminate the infection of TMMV from tuberoses, a constant supply of specific antiserum for indexing is necessary. However, tuberose is the only host plant found to be infected by TMMV, and the virus concentration in infected tissue is so low that purification of virus particle is difficult (Chen and Chang, 1998). It has been shown that plant virus antiserum can be prepared by immunizing the viral coat protein expressed in bacteria (Li et al., 1998; Nagel and Hiebert, 1985; Nikolaeva et al., 1995). In this study, we also successfully introduced a molecular technique to overcome the obstacle encountered in the purification of TMMV. A 39-kDa fusion protein containing complete CP sequence of TMMV (Figure 4) was efficiently expressed in bacterial culture and subsequently purified. It was shown that the bacteria expressed protein preserved an antigenicity indistinguishable from the native viral CP, although the antiserum prepared against the fusion protein did contain some antibodies specific to the amino acid sequence originated from the expression vector. However, ELISA, tissue blotting and SDS-immunodiffusion tests showed that the prepared antiserum was equivalent in reactivities and detected TMMV antigens to the traditionally made antiserum against TMMV virions.

Therefore, our results confirm that cloning and expressing the viral CP gene in a bacterial system can be applied in the purification of viral antigens for antiserum preparation or for other possible studies. Furthermore, we would particularly like to point out that the directional cloning approach in the construction of the CP gene expression cassette is more efficient than those applied by previous workers (Li et al., 1998; Nagel and Hiebert, 1985). We believe that the protocol developed in this study can easily and efficiently produce viral antigens in good quantities for advanced studies.

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夜來香微嵌紋病毒 3′ 端基因之序列分析及利用細菌表現之病毒鞘蛋白製備抗血清

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利用對應 *Potyvirus* 屬病毒基因體 RNA 3′ 端之簡併式引子對，可將夜來香微嵌紋病毒（*Tuberose mild mosaic virus*，TMMV）之 RNA 模版在反轉錄聚合酶連鎖反應下增幅出一個約 2 kb 之核酸片段。此核酸片段經選殖及核苷酸序列分析後，共解得全長含 1947 個核苷酸（nucleotide, nt）之序列。經分析比對後證實此序列的確符合 *Potyvirus* 屬病毒 3′ 端基因體之典型架構，由 5′ 端起此序列分別含 957 nt 之細胞核內含體 b（nuclear inclusion b, NIb）基因之 3′ 端部分序列、837 nt 之全長度鞘蛋白（coat protein, CP）基因及一個 136 nt 之 3′ 端非轉譯區（3′ non-coding region，3′-NCR）及 poly A 尾端。此序列經解析後只發現含有一個轉譯架構，其中所含之部份細胞核內含體 b 蛋白及全長鞘蛋白乃分別由 319 及 279 個氨基酸所組成，預估二者間之蛋白酶切位應落於符合 potyviruses 特質之 Q/S 之間，且由鞘蛋白 N 端起第 19-21 個氨基酸位置具有代表 potyviruses 蚜蟲傳播能力之 DAG 序列。與已登錄於 GenBank 之 potyviruses 基因比對，與 TMMV 親緣最接近之病毒其 CP 及 3′-NCR 區域中之核苷酸序列相同度分別只有 62 及 39%；CP 之氨基酸序列相同度則低於 58%。此結果確立 TMMV 為 *Potyvirus* 屬之新種病毒，同時也印證本實驗室過去以血清學方式證明 TMMV 乃獨立 potyviruses 的結果之正確性。根據解得之 TMMV CP 基因序列，本研究設計對應完整 CP 基因之專一性引子對，將其順利選殖於表現載體 pET-30b 上，並使其於 *E. coli* 宿主內大量表現一含有部份載體氨基酸序列之融合性 TMMV 鞘蛋白，並以親和性色層分析法回收純化，經免疫注射製成多元抗體。實驗證明此抗體可應用於 ELISA，雙重免疫擴散反應及免疫點漬法，順利偵測感病夜來香葉片及種球組織內之 TMMV，其偵測效果與傳統利用純化病毒顆粒所製備之抗體相當。

關鍵詞：夜來香微嵌紋病毒；3′端基因區域；基因序列；表現載體；融合性蛋白；抗血清。