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Construction of a Plant Expression Vector for the Coat Protein Gene of Cucumber Mosaic Virus-As Strain for Plant Transformation

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오이 모자이크 바이러스 As계통 외피단백질 유전자의 식물체 형질전환을 위한 발현벡타의 구축

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ABSTRACT : The coat protein (CP) gene of cucumber mosaic virus-As (CMV-As) strain was engineered for expression in the plant by using the cauliflower mosaic virus 35S transcript regulatory sequences. The CP gene was cloned into an *Agrobacterium*-derived binary vector. A chimeric gene was constructed by the cDNA of CMV-As CP and plant expression vector pBI121. The clone, pCMAS66, was first introduced into the phagemid vector pSPORT1 for situating sense orientation for translation and making restriction sites in order to re-introduce plant expression vector, pBI121. The resulting subclone pCASCP02 and plant expression vector pBI121 were treated with *Bam*HI-*Sac*I for excising the target gene and removing GUS gene, respectively. After *Agrobacterium* transformation by freeze-thaw technique, the clone, pCMASCP121-123 which contains sense orientation of the target gene, was selected and confirmed by restriction endonuclease analysis. The CMV-As CP gene was introduced into *A. tumefaciens*. The results on tobacco plant transformation with the vector system revealed that the system could be successfully intoduced and showed high frequency of selection to putative transformants.

Key words **: cucumber mosaic virus, As strain, coat protein gene, cDNA, *Agrobacterium*, plant expression vector.**

With the development of molecular biological techniques for introduction of a foreign gene into plant cell and the regeneration of transgenic plant, a number of different approaches have been taken to attempt to produce resistant plants to virus diseases (2, 3, 4, 7, 10, 11, 13, 14, 16, 17, 19, 22, 28, 30). Those most commonly used have been (1) the expression of antisense RNAs; (2) the expression of satellite RNAs and defective interfering RNAs and DNAs; (3) the expression of genes encoding viral coat proteins (CP); (4) the expression of ribozymes; and (5) the expression of virus-derived genes such as replication-associated or cell-to-cell movement protein-associated virus genes. Of these the expression of viral CP, so called "coat protein-mediated resistance" (CP-MR) strategy (4), has been generally accepted because of its ready application to control a variety of virus diseases in a number of different crops.

Cucumber mosaic virus (CMV) was first reported by Doolittle in 1916 (8). CMV is the type member of the cucumovirus group in the family Bromoviridae with Bromovirus, Ilarvirus, and Alfamovirus (9). It is one of the best characterized and the most economically important plant viruses all over the world. CMV is known to have a very broad host range including both mono- and dicotyledonous plants. The virus is widely distributed throughout the world (9). The genome of CMV consists of three positive-sense single-stranded RNAs (RNA1, 2, and

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3) and a forth RNA (RNA4), a subgenomic RNA derived from RNA3. Their expressions provide production of four proteins. RNA1 and RNA2 encode the 1a and 2a proteins, involved in virus replication, respectively. RNA3 encodes the 3a protein and coat protein. The 3a protein is thought to be involved in cell-to-cell movement function. The coat protein is translated from subgenomic RNA (RNA4) transcribed from RNA3. These characteristics are shared with bromovirus, ilarvirus, and alfalfa mosaic virus. They are called the family Tricornaviridae (29).

The properties of CMV-As strain, the Korean isolate, were characterized and reported previously (6, 20, 21, 23, 24). The CMV-As belongs to subgroup I (20), and the nucleotide sequence of the coat protein gene has been determined (23). We report here construction of plant expression vector of CP of CMV-As strain for *Agrobacterium* mediated plant transfer system.

MATERIALS AND METHODS

Virus source and extraction of viral RNA. The CMV-As strain, originally obtained from *Aster yomena* Makino, was purified and the viral genomic RNA was extracted from purified virus particles by-using SDS/proteinase K disruption and phenol extraction method (18, 25).

The resulting precipitates of viral RNAs were dissolved in an appropriate volume of 0.1 strength of TE buffer (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA). The purity and yield of the recovered viral RNAs were determined by spectrophotometry (1 $A_{260}=40$ μg/ml). It was adjusted to the final concentration of 0.5 μg/μl, divided into small aliquots, and then stored at −70℃ until use.

Coat protein gene cloning. The purified viral RNAs were polyadenylated by using *Escherichia coli* poly (A) polymerase (GIBCO BRL) as described previously (26). The first-strand cDNA was synthesized with oligo $(dT)_{12-18}$ and AMV reverse transcriptase (Promega), and second-strand cDNA was synthesized by using *E. coli* RNase H, *E. coli* DNA ligase and *E. coli* DNA polymerase I. The resulting cDNA fragments were ligated to pT7T3 18U (Pharmacia) using *Eco*RI adapter system, and then transformed into competent *E. coli* strain NM522 cells (Stratagene). A clone containing about 1 kb insert was selected. The coat protein (CP) gene of the virus RNA in the clone was determined by nucleotide sequencing. The nucleotide sequencing was performed by dye terminator protocol by the manufacture's recommendations (Applied Biosystems). A 21-mer M13 forward and a 20-mer T7 universal dye-labeled primers (Applied Biosystems) were used to prime the reactions. Using a DNA thermal cycler (Perkin Elmer Cetus), we performed 15 cycles of amplification using the thermal cycling program (95℃ 30 seconds (s); 55℃ 30s; 70℃ 1 min), and continued another 15 cycles of thermal cycling program (95℃ 30s and 70℃ 1 min) followed by a 4℃ cooling. After the reaction was terminated, the products in the four tubes (ddATP, ddCTP, ddGTP, and ddTTP) were combined into one tube. The PCR products were precipitated with ammonium acetate and ethanol. The nucleotide sequence was determined by automated DNA Sequencer (model 373A, Applied Biosystems).

Construction of plant transformation vector. The cDNA of CP gene in pCMAS66 was excised by *Bam*HI and was subcloned into pSPORT1 vector (GIBCO BRL) to make pCASCP02 for translation and making restriction sites in order to re-introduce plant expression vector. The subclone pCASCP02 was digested with *Bam*HI and *Sac*I by complete reaction. The digested materials were separated by agarose gel electrophoresis (27) and ethidium bromide stained cDNA fragment was recovered from the agarose gel by using Gene Clean Kit (BIO 101). Plant expression plasmid was derived from the pBI 121 plant expression vector (Clontech Labs) by replacing the *Bam*HI-*Sac*I DNA fragment corresponding to the GUS sequence, with the CMV-As CP sequence. The GUS gene (−) pBI121 vector was also eluted from the agarose gel by using low-melting agarose (27). The vector and the insert cDNA fragment were religated, and then the resulting recombinants were transformed into competent *E. coli* strain HB101. The clone of sense-oriented target gene was selected and it was multiplied in *E. coli*. The recombinant DNA was reisolated from the transformed bacteria by alkaline-lysis preparation and polyethylene glycol precipitation (27). This plant transformation vector was introduced into *Agrobacterium tumefaciens* strain LBA4404 by freeze-thaw method (1). Recombinant clone of the *A. tumefaciens* was selected on YEP agar medium (nutrient broth 8 g, bacto-yeast extract 1 g, bacto-peptone 5 g, suc-

rose 5 g, $MgSO_4 \cdot 7H_2O$ 0.493 g, bacto-agar 16 g per liter, pH 7.2) supplemented with Rifampicin (30 μg/ml) and kanamycin (50 μg/ml). Isolation of plasmid from the *A. tumefaciens* was carried out by the method of An *et al.* (1).

RESULTS

CMV coat protein gene cloning. Several cDNA clones encoding RNA4 of CMV-As were synthesized and selected. The clone containing about 1 kb insert was selected, denoted as pCMAS66. It contained one restriction recognition site for *Acc*I, *Hinc*II, *Aat*II, *Pvu*II, *Eco*RI, *Eco*RV, *Ava*I, *Xho*I and *Sca*I, respectively. The partial nucleotide sequence corresponding to both 3′ and 5′ terminal regions of the cDNA insert was shown in Fig. 1. The clone contained a full-length open reading frame (ORF) of CMV-As CP. The ORF containing the translational initiation (AUG) and termination (UGA) codon, was used for cDNA source. About 52 bases poly (A) tail for the synthesis of cDNA was also contained in the pCMAS66 plasmid.

Construction of plant expression vector. For cloning purpose, cDNA of the CP gene of CMV-As was reconstructed in plant binary vector. The clone, pCMAS66, was first introduced into the phagemid vector pSPORT1 for sense orientation and making restriction sites in order to introduce next plant expression vector, pBI121 (Clonetech). The resulting subclone pCASCP02 and plant expression vector pBI121 were treated with *Bam*HI-*Sac*I for removing GUS gene, respectively. They were transformed into *E. coli* strain HB101 to create a chimeric gene containing 35S promoter from cauliflower mosaic virus (CaMV) and a polyadenylation signal sequence from the nopaline synthase gene (NOS) after annealing step. The selected recombinants were denoted as pCMASCP/pBI121-1-14, and were screened by restriction endonuclease digestion. The bands of insert cDNA about 1 kb were shown in Fig. 2 after *Kpn*I digestion. Among there combinants, pCMASCP/pBI121-12 was used for transformation into the *A. tumefaciens*. The design of plant expression vector for CPof CMV-As was shown in Fig. 3. After *Agrobacterium* transformation and screening the clone, pCMASCP121-123 which contains sense orientation of the target gene (977 bp) was selected and confirmed by restriction endonuclease analysis (Fig. 4). The CMV-As CP gene was successfully introduced into *A. tumefaciens*.

66 GUCGAGUCAUGGACAAAUCUGAAUCAACCAGUGCUGGUCGUAACCGUCGACGUCG

121 UCCGCGUCGUGGUUCCCGCUCCGCCUCCUCCUCCGCGGAUGCUAACUUUAGAGUCCUGUC

181 GCAGCAACUUUCGCGACUCAAUAAGACGUUAGCAGCUGGUCGUCCUACCAUUAACCACCC

241 AACCUUUGUGGGGAGUGAACGUUGUAAACCUGGGUACACGUUCUCAUCUAUUACCCUGAA

* *

661 GGAUGAACUAGUGCUUCAUGUCGACGUCGAGCACCAACGCAUUCCCACAUCUGGGGUGCU

721 CCCAGUUUGAAUCCGUGUUUUCCCAGAACCCUCCCUCCAGUCCUGAGGCGGGAGCUGAGU

*

781 UGGCAGUGUUGCUAUAAACUGUCUGAAGUCACUAAAGCGGUUUGCUGAACGGGUUGUCCA

841 UCCAGCUUACGGCUAAAAUGGUCAGUCGUGGAGAAAUCUACGCCAGUAGACUUACAAGUC

901 UCUGAGGCACCUUUGAAACCAUCUCCUGGGUUUCUUCGGAAGGACUUCGGUCCGUGUACU

961 UCUAGCACAAUGAGCUACUUCUAGCACAAGAUGCUAGUUUUAGAGUACGGGUGUGCUUUG

1021 CGGGGGUCUCUCUAAGGAGACCA-Poly A_{52}

Fig. 1. The partial nucleotide sequence of coat protein gene from cucumber mosaic virus-As strain. The numbering of nucleotide is starting from 5′ end. Thick underline and asterisk with bold character indicate the initiation and termination codons, respectively. The sequence represented was used for construction of plant expression vector in this study.

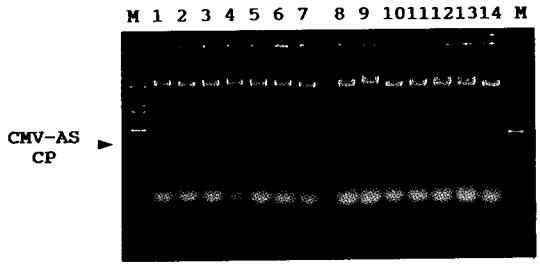

Fig. 2. Electrophoretic pattern of pCMASCP/pBI121 from *E. coli* transformants on 1.0% agarose gel to determine insert fragment of coat protein gene of cucumber mosaic virus-As strain. The upper number of each lane indicate the clone, and they were treated with *Kpn*I before electrophoresis. Lane M : 1 kb DNA ladder (GIBCO BRL) for molecular size marker. Arrow-head indicates the insert cDNA.

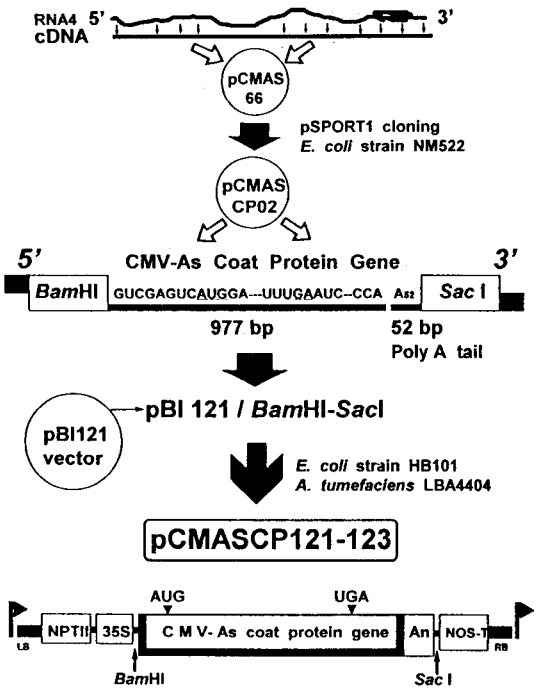

Fig. 3. Schematic representation of flow chart for construction of plant expression vector for coat protein gene of cucumber mosaic virus-As strain in this study. Translational initiation (AUG) and termination (UGA) codon were marked at their corresponding sites. Abbreviations used were as follow; LB and RB : left and right border sequences of Ti plasmid, NPT II : Neomycin phosphotransferase II, 35S : cauliflower mosaic virus (CaMV) 35S transcript regulatory sequences (CaMV 35S promoter), and NOS-T : nopaline synthase terminator. Vectors used for cloning purposes were pSPORT1 (GIBCO BRL) and pBI121 (Clontech Lab.).

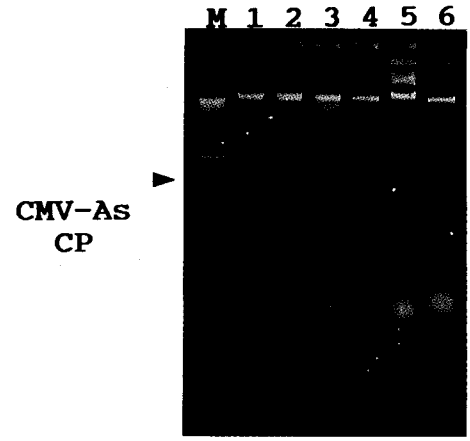

Fig. 4. Electrophoretic pattern of pCMASCP121-123 from *Agrobacterium tumefaciens* strain LBA4404 on 1.0% agarose gel to confirm insert fragment of coat protein gene of cucumber mosaic virus-As strain. Lane 1 : *Bam*HI, 2 : *Kpn*I, 3 : *Bam*HI-*Kpn*I, 4 : *Bam*HI-*Sac*I, 5 : not treated, 6 : pBI121 vector/*Bam*HI-*Sac*I, and M : 1 kb DNA ladder (GIBCO BRL) for molecular size marker. The GUS gene was shown in lane 6. Arrow-head indicates the insert cDNA.

DISCUSSION

The CMV-As strain coat protein (CP) gene was engineered for expression in plant using the cauliflower mosaic virus (CaMV) 35S transcript regulatory sequences, cloned into an *Agrobacterium*-derived binary vector. We have cloned the CP gene of CMV-As for this purpose and constructed a chimeric gene by using the gene for plant transformation and expression by using cDNA of CMV-As CP and plant expression vector pBI121.

Many studies have demonstrated the great potential of genetically engineered plants with virus-related genes to control virus diseases.

Virus-derived genes have been important tools for the production of transgenic plant lines resistant to viral pathogens in the past several years. In 1986, Powell-Abel *et al.* firstly reported that the transgenic tobacco plant expressing the CP gene of tobacco mosaic virus (TMV) became resistant to TMV infection (22). Transformation with DNA which encodes a virus CP can result in transgenic plants that are resistant to infection by the same virus. The coat protein-mediated resistance (CP-MR) transgenic plants have led to the development of plant lines resistant to alfalfa mosaic virus (28), CMV (7,

30), potato virus X (11), potato leafroll virus (3, 13), tomato yellow leaf curl virus (14) and several different plant viruses. In the CP-MR the transgenic plants harbor a gene that encodes the CP indicated providing resistance against the virus from which the gene was taken. The effectiveness of CP-MR largely depends upon the inoculum concentrations and the viral strains for challenging inoculation.

In some cases, the gene that gives resistance to viruses is closely related in structure to the virus from which the gene was taken. For example the CP gene of TMV gave resistance with the proportion to the amino acid homology between TMV CP and challenging virus against the following tobamoviruses: tomato mosaic virus, pepper mild mottle virus, tobacco mild green mosaic virus, odontoglossum ringspot virus, and ribgrass mosaic virus, but much less resistance against the distantly related tobamovirus such as sunnhemp mosaic virus (cowpea strain of TMV) (17). This resistance is useful because it is expressed throughout the life of an infected plant, even in plants with secondary infection, which are potent sources of inoculum for further spread of the virus (4).

An alternative method to produce CMV resistant plant is the use of a satellite RNA (satRNA) of CMV, which interferes with CMV replication, leading to attenuation of CMV symptoms (10, 15). In Korea, Lee *et al.* (15) reported construction of plant binary vector for satRNA of CMV-As, and Paek and Hahn (19) reported that the transgenic tobacco plant expressing the satRNA of CMV revealed resistant to CMV infection (19). This method is considered to have some limitations, such as a potential risk of mutation in transgenic plants during CMV infection and expression of mild symptoms, but it is effective in reducing CMV replication during later stages after infection has occurred. Yie *et al.* (30) reported that the transgenic plants expressing both a CP gene and a satRNA of CMV showed high resistance compared with the plants expressing either gene alone.

Recently, nonviral, *Pac*I gene, double-stranded RNA specific ribonuclease (ds-RNase) which is similar to Ribonuclease III, from *Schizosaccharomyces pombe* (12), has been introduced into the tobacco plant with *Agrobacterium*-mediated system. The transgenic plant expressing the gene was resistant or delay of symptom expression in several single-stranded RNA plant viruses (TMV, CMV, and PVY) (18). This approach would be widely used for production of virus-resistant crops in the near future.

As the following steps in this study, tobacco plants were transformed with this constructed chimeric plant expression vector by *Agrobacterium*-mediated system by using the leaf-disc method. About 1,000 kanamycin resistant, putative transgenic tobacco plants were selected. Among the putative lines, about 200 lines have been investigated. Preliminary results have shown that some transgenic plant lines represented delay of symptom development and were resistant as compared with control plant at 1.0 μg/ml virus concentration challenged. Our preliminary results demonstrate that the transgenic plants expressing a CP have potential resistance in disease incidence.

요 약

오이 모자이크 바이러스 As(CMV-As)계통의 외피단백질을 식물체에서 발현시키기 위해서 컬리플라워 모자이크 바이러스(CaMV) 35S 전사조절 염기부위와 함께 CMV-As 외피단백질 유전자를 *Agrobacterium* 벡타에 클로닝시켰다. CMV-As 계통의 외피단백질 유전자를 클로닝한 후 식물체 발현벡타인 pBI121을 사용하여 식물체 형질전환용 재조합 유전자를 구축하였다. CMV-As 계통 외피단백질 유전자를 포함하는 pCMAS66 클론을 pBI121벡타에 도입시키기 위한 제한효소 인식부위를 위해서 phagemid 벡타인 pSPORT1에 삽입시켰다. 선발된 pCASCP02를 확인하고 pBI121 벡타의 GUS유전자 위치에 CMV-As 외피단백질 유전자를 클로닝시키기 위해서 이들을 각각 *Bam*HI 및 *Sac*I 처리 후 클로닝시켰다. 클로닝 후 선발된 pCMASCP121-123은 CMV-As 외피단백질 유전자가 해독방향으로 삽입되었으며 이를 급냉해빙(Freeze-thaw)법으로 *Agrobacterium*에 도입시켰다. 도입된 클론을 제한효소분석으로 확인한 결과, 오이 모자이크 바이러스 As 계통의 외피단백질 유전자가 *Agrobacterium tumefaciens*에 형질전환되었음을 확인할 수 있었다. 이들 벡터시스템을 사용하여 성공적으로 CMV-As 외피단백질 유전자를 담배에 도입시켰으며, 높은 빈도의 임의 형질전환 개체를 선발할 수 있었다.

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