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Short Communication

Immunogenicity of Tobamovirus Universal Antibodies Raised Against Expressed Protein Encoded by a Synthetic Polynucleotide Containing Epitope Genes of ToMV, ORSV, and CGMMV

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Abstract

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Using of synthetic peptide immunogens to produce antibodies with universal immunogenicity are a useful tool for diagnosis of plant viral diseases. Addition to the coat protein (CP) gene of *Tomato mosaic virus* (ToMV), we adopted the CP gene containing the predicted epitope sequences of *Odontoglossum ringspot virus* (ORSV) and *Cucumber green mottle mosaic virus* (CGMMV) to assemble into a fused polynucleotide. A total of 1,136 bases of synthetic nucleotides were joined covalently and created a recombinant plasmid cloned in the vector of pUC57 in *Escherichia coli*. The protein containing peptides encoded by the synthetic polynucleotide was expressed by the vector pET-28a. After purification, this expressed protein was used to immunize a New Zealand rabbit for production of a polyclonal antiserum. By Western blot, such an antiserum reacted with corresponding virus was examined for immunological properties and the coverage of antigenic universe. As the antiserum was applied to indirect enzyme-linked immunosorbent assay (ELISA), every tobamovirus tested in this study was detectable by the universal antibodies, but *Hibiscus latent Singapore virus* (HLSV) was an exception. A single reagent can detect a variety of tobamoviruses simultaneously; therefore, it can be practically applied to quarantine inspection and productions of Solanaceous seeds, Cucurbitaceous seeds, and some ornamental vegetative propagules.

Key words: Virus, Coat portion, Immunogen, Polyclonal antiserum, Western blot.

INTRODUCTION

The genus *Tobamovirus* of the family *Virgaviridae* consists of 37 accepted species of plant viruses [ICTV Online (10th) Report, https://talk.ictvonline.org/ictv-reports/ictv_online_report/]. Most species are pathogens to important crops and cause yield losses in nature. Some of tobamoviruses previously known as strains of *Tobacco mosaic virus* (TMV) are now defined as separate species according to their nucleotide sequence identities, host ranges, and antigenic relationships (Adams *et al*. 2017). Co-diverged with their hosts, tobamoviruses have been classified into 3 clusters: subgroup 1 includes those infecting Solanaceous plants,

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subgroup 2 includes those infecting cucurbits and legumes, and subgroup 3 includes those infecting crucifers (Lartey *et al*. 1996). Tobamoviruses are not practically vector-transmissible, however, they have been spread globally and cases of seed-transmission occur very often. Therefore, certifications of 'tobamovirus-free' are required by quarantine authorities when the plant materials, especially seeds, move internationally. There are 7 tobamoviruses listed as A2 quarantine pests in Taiwan. In border inspection, a broad-spectrum, simple, rapid, sensitive, and accurate method for detection of tobamoviruses is an ongoing requirement. Seed producers also need the user-friendly, liable, and wide effective detection tools for self-inspection during the growing and after the harvesting of seeds. For this purpose the universal antibodies may be provided as the diagnostic reagents.

Tobamovirus generally has stable rod-shaped virions and strong immunogenicity, such as TMV, which elicits antibodies to cross-react with many heterologous viral antigens (Bar-Joseph & Salomon 1980). However, some other species of this genus are still serologically distinct (Adkins *et al*. 2003). Tobamoviruses have a single-stranded RNA non-segmented genome with 6.3–6.6 kb in size. The downstream open reading frame (ORF) encodes the 17–18 kDa coat protein (CP), which is expressed from 3'-terminal subgenomic mRNAs. In this study, the predicted epitopes encoded by CP gene of *Odontoglossum ringspot virus* (ORSV) and that of *Cucumber green mottle mosaic virus* (CGMMV) were adopted to assemble into the CP gene of *Tomato mosaic virus* (ToMV) to produce multivalent antibodies. The immunological properties and the coverage of antigenic universe of the antibodies are revealed in the experiment.

SCHEME OF SYNTHETIC POLYPEPTIDE

The coat protein sequences of ToMV (Accession No. AJ132845.1), ORSV (Accession No. AF405727.1), and CGMMV (Accession No. KJ754196.1) were adopted from the National Center for Biotechnology Information (NCBI) for this study. From the Immune Epitope Database and Analysis Resource (IEDB), the sequences of epitope peptides of ORSV and CGMMV were searched by program of "Antibody Epitope Prediction" (http://tools.immuneepitope.org/bcell/) and the Kolaskar & Tongaonkar Antigenicity results shown in Table 1.

Table 1. Epitope peptides found in coat proteins of *Odontoglossum ringspot virus* (ORSV) and *Cucumber green mottle mosaic virus* (CGMMV)[z].

Virus	Start position	End position	Peptide sequence[y]	Peptide length
ORSV	10	17	KLAYLSSA	8
	24	34	LINLCTN	7
	46	59	QQQFADVWQPVPTL	14
	69	85	YFRVYRYDPILDPLITF	17
	119	126	DATVAIRS	8
	143	148	NQVSFE	6
CGMMV	8	32	PSKLIAFSASYVPVRTLLNFLVASQ	25
	49	59	LSALPSSVVDI	11
	69	86	YAFLNGPVLRPIFVSLLS	18
	94	100	VIEVVDP	7
	136	142	GFDVYDR	7
	148	154	AFSVVWS	7

[z] Searched by program of "Antibody Epitope Prediction" (http://tools.immuneepitope.org/bcell/).
[y] Epitope sequences assembled into the synthetic polypeptide (shown in Fig. 1) are marked with underlines.

The fragments of coat protein genes located at amino acid sequence of 82–378 of ORSV and that of 118–450 of CGMMV were adopted to join covalently with CP gene of ToMV. Resultant synthetic polynucleotide as shown in Fig. 1, in a total of 1,136 bases, nucleotides before sequence 485 were derived from ToMV CP gene, sequence 492–788 were from ORSV, and sequence 795–1,127 were from CGMMV. The recombinant plasmid was created with the vector of pUC57 then cloned in *Escherichia coli*. The vector is 2,710 bp in length, containing a

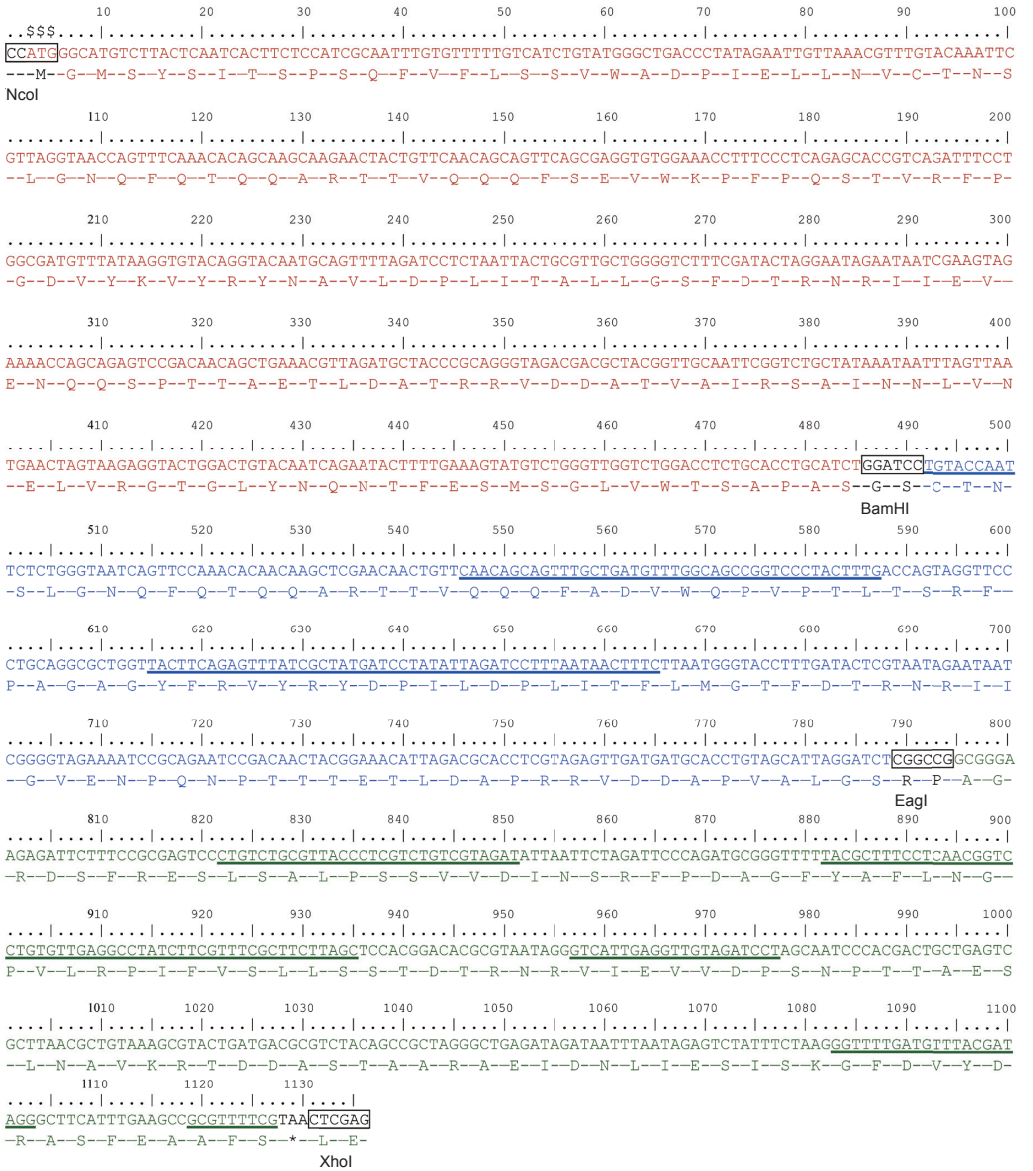

Fig. 1. Scheme of 1,136 bases of synthetic nucleotides encoding the polypeptide as immunogens used in this study. Nucleotide sequences before 485 are derived from coat protein (CP) gene of *Tomato mosaic virus* (ToMV), sequences 492–788 are from *Odontoglossum ringspot virus* (ORSV), and sequences 795–1,127 are from *Cucumber green mottle mosaic virus* (CGMMV). Sequences encode epitopes presented in each fragments are marked with underlines, and restriction sites are marked with boxes.

bla gene for ampicillin resistance and a *lacZ* gene for white/blue selection. In the study, the synthetic polynucleotide was purchased from Protech Technology Enterprise Co., Taipei, Taiwan by a specialized contract.

PROTEIN EXPRESSION AND ANTISERUM PRODUCTION

Followed by an overnight digestion with NcoI and XhoI at 37℃, the synthetic polynucleotide in the vector of pUC57 was cleaved and transferred to the vector of pET-28a. The subcloned plasmid in DH5α competent cell was cultured by lysogeny broth (LB) medium with adding of Kanamycin 50 μg mL^{-1}. After an overnight culture and transformation, the plasmids digested with NcoI and XhoI were checked for nucleotide sequence. A total of 1,128 bases of nucleotide cleaved from the vector were identical to that in pUC57 as original design. The schemed proteins encoded by these sequences were expressed by the vector pET-28a. The cloning and the expression systems were provided by Protech Technology Enterprise Co., Taipei, Taiwan. After purification, this expressed protein was used as an immunogen to immunize the New Zealand rabbit for production of polyclonal antiserum by Protech Technology Enterprise Co. From the resultant antiserum, immunoglobulin G (IgG) was purified and used in subsequent experiments.

VIRUS ISOLATES

The following tobamovirus isolates and their corresponding antisera shown in previous studies were collected, including CGMMV (Deng *et al*. 2013), *Hibiscus latent Singapore virus* (HLSV) (Deng *et al*. 2011), ORSV (Chang & Pang 1990), *Pepper mild mottle virus* (PMMoV) (Deng *et al*. 2014), *Plumeria mosaic virus* (PluMV) (Deng *et al*. 2015), TMV (Liao *et al*. 2007), ToMV (Deng *et al*. 2012), and *Wasabi mottle virus* (WMoV) (Deng *et al*. 2016). All the tobamovirus isolates were inoculated to *Chenopodium quinoa* Willd. and the virus-infected sap extracts were used for antigen preparation. The antisera tested were prepared in a laboratory at Taiwan Agricultural Research Institute except the diagnostic kits of PMMoV which was supplied by AC Diagnostics, Inc., Fayetteville, AR, USA.

WESTERN BLOT

As described by Towbin *et al*. (1979), tested virus coat proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to a nitrocellulose membrane. The membrane with transferred proteins was blocked, incubated with a primary antibody, washed, incubated with a conjugated secondary antibody, and washed again. The primary antibody is specific for the virus species tested and the secondary antibody was that of goat anti-rabbit antibody conjugated to alkaline phosphatase (AP) for subsequent detection. Colorimetric detection of alkaline phosphatase activity was tested by 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in conjunction with nitro blue tetrazolium (NBT). Blot images of various viruses detected by antibodies to PMMoV, TMV, ToMV, CGMMV, ORSV, WMoV, and HLSV, and the expressed immunogen was documented and shown in Fig. 2. Analyses of the relative signal intensities and molecular weights in these images reveal the antigenic property of each antibody reacted with 8 different tobamoviruses. Although CGMMV is serologically distinct from other tobamoviruses tested, the virus has become to be detectable by the antibodies raised from the expressed protein as the original scheme. Moreover, the universal antibodies have extended the coverage of antigenic universe to PMMoV, TMV, ToMV, WMoV, CGMMV, ORSV, HLSV, and PluMV with equivalent intensities, except HLSV. The unique serological relationships between HLSV and other tobamoviruses had also been indicated by Adkins *et al*. (2003).

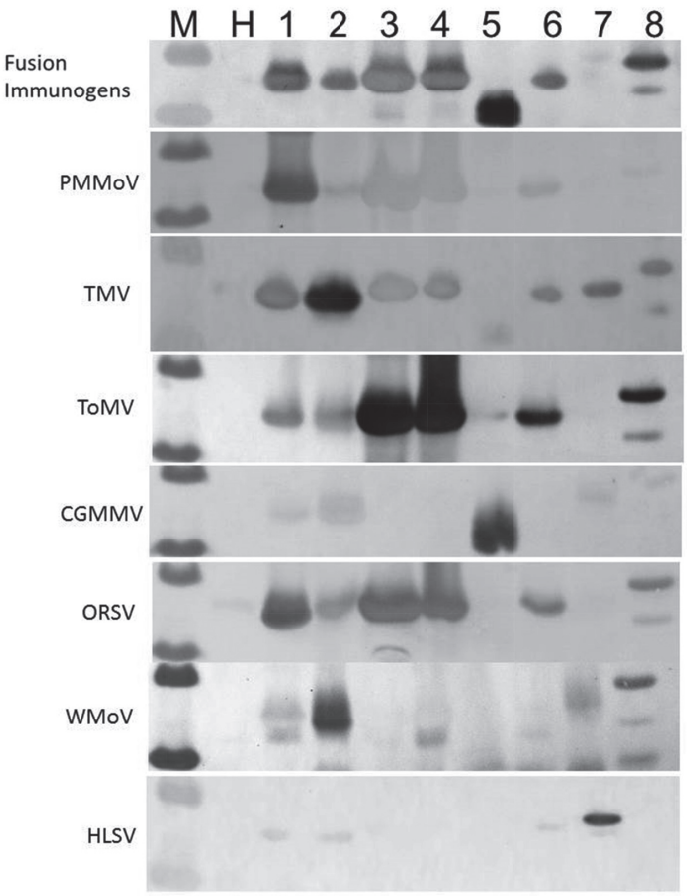

Fig. 2. Results of Western blot using antibody to "Fusion immunogens," *Pepper mild mottle virus* (PMMoV), Tobacco mosaic virus (TMV), *Tomato mosaic virus* (ToMV), *Cucumber green mottle mosaic virus* (CGMMV), *Odontoglossum ringspot virus* (ORSV), *Wasabi mottle virus* (WMoV), and *Hibiscus latent Singapore virus* (HLSV) on each row. The tested antigens are indicated on the top of each lane: M (molecular marker), H [healthy creatine kinase (CK)], 1 (PMMoV), 2 (TMV), 3 (ToMV), 4 (WMoV), 5 (CGMMV), 6 (ORSV), 7 (*Hibiscus latent Singapore virus*, HLSV), and 8 (*Plumeria mosaic virus*, PluMV).

INDIRECT ELISA

The indirect ELISA was performed as previously described by Koenig (1981) using 96-well polyvinyl microtiter plates. Per well was coated with 0.1 mL of antigen prepared by extracting crude sap of virus infected leaves in coating buffer. The primary antibody is specific for the virus species tested and the secondary antibody was the same as used by Western blot. ELISA set for detection of *Ribgrass mosaic virus* (RMV) was used as reference control, which was provided by DSMZ, Braunschweig, Germany. All of the antigens were tested at the same time with duplications. Nearly, every tobamovirus tested in this study is detectable by the universal antibodies, but HLSV is an exception (Table 2).

CONCLUSIONS

The immunological properties of resultant antiserum developed in this study showed that the synthetic polynucleotide containing epitope genes of 3 tobamoviruses had extended the coverage of antigenic universe as original

Table 2. Enzyme-linked immunosorbent assay (ELISA) values (A_{405nm}) of various tobamoviruses reacted with universal antibodies.

	Antigen									
Antibody to	HCK[z]	PMMoV	TMV	ToMV	CGMMV	ORSV	WMoV	HLSV	PluMV	RMV
Tobamo	0.000	0.243	0.204	0.878	1.438	0.878	1.305	0.096	1.054	0.616
PMMoV	0.000	0.611	0.012	0.173	0.059	0.111	0.252	0.079	0.030	0.067
TMV	0.177	0.257	0.149	0.222	0.031	0.163	0.363	0.281	0.229	0.787
ToMV	0.037	0.168	0.363	1.926	0.042	1.587	2.216	0.000	1.131	0.825
CGMMV	0.000	0.000	0.000	0.000	2.233	0.000	0.000	0.082	0.051	0.013
ORSV	0.000	0.187	0.096	0.911	0.004	0.585	1.333	0.014	0.208	0.353
WMoV	0.105	0.114	0.152	0.150	0.197	0.197	0.301	0.277	0.191	1.103
HLSV	0.010	0.141	0.048	0.046	0.006	0.050	0.042	0.712	0.012	0.006
RMV	0.057	0.005	0.057	0.073	0.010	0.082	0.114	0.148	0.084	0.780

[z] Abbreviation, HCK: healthy creatine kinase; PMMoV: *Pepper mild mottle virus*; TMV: *Tobacco mosaic virus*; ToMV: *Tomato mosaic virus*; CGMMV: *Cucumber green mottle mosaic virus*; ORSV: *Odontoglossum ringspot virus*; WMoV: *Wasabi mottle virus*; HLSV: *Hibiscus latent Singapore virus*; PluMV: *Plumeria mosaic virus*; and RMV: *Ribgrass mosaic virus*.

scheme. Species of *Tobamovirus*, especially those with seed-transmissibility, are quarantine important in the world. A broad-spectrum, sensitive and accurate detection method is required by quarantine authorities and also by seed producers. If the universal antibodies were applied, tobamoviruses such as PMMoV, TMV, ToMV, WMoV, CGMMV, ORSV, HLSV, PluMV, and RMV were all detectable by indirect ELISA. As a single reagent can detect a variety of tobamoviruses simultaneously, it is a cost-effective way to test the existence of these viruses. Therefore, it will be practically applied to virus inspection in quarantine inspection and productions of Solanaceous seeds, Cucurbitaceous seeds, and some ornamental vegetative propagules.

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REFERENCES

Adams, M. J., S. Adkins, C. Bragard, D. Gilmer, D. Li, S. A. MacFarlane, S. M. Wong, U. Melcher, C. Ratti, K. H. Ryu, and ICTV Report Consortium. 2017. ICTV virus taxonomy profile: *Virgaviridae*. J. Gen.Virol. 98:1999–2000.

Adkins, S., I. Kamenova, D. Achor, and D. J. Lewandowski. 2003. Biological and molecular characterization of a novel tobamovirus with a unique host range. Plant Dis. 87:1190–1196.

Bar-Joseph, M. and R. Salomon. 1980. Heterologous reactivity of tobacco mosaic virus strains in enzyme-linked immunosorbent assays. J. Gen. Virol. 47:509–512.

Chang, C. A. and J. H. Pang. 1990. Preparation of antisera against *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* and their uses in serological indexing for orchid industry in Taiwan. Plant Prot. Bull. 32:336. (abstract in Chinese)

Deng T. C., C. T. Chen, C. H. Tsai, F. Y. Ning, and Y. H. Cheng. 2014. *Pepper mild mottle virus* detected in pepper seeds for exporting quarantine test. Plant Pathol. Bull. 23:196–197.

Deng, T. C., M .C. Chen, C. H. Tsai, Y. T. Lin, and J. Y. Liao. 2011. The occurrence of *Hibiscus chlorotic ringspot virus* and *Hibiscus latent Singapore virus* in Malvaceae plants in Taiwan. p.V05. *in*: 2010 Annual Meeting of Taiwan Phytopathological Society. March 25–26, 2011. Taichung, Taiwan. Taiwan Phytopathological Society, Taichung, Taiwan. (in Chinese)

Deng, T. C., Y. T. Lin, C. H. Tsai, J. Y. Liao, Y. H. Cheng, Z. H. Lee, C. C. Shih, and Y. C. Chen. 2012. Identification and occurrence of a *Tomato mosaic virus* infecting pepino (*Solanum muricatum*) plants in Penghu,

Taiwan. Plant Protect. Bull. 54:47–64. (in Chinese with English abstract)

Deng, T. C., C. H. Tsai, Y. H. Chien, M. J. Lin, and M. Y. Chen. 2015. Chacterization of a tobamovirus isolated from desert rose (*Adenium obesum*) in Kaohsiung, Taiwan. Plant Pathol. Bull. 24:152–153. (in Chinese)

Deng, T. C., C. H. Tsai, and F. Y. Ning. 2016. Identification of *Wasabi mottle virus* infecting wasabi in Taiwan. J. Taiwan Agric. Res. 65:103–108. (in Chinese with English abstract)

Deng, T. C., C. H. Tsai, H. L. Tsai, J. Y. Liao, and Y. H. Cheng. 2013. Detection of *Cucumber green mottle mosaic virus* carried by seeds of bottle gourd (*Lagenaria siceraria* L.). Seed Nursery (Taiwan) 15:37–56. (in Chinese with English abstract)

Koenig, R. 1981. Indirect ELISA methods for the broad specificity detection of plant viruses. J. Gen. Virol. 55:53–62.

Lartey, R. T., T. C. Voss, and U. Melcher. 1996. Tobamovirus evolution: Gene overlaps, recombination, and taxonomic implications. Mol. Biol. Evol. 13:1327–1338.

Liao, J. Y., C. C. Hu, J. L. Kao, and T. C. Deng. 2007. Identification of *Tobacco mosaic virus* infecting *Rehmannia glutinosa*. Plant Pathol. Bull. 16:61–69. (in Chinese with English abstract)

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350–4354.

以含 ToMV、ORSV 及 CGMMV 表位基因的合成聚核苷酸所表現的蛋白製備 Tobamovirus 廣效性抗體的抗原性

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摘要

周建銘、陳君弢、蔡錦慧、陳金枝、鄧汀欽。2018。以含 ToMV、ORSV 及 CGMMV 表位基因的合成聚核苷酸所表現的蛋白製備 Tobamovirus 廣效性抗體的抗原性。台灣農業研究 67(3):301–308。

利用合成肽當免疫體原製備具有廣效的抗體，是一種實用的植物病毒診斷工具。我們設計以番茄嵌紋病毒 (*Tomato mosaic virus*; ToMV) 鞘蛋白基因序列為主體，加上齒舌蘭輪斑病毒 (*Odontoglossum ringspot virus*; ORSV) 及胡瓜綠斑嵌紋病毒 (*Cucumber green mottle mosaic virus*; CGMMV) 含表位基因的部分核苷酸序列，組裝成一聚核苷酸。合成聚核苷酸總共由 1,136 個鹼基共價連接，以質體轉殖到大腸桿菌中的 pUC57 載體中，再轉殖到 pET-28a 載體生產表現蛋白，經純化的表現蛋白用以注射白兔生產多原抗血清。利用西方墨漬法 (Western blot) 分析，可檢驗本研究所生產的抗血清與相對應病毒反應的免疫特性及抗原廣效性。製成酶聯抗體免疫酵素分析 (enzyme-linked immunosorbent assay; ELISA) 試劑，以間接法試驗，除 *Hibiscus latent Singapore virus* (HLSV) 以外，所有供試 tobamoviruses 都可有效被檢出。由於一種試劑即可同時檢出多種病毒，將可應用在檢疫或種苗生產時，茄科及葫蘆科種子或蘭花等觀賞作物繁殖體的病毒檢查。

關鍵詞：病毒、鞘蛋白、免疫體原、多原抗體、西方墨漬法。

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