Identification and Safety Assessment of *Cucumber Mosaic Virus* Coat Protein in Genetically Modified Pepper (*Capsicum annuum*)

Eunji Kim[1†], Hee Min Noh[1†], Chanvorleak Phat[1], Gung Pyo Lee[2], Jun Hong Kim[3], Tae-Sung Park[4], and Chan Lee[1*]

[1]Advanced Food Safety Research Group in BK21, Department of Food Science and Technology, Chung-Ang University, Anseong, Gyeonggi-Do 17546, Korea
[2]Department of Integrative Plant Science, Chung-Ang University, Anseong, Gyeonggi-Do 17546, Korea
[3]Research and Development Coordination Division, Research Policy Bureau, Rural Development Administration, Jeonju, Jeollabuk-Do 54875, Korea
[4]Vegetable Research Division, National Institute of Horticultural & Herbal Science, Wanju, Jeollabuk-Do 55365, Korea

*Corresponding author: chanlee@cau.ac.kr
†These authors contributed equally to this work

Abstract

The great economic losses caused by *Cucumber mosaic virus* (CMV) infection of peppers has led to the development of genetically modified (GM) CMV-resistant peppers. We developed virus-resistant pepper plants using *Agrobacterium tumefaciens*-mediated transformation. The expressed recombinant protein was purified using nickel-nitrilotriacetic acid resin and immunoaffinity chromatography, and purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblot analysis revealed the purified CMV coat protein (CMV-CP) had a molecular mass of 25 kDa. After in-gel digestion and desalting, the internal peptide fragments of CMV-CP were sequenced by matrix-assisted laser desorption/ ionization–time of flight. Most GM pepper and *Escherichia coli* BL21 internal peptides had identical peptide sequences and contained 137 of 183 whole peptides in CMV-CP. A quantitative enzyme-linked immunosorbent assay was performed to detect CMV-resistant GM peppers. We also provide basic information about the expressed protein in GM peppers for further safety assessment. The contents of soluble protein and CMV-CP were measured in GM and control peppers cultivated in three different areas of Korea. Statistical significance in terms of cultivation areas, harvest times, generations, and plant tissue origin were determined based on a P value of 0.05. The highest amount of CMV-CP was detected at the seedling stage from plant grown in each region. T3 and T5 showed significantly different levels of CMV-CP from T4 in leaves in the whorl stage. No statistical differences were observed among GM peppers at different stages of maturity in any cultivation area. The results from this study contribute to the safety evaluation of newly designed CMV-resistant GM peppers and provide a standard against which to compare other virus-resistant GM peppers.

Additional key words: CMV-resistant peppers, soluble protein, enzyme-linked immunosorbent assay (ELISA), safety evaluation

Introduction

Pepper (*Capsicum* sp.) is used internationally as a food source and for seasonings, dyes, and medicines. Because of its various flavors and colors, it is an integral part of many world cuisines. Because of its high palatability, commercial value, and adaptability (these plants tolerate nearly every climate), the species has become one of world's staple vegetables fruit. Approximately 22% of the total vegetable fruit cultivation area in Korea is given to chili peppers (Statistics Korea, 2015). The annual commercial value of peppers in Korea has reached nearly $2 billion dollars, and continues to grow. However, virus infection of peppers has caused heavy losses in the rural economy. According to Choi et al. (2015), chili peppers were infected at the highest level (51.6%) of all vegetablesfruit cultivated in outfield systems in Korea. Several studies have reported that over 60 species of viruses can infect peppers (Kenyon et al., 2014). The major viruses found in peppers are *Cucumber mosaic virus* (CMV), *Pepper mild mottle virus* (PMMoV), *Broad bean wilt virus* 2 (BBWV2), *Tomato spotted wilt virus* (TSWV), and Impatiens necrotic spot virus (INSV) (Kaper et al., 1981).

CMV, the most frequently occurring virus in peppers, belongs to the genus *Cucumovirus* in the plant virus family *Bromoviridae*, and it infects over 1000 plant species in 85 families (El-Borollosy and Oraby, 2012). Since it was discovered as the causal disease agent of cucurbit hosts in 1916 (Doolittle, 1916; Jagger, 1916), CMV has been reported to infect cucumber, melon, zucchini, soybean, and tobacco (Baskaran et al., 2016; Mochizuki et al., 2014; Roossinck et al., 1990; Thompson et al., 2015). In Korea, the virus is associated with over 33% of chili pepper infections, and causes losses valued at approximately $200 million dollars each year. Many studies have sought to develop virus-resistant plants to prevent plant viral diseases.

One technique used to create disease resistance involves insertion of the viral coat proteins (CP) of a specific virus into the plant genome. CP-mediated protection shows a common phenomenon similarities with viral cross-protection (Ivanov and Mäkinen, 2012). The mechanism is not clearly understood, but it may involve interference with viral uncoating, or in virus replication. Several studies have shown that plants expressing viral coat protein genes are protected against viral infection; these genetically modified (GM) plants include tomato (Zrachya et al., 2007), tobacco (Nida et al., 1992), orange (Iwanami et al., 2004), sugarcane (Jain et al., 2007), and papaya (Krubphachaya et al., 2007). Insertion of CMV-CP genes in numerous pepper plants has successfully created CMV and *tomato mosaic virus* (ToMV)-resistant hot peppers (Shin et al., 2002), CMV-resistant sweet peppers (Chen et al., 2003), and CMV and *tobacco mosaic virus* (TMV)-resistant chili peppers (Cai et al., 2003).

However, driven by consumer demand for GM crops to be controlled and discriminated from other natural crops, the need for safety assessment and labeling information is increasing. Reliable analysis to qualify and quantify inserted and expressed proteins are forming a greater part of the safety assessment and management of GMOs. In the same way, optimization of qualification and quantification methods for viral CPs in the wild-type virus is required to assess the safety of these crops. In this study, we applied recently established analytical methods to detect viral CPs in virus-resistant GM crops using a peptide polyclonal antibody (Choi et al., 2015). An enzyme-linked immunosorbent assay (ELISA) was used to quantify the inserted protein in GM crops. In addition, we also confirmed variation in CP protein levels in GM peppers of different cultivation areas, harvest times, generations, or in different plant tissues.

Materials and Methods

Capsicum species

Virus-resistant pepper plants were developed by *Agrobacterium tumefaciens*-mediated transformation. The pCambia vector 2300 (CambiaLabs, Canberra, Australia) contains expression cassettes of CMV-CP and neomycin phosphotransferase II (NPT II) genes, each of which is flanked by the *Cauliflower mosaic virus* 35S promoter and the kanamycin selection cassette with a $2 \times 35S$ promoter and 35S poly A terminator (Fig. 1). Seeds of GM pepper (*Capsicum annuum* var. *Longum*) cultivar 'H15' and its control line 'P2377', provided by the Biotechnology Institute of Nongwoo Bio Company Ltd., Korea, were cultivated at Chun-Ang University (Anseong, Gyeonggi-do, Korea), Korea University (Namyangju, Gyeonggi-do, Korea), and the Korea Research Institute of Bioscience and Biotechnology (KRIBB; Cheongju, Chungchung-do, Korea). Pepper samples were maintained at −70℃ until required for analysis.

Expression and purification of rCMV-CP from *E. coli*

The expression plasmid was constructed using the pET-22(+) vector to express recombinant CMV-CP (rCMV-CP) in bacteria (Sørensen et al., 2005). CMV-CP was positioned downstream of the bacteriophage T7 promoter in a medium copy number plasmid. Polymerase chain reaction (PCR) was performed using CMV-CP-pCAMBIA-2300 .

For expression, *E. coli* BL21 strain was grown overnight at 37℃ on lysogeny broth (LB medium) containing 0.1% ampicillin, and was then used as a seed culture to inoculate fresh cultures. When the optical density reached 0.5 at 600 nm, the cells were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM final concentration) and cultivated for 4 h at 25℃ at 200 rotations per minute (rpm). Induced *E. coli* culture was centrifuged at 8000 rpm for 20 min at 4℃, and the precipitate was suspended in imidazole (20 mM) with 0.1% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA). A French pressure cell press (SLM Aminco, Waltham, MA, USA) was used for cell lysis. Cell debris was separated by centrifugation at 10 000 rpm for 30 min at 4℃. The resultant protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen, Valencia, CA,

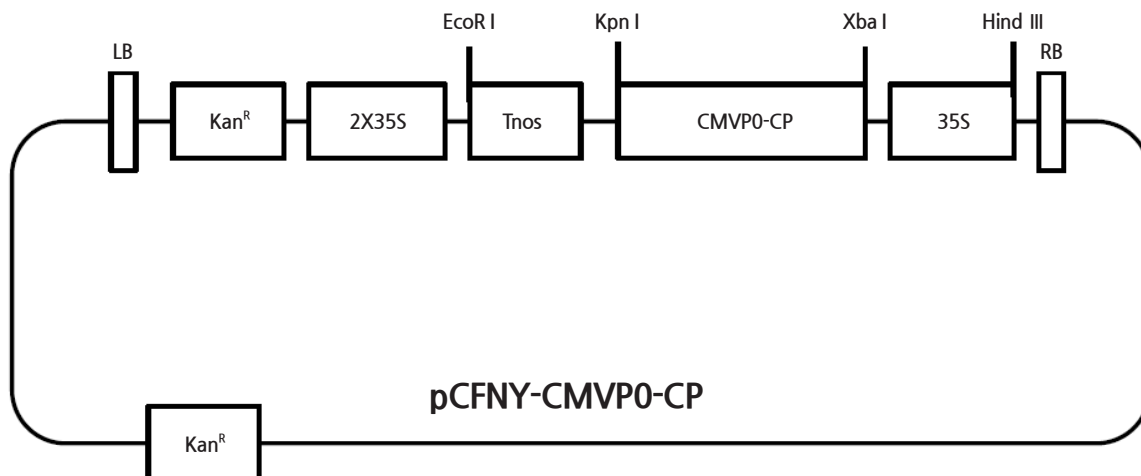Fig. 1. Vector used for genetic transformation n[R]; the coat protein-encoding gene was cloned from the *cucumber mosaic virus* (CMV)-P0 pathogen and subcloned into a pCAMBIA 2300 vector.
(Kan[R]: Kanamycin resistant gene; 2X35S and 35S: Promoter; Tnos: Nos terminator; CMVP0-CP: Cucumber mosaic virus resistant coat protein gene; pCFNY: The binary vector pCAMBIA2300; LB and RB: The left and right borders)

USA) pre-equilibrated with lysis buffer (50 mM NaH_2PO_4, 10 mM imidazole, 300 mM NaCl, 0.05% Tween 20, pH 8.0). After loading 10 mL of cell-free extract, the column was washed once (5 mL matrix volume) with five bed volumes of washing buffer (50 mM NaH_2PO_4, 20 mM imidazole, 300 mM NaCl, 0.05% Tween 20, pH 8.0). Elution buffer (50 mM NaH_2PO_4, 250 mM imidazole, 300 mM NaCl, 0.05% Tween 20, pH 8.0) was used to elute bound proteins from the column.

Preparation of the immunoaffinity column for CMV-CP purification

In previous studies, Choi et al. (2015) developed antibodies that were designed from the peptide sequence of CMV-CP. Using peptide polyclonal antibodies, an immunoaffinity column was produced to purify CMV-CP. Ten milligrams of peptide polyclonal antibody was coupled to the AminoLink Plus column according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA).

Expression and purification of CMV-CP in GM peppers

GM pepper fruit (100 g) was ground in 200 mL chilled phosphate-buffered saline (PB; 0.1 M phosphate, 0.15 M sodium chloride, pH 7.2, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% protease inhibitor) and the slurry was centrifuged at 10 000 rpm for 20 min. Protein pellets were precipitated by adding 70% saturated ammonium sulfate solution and dissolved in a minimal volume of PBS. The suspended solution was centrifuged at 10 000 rpm for 20 min, and the supernatant was concentrated using a Centriprep (Millipore, Bedford, MA, USA). To remove salts, the concentrated protein was dialyzed overnight; all procedures were proceeded carried out at 4°C.

The affinity column was equilibrated at room temperature. Six milliliters of PBS and 2 mL of dialyzed protein sample were poured into the column, which was then rocked for 60 min at room temperature. Subsequently, 2 mL of wash buffer was passed through the column four times, and 2 mL of elution buffer (0.1 M Glycine·HCl, pH 2.5) was passed through the column three times. All fractions were collected and used as protein samples directly for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.

Determination of protein concentration

Protein was quantified using the Coomassie protein assay (Thermo Scientific, Rockford, IL, USA) with bovine serum albumin (BSA) as the protein standard. BSA concentrations were 0.2, 0.4, 0.6, 0.8 mg·mL^{-1}. Protein (10 μL) was mixed with 90 μL of Bradford reagent, and the mixture was measured at 595 nm using a microplate reader (Molecular Devices, Uppsala/Stockholm, Sweden).

SDS-PAGE and immunoblot analysis

Protein samples were heated for 5 min in 5× loading buffer (1 M Tris·HCl at pH 6.8, 50% glycerol, 10% SDS, 0.5% 2-mercaptoethanol, 1% bromophenol blue) and separated using a 12% acrylamide gel with a molecular weight standard marker (Bio-Rad, CA, USA). Proteins were visualized using Coomassie brilliant blue R250. Protein blots were transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore) at 100 V for 2 h. After blocking the PVDF membrane with 5% skim milk/Tris-buffered saline (TBS) for 2 h, it was then incubated with peptide polyclonal antibody for 2 h at room temperature. The anti-immunoglobulin G (IgG) alkaline phosphatase conjugate (Sigma–Aldrich, St. Louis, MO, USA) was used as a secondary

antibody. Nitroblue tetrazolium (Promega, Madison, WI, USA) was used to stain the membrane and 5-bromo-4-chloro-3-indoyl phosphate (Promega) was used as a substrate in alkaline phosphatase buffer (100 mM Tris·HCl, pH 9.5). The reaction was stopped when a desirable color density was achieved.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis

Following SDS-PAGE, the protein was separated and the gel was stained with Coomassie blue. Protein spots in the gel of interest were analyzed at the E-mass Institute Protein Mass Spectrometry Facility. Briefly, sequencing-grade modified trypsin was used to excise the protein of interest for in-gel trypsin digestion and the proteins were alkylated with iodoacetamide (Sigma–Aldrich) as part of the digestion protocol. The digestion was performed overnight at 37°C. The peptide solution was desalted using ZipTip C18 (Pierce). The solution was re-suspended in 2 μL of liquid chromatography (LC) water (0.2% trifluoroacetic acid (TFA)) and loaded onto the MALDI-TOF plate (Agilent).

The resulting database file was loaded to the databases of a locally installed (University of Liverpool, UK) Mascot search engine (Matrix Science; http://www.maxtrixscience.com). The identified CMV-CP sequences were submitted to the National Center for Biotechnology Information (NCBI) nr protein database using Protein BLAST (http://www.ncbi.nlm.nih.gov/blast) (Millares et al., 2012; Zolla et al., 2008).

Edman sequencing of CMV-CP expressed in GM pepper and *E. coli* BL21

Purified protein was separated using SDS-PAGE, and transferred to PVDF membranes at 100 V for 2 h. The membrane was stained using Coomassie blue for 2 min and destained with 50% methanol. N-terminal sequencing analysis used phenylisothiocyanate (PTH; Edman's reagent). This solution, in conjunction with the N-terminal peptide of PTH, formed phenylthiohydantoin. The peptide bond was broken because the structure was unstable. Amino acids were then detected by mass spectrometry.

Glycosylation analysis

A Glycoprotein Detection Kit (Sigma-Aldrich) was applied to the gel containing separated proteins and treated with the oxidation reagent according to the manufacturer's instructions. This treatment induced the oxidization of cis-diol sugar groups in the glycoproteins. The resulting aldehyde groups reacted with the Sigma Stain Reagent to form Schiff-base bonds that developed magenta bands. This detection system was a modification of the periodate-acid-Schiff (PAS) reagent method.

Quantification of inserted proteins in plant tissues using ELISA

Production of standard protein

rCMV-CP purified from *E. coli* BL21 was used as the standard substrate for ELISA analysis. The standard protein was loaded on to the same plate with protein concentrations of 0.5, 5, 15, 25, and 50 ng·mL^{-1}. PBS was used as the dilution buffer (4.3 mM Na$_2$HPO$_4$, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH$_2$PO$_4$).

Limit of detection (LOD) and limit of quantification (LOQ)

The ELISA limit of detection (LOD) and limit of quantification (LOQ) were determined according to the ICH Harmonized Tripartite Guideline (1994). Absorbances of the three most dilute standards were measured using ELISA, and a calibration curve

was calculated. The middle concentration (5 ng·mL^{-1}) was detected seven times, and the standard deviation was calculated. The ICH Harmonized Tripartite Guideline provided the formula to calculate LOD and LOQ values as follows:

LOD = 3.3 x σ/S;

LOQ = 10 x σ/S (σ: standard deviation, S: slope of calibration curve.)

Sources of plants for evaluation of expression

To confirm the variation of CMV-CP expression in GM pepper, all portions of the GM pepper were analyzed. Cultivation areas were Chun-Ang University (Anseong, Korea), Korea University (Namyangju, Korea), and KRIBB (Cheongju, Korea). Peppers of the same cultivar were planted in three cultivation areas. Sampling times were at the whorl (root, stem, and leaf) and maturation stage (root, stem, leaf, fruit, and pollen). Samples were weighed and stored at −70°C until required for extraction and analysis.

Plant tissue processing

Each sample was mixed with PBS (0.1 M Phosphate, 0.15 M sodium chloride, pH 7.2, 1 mM PMSF, 1 mM EDTA, 0.1% protease inhibitor), and centrifuged at 10 000 rpm for 20 min. The supernatant was then filtered using Whatman filter paper No. 4 and subjected to ELISA analysis. The percentage dry-weight of each sample was determined by recording the weight of the sample before and after drying.

Indirect ELISA

Optimum conditions for the detection of CMV-CP are shown in Table 1. A 96-well microplate (Nunc, Roskilde, Denmark) was coated with 200 μL of the extracts per well. The plate was incubated overnight at 4°C. Next, the plate was washed and 3% BSA dissolved in PBS supplemented with Tween 20 (PBST) was added to block the uncoated surfaces in the well. Plates were incubated for 2 h at 37°C. As the primary antibody, polyclonal antibody was diluted in 1% BSA in PBST and incubated with the samples for 3 h at 37°C. After washing, goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma–Aldrich) was diluted with 1% BSA in PBST, and added to all wells. Plates were incubated at 37°C for 2 h. After unbound antibody was removed by washing, color was developed using 4-nitrophenyl phosphate (Sigma–Aldrich, 1 mg/ml) as a substrate in PBS. The substrate changed color by reacting with the enzyme. The color change was measured at 405 nm using a microplate reader (Molecular Devices), and the optical density of the sample was compared to the standard curve for quantification (Choi, 2011).

Table 1. Optimal ELISA conditions.

Reagents	Materials	Incubation conditions
PBS[*]	4.3 mM sodium phosphate, dibasic (Na$_2$HPO$_4$),	Coating buffer: overnight at 4°C
	137 mM sodium chloride (NaCl),	
	2.7 mM potassium chloride (KCl),	
	1.4 mM potassium chloride, monobasic (KH$_2$PO$_4$), pH 7.2	Washing buffer
PBST[**]	PBS + 0.05% Tween 20	Blocking step: 2 h at 37°C
3% BSA[***]/PBST	PBST + 3% BSA	3 h at 37°C
1st antibody	1:5,000 in 1% BSA/PBST	2 h at 37°C
2nd antibody	1:5,000 in 1% BSA/PBST	Color developing step: determination every 10 min
Substrate	4-nitrophenyl phosphate (pNPP)	

[*] Phosphate buffer saline
[**] Phosphate buffer saline tween-20
[***] Bovine serum albumin

Calculation and explanation of ELISA data

All ELISA data were analyzed using Microplate Analysis Software. The mean absorbance per sample was read and plotted against the standard curve to give a value for the concentration of the protein of interest (POI) per mL of extract, without accounting for the dilution factor. The POI concentration ($\mu g \cdot mL^{-1}$) in plant tissue was calculated as follows:

ng POI·mL^{-1}= diluted ng POI·mL^{-1} × dilution factor;

μg POI·g^{-1} FW= ng POI·mL^{-1} × extraction buffer / amount extracted (g) / 1000;

μg POI·g^{-1} DW = μg POI·g^{-1} FW / % dry weight.

Statistical methods to compare the CMV-CP levels

SPSS software version 21 (IBM Corp., USA) was used for statistical analyses to compare group means. The statistical significance was determined using the Duncan model.

Results and Discussion

Expression of rCMV-CP in *E. coli* BL21

E. coli BL21 was incubated on an LB plate containing 0.1% ampicillin, and ampicillin resistant colonies were selected. After harvesting the cells, protein was extracted using a French pressure cell press. Extracted proteins were purified using a Ni-NTA chromatography column. The His-tagged CMV-CP was eluted with elution buffer containing a high concentration of imidazole. In all steps, the fractions were loaded onto an acrylamide gel and an immunoblot assay was performed. The purified CMV-CP produced a single band by SDS-PAGE, and this was further confirmed by immunoblotting using a peptide polyclonal antibody. As shown in Fig. 2, the presence of CMV-CP was confirmed as a single band at 25 kDa through SDS-PAGE and immunoblot analysis.

According to Pina et al. (2014), the hexapeptide 6 × His was genetically fused to the N-terminus of proteins produced in *E. coli* and purified by immobilized metal affinity chromatography (IMAC) using a Ni-NTA column. Subsequently, protein elution was attempted using a pH gradient in the presence of denaturing agents. Janknecht et al. (1991) reported that SRF-His6-tagged protein was found in a broad range of imidazole concentrations. Normally, elution of SRT-His6-tagged protein with 0.8–40 mM imidazole leads to high contamination. Thus, elution with 80 mM imidazole was used for the majority of tagged proteins.

Expression of CMV-CP in GM peppers

Extraction of GM pepper proteins was investigated after precipitating soluble protein in 70% ammonium sulfate. Extracts were purified using an immunoaffinity chromatography column. During purification, all fractions were analyzed using SDS-PAGE; a single band was present in eluted fractions. The single protein band was confirmed by immunoblot analysis. This band exhibited the same molecular weight (approximately 25 kDa) of CMV-CP in *E. coli* according to SDS-PAGE and immunoblotting (Fig. 2). Choi et al. (2015) observed that, in immunoblot analysis, the expression of CMV-CP in GM peppers appeared as a major band at 26 kDa. Also in immunoblot analysis, Zein et al. (2009) used specific monoclonal antibodies (mAbs) for Pepo- and M2-CMV to detect CMV-CP in infected tobacco plants, and revealed the presence of 26
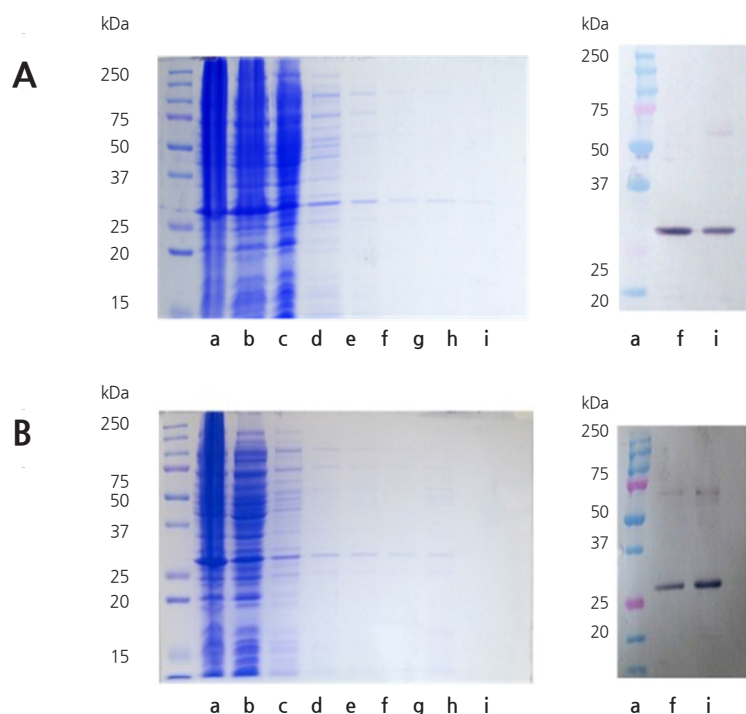
Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (left) and immunoblot (right) of purified expressed *cucumber mosaic virus* coat protein (CMV-CP) from (A) genetically modified pepper and (B) *Escherichia coli* BL21, purified with a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column. a: size marker, b: fraction extracted before passing through the column, c-e: washing fractions 1-3, f-i: elution fractions 1-4.

and 52 kDa viral proteins. The 26 kDa protein band closely matches the molecular weight of CMV-CP, whereas the other band could be a dimer of the 26 kDa protein.

Identification of CMV-CP expressed in E.coli BL21 and GM peppers

MALDI-TOF analysis

After hydrolysis with trypsin, the internal peptide fragments of CMV-CP from *E. coli* BL21 and GM pepper were sequenced by MALDI-TOF (Fig. 3). A mass chromatogram is presented in Table 2. For CMV-CP expressed in *E. coli* BL21, the intensity coverage was 98.8%. The peptide sequence list is shown in Fig. 4. Of these sequences, 65.1% were found to have significant identity to CMV-CP. For CMV-CP expressed in GM pepper, the intensity coverage was 52.7%. Of these sequences, 69% were found to have significant identity to CMV-CP.

Using MALDI-TOF, Corpillo et al. (2004) reported differences in protein expression between wild-type and transgenic tomatoes. ATP synthase, RuBisCO, and oxygen-evolving enhancer protein were respectively found to comprise 24.5, 43.2, and 63.6% of sequence coverage. Food Standards (2008) reported that the Vip3Aa20 proteins isolated from MIR162 and recombinant *E. coli* were digested with trypsin. Mass spectrometry analysis was then performed using a quadrupole time-of-flight mass spectrometer. To identify a protein using this method, it was essential to match a sufficient number of observed tryptic mass fragments to expected mass fragments. Generally, if the measured coverage of the sequence is 15% or higher with a minimum of five matching peptides, it is considered to be reliable for protein identification (Lim et al., 2003;

Jensen et al., 1997). The tryptic mass fragments identified from plant and bacterially derived Vip3Aa20 matched the expected tryptic peptides generated in silico, with coverage of 40% and 45%, respectively.

In this study, we obtained more than 40% sequence coverage; therefore, we could confirm the proteins identified in both *E. coli* and GM pepper (Fig. 4).

Edman sequencing of CMV-CP expressed in GM pepper and E. coli BL21

After modification to a more stable phenylthiohydantoin (PTH) derivative, the derivatized amino acid was subjected to chromatography. The PTH amino acid was identified by its unique intensity in the chromatogram. This process was repeated iteratively for each subsequent terminal amino acid of the protein (Thoma et al., 2009). In a study by Food Standards Australia New Zealand (2008), technical difficulties meant that the N-terminal sequence of plant-derived proteins
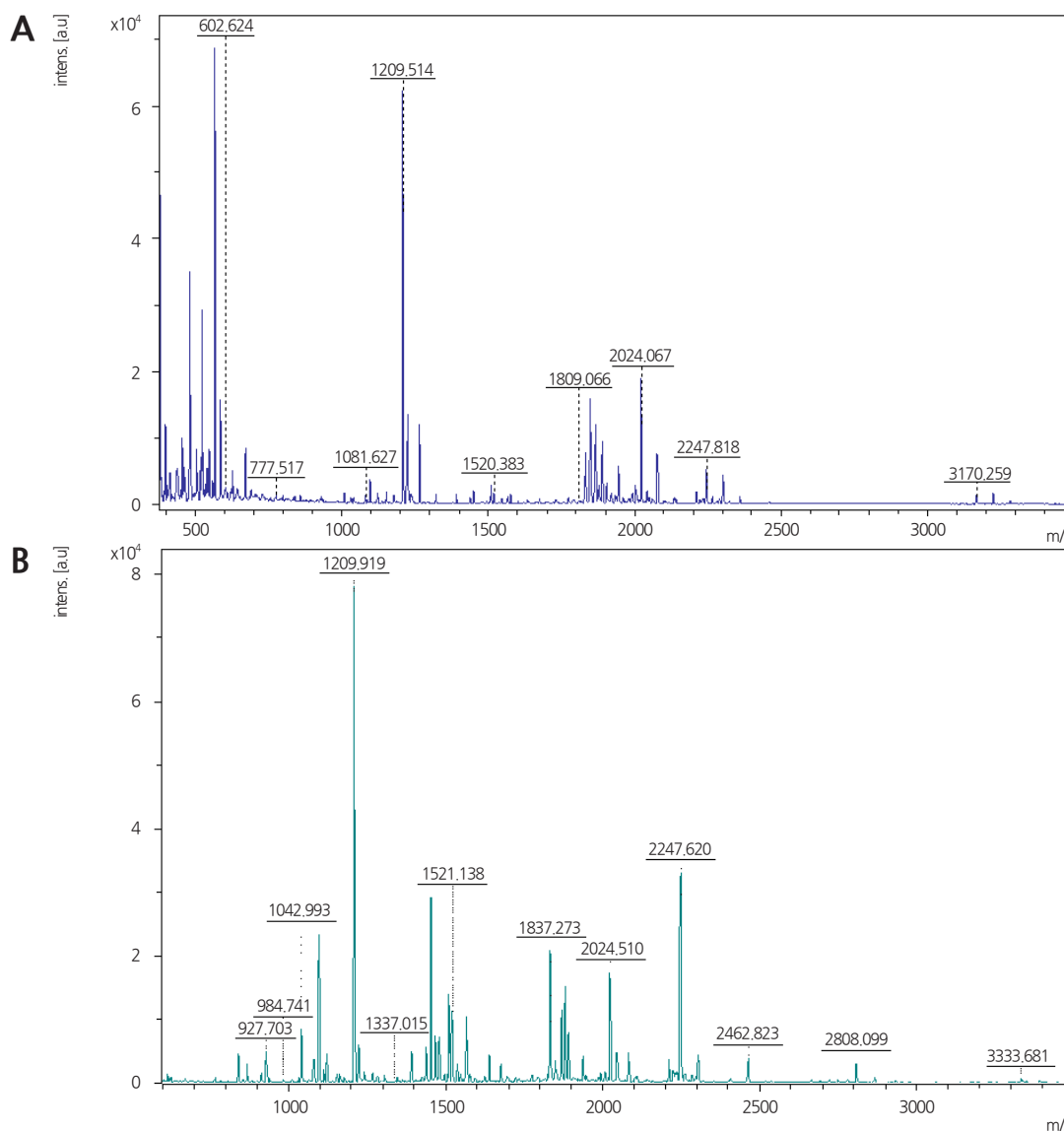

Fig. 3. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) data of trypsin hydrolysate of *cucumber mosaic virus* coat protein (CMV-CP) from (A) *Escherichia coli* BL21 and (B) genetically modified pepper.

Table 2. Tryptic digested peptides of cucumber mosaic virus coat protein (CMV-CP) from (A) *Escherichia coli* BL21 and (B) genetically modified pepper.

	Start-End	Observed	Mr (expt)	Mr (calc)	Delta	M	Peptide
A	12 - 14	444.5503	443.5431	444.2557	-0.7126	1	R.NRR.R
	16 - 18	428.6966	427.6893	427.2655	0.4238	0	R.RPR.R
	16 - 19	584.6344	583.6271	583.3666	0.2604	1	R.RPRR.G
	23 - 33	1122.5185	1121.5112	1121.5101	0.0011	0	R.SAPSSADANFR.V
	23 - 41	2034.9491	2033.9418	2033.0290	0.9128	1	R.SAPSSADANFRVLSQQLSR.L
	34 - 41	930.6638	929.6565	929.5294	0.1271	0	R.VLSQQLSR.L
	45 - 63	2024.0671	2023.0598	2023.0599	-0.0001	0	K.TLAAGRPTINHPTFVGSER.C
	83 - 89	830.4294	829.4221	829.4082	0.0139	1	R.GSYYGKR.L
	89 - 101	1548.3023	1547.2950	1547.8195	-0.5245	1	K.RLLLPDSVTEYDK.K
	90 - 102	1520.3834	1519.3761	1519.8134	-0.4372	1	R.LLLPDSVTEYDKK.L
	102 - 106	602.6235	601.6162	601.3911	0.2251	1	K.KLVSR.I
	107 - 110	529.7413	528.7341	528.3384	0.3957	0	R.IQIR.V
	107 - 116	1178.4666	1177.4594	1176.7343	0.7251	1	R.IQIRVNPLPK.F
	117 - 126	1209.5139	1208.5067	1208.6190	-0.1123	0	K.FDSTVWVTVR.K
	166 - 174	1081.6271	1080.6198	1080.5637	0.0560	0	K.LLYDLSAMR.A
	175 - 181	777.5167	776.5094	776.3487	0.1607	0	R.ADIGDMR.K
	175 - 182	905.4559	904.4487	904.4436	0.0050	1	R.ADIGDMRK.Y
	183 - 209	3170.2592	3169.2519	3169.5931	-0.3413	1	K.YAVLVYSKDDALETDELVLHVDIEHQR.I
	191 - 209	2247.8183	2246.8110	2246.0815	0.7295	0	K.DDALETDELVLHVDIEHQR.I
	210 - 218	883.4613	882.4540	881.5222	0.9317	0	R.IPTSGVLPV.-
B	1 - 11	1168.8962	1167.8889	1167.5190	0.3699	1	-MDKSESTSAGR.N
	4 - 11	794.5525	793.5453	793.3566	0.1887	0	K.SESTSAGR.N
	23 - 41	2040.4815	2039.4742	2039.0032	0.4710	1	R.SASSSSDANKRVLSQQLSR.L
	34 - 41	930.7417	929.7344	929.5294	0.2050	0	R.VLSQQLSR.L
	34 - 44	1285.9552	1284.9479	1284.7514	0.1965	1	R.VLSQQLSRLNK.T
	45 - 63	2024.5101	2023.5028	2023.0599	0.4429	0	K.TLAAGRPTINHPTFVGSER.C
	80 - 88	1058.8398	1057.8325	1057.5192	0.3133	1	K.IDRGSYYGK.R
	83 - 88	674.3556	673.3483	673.3071	0.0412	0	R.GSYYGK.R
	89 - 101	1549.1537	1548.1464	1547.8195	0.3269	1	K.RLLLPDSVTEYDK.K
	90 - 101	1393.0219	1392.0146	1391.7184	0.2962	0	R.LLLPDSVTEYDK.K
	90 - 102	1521.1377	1520.1305	1519.8134	0.3171	1	R.LLLPDSVTEYDKK.L
	103 - 110	984.7411	983.7338	983.6240	0.1098	1	K.LVSRIQIR.V
	107 - 116	1177.8225	1176.8152	1176.7343	0.0809	1	R.IQIRVNPLPK.F
	111 - 116	666.5973	665.5900	666.4064	-0.8165	0	R.VNPLPK.F
	111 - 116	667.3611	666.3538	666.4064	-0.0526	0	R.VNPLPK.F
	111 - 126	1858.2806	1857.2733	1857.0149	0.2584	1	R.VNPLPKFDSTVWVTVR.K
	117 - 126	1209.9185	1208.9113	1208.6190	0.2923	0	K.FDSTVWVTVR.K
	117 - 127	1337.0145	1336.0073	1336.7140	-0.7067	1	K.FDSTVWVTVRK.V
	166 - 174	1080.7443	1079.7370	1080.5637	-0.8267	0	K.LLYDLSAMR.A
	166 - 174	1081.7600	1080.7527	1080.5637	0.1890	0	K.LLYDLSAMR.A
	175 - 181	776.4211	775.4138	776.3487	-0.9349	0	R.ADIGDMR.K
	183 - 190	942.7436	941.7363	941.5222	0.2141	0	K.YAVLVYSK.D
	191 - 209	2247.6204	2246.6131	2246.0815	0.5316	0	K.DDALETDELVLHVDIEHQR.I
	210 - 218	883.5706	882.5633	881.5222	1.0411	0	R.IPTSGVLPV.-

could not be determined. However, we were able to successfully sequence the N-terminal of CMV-CP from *E. coli* BL21 and GM pepper, and the results showed that two N-terminal sequences were the same. Smith et al. (2000) reported the

A
```
MDKSESTSAG  RNRRRRPRRG  SRSAPSSSDA  NFRVLSQQLS  RLNKTLAAGR
PTINHPTFVG  SERCRPGYTF  TSITLKPPKI  DRGSYYGKRL  LLPDSVTEYD
KKLVSRIQIR  VNPLPKFDST  VWVTVRKVPA  SSDLSVAAIS  AMFADGASPV
LVYQYAASGV  QANNKLLYDL  SAMRADIGDM  RKYAVLVYSK  DDALETDELV
LHVDIEHQRI  PTSGVLPV
```

B
```
MDKSESTSAG  RNRRRRPRRG  SRSASSSSDA  NFRVLSQQLS  RLNKTLAAGR
PTINHPTFVG  SERCRPGYTF  TSITLKPPKI  DRGSYYGKRL  LLPDSVTEYD
KKLVSRIQIR  VNPLPKFDST  VWVTVRKVPA  SSDLSVAAIS  AMFADGASPV
LVYQYAASGV  QANNKLLYDL  SAMRADIGDM  RKYAVLVYSK  DDALETDELV
LHVDIEHQRI  PTSGVLPV
```

Fig. 4. Peptide sequencing coverage of *cucumber mosaic virus* coat protein (CMV-CP) from (A) *Escherichia coli* BL21 and (B) genetically modified pepper. (Underline: Same sequences analyzed by MALDI-TOF analysis)

N-terminal region of CMV from strain Fny as having a sequence alignment of M-D-K-S-E-S-T-S-A-G, which is identical to that of CMV-CP from *E. coli* BL21 in our study. Similar results were also reported by Maneechoat et al. (2015), where the N-terminal sequence of CMV strain Z1 segment RNA-3 from South Korea was M-D-K-S-E-S-T-S-A-G.

Glycosylation analysis

According to Albert (2003), deglycosylation of glycoproteins with trifluoromethanesulphonic acid (TFMS) is a powerful technique that can be used to determine the size of the peptide portion of a glycoprotein, and to reduce all glycosylation variants to a single parent molecule. However, a previous study using TFMS (Jung, 2011) did not provide an accurate basis for glycosylation. In SDS-PAGE, the bands of purified CMV-CP and TFMS-treated CMV-CP were not clear. The periodic/Schiff (PAS) method, another glycosylation detection method, was specific for glycoproteins separated by SDS-PAGE (Devine et al., 1990; Egito et al., 2001). The detection limit for carbohydrates was found to be in the range of 25–100 ng depending on the nature and degree of protein glycosylation. Hu et al. (1996) detected the glycosylation of YKL-40, a novel human chondrocyte protein, using the PAS-based Sigma glycoprotein detection kit. Here, staining of the gel for carbohydrates showed that YKL-40-related proteins are not glycoproteins.

The glycosylation of CMV-CP expressed in GM pepper and *E. coli* was detected using the glycosylation detection kit (Sigma–Aldrich, St. Louis, MO, USA), and the band was detected by SDS-PAGE (Fig. 5). Consequently, CMV-CP was not considered to be glycosylated.

## Quantification of CMV-CP in GM peppers

ELISA was used to quantitatively analyze CMV-CP in plant material. CMV-CP in the protein extract of GM peppers was used as the antigen, and protein extract from non-GM peppers was applied as the control antigen. A standard curve was created using serial dilutions of CMV-CP from *E. coli* BL21.

A calibration curve was obtained (Fig. 6), and the absorbance range was measured from 0.1 to 0.8. Then, LOD and LOQ values were calculated using the proposed ICH Harmonized Tripartite Guideline (Fig. 6). LOD and LOQ were determined as 0.022 and 0.0068 μg·mL^{-1}, respectively. Zein and Miyatake (2009) applied triple antibody sandwich (TAS)-ELISA to detect CMV in infected tobacco leaves using MAbs, and detected a concentration of 10 pg per well (10 ng·mL^{-1}) for purified Pepo-CMV, and 1 ng per well (1 μg·mL^{-1}) for M2-CMV.

Results for protein amount by cultivation area, harvest time, and tissue type are shown in Tables 3 and 4. The highest
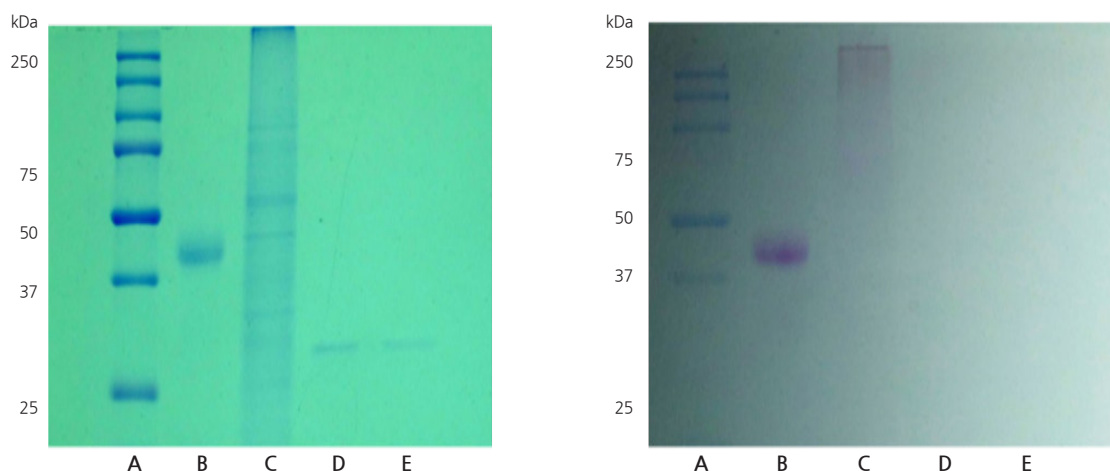
Fig. 5. Confirmation of glycosylation. Left: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomasie blue. Right: glycosylation detection after SDS-PAGE. A: molecular weight marker, B: positive control (peroxidase), C: crude extract of GM protein, D: *cucumber mosaic virus* coat protein (CMV-CP) standard (genetically modified), E: CMV-CP standard (*Escherichia coli* BL21).
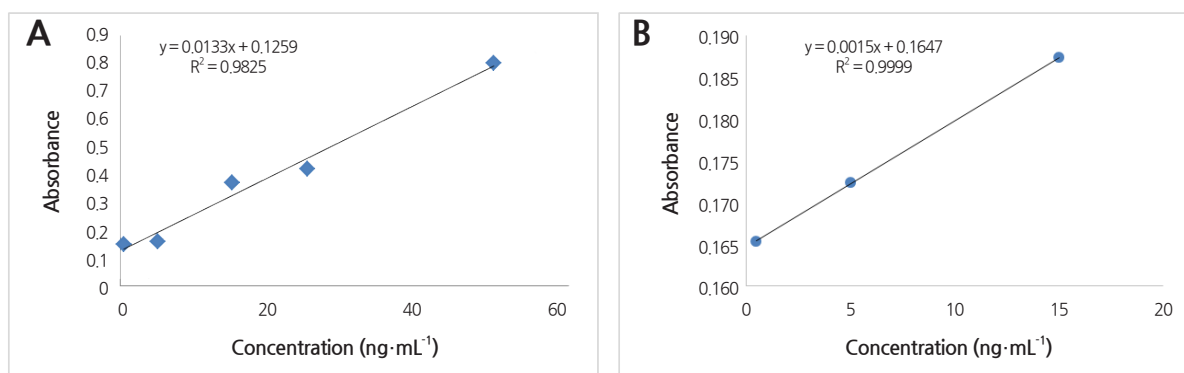
Fig. 6. Standard curve of (A) *cucumber mosaic virus* coat protein (CMV-CP) purified from Escherichia coli BL21 (0.5, 5, 15, 25, 50 ng·mL⁻¹) and (B) CMV-CP at low concentrations determined by enzyme-linked immunosorbent assay (ELISA) (Limit of detection (LOD) and limit of quantification (LOQ)).

amount of CMV-CP was detected at the seedling stage from plant grown in each region. T3 and T5 showed significantly different levels of CMV-CP from T4 in leaves in the whorl stage. Moreover, the levels of CMV-CP in GM peppers cultivated at Korea University and KRIBB were different in the stem of the whorl, but were not significantly different in other tissue types. No statistical differences were observed among GM peppers at different stages of maturity in any cultivation area. However, the root, fruit, and pollen had significantly different protein concentrations among generations in the maturity stage. Variation in expression was inconsistent according to place, generation, or crop site. Therefore, the variation in expression levels of CMV-CP was determined by the different among the growths of the plantation.

Using ELISA, Roggero and Pennazio (1984) reported the concentrations of *tobacco necrosis virus* from necrotic lesions in tobacco leaves, and found less than 10% variation among different plates. According to Sun et al. (2001), detection of CMV using electrochemical ELISA with a detection limit of 0.5 ng·mL⁻¹, was ten times more sensitive than the colorimetric ELISA method. FSANZ (2008) analyzed novel protein levels in the corn line MIR162 using ELISA. Protein was extracted

Table 3. CMV-CP content in GM peppers (whorl stage) from different cultivation areas.

		H15 T3 (µg·g⁻¹ dried weight)	H15 T4 (µg·g⁻¹ dried weight)	H15 T5 (µg·g⁻¹ dried weight)
		$(\mu g \cdot g^{-1} \text{ dried weight})$	$(\mu g \cdot g^{-1} \text{ dried weight})$	$(\mu g \cdot g^{-1} \text{ dried weight})$
Whorl root	Korea University	0.508 ± 0.109	0.514 ± 0.292	0.667 ± 0.253
	Chung-Ang University	0.792 ± 0.332	0.421 ± 0.131	0.849 ± 0.191
	KRIBB	0.877 ± 0.162	0.294 ± 0.170	0.811 ± 0.236
Whorl stem	Korea University	0.151 ± 0.029	0.176 ± 0.038	0.254 ± 0.097
	Chung-Ang University	0.792 ± 0.332	0.421 ± 0.131	0.849 ± 0.191
	KRIBB	0.740 ± 0.049	0.336 ± 0.258	0.695 ± 0.089
Whorl leaf	Korea University	0.508 ± 0.109	0.514 ± 0.292	0.667 ± 0.253
	Chung-Ang University	0.406 ± 0.087	0.413 ± 0.068	0.288 ± 0.014
	KRIBB	0.640 ± 0.093	0.407 ± 0.168	0.497 ± 0.085

Table 4. CMV-CP content in GM peppers (maturity stage) from different cultivation areas.

		H15 T3 (µg·g⁻¹ dried weight)	H15 T4 (µg·g⁻¹ dried weight)	H15 T5 (µg·g⁻¹ dried weight)
		$(\mu g \cdot g^{-1} \text{ dried weight})$	$(\mu g \cdot g^{-1} \text{ dried weight})$	$(\mu g \cdot g^{-1} \text{ dried weight})$
Maturity root	Korea University	0.161 ± 0.043	0.101 ± 0.028	0.162 ± 0.006
	Chung-Ang University	0.270 ± 0.071	0.172 ± 0.035	0.202 ± 0.012
	KRIBB	0.351 ± 0.098	0.328 ± 0.082	0.423 ± 0.076
Maturity stem	Korea University	0.370 ± 0.056	0.225 ± 0.057	0.260 ± 0.026
	Chung-Ang University	0.297 ± 0.105	0.080 ± 0.043	0.250 ± 0.009
	KRIBB	0.283 ± 0.074	0.206 ± 0.074	0.263 ± 0.070
Maturity leaf	Korea University	1.606 ± 0.048	0.564 ± 0.342	2.076 ± 0.026
	Chung-Ang University	4.120 ± 0.958	3.184 ± 0.752	3.485 ± 0.359
	KRIBB	2.946 ± 0.166	2.140 ± 0.255	3.304 ± 0.593
Maturity fruit	Korea University	3.698 ± 1.330	1.300 ± 1.081	5.359 ± 1.266
	Chung-Ang University	4.615 ± 0.252	4.721 ± 0.652	5.773 ± 1.829
	KRIBB	7.116 ± 0.934	3.119 ± 0.702	6.508 ± 1.189
Maturity pollen	Korea University	0.510 ± 0.024	0.368 ± 0.056	0.387 ± 0.041
	Chung-Ang University	0.189 ± 0.072	0.077 ± 0.009	0.107 ± 0.043
	KRIBB	0.302 ± 0.142	0.097 ± 0.069	0.325 ± 0.094

from samples of leaves, whole plant, kernels, roots, silk, pollen, and silage, from plants cultivated in two fields (Illinois and Nebraska). The plants from each control genotype were sampled at each of four growth stages. Here, it was reported that the average Phosphomannose-isomerase (PMI) level in mature MIR162 corn kernels was 1.93 µg·g⁻¹ dry weight (DW). The amounts of PMI in leaves, roots, and pith were 5.77, 1.99, and 2.75 µg·g⁻¹ DW, respectively.

This study identified CMV-CP extracted from *E. coli* and GM peppers for food safety assessment for the commercialization of CMV-resistant GM peppers. CMV-CP was purified from *E. coli* and GM peppers, and its purity was tested by SDS-PAGE and immunoblotting. N-terminal sequences of purified CMV-CP expressed in *E. coli* and GM pepper were analyzed and compared using MALDI-TOF and electrospray ionization tandem mass spectrometry (ESI-MS/MS). Both proteins expressed the same amino acid sequences and the same biochemical characteristics.

An affinity column bound with CMV-CP polyclonal antibody induced by peptide-antigen was prepared, and ELISA conditions were optimized determine CMV-CP content in GM-peppers. The contents of CMV-CP were measured in root, leaves, stem, fruit, and pollen of GM peppers cultivated in three different fields (Korea University, Chung-Ang University,

and KRIBB). The highest expression of CMV-CP was 0.849 μg·g^{-1} in leaves from GM peppers. The cultivation areas, harvest time, generation, and plant tissues did not affect the expression of CMV-CP and there were no statistically significant differences among all tested parameters.

CMV-CP expressed in GM pepper showed same biochemical characteristics compared with the expressed coat protein in *E. coli*. These results showed that CMV-CP gene was expressed successfully in GM Pepper with the highest expression level. The cultivation areas, harvest time, generation, and plant tissues did not affect the expression of CMV-CP, because there were no statistically significant differences among all tested parameters. This may justify the potential application of new GM pepper with CMV-CP gene in food industry

Literature Cited

Astwood JD, Leach JN, and Fuchs RL (1996) Stability of food allergens to digestion *in vitro*. Nat Biotechnol 14(10):1269-1273. doi: 10.1038/nbt1096-1269

Baskaran P, So s V, Bal zs E, and Staden JV (2016) Shoot apical meristem injection: A novel and efficient method to obtain transformed cucumber plants. S Afr J Bot 103:210-215. doi: 10.1016/j.sajb.2015.09.006

Cai WQ, Fang RX, Shang HS, Wang X, Zhang FL, Li YR, Zhang JC, Cheng XY, Wang GL, and Mang KQ (2003) Development of CMV- and TMV-resistant transgenic chili pepper: field performance and biosafety assessment. Mol Breeding 11:25-35. doi: 10.1023/A:1022655204552

Chen ZL, Gu H, Li Y, Su Y, Wu P, Jiang Z, Ming X, Tian J, Pan N, and Qu LJ (2003) Safety assessment for genetically modified sweet pepper and tomato. Toxicology 188:297-307. doi: 10.1016/S0300-483X(03)00111-2

Choi J, Phat C, Kim E, Kim M, Lee GP, Ryu KH, and Lee C (2015) Improved detection of *Cucumber Mosaic Virus* coat protein (CMV-CP) in genetically modified pepper (*Capsicum annuum*) using a polyclonal antibody to a synthetic CP peptide. Hort Environ Biotechnol 56(3):316-323. doi: 10.1007/s13580-015-0139-5

Corpillo D, Gardini G, Vaira AM, Basso M, Aime S, Accotto GP, and Fasano M (2004) Proteomics as a tool to improve investigation of substantial equivalence in genetically modified organisms: the case of a virus–resistant tomato. Proteomics 4(1):193-200. doi: 10.1002/pmic.200300540

Devine P, Warren J (1990) Glycoprotein detection on immobilon PVDF transfer membrane using the periodic acid/Schiff reagent. Biotechniques 8(5):492-495

Doss VA, Kumar AK, Jayakumar R, and Sekar V (2002) Cloning and expression of the vegetative insecticidal protein (vip3V) gene of *bacillus thuringiensis* in *Escherichia coli*. Protein Expr Purif 26(1):82-88. doi: 10.1016/S1046-5928(02)00515-6

Doolittle SP (1916) A new infectious mosaic disease of cucumber. Phytopathology 6:145-147

Edge ASB (2003) Deglycosylation of glycoproteins with trifluoromethanesulphonic acid: elucidation of molecular structure and function. Biochem J 376:339-350. doi: 10.1042/bj20030673

Egito AS, Girardet JM, Miclo L, and Gaillard JL (2001) Highly sensitive periodic acid/Schiff detection of bovine milk glycoproteins electrotransferred after nondenaturing electrophoresis, urea electrophoresis, and isoelectric focusing. Lait 81:775-785. doi: 10.1051/lait:2001104

El-Borollosy AM, Oraby MM (2012) Induced systemic resistance against *Cucumber mosaic cucumovirus* and promotion of cucumber growth by some plant growth-promoting rhizobacteria. Ann Arg Sci 57(2):91-97. doi: 10.1016/j.aoas.2012.08.001

Food Standards (2008) Application A1001 food derived from insect-protected corn line mir162 assessment report. Australia New Zealand

FSANZ (2008) Application A1001 Food derived from insect-protected corn line MIR162 assessment report. Food Standard Australia New Zealand

Ha M, In Y, Maeng H, Zee OP, Lee J, and Kim Y (2011) Development of a matrix-prespotted plate for enhancing the reproducibility of serum glycan analysis by MALDI-TOF-MS. Mass Spectrom Lett 2(3):61-64. doi: 10.5478/MSL.2011.2.3.061

Hu B, Trinh K, Figueira WF, and Price PA (1996) Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. J Biol Chem 271(32):19415-9420. doi: 10.1074/jbc.271.32.19415

ICH Harmonized Tripartite Guideline (1994) Validation of analytical procedures: Text and methodology-Q2(R1)

Ivanov KI, M kinen K (2012) Coat protein, host factors and plant viral replication. Curr. Opin Virol 2:712-718. doi: 10.1016/j.coviro.2012.10.001

Iwanami T, Shimizu T, Ito T, and Hirabayashi T (2004) Tolerance to citrus mosaic virus in transgenic trifoliate orange lines harboring capsid polyprotein gene. Plant Dis. doi: 88:865-868. 10.1094/PDIS.2004.88.8.865

Jagger IC (1916) Experiments with the cucumber mosaic disease. Phytopathology 6:148-151

Jain M, Chengalrayan K, Abouzid A, and Gallo M (2007) Prospecting the utility of a PMI/mannose selection system for the recovery of

transgenic sugarcane (*Saccharum* spp. hybrid) plants. Plant Cell Rep 26:581-590. doi: 10.1007/s00299-006-0244-0

Janknecht R, Martynoff GDE, Lou J, Hipskind RA, Nordheim A, and Stunnenberg HG (1991) Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. Proc. Natl Acad Sci USA 88(20):8972-8976. doi: 10.1073/pnas.88.20.8972

Jensen ON, Podtelejnikov AV, and Mann M (1997) Identification of the components of simple protein mixtures by high-accuracy peptide mass mapping and database searching. Anal Chem 69(23):4741-4750. doi: 10.1021/ac970896z

Kaper JM, Waterworth HE (1981) Cucumoviruses, p 257-332. In: E. Kurstak (ed.). Handbook of plant virus infections and comparative diagnosis. Elsevier North-Holland biomedical press, NY USA

Kenyon, L, Kumar S, Tsai WS, and Hughes Jd'A (2014) Virus diseases of peppers (Capsicum spp.) and their control. Adv. Virus Res 90:297-354. doi: 10.1016/B978-0-12-801246-8.00006-8

Kothari SL, Joshi A, Kachhwaha S, and Ochoa-Alejo N (2010) Chilli peppers — A review on tissue culture and transgenesis. Biotechnol Adv 28(1):35-48. doi: 10.1016/j.biotechadv.2009.08.005

Krubphachaya P, Juricek M, and Kertbundit S (2007) Induction of RNA-mediated resistance to papaya ringspot virus type W. J Biochem Mol Biol 40:404-411. doi: 10.5483/BMBRep.2007.40.3.404

Lee JS, Kim YJ, Ryu KH, Han TH, Park KW, Chung KH, Lee C, Lee GP, Kim SH, Hong JS, Park YD, Woo ET, Park SC and Son DY (2012) Pollen allergic risk assessment of genetically modified virus resistant pepper and functional Chinese cabbage. Hort Environ Biotechnol 53(2):167-174. doi: 10.1007/s13580-012-0092-5

Lim H, Eng J, Yates JR3rd, Tollaksen SL, Giometti CS, Holden JF, Adams MW, Reich CI, Isen GJ, and Hays LG (2003) Identification of 2D-gel proteins: a comparison of MALDI/TOF peptide mass mapping to mu LC-ESI tandem mass spectrometry. J Am Soc Mass Spectrom 14(9):957-970. doi: 10.1016/S1044-0305(03)00144-2

Lin CH, Sheu F, Lin HT, and Pan TM (2010) Allergenicity assessment of genetically modified *cucumber mosaic virus* (CMV) resistant tomato (*solanum lycopersicon*). J Agric Food Chem 58:2302-2306. doi: 10.1021/jf903487f

Maneechoat P, Takeshita M, Uenoyama M, Nakatsukasa M, Kuroda A, Furuya N, and Tsuchiya K (2015) A single amino acid at N-terminal region of the 2b protein of *cucumber mosaic virus* strain m1has a pivotal role in virus attenuation. Virus Res 197:67-74. doi: 10.1016/j.virusres.2014.12.020

Millares P, LaCourse EJ, Perally S, Ward DA, Prescott MC, Hodgkinson JE, Brophy PM, and Rees HH (2012) Proteomic profiling and protein identification by MALDI-TOF mass spectrometry in unsequenced parasitic nematodes. PloS One 7(3):e33590. doi: 10.1371/journal.pone.0033590

Mochizuki T, Yamazaki R, Wada T, and Ohki ST (2014) Coat protein mutations in an attenuated *Cucumber mosaic virus* encoding mutant 2b protein that lacks RNA silencing suppressor activity induces chlorosis with photosynthesis gene repression and chloroplast abnormalities in infected tobacco plants. Virology 456-457:292-299. doi: 10.1016/j.virol.2014.04.010

Nakajima O, Teshima R, Takagi K, Okunuki H, and Sawada J (2007) ELISA method for monitoring human serum IgE specific for Cry1Ab introduced into genetically modified corn. Regul Toxicol Pharmacol 47(1):90-95. doi: 10.1016/j.yrtph.2006.08.003

Nida DL, Anjos JR, Lomonossoff GP, and Ghabrial SA (1992) Expression of cowpea mosaic virus coat protein precursor in transgenic tobacco plants. J Gen Virol 73:157-163. doi: 10.1099/0022-1317-73-1-157

Pina AS, Lowe CR, and Roque ACA (2014) Challenges and opportunities in the purification of recombinant tagged proteins. Biotechnol Adv 32(2):366-381. doi: 10.1016/j.biotechadv.2013.12.001

Quemada HD, Gonsalves D, and Slightom JL (1991) Expression of coat protein gene from *cucumber mosaic virus* stran C in tobacco: protection against infections by CMV strains transmitted mechanically or by aphids. Phytopathology 81:794-802. doi: 10.1094/Phyto-81-794

Roggero P, Pennazio S (1984) Quantitative determination by ELISA of *tobacco necrosis virus* from necrotic local lesions in tobacco. J Virol Methods 8(4):283-291. doi: 10.1016/0166-0934(84)90065-X

Roossinck MJ, Palukaitis P (1990) Rapid induction and severity of symptoms in zucchini squash (*Cucurbita pepo*) map to RNA 1 of cucumber mosaic virus. Mol Plant Microbe In 3(3):188-192. doi: 10.1094/MPMI-3-188

Shin R, Han JH, Lee GJ, and Peak KH (2002) The potential use of a viral coat protein gene as a transgene screening marker and multiple virus resistance of pepper plants coexpressing coat proteins of *cucumber mosaic virus* and tomato mosaic virus. Transgenic Res 11:215-219. doi: 10.1023/A:1015200622716

Smith TJ, Chase E, Schmidt T, and Perry KL (2000) The structure of *Cucumber Mosaic Virus* and comparison to Cowpea Chlorotic Motte Virus. J Virol 74(16):7578-7586. doi: 10.1128/JVI.74.16.7578-7586.2000

Sørensen HP, Mortensen KK (2005) Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. J Biotechnol 115(2):113-128. doi: 10.1016/j.jbiotec.2004.08.004

Statistic Korea (2009) Cultivated Area of Rice and Pepper in 2015. http://kostat.go.kr/portal/english/news/1/1/index.board?bmode=read&aSeq=348324

Sun W, Jiao K, and Zhang S (2001) Electrochemical ELISA for the detection of *cucumber mosaic virus* using o-pheneylenediamine as substrate. Talanta 55(6):1211-1218. doi: 10.1016/S0039-9140(01)00530-6

Takagi K, Teshima R, Okunuki H, and Sawada J (2003) Comparative study of in vitro digestibility of food proteins and effect of preheating on the digestion. Biol Pharm Bull 26(7):969-973. doi: 10.1248/bpb.26.969

Thoma RS, Smith JS, Sandoval W, Leone JW, Hunziker P, Hampton B, Linse KD, and Denslow ND (2009) The ABRF edman sequencing research group 2008 study: investigation into homopolymeric amino acid N-terminal sequence tags and their effects on automated edman degradation. J Biomol Tech 20(4):216-225

Thompson JR, Langenhan JL, Fuchs M, and Perry KL (2015) Genotyping of *Cucumber mosaic virus* isolates in western New York State during epidemic years. Virus Res 210:169-177. doi: 10.1016/j.virusres.2015.07.028

United States Pharmacopeia and National Formulary (2009) General notices and requirements. USP 33-NF 28 Reissue

Wang W, Scali M, Vignani R, Spadafora A, Sensi E, Mazzuca S, and Cresti M (2003) Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. Electrophoresis 24(14):2369-2375. doi: 10.1016/j.virusres.2015.07.028

Zein HS, Miyatake K (2009) Development of rapid, specific and sensitive detection of *Cucumber mosaic virus*. Afr J Biotechnol 8(5):751-759

Zein HS, Teixeira da Silve JA, and Miyatake K (2009) Antigenic properties of the coat of *cucumber mosaic virus* using monoclonal antibodies. J Virol Methods 162:223-230. doi: 10.1016/j.jviromet.2009.08.014

Zhu YX, Ou-Yang WJ, Zhang YF, and Chen ZL (1996) Transgenic sweet pepper plants from Agrobacterium mediated transformation. Plant Cell Rep 16:71-75. doi: 10.1007/BF01275453

Zolla L, Rinalducci S, Antonioli P, and Righetti PG (2008) Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. J Proteome Res 7(5):1850-1861. doi: 10.1021/pr0705082

Zrachya A, Kumar PP, Ramakrishnan U, Levy Y, Loyter A, Arazi T, Lapidot M, and Gafni Y (2007) Production of siRNA targeted against TYLCV coat protein transcripts leads to silencing of its expression and resistance to the virus. Transgenic Res 16:385-398. doi: 10.1007/s11248-006-9042-2