Plant Pathol. J. 20(3) : 206-211 (2004)

Virus Resistant and Susceptible Transgenic *Nicotiana benthamiana* Plants Expressing Coat Protein Gene of *Zucchini green mottle mosaic virus* for LMO Safety Assessment

Minjea Kim[1], Sun Hee Choi[1], Taesung Kim[2,3], Min Hye Park[1], Hee Rae Lim[1], Kyung Hee Oh[2], Taesan Kim[4], Min Hyo Lee[2] and Ki Hyun Ryu[1]*

[1]*Plant Virus GenBank,Division of Environmental and Life Sciences, Seoul Womens University, Seoul 139-774, Korea*
[2]*School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea*
[3]*Biological Resources Division, Biodiversity Research Department, National Institute of Environmental Research, Incheon 404-170, Korea*
[4]*National Institute of Agricultural Biotechnology, RDA, Suwon 441-707, Korea*

(Received on January 26, 2004; Accepted on July 14, 2004)

Transgenic *Nicotiana benthamiana* plants harboring coat protein (CP) gene of *Zucchini green mottle mosaic virus* (ZGMMV) were generated for virus-resistant screening and complementation analysis of related viruses for environmental safety assessment (SA) of living modified organism (LMO) purposes. Transformation of leaf disc of *N. benthamiana* was performed by using *Agrobacterium*-mediated method and the pZGC-PPGA748 containing the ZGMMV CP and NPTII genes. Two kinds of transgenic homozygous groups, virus-resistant and virus-susceptible *N. benthamiana* lines, were obtained by screening of challenging homologous virus for T1 generations. These two pathologically different lines can be useful for host-virus interactions and LMO environmental SA.

Keywords : living modified organism (LMO), *Tobamovirus*, transformation, transgenic plant, *Zucchini green mottle mosaic virus*

The genus *Tobamovirus* is one of the biologically and molecularly best-characterized plant virus groups with host plants (Lewandowski et al., 2000). Tobamoviruses are widespread in a wide host range throughout the world, and cause substantial crop losses. Species of the genus cause diseases in tobacco, tomato, pepper, orchid, cucumber, melon, bean, and cactus or crucifer plants (Lewandowski et al., 2000). These viruses are easily transmitted mechanically, and are very stable physically and chemically. Tobamoviruses infect almost all cells within the systemic host plant, reaching high titers. Tobamoviruses usually cause disease by preventing proper chloroplast development, resulting in leaves and fruit with a mottle and mosaic pattern of light and dark green on stunted plants.

There are at least 4 different Tobamoviruses: *Cucumber green mottle mosaic virus* (CGMMV), *Cucumber fruit mottle mosaic virus* (CFMMV), *Kyuri green mottle mosaic virus* (KGMMV), and *Zucchini green mottle mosaic virus* (ZGMMV) in cucurbit crops in the world (Antignus et al., 2001; Ryu et al., 2000; Tan et al., 2000; Ugaki et al., 1991; Yoon et al., 2001). Information on their genome sequences and comparative analysis has been determined (Antignus et al., 2001; Ryu et al., 2000; Tan et al., 2000; Ugaki et al., 1991; Yoon et al., 2001).

CGMMV is the first known cucurbit-infecting *Tobamovirus*. CGMMV was first reported from the UK and Europe. Francki et al. (1986) reported that CV3, CV4, and CGMMV-W are serologically closely related and have a high degree of RNA sequence similarity. They also found the lack of detectable sequence similarity and the remote serological relationship among the three CGMMV isolates and CGMMV-C. This led them to consider that the latter is a distinct virus, and they renamed CGMMV-C as KGMMV.

Moreover, CGMMV-Y has been reclassified as KGMMV-Y based on the sequence analysis. Subsequently, Ryu et al. (2000) reported that the virus isolated from zucchini squash and KGMMV was a different species in the genus *Tobamovirus* based on CP sequence homology and serological specificity among the Tobamoviruses, including CGMMV and KGMMV, and was renamed as ZGMMV. Very recently, Yoon et al. (2002) reported the complete nucleotide sequence and overall properties of type strain of ZGMMV.

We are interested in the evolution of cucurbit-infecting tobamoviruses and resistance mechanism and their pathogenicity. Thus, in this study, transgenic *Nicotiana benthamiana* plants expressing coat protein gene of ZGMMV were screened for their resistance against homologous pathogen.

Some transgenic lines show susceptibility to target virus,

*Corresponding author.
Phone) +82-2-970-5618, FAX) +82-2-970-5610
E-mail) ryu@swu.ac.kr

and this study is useful for learning virus-host interactions and complementary analysis of defected viral cDNA constructs of ZGMMV. Here, we report and discuss the construction of model plants for virus-host interactions and living modified organism (LMO) environmental safety assessment.

Materials and Methods

Source of virus. ZGMMV-Zu, a type isolate of the virus, was used in this study (Ryu et al., 2000). The virus was purified from zucchini squash cv. Black Beauty plants inoculated with the virus. Viral genomic RNA was extracted from purified virion particles by using SDS/proteinase K and phenol extraction followed by ethanol precipitation. *Nicotiana benthamiana*, used as plant materials for transformation, was sown in sterilized tissue culture glass.

Coat protein gene cloning. Purifed viral RNAs were used for coat protein (CP) gene amplification. The 3' termini of the purified genomic viral RNAs were polyadenylated by using *Escherichia coli* poly (A) polymerase (Gibco BRL, MD). cDNA was synthesized from 10 μg of polyadenylated viral genomic RNA by using the Plasmid Choice cDNA Synthesis & Cloning Kit(Gibco BRL, MD). Double-stranded cDNA molecules were cloned into *Not*I/*Sal*I site of pSPORT1 vector (Gibco BRL, MD). The recombinant plasmids were transfected into *E. coli* JM109 strain. Subclones containing CP genes of ZGMMV were sequenced by the dideoxynucleotide chain termination method using the Model 377 automated DNA sequencer (ABI Perkin-Elmer, PVGABC).

Construction of plant transformation vector. A cDNA clone of the ZGMMV CP gene was excised by *Eco*RI and inserted in the sense orientation between the CaMV 35S promoter and NOS-terminator site of the pGA748 plant transformation vector (Ryu and Park, 1995). The resulting recombinant pZGCPPGA748 was transformed into *E. coli* JM109 strain. The recombinant DNA was isolated from the transformed bacteria and was introduced into *Agrobacterium tumefaciens* LBA4404 by freeze-thaw method (An et al., 1988). Recombinant clone of the *A. tumefaciens* was selected on YEP agar medium supplemented with kanamycin (50 μg/ml). Isolation of plasmid from the *A. tumefaciens* was carried out by the method of An et al. (1988).

Transformation of *Nicotiana benthamiana*. Transformation and regeneration were carried out as described by Horsch et al. (1985) and by Ryu et al. (1998). Construct of pZGCPPGA748 was transferred into *N. benthamiana* plants by the *Agrobacterium*-mediated transformation method (An et al., 1988). Leaf tissues from *in vitro* cultured *N. benthamiana* were used for transformation. The tissues were cut into small pieces and the sections were incubated overnight in a MS medium. The sections were soaked in a fresh overnight culture of *A. tumefaciens* for 20 min. Explants were then blotted dry on a sterilized filter paper and placed onto solidified media in disposable plastic petri dishes for cocultivation with the bacteria. Calli were induced from the leaf tissues treated with the bacteria on a MS selective medium containing 300 μg/mL kanamycin.

After transfers for 10 weeks, the calli were transferred to a MS medium not supplemented with kanamycin to promote callus proliferation. Shoots were generated, and these were subsequently induced to establish whole plants. Regenerated plants were selected on kanamycin and then rooted. Young plantlets with roots were transferred to soil and grown in a greenhouse. Transgenic *N. benthamiana* plants were screened for the presence of the ZGMMV CP gene by using PCR (Sambrook et al., 1989).

Virus challenge inoculation. Two leaves of each plant were dusted with Carborundum (300 mesh) and inoculated with purified virus diluted with a 50 mM phosphate buffer (pH 7.0). Four to five leaf-stage transgenic *N. benthamiana*, transformed with the ZGMMV CP gene and nontransformed control plants, were challenged with ZGMMV by mechanical inoculation. The ZGMMV was used as an inoculum. Plants were observed daily for symptom development after inoculation of the virus. Genomic DNA of *N. benthamiana* was extracted from leaf tissues by using the CTAB method for PCR analysis of transgene. Total proteins were extracted from whole leaf tissues by homogenizing the samples in a 0.01 M phosphate buffer.

Antibody against ZGMMV was produced in New Zealand white rabbits and used for western blot analysis. ZGMMV CP, extracted from infected plant tissue, was separated on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose (NC) membrane by electro-blotting using a transfer electroblot unit (Bio-Rad Laboratories, Hercules, CA). The membrane was washed three times with TBS-T buffer (20 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween20) and blocked for 4h at 25°C in the same solution with 5% nonfat milk. After it was washed and blocked, the membrane was probed with rabbit antibody (1:1,500 dilutions; immunoglobulin G (IgG) fraction; 1 mg/ml) against ZGMMV CP).

It was again washed three times in TBS-T buffer and incubated with an alkaline phosphatase (AP)-conjugated secondary antibody (1:7,500 dilution; Promega Corp., Madison, WI). The membrane was washed three times with TBS-T buffer and rinsed once in AP-substrate buffer (0.1 M Tris (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$). To make the antibody-specific proteins visible, the membrane was exposed to AP-substrate solution (Western Blue Stabilized Substrate Solution; Promega Corp.).

Results and Discussion

Construction of plant transformation vector. For cloning purposes, cDNA of the CP gene of ZGMMV was reconstructed in plant binary vector. The clone, pZGCP, was first introduced into the vector, pGEM-T-Easy, for sense orientation and site restriction in order to introduce the next plant expression vector, pGA748. They were transformed into *E. coli* JM109 strain to create a chimeric gene containing 35S promoter from cauliflower mosaic virus, and a polyadenylation signal sequence from the nopaline synthase gene (NOS) after the annealing step. The selected recombinants were denoted as pZGCPGA748, and were screened by restricting endonuclease digestion. The bands

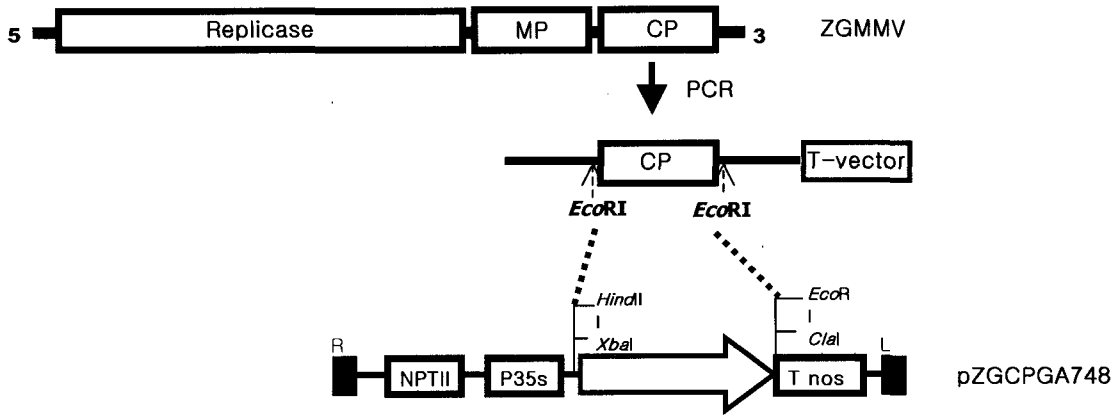

Fig. 1. Construction of plant transformation vector (pZGCPGA748) containing ZGMMV CP gene for *Nicotiana benthamiana* transformation in this study.

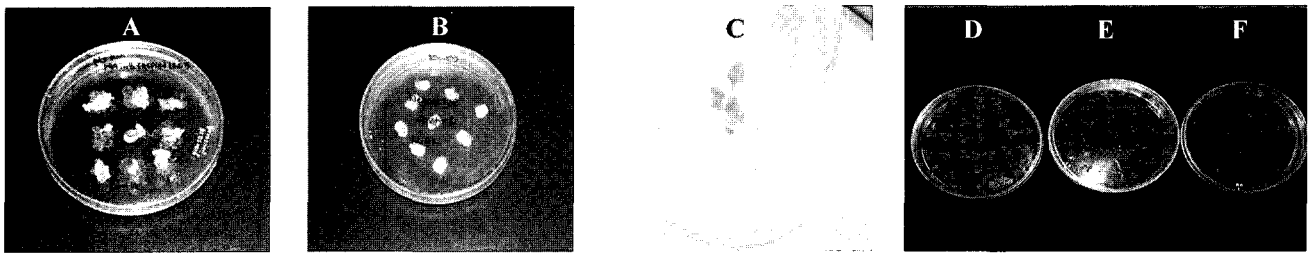

Fig. 2. Generation of *N. benthamiana* plants harboring ZGMMV CP gene, and primary selection of kanamycin-resistant *N. Benthamiana* T1 seeds. (**A**) Plant regeneration from transgenic callus tissue. (**B**) Wild type *N. benthamiana* leaf disc exhibits uniform susceptibility to Kanamycine. (**C**) Rooting of transgenic *N. benthamiana* shoots. (**D**) Seeds of transgenic T1 plants transferred to MS-kanamycin medium. (**E**) Seeds of wild type *N. benthamiana* transferred to MS medium. (**F**) Seeds of wild type *N. benthamiana* transferred to MS-kanamycin medium.

of inserted cDNA, about 0.5kb, were shown after *Eco*RI digestion (data not shown). pZGCPGA748 was used for transformation into the *A. tumefaciens*.

The construct design of plant expression vector is shown in Fig. 1. After *Agrobacterium* was transformed and the clone was screened, pZGCPGA748 which contains sense orientation of the target gene(486bp) was selected and confirmed by PCR analysis. The ZGMMV CP gene was successfully introduced into *A. tumefaciens*.

Transgenic plant selection. Transgenic callus tissues of *N. benthamiana* plants were regenerated. Shoot regeneration was observed approximately two weeks after incubation on kanamycin-containing tissue culture medium (Fig. 2). Regenerated *N. benthamiana* plants were screened for

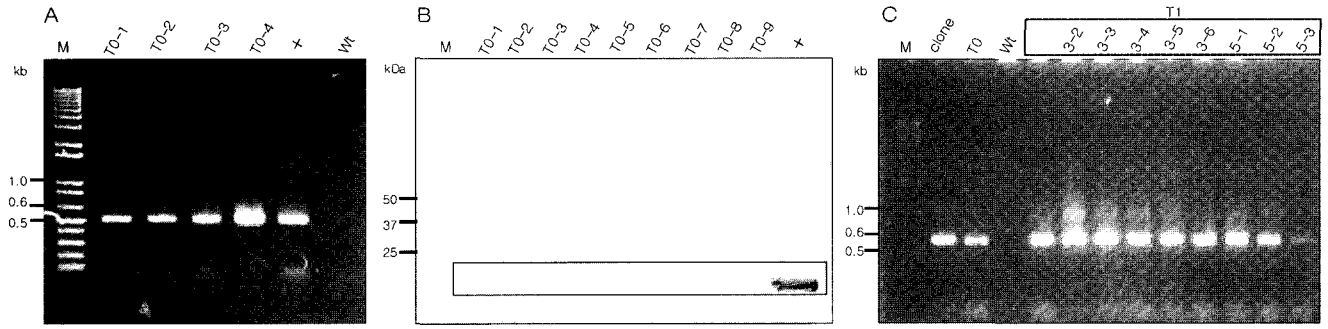

Fig. 3. Detection of transgene and expressed proteins from transgenic T0 and T1 plants. (**A**) PCR analysis with a primer pair (ZGCP UP/ ZGCP DN) flanking the transgene insertion site in the *N. Benthamiana* genome to confirm transgene. (**B**) Western blot analysis confirmation of stable expression of the transgene in T0 plant. (**C**) PCR analysis with a primer pair (35SC UP/ZGMMV CP DN) flanking the transgene insertion in the *N. benthamiana*.

Table 1. Summary of resistance screening and PCR analysis of ZGMMV CP transgenic T1 *N. benthamiana* plants

Plant	Tested sample number	Number of ZGMMV resistant T1 plant	PCR
T1-1	47	0	0
T1-2	16	0	0
T1-3	20	6	6
T1-4	13	0	0
T1-5	44	3	3
Wt[a]	10	0	0

[a]wild type, nontransformed *N. benthamiana* plant.

transgenic incorporation in the plant genome by using PCR. ZGMMV CP-specific primers (ZGCP UP/ZGCP DOWN) were used to amplify the ZGMMV CP gene (Ryu et al., 2000). Amplification of the transgene from genomic DNA containing the ZGMMV CP with the primers resulted in a 486bp product (Fig. 3A). Nontransformed *N. benthamiana* plant DNA yielded no PCR product (Fig. 3A, lane 6).

ZGMMV CP in transgenic *N. benthamiana* plants. Immunological analysis by SDS-PAGE indicated that the ZGMMV CP gene was expressed in T0 generation in the transgenic *N. benthamiana* plants (Fig. 3B). Initial experiments were effective in monitoring the relative expression levels of the transgene (Namba et al., 1995). A total of 150 kanamycin-resistant, putative T1 generation of transgenic *N. benthamiana* plants were tested to amplify CP gene by PCR; 9 plants were amplified for ZGMMV CP gene by PCR with the primers 35S UP (5'-TTGTGCGTCATCCC-TTACG-3')/ZGCP DOWN (Table 1, Fig. 3C).

Resistant assessment assay. Selected CP transgenic lines

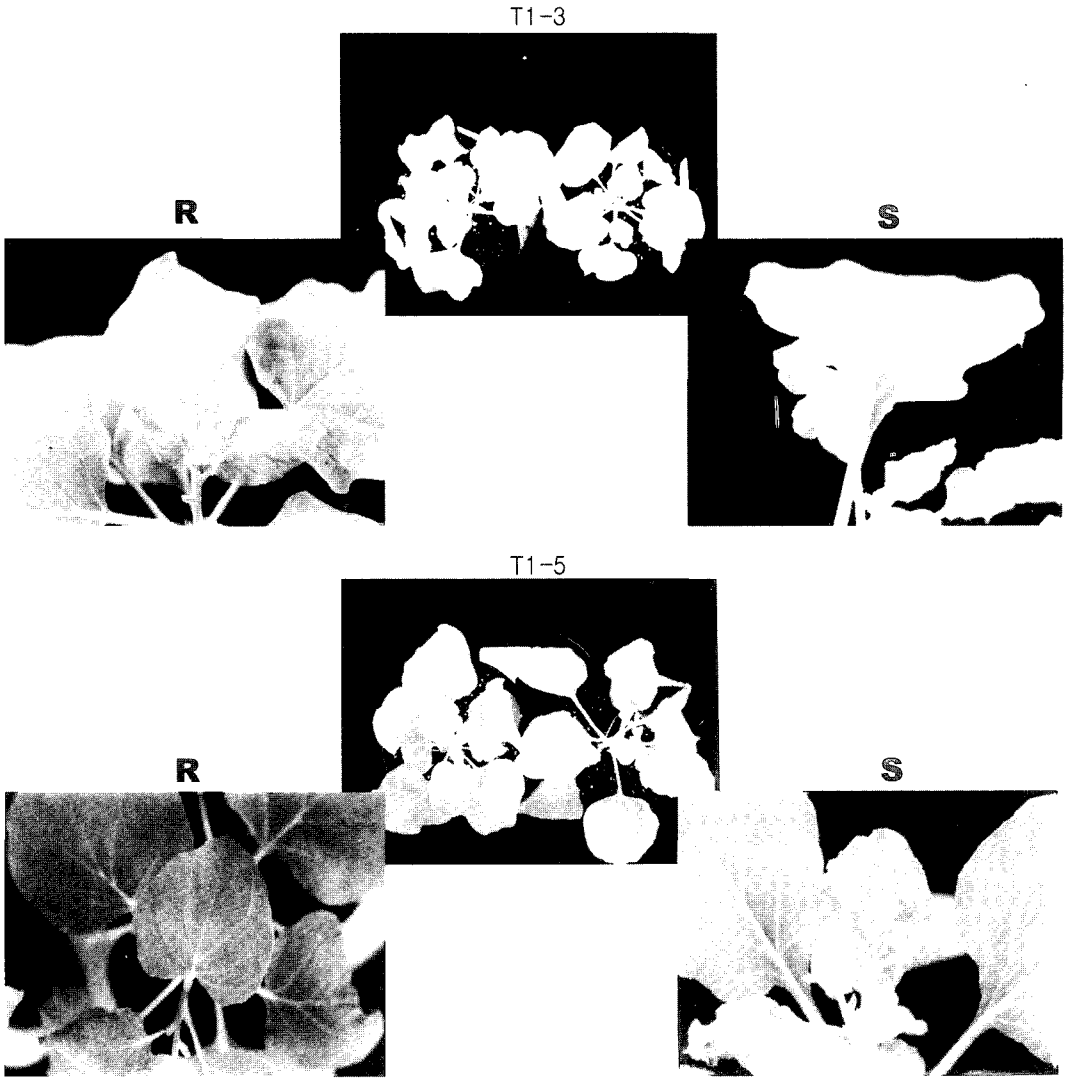

Fig. 4. Transgenic *N.benthamiana* plants (T1-3, T1-5) inoculated with ZGMMV for screening of virus-resistant and susceptible lines. R; Resistant plant without symptoms caused by ZGMMV. S; Susceptible plant with typical severe mosaic symptoms caused by ZGMMV.

(T1-3 and T1-5) were analyzed for protection effects against ZGMMV (Fig. 4). Transgenic lines revealed the incorporation of the transgene among the tested transgenic *N. benthamiana* plants which showed resistance against homologous virus infection. These *N. benthamiana* plants were morphologically normal, and were visually indistinguishable from the wild type of *N. benthamiana* plants which were used as the nontransformed control (Fig. 4).

Some transgenic *N. benthamiana* plants showed severe systemic mosaic symptom development at 21 days after inoculation, while others showed a significant level of resistance. Of the nine CP transgenic plants, 4 plants showed significant delays in systemic symptom development after inoculation with the virus. These transgenic plants developed milder mosaic symptoms, with more delayed symptom development for 20-25 days compared to those of the controls.

When the concentration of ZGMMV was increased to 100 μg/mL, they retained resistance to the infected virus. No comprehensive correlation occurred in such CP transgenic plants between the expression level of CP and the virus resistance. The progenies of the resistant transgenic *N. benthamiana* plants expressing the gene were protected from ZGMMV infection.

The results of this study would form part of the baseline research data that would be useful for researchers conducting environmental risk assessment of LMOs, such as specific virus-resistant transgenic LM crop and genetically engineered LM virus. Virus resistance is among the most useful traits to be introduced into transgenic crops, and many virus resistant transgenic cultivars have already been commercially released in several countries (Namba et al., 1991; Ryu et al., 1998).

In order to provide the necessary science-based risk assessment of such LM crops before release into the field, it is essential to clarify several points concerning potential ecological impact. Baseline information, like the virus genome stability of serial passage in systemic hosts, will fill a significant gap in current knowledge and will provide the natural biological context against which previous laboratory studies can be assessed.

Acknowledgments

This study was supported in part by the research grant from the National Institute of Environmental Research of the Ministry of Environment in Korea, in part by grant from the BioGreen 21 project from the Rural Development Administration in Korea.

References

An, G., Paul, R. E., Mitra, A. and Ha, S. B. 1988. Binary vectors. In: Plant Molecular Binary Manual A3, ed. By B. G. Stanton, A. S. Robbert and P. S. V. Desh, pp. 1-19. Kluwer Academic Publishers.

Antignus, Y., Wang, Y., Pearlsman, M., Lachman, O., Lavi, N. and Gal-on, A. 2001. Biological and molecular characterization of a new cucurbit-infecting Tobamovirus. *Phytopathology* 91: 567-571.

Choi, G. S., Kim, J. H., Chung B. N., Kim, H. R. and Choi, Y. M. 2001. Simultaneous detection of three tobamoviruses in cucurbits by rapid immunofilter paper assay. *Plant Pathol. J.* 17:106-109.

Francki, R. I. B., Hu, J. and Palukaitis, P. 1986. Taxonomy of cucurbit-infecting tobamoviruses as determined by serological and molecular hybridization analyses. *Intervirology* 26:156-163.

Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, S. G. and Fraley, R. T. 1985. A simple and general method for transferring genes into plants. *Science* 227:1229-1231.

Lewandowski, D. J. 2000. Genus *Tobamovirus*. Pages 889-894 In: Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses. M. H. V. Van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner, eds. Academic Press, San Diego.

Namba, S., Ling, K., Gonsalves, C., Gonsalves, D. and Slightom, J. L. 1991. Expression of the gene encoding the coat protein of cucumber mosaic virus CMV strain WL appears to provide protection to tobacco plants against infection by several different CMV strains. *Gene* 107:181-188.

Ryu, K. H. and Park, W. M. 1995. Construction of a plant expression vector for the coat protein gene of cucumber mosaic virus-As strain for plant transformation. *Korean J. Plant Pathol.* 11:66-72.

Ryu, K. H., Lee, G. P., Park, K. W., Lee, S. Y. and Park, W. M. 1998. Transgenic tobacco plants expressing the coat protein of cucumber mosaic virus show different virus resistance. *J. Plant Biol.* 41:255-261.

Ryu, K. H., Min, B. E., Choi, G. S., Choi, S. H., Kwon, S. B., Noh, G. M., Yoon, J. Y., Choi, Y. M., Jang, S. H., Lee, G. P., Cho, K. H. and Park, W. M. 2000. Zucchini green mottle mosaic virus is a new tobamovirus; comparison of its coat protein gene with that of kyuri green mottle mosaic virus. *Arch. Virol.* 145:2325-2333.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Springer Harbor Laboratory Press, New York.

Tan, S. H., Nishiguchi, M., Murata, M. and Motoyoshi, F. 2000. The genome structure of kyuri green mottle mosaic tobamovirus and its comparison with that of cucumber green mottle mosaic tobamovirus. *Arch. Virol.* 145:1067-1079.

Ugaki, M., Tomiyama, M., Kakutani, T., Hidaka, S., Kiguchi, T.,

Nagata, R., Sato, T., Motoyoshi, F. and Nishiguchi, M. 1991. The complete nucleotide sequence of cucumber green mottle mosaic virus (SH strain) genomic RNA. *J. Gen. Virol.* 72:1487-1495.

Yoon, J. Y., Min, B. E., Choi, S. H. and Ryu, K. H. 2001 Completion of nucleotide sequence and generation of highly infectious transcripts to cucurbits from full-length cDNA clone of *Kyuri green mottle mosaic virus. Arch Virol.* 146:2085-2096.

Yoon, J. Y., Min, B. E., Choi, J. K. and Ryu, K. H. 2002. Genome structure and production of biologically active in vitro transcripts of cucurbit-infecting *Zucchini green mottle mosaic virus. Phytopathology* 92:156-163.