Study on Environmental Risk Assessment for Potential Effect of Genetically Modified *Nicotiana benthamiana* Expressing ZGMMV Coat Protein Gene

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Transgenic *Nicotiana benthamiana* plants harboring the coat protein (CP) gene of *Zucchini green mottle mosaic virus* (ZGMMV) were chosen as a model host for the environmental risk assessment of genetically modified plants with virus resistance. This study was focused on whether new virus type may arise during serial inoculation of one point CP mutant of ZGMMV on the transgenic plants. *In vitro* transcripts derived from the non-functional CP mutant were inoculated onto the virus-tolerant and -susceptible transgenic *N. benthamiana* plants. Any notable viral symptoms that could arise on the inoculated transgenic host plants were not detected, even though the inoculation experiment was repeated a total of ten times. This result suggests that potential risk associated with the CP-expressing transgenic plants may not be significant. However, cautions must be taken as it does not guarantee environmental safety of these CP-mediated virus-resistant plants, considering the limited number of the transgenic plants tested in this study. Further study at a larger scale is needed to evaluate the environmental risk that might be associated with the CP-mediated virus resistant plant.

Keywords : Coat protein, environmental risk assessment, genetically modified, living modified organism, *Nicotiana benthamiana*, *Tobamovirus*, transgenic plant, *Zucchini green mottle mosaic virus*

Since pathogen-mediated virus resistance is the most useful modern technique to be introduced into transgenic plants to protect pathogenic plant virus, coat protein gene-mediated protection is one of the best known approaches developed for production of virus resistant transgenic plants (Powell Abel et al., 1986; Matthews, 1991).

Powell Abel et al. (1986) first reported that transgenic tobacco plants expressing the coat protein (CP) of *Tobacco mosaic virus* (TMV) imparted resistance against TMV. Since that time, over 30 genetically modified (GM) plants,

both monocots and dicots, have been engineered with more than 50 viral CP genes from ten taxa. Many of these have been field-tested (http://webdomino1.oecd.org/ehs/biotrack. nsf) and several virus-resistant transgenic plants such as papaya and zucchini squash have been commercially released in several countries such as USA and Taiwan (Fuchs et al., 1998; Bau et al., 2004).

In order to provide the necessary science-based environmental risk assessment of such kind of biotic-stress resistant plants before contemplating commercial release into environment, it is essential to clarify several points concerning potential ecological impact. The most important potential impacts in transgenic plants expressing the viral CP gene can be categorized as gene transfer, transcapsidation, recombination and synergistic effects (OECD, 1996).

With the natural evolution of cucurbit-infecting *Tobamoviruses* and resistance mechanism, their pathogenicity and environmental risk assessment of virus-resistant genetically modified (GM) plants were the main interests in this work. Therefore, we reported the transgenic *Nicotiana benthamiana* plants expressing the CP gene of *Zucchini green mottle mosaic virus* (ZGMMV) were screened for their resistance against ZGMMV, and some transgenic lines showed susceptibility to the target virus (Kim et al., 2004).

In this study, to assess potential risk of new virus types arising through recombination with the transgene expressed in the host plants, non-functional CP mutant of ZGMMV was constructed and inoculated onto *N. benthamiana* expressing the CP gene of ZGMMV.

Materials and Methods

Source of virus and plants. ZGMMV-Zu, a type isolate of the virus belonging to the genus *Tobamovirus*, originally obtained from the Plant Virus GenBank (PVGB PV001), 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 RNA was extracted from

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purified virion particles using SDS/proteinase K and phenol extraction, followed by ethanol precipitation. Zucchini squash (*Cucumbita pepo* cv. Black Beauty) and cucumber (*Cucumis sativus* cv. Baekdadagi) were generally used as systemic host plants for the infectivity tests of ZGMMV and CP mutated ZGMMV. *N. benthamiana* wild type and transgenic *N. benthamiana* plants harboring the CP of ZGMMV were used as plant materials for risk assessment in this study (Kim et al., 2004).

Construction of CP mutation ZGMMV full-length cDNA clone. A full-length infectious cDNA clone of the ZGMMV in pUC18 (pZGT7ZT1) was used as a template for construction of CP mutated ZGMMV. To construct a

point mutant of ZGMMV CP, a base of the CP start codon (A**T**G → A**G**G) was replaced by primer 2 and primer containing an *Stu* I recognition site (underlined).

Two new designed primers (upstream primer, 5'-GA-GCTACCCGGTTTATAACA**AGGCCT**CTTA-3', 30 nts; Downstream primer, 5'-GAATACCGCTGGTAGAGTA-AG**AGGCCT**TGT-3', 30 nts) were amplified by PCR mutagenesis method (Ito et al., 1991), purified using PCR purification kit (QIAGEN, Valencia, CA, USA) and digested with *Stu* I (Promega, Madison, WI, USA), then self-ligated at 16°C for 12 hours with T4 DNA ligase (Promega). The ligated DNAs were transformed into competent *Escherichia coli* strain JM109 cells. Selected colonies were cultured in LB broth containing ampicilline
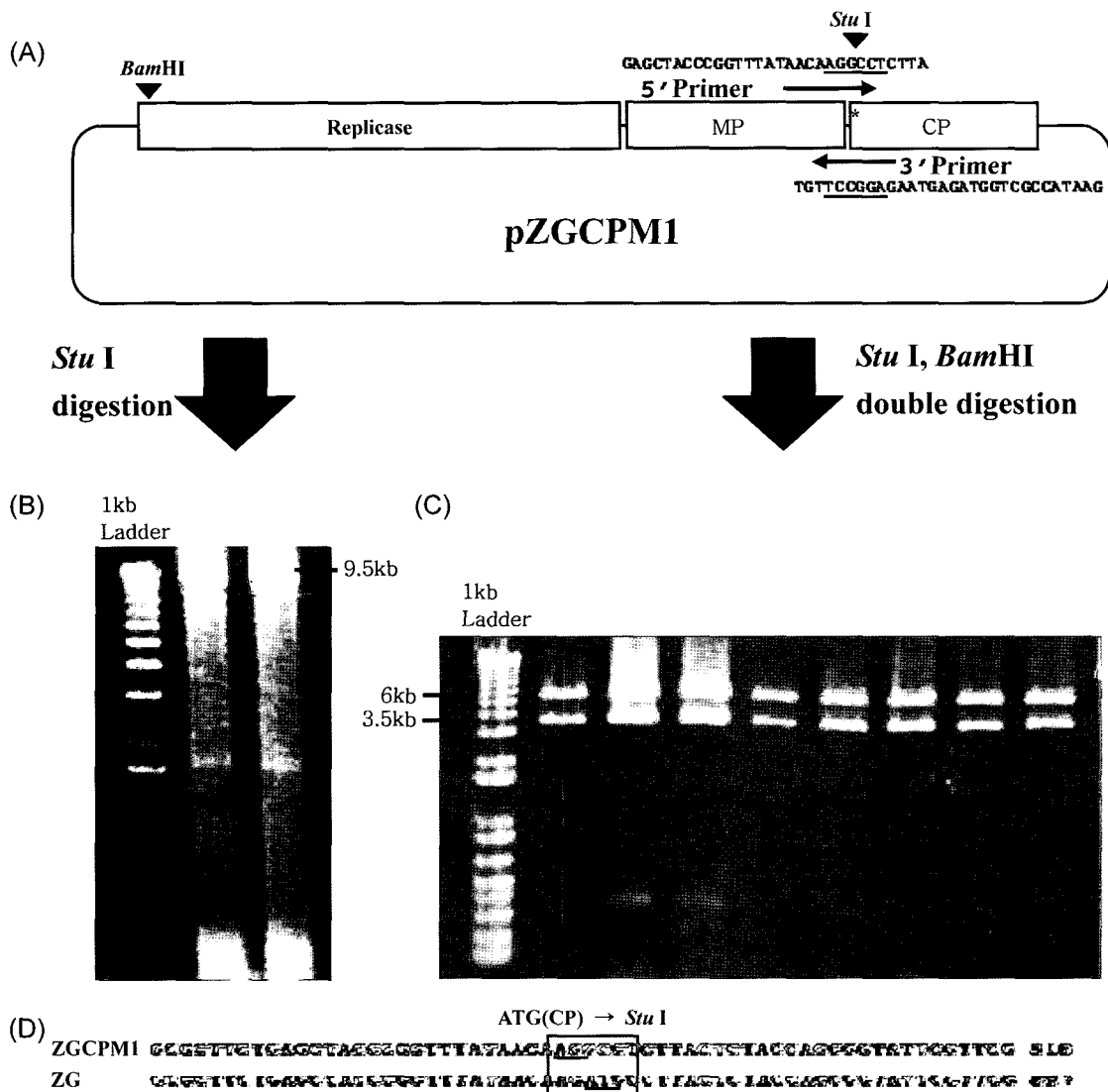

Fig. 1. Construction of CP-point mutated cDNA clones of ZGMMV by PCR mutagenesis methods (Ito et al., 1991). (**A**) Two pairs of primer containing and *Stu*I site (upper lined) for replace of a base of the CP start cordon (ATG → AGG), (**B**) Conformation of CP mutant ZGMMV cDNA clone by digestion with *Stu*I, (**C**) Conformation of CP mutant ZGMMV cDNA clone by double digestion with *Stu*I and *Bam*HI, (**D**) Conformation of one point CP mutant by DNA sequencing.

and recombinant plasmid DNA was prepared. The selected clones were digested with *Stu* I and double digested with *Stu* I and *Bam*HI to select those containing the full-length CP mutated ZGMMV cDNA.

DNA sequencing was performed to identify one-point CP mutant by the dideoxynucleotide chain termination method using the Model 377 automated DNA sequencer (ABI Perkin-Elmer, PVGABC).

In vitro transcription and infectivity test.

RT-PCR products of full-length cDNA of ZGMMV and CP mutation ZGMMV full-length cDNA clone were used as template DNAs for cell-free *in vitro* transcription. Transcription reaction was done with the linearized clones with *Sph*I. *In vitro* transcription reaction was performed in the presence or absence of cap analog (m G [5'] ppp [5'] G; New England Biolabs). One microgram of *Sph*I-linearized plasmid DNA was mixed with 5 μl of 100 mM DTT, 10 μl of 2.5 mM rNTPs (rATP, rUTP, rCTP) mix, 10 μl of 0.5 mM rGTP, 10 μl of 5X transcription buffer, 5 μl of 2.5 mM Cap analog (New England Biolabs), 1 unit of RNase inhibitor (Promega) and 4-7 units of T7 transcriptase (Promega), and then was incubated at 37°C for 1 hr. Five microliter of 2.5 mM rGTP was added to the mixture and incubated at 37°C for 20 min.

In vitro transcripts of ZGMMV and CP mutation ZGMMV were directly inoculated onto host plants for infectivity test. Inoculum applied to zucchini squash, cucumber and *N. benthamiana* seedlings was prepared by mixing 50 μl of *in vitro* transcription reaction mixture with 150 μl of 10 mM sodium phosphate buffer (pH 7.5). Cotyledons of zucchini squash and cucumber plants and 3rd-to-4th leaves of *N. benthamiana* plants were mechanically inoculated with 20 μl of the mixture. Control seedlings were mock-inoculated with the buffer and with 0.5 μl of ZGMMV RNA in 20 μl of phosphate buffer.

Infectivity of mutant clone for potential effects on transgenic host.

In vitro transcripts derived from ZGMMV CP mutants and ZGMMV were directly to identify potential effects onto virus-tolerant line 10, -susceptible line 20 and non-transgenic *N. benthamiana*, respectively, as above infectivity test method. The inoculation experiment was repeated a total of ten times as mentioned above. Plants were observed daily for 30 days for symptom and infectivity after challenged inoculation.

Detection of ZGMMV Replicase (RP) in transgenic *N. benthamiana* plants.

Viral RNAs from inoculated *N. benthamiana* leaves with CP mutated ZGMMV were used as templates for RT-PCR. RT was performed in a reaction mixture (20 μl) containing 2.5 mM MgCl₂, 10 mM of each

dNTP, 50 pM of primer (5'-TCTACTTTACATTATAC-GCATAGTTTTTC-3': 29 nts) 1 unit RNasin (Promega, USA), 1 unit MuLV reverse transcriptase (Clonetech) at 42°C for 60 min. PCR amplification was performed in an i-cycler (Bio-Rad) with *Taq* polymerase (Promega) and carried out for 25 cycles (Kim et al., 2004). Finally, the mixtures were incubated at 72°C for 20 min for extension. Amplified products were separated and visualized on 1.2% agarose gels in 0.5X TAE buffer (Sambrook et al., 2001).

Results

Construction of CP-point mutated cDNA clone of ZGMMV and infectivity test.

To develop the method of virus resistant transgenic plant expressing CP gene for environmental risk assessment, one point CP mutant of ZGMMV was constructed (Fig. 1A). This CP mutant was identified by cutting with restriction enzymes (Fig. 1B and 1C) and then confirmed by DNA sequencing (Fig. 1D).
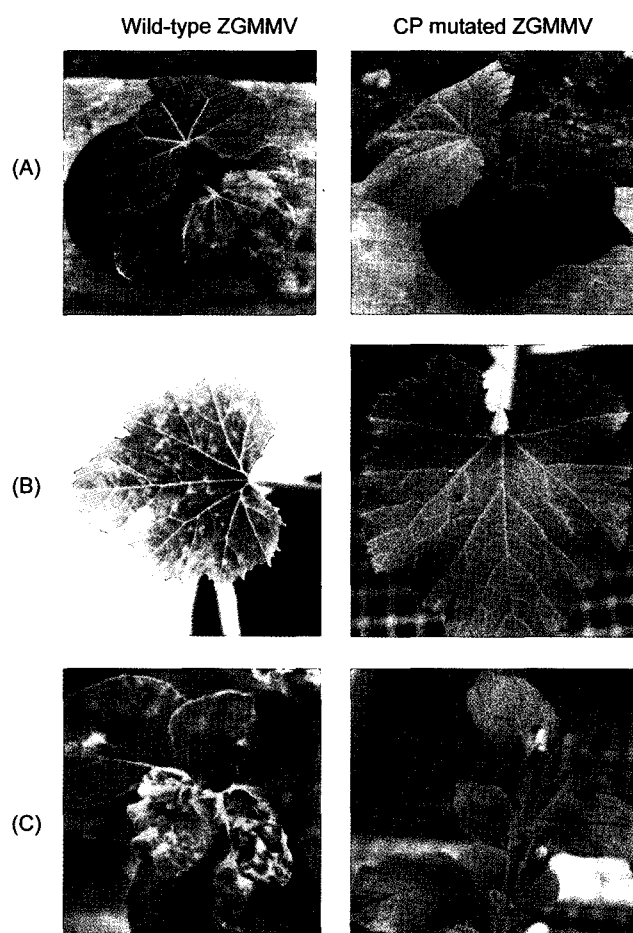

Fig. 2. Observation of systemic symptoms for infectivity tests with CP one point mutated ZGMMV and wild-type ZGMMV at 14 days post-inoculation on different host plants. (A) Cucumber, (B) Zucchini squash, and (C) *Nicotiana benthamiana*.

This CP mutant did not cause any symptoms in infectivity test in three systemic hosts such as cucumber, zucchini squash and *N. benthamiana* (Fig. 2). It means the CP mutant was non-functional virus which could not replicate. For this reason, if any symptoms were detected by inoculation with the CP mutant, it could be arise through recombination or transcapsidation. We could conclude that the one-point CP mutant ZGMMV cDNA clone, can be useful material for the environmental risk assessment of virus- resistant CP mediated GM plants.

Potential impact of virus resistant transgenic plant expressing CP gene. In *vitro* transcripts derived from the non-infectious CP mutants were inoculated onto two kinds of transgenic *N. benthamiana*, virus-tolerant and -susceptible lines, and wild-type plants. Any notable viral symptoms that could arise through transcapsidation or recombination on the inoculated transgenic host plants were not detect, even though the inoculation experiment was repeated a total of 10 times (Fig. 3, Table 1). More than 400 independent experiments of *in vitro* transcription and challenge-

Fig. 3. Observation of systemic symptoms for potential impact from upper leaves of transgenic *Nicotiana benthamiana* (susceptible and tolerant lines) and wild-type plants infected with the CP mutants or wild-type ZGMMV at 30 days post-inoculation.

Table 1. Systemic symptom development from upper leaves of transgenic *N. benthamiana* and wild type plants infected with the CP mutants or wild-type ZGMMV at 30 days post-inoculation

CP Mutant \ Line	Transgenic *N. benthamiana*		Wild type (*N. benthamiana*)
	Susceptible	Tolerant	
Point mutant	0/200	0/100	0/20
ZGMMV	30/30	25/30	20/20
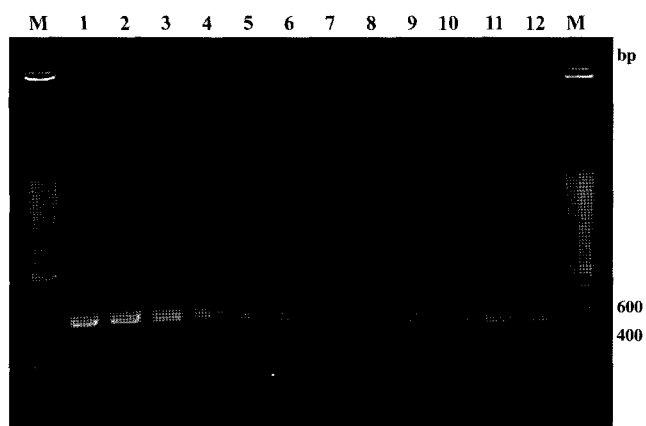
Fig. 4. RT-PCR analysis (replicase region) of RNA extracted for potential impact from upper leaves of transgenic *N. benthamiana* infected with CP mutants and ZGMMV at 7 days post-inoculation. Lane M: DNA size marker (200 bp ladder); lanes 1 to 3: ZGMMV inoculated onto wild-type, susceptible and tolerant plants; lanes 4 and 6: one point CP mutant ZGMMV inoculated onto wild-type plants; lanes 7 and 9: one point CP mutant ZGMMV inoculated onto susceptible plants; lanes 10 and 12: one point CP mutant ZGMMV inoculated onto tolerant plants.

inoculation were performed for identification of potential impact in this study. It was not small scale test compared with other experiments if these mutants be considered as non-functional virus (Soledad et al., 2003; Li et al., 2004).

To corroborate the result of the infectivity assays, RT-PCR analysis was performed on total RNA from upper leaves of the challenged plants. Replicase-specific primers were used to detect recombination, transcapsidation or ZGMMV accumulation. RT-PCR analysis indicated that replicase of ZGMMV was expressed only in wild-type ZGMMV inoculated transgenic *N. benthamiana* (both susceptible and tolerant lines), but not in any of the plants inoculated with the CP mutants (Fig. 4). Although this result suggests that potential risk associated with the CP-expressing transgenic plants may not be significant, we could not exclude potential environmental risk of these GM plants, considering the limited number of the model plants being tested in this study.

Discussion

Recombination is considered to play a key role in RNA

virus evolution (Nagy and Simon, 1997). However, little is known about its occurrence under natural conditions. Recently, recombination between invading wild-type viruses and transgenes has been demonstrated (Aaziz and Tepfer, 1999b; Hammond et al., 1999).

Gene transfer can result either from plant to virus by recombination or from plant to plant by sexual outcrossing. Synergy is a phenomenon when two different viruses simultaneously infect a plant naturally, causing more severe symptoms than those by either virus alone (Matthews, 1991). Transcapsidation is a phenomenon that the genome of one virus is to be encapsidated by coat protein of the second virus when a single plant cell is simultaneously infected by different strains of one or two viruses (Falk et al., 1995). Recombination is defined as an exchange of nucleotide sequences between two nucleic acid molecules. Recombination between viral genomes results in heritable and permanent change (Lai, 1992). The persistence of a recombined viral genome will depend on its fitness with respect to its ability to replicate within the original host cell, its ability to replicate in the presence of parental viruses, its ability to spread systemically within the host, or its successful transmission to other host plants.

Although most of the studies demonstrating such recombination have designed the experimental conditions to select for recombinants, few studies have shown significant amounts of virus-transgene recombination even under conditions of moderate to weak selection (Wintermantel and Schoelz, 1996; Borja et al., 1999). Similarly, Aaziz and Tepfer (1999a) showed that recombination among viruses can occur even under weak selection. In a monoculture of transgenic plants expressing viral genes, every infection with an invading virus provides the potential for recombination. This implies that the number of recombinants is likely to rise, even if the rate of recombination is equal to or less than the rate of natural recombination among viruses (de Zoeten, 1991).

The majority of experiments for ecological risks of transgenic virus-resistant plants were used with wild type viruses by sap inoculation (Thomas et al., 1998; Fuchs et al., 1998). Actually, we supposed that probability of recombination arose on the inoculated susceptible-line more than tolerant line because susceptible-line showed much severe symptoms in resistance assessment assay. However, we do

not know exact mechanism about severe symptoms of susceptible-line.

Transcapsidation and recombination was not detected through a small-scale green house test. This result suggests that potential risk associated with the CP-expressing transgenic plants may not be significant. However, cautions must be taken as it does not guarantee environmental safety of these CP-mediated virus-resistant plants, considering the limited number of the transgenic plants tested and probability enough to cause transcapsidation and recombination in this study.

One recent study was attempted to examine the probability of recombinant viruses arising under field conditions, by screening for changes in the characteristics of viruses found in infected transgenic potato plants (Thomas et al., 1998). Over 6 years, Thomas et al. (1998) exposed 65,000 transgenic potatoes expressing the CP or replicase genes of potato leaf roll virus to field infections of wild-type viruses. Viruses found in the infected transgenic plants were examined for changes in symptoms, serology, host range, and transmission characteristics. However, unusual variants were not detected. Although this does not rule out recombination having occurred, it does suggest that the probability is rather low, as recombinants with significant effects on virus fitness will persist in this system. Similarly, Fuchs et al. (1998, 1999) were unable to detect recombinant viruses in fields of transgenic squash or melon.

In order to evaluate any potential biosafety concerns posed by the use of viral genes, viral sequences engineered into the plant should be well-characterized sequences that are derived from well-characterized viruses, and the specific biological properties of the actual strain utilized should be known. Further information is needed to evaluate the environmental risk that might be associated with CP-mediated virus resistant plants.

Acknowledgements

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