Cysteine-Added Mutants of Turnip Yellow Mosaic Virus

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INTRODUCTION

Turnip yellow mosaic virus (TYMV) is a nonenveloped icosahedral plant virus which has a single (+)-strand RNA as a genome. TYMV capsid is ca. 30 nm in diameter and is composed of 180 identical 20 kDa coat proteins (1). By now, there have been efforts to use plant viruses for medical applications (2, 3). The advantages of using plant viruses as drug delivery vehicles include their safety in terms of handling and their abundance in infected tissue (> 1 mg/g tissue). For these reasons, plant viruses are considered to be ideal building blocks for nanocarriers. Many plant viruses, including tobacco mosaic virus (TMV), can be purified following simple isolation protocols. Furthermore, in vitro assembly protocols using purified coat proteins have been established for many viruses (2).

In viruses, coat proteins are regularly arranged and many functional groups exist on the surface. Therefore, viral capsids can be decorated with diverse molecules, such as drugs, peptides, fluorescent dyes, MRI contrast agents, and gold nanoparticles. In this respect, Wang et al. (4) showed that a variety of molecules-ranging from small molecules, such as fluorescent dyes, to large structures, such as quantum dots-could be conjugated to cowpea mosaic virus (CPMV). Other plant viruses including TMV

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have been investigated for this purpose (5).

Similarly to many other plant viruses, TYMV is robust under a variety of conditions. TYMV is stable from 4°C to RT for months and 60°C for several hours. It is stable in a wide pH range (4–10), up to 50% organic solvent, and in a variety of reaction conditions (6). Furthermore, TYMV has unique properties that can be exploited for ideal drug delivery. Specifically, empty particles can be easily prepared by simple physical and chemical treatments, such as high pressure treatment, a freeze-thaw process and treatment with alkaline solution (7). The freeze-thaw process is known to result in a hole of 6–9 nm (8). Recently, it was demonstrated that the empty particles prepared by the freeze-thaw process were suitable to load fluorescent dyes. In addition, it was observed that TYMV efficiently entered mammalian cells when the particle was conjugated with cell penetrating peptide, such as Tat (9). Therefore, TYMV has a great potential as a drug delivery vehicle.

Commonly used functional groups in surface modification of viral nanoparticles are primary amines and carboxyl moieties (10). Barnhill et al. (6) reported that TYMV has 60 amine groups reacting with N-hydroxysuccinimide ester and 90–120 carboxyl groups reacting with amines per virus particle. These functional groups can be used to conjugate fluorescent dyes and other molecules using simple chemistry. Another popular functional group for conjugation is a sulfhydryl group of solvent-exposed cysteine residue. The reactive thiols provide coupling sites for molecules with maleimides or halogen-substituted acetamides (11). Thiol groups also react with gold nanoparticles. Unfortunately, however, TYMV does not have reactive cysteine residues on the surface.

In the present study, we sought to construct Cys mutants that have reactive thiol groups. To this end, a random sequence of amino acids was introduced into the C-terminus of coat protein (CP) using the genetic engineering approach. One of the inserted residues was cysteine. After three rounds of propagation in Nicotiana benthamiana, the TYMV RNA sequence was analyzed using high-throughput sequencing strategy. Based on the sequence data, Cys mutants were constructed and then examined for their reactivity to fluorescein-5-maleimide. The results showed that several Cys mutants hold promise for further examination.

MATERIALS AND METHODS

Construction of Cys library

To prepare a TYMV Cys mutant library, the following two primers were used to amplify a part of CP as well as to introduce a random sequence of amino acids including a cysteine at the C-terminus of CP: 5'-TCGATTCCCGGGGTCAAAAGATT-3' (upstream primer; the XmaI site is underlined), 5'-CCTGAATTCANNNACAANNANNACCACCTAGTGTCGGTGATGAGC-3' (downstream primer; the EcoRI site is underlined. N represents any nucleotides). The PCR product was digested with XmaI and EcoRI, and inserted into a TYMV replicon TY-eGFP2 (12). The TYMV replicon DNA was introduced to E. coli to prepare the 1st Cys mutant library. Thereafter, the library DNA was used to transform Agrobacterium tumefaciens. Then, A. tumefaciens bacteria were introduced to Nicotiana benthamiana leaves by agroinfiltration. After seven days, TYMV RNA was extracted from the agroinfiltrated leaves. The CP sequence of the viral RNA was amplified by reverse transcription-PCR (RT-PCR) and used to create the 2nd library. The upstream primer used was as the same as the primer used in the 1st library construction. The downstream primer used was 5'-GGAGAATTCTCTAGATGGCTCTCCCTAG-3' (the XbaI recognition site is underlined). The PCR product was digested with XmaI and XbaI, and inserted into TY-eGFP2. For the 3rd library construction, encapsidated viral RNA was used.

Construction of Cys mutants

Cys mutants were prepared by replacing the sequence between XmaI and EcoRI sites of TY-eGFP2 with PCR-amplified DNA. The upstream primer used in the PCR amplification was as follows: 5'-TCGATTCCCGGGTCAAAGATT-3' (the XmaI site
is underlined). Downstream primers contained mutant sequences. For example, the downstream primer for Cys 2 construction was as follows: 5′-GCTGAATTCATTAACAAGGACGACC-3′ (the EcoRI site is underlined). For the construction of Cys 1A, the following downstream primer was used: 5′-CTGAATTCAAAGACAAGGACGACCACCATAGTGCCGTGATG-3′ (the EcoRI and SpeI sites are underlined).

Isolation and purification of TYMV

Agroinfiltration was performed following the procedure described in Cho and Dreher (13). Seven days after agroinfiltration, the infiltrated leaves were collected and homogenized in 4 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 20 mM β-mercaptoethanol (β-ME) per g tissue. The mixture was extracted with 0.2 ml chloroform per g tissue. After centrifugation at 7,000 rpm in a microfuge for 10 min, virus particles were precipitated by adding 0.5 volume of 30% polyethylene glycol (PEG) 8000. After overnight incubation at 4℃, the sample was centrifuged at 5,000 g for 10 min, and the pellet was dissolved in distilled water containing 10 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The suspension was again extracted with 0.4 volume of chloroform. TYMV in the aqueous phase was precipitated by adding 0.5 volume of 30% PEG and 0.05 volume of 5 M NaCl. The pellet obtained after centrifugation was dissolved in distilled water containing 10 mM TCEP. For TYMV RNA and protein extraction, the leaf samples were frozen in liquid nitrogen immediately after collection, and then stored at -80℃.

Analysis of RNA

Total RNA was isolated from frozen *N. benthamiana* leaf samples using Easy-Red (iNtRON Biotechnology, Sungnam-si, Korea). The ribonuclease protection assay for encapsidated RNA in TYMV virions and the Northern blot analysis were performed using a DIG-labeled DNA probe representing the TYMV CP ORF as previously described (13). For the RNA sequence analysis, nucleotide sequence corresponding to the C-terminal part of TYMV CP was amplified by RT-PCR using the same primers as used in the 2nd and 3rd library construction. The DNA sequence was analyzed by a high-throughput sequencing method at ebiogen (Seoul, Korea). Briefly, sample preparation for sequencing was performed using NEBNext™ Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Beverly, MA, USA) according to the manufacturer’s instructions, and high-throughput sequencing was performed as single-end 100 sequencing using NextSeq 500 (Illumina, San Diego, CA, USA).

Analysis of coat protein

To examine the TYMV coat protein expression, leaf samples (0.1 g) were ground in 400 μl of 0.1 M sodium phosphate buffer (pH 7.0) containing 20 mM β-ME. Protein concentration of the leaf extract was determined by Bradford assay using bovine serum albumin as the standard. In the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), an equal volume of 2X SDS sample buffer was added to the leaf extract, which was boiled for 10 min and then loaded into 12.5% SDS-polyacrylamide gel. The gel was run at 30 mA for 80 min, and the proteins in the gel were examined by staining with Coomassie. Alternatively, TYMV CP was detected by a chemiluminescent method using Luminata™ Forte, employing anti-TYMV rabbit antiserum and HRP-conjugated goat anti-rabbit IgG.

Fluorescein labeling

Before the conjugation reaction, 100-fold excess of TCEP was added to the TYMV samples. The TYMV particles were made react with a 25-fold molar excess of fluorescein-5-maleimide (Sigma, USA) in 20 mM sodium phosphate buffer (pH 7.2), 150 mM NaCl at room temperature (RT) for 2 h. After the reaction, the unreacted fluorescein-5-maleimide was removed using a 7 K Zeba spin desalting column (Thermo Fisher Scientific, Waltham, MA, USA).
RESULTS

Preparation of Cys-added TYMV mutant library

According to an X-ray crystallographic study of TYMV, C-terminus of the coat protein is exposed to solvent environment (14). Accordingly, the C-terminal region could be an appropriate site for the introduction of a reactive cysteine residue. Previously, we examined whether the N- or C-terminus of TYMV CP could accommodate an extension of 15 amino acids representing a c-Myc epitope peptide (15). The results showed that N-terminus could be modified without compromising the CP assembly. However, C-terminal extension turned out to be detrimental to the CP assembly and replication. In another study of a TYMV mutant with a five amino acid (Tyr-Val-Leu-Asp-Arg) extension at the C-terminus, Bransom et al. (16) reported that the mutant replicated normally and showed systemic symptom, although the yield of the mutant was slightly lower than wildtype.

In a preliminary experiment, we constructed two mutants that had five additional amino acid residues at the C-terminus. The C-terminal extension included a cysteine. One of the two mutants did not replicate at all. The other replicated, but the viral products were about 10–20% of wildtype (data not shown). Therefore, in the hope to obtain cysteine-added mutants that would efficiently replicate as wildtype, we decided to make a library in which a random sequence of amino acid residues was introduced at the C-terminus of TYMV CP (see Fig. 1A). These mutants have a cysteine residue near the C-terminus and three random amino acid residues around cysteine. The DNA containing a random sequence of C-terminal extension was prepared by PCR and used to replace the C-terminal part of CP in a TYMV replicon TY-eGFP2 (12), which was constructed using a Ti plasmid binary vector pCB302-3.

The Cys mutant library DNA was introduced to E. coli, resulting in over 100,000 bacterial colonies. Plasmid DNA from all the transformed bacteria was then used to transform A. tumefaciens. The bacterial colonies produced were again at least 100,000 in number. Transformed A. tumefaciens grown on agar plates were collected by scraping, and the mixture was used to inoculate N. benthamiana leaves by agroinfiltration.

To screen for the efficiently replicating TYMV Cys mutants, total RNA was extracted from the inoculated leaves at seven days post-inoculation and used to prepare a next library (the 2nd library) by amplifying the sequence corresponding to the C-terminal CP by RT-PCR and inserting into the TYMV replicon (see Fig. 1B). The library was re-introduced to N. benthamiana. In order to enrich TYMV variants which were good at viral RNA packaging and at replication, encapsidated viral RNA was used to prepare the 3rd library. The leaves were collected after 7 days of inoculation, and the leaf extract was examined by SDS-PAGE and Western blot analysis using anti-TYMV CP antibody (see Fig. 1C). The results showed that an additional band of about 40 kDa appeared in the case of the Cys mutant library. The ‘40 kDa’ band was not intense in wildtype. Since the ‘40 kDa’ band corresponds to the size of a TYMV CP dimer and disappears in the presence of β-ME, the ‘40 kDa’ band was assumed to be generated by cross-linking between the CP subunits. This could be indicative of the reactivity of the newly introduced cysteine residue. Cysteine residues exposed on the surface can cause cross-linking of viral particles, leading to aggregation of viral particles. However, in our samples, heavy precipitation was not observed. Therefore, the ‘40 kDa’ band was assumed to be mostly ascribed to cross-linking between neighboring CP subunits within viral particles.

Preparation and analysis of Cys mutants

A. tumefaciens harboring the 3rd library was introduced into N. benthamiana leaves by agroinfiltration, and the encapsidated TYMV RNA extracted from the agroinfiltrated leaves was analyzed using high-throughput sequencing. Of the 11,584 sequences analyzed, occurrences of all sequences in the sequencing data were counted. Based on the frequency, we initially selected 10 variant CP sequences whose frequencies were above 20. Considering that the chance of occurrence of a random sequence is 1/16,384, the frequency of each of the selected sequences was thus more than 30 times compared to random chance. Among the 10 Cys mutants, four mutants (Cys mutants 1A, 2, 3 and 5) were chosen for further study primarily based on the CP expression level. The nucleotide and amino acid sequences of the mutants are shown in Fig. 2A. As for Cys 1A, the sequence upstream
of the ACTAGT sequence (SpeI recognition site) was found to be changed from GAC to GCC. This resulted in a change in amino acid from Asp to Ala. This substitution was not observed in other Cys mutants. Therefore, the change was not derived from the primer used in the mutant library construction. Rather, it would indicate natural variation (mutation) and selection.

Figure 1. TYMV Cys mutant library. (A) Library construction. A random sequence of three amino acids and one cysteine was introduced to the C-terminal part of TYMV CP by PCR-amplification and insertion of the PCR product into Xmal/EcoRI sites of TY-eGFP26203 (for further details, see MATERIALS AND METHODS). The C-terminal extension is highlighted in bold face. (B) Screening of a Cys mutant library. *Agrobacterium tumefaciens* containing the library was introduced to *N. benthamiana*. In a week, RNA was extracted from the inoculated leaf and the TYMV CP sequence was amplified by RT-PCR. The amplified DNA was used to generate the 2<sup>nd</sup> library, which was introduced again to *N. benthamiana*. After this process was repeated, the TYMV CP sequence was examined by high-throughput sequencing analysis using encapsidated TYMV RNA. (C) Examination of CP. Leaf extract was prepared in the presence (+) or absence (−) of 20 mM β-mercaptoethanol (β-ME) from the leaf inoculated with the 3<sup>rd</sup> library or wild-type TYMV. 2 μl of each leaf extract sample (1:10 diluted sample for wildtype) were loaded onto 12.5% SDS-polyacrylamide gel. After electrophoresis, the proteins were examined by Western blot analysis using TYMV CP antibody.
Table 1: Nucleotide and amino acid sequences of Cys mutants.

| Number | Nucleotide Sequence | Amino acid sequence | Occurrence* |
|--------|---------------------|---------------------|-------------|
| 1A     | GCCACTAGTGGTGTCCTCTTCATTCAAGTAC | ATSGGRPCCStop | 182         |
| 2      | GACACTAGTGGTGTCCTCTTCATTCAAGTAC | DTSGGRPCCStop  | 77          |
| 3      | GACACTAGTGGTGTCATCTCTTCATTCAAGTAC | DTSGHPCTStop    | 48          |
| 5      | GACACTAGTGGTGTCATCTCTTCATTCAAGTAC | DTSGRARCSStop   | 47          |

*Occurrence among the 11,584 CP sequences analyzed.

Figure 2. Cys mutants. (A) Sequence of the Cys mutants. Nucleotide and amino acid sequences of Cys mutants are shown. In Cys1A, the nucleotide sequence upstream of the ACTAGT is GCC, whereas, in other mutants, the sequence is GAC. (B) Western blot analysis of CP in leaf extracts. 1 μg of total protein for each sample was loaded onto 12.5% SDS-polyacrylamide gel. After electrophoresis, the proteins were examined by Western blot analysis using TYMV CP antibody. (C) Northern blot analysis of TYMV RNA. 500 ng of total RNA were size-fractionated in a 1% agarose gel and examined by Northern blot analysis using the DIG-labeled TYMV CP DNA as a probe. The blots were developed by chemiluminescent immunodetection of DIG. Upper and lower arrowheads indicate genomic and subgenomic RNAs of TYMV, respectively. The panel below the Northern blot shows 25S rRNA stained with EtBr. (D) Systemic infectivity of the Cys mutants. Chinese cabbage was inoculated with the TYMV constructs containing wildtype or the Cys mutants, and young systemically infected leaf (5th leaf from the inoculated leaf) was collected two weeks post-inoculation. Total protein (1 μg) in the leaf extract prepared in the presence of β-ME was examined for its CP levels by Western analysis as described in Fig. 2B.
Individual Cys mutant construct was prepared and introduced into *N. benthamiana*, and the inoculated leaves were examined after 7 days using the Western blot analysis of CP expression (Fig. 2B). Here, protein concentration was determined by Bradford assay for each leaf extract, and equal amounts in terms of protein were loaded into the SDS-polyacrylamide gel. The results showed that the Cys mutants produced nearly as much CP as wildtype. Cys mutants 1A and 2 showed a strong '40 kDa' band in the absence of β-ME in the extraction buffer. The intensity of the '40 kDa' band decreased in the presence of β-ME. In the cases of Cys 3 and Cys 5, the '40 kDa' bands were about the same, irrespective of the presence or absence of β-ME; however, an increased amount of β-ME or TCEP was found to lower the level of the upper band (data not shown). The analysis of leaf RNA by Northern blot hybridization using DIG-labeled TYMV CP DNA showed that the Cys mutants replicated nearly as efficiently as wildtype (see Fig. 2C).

**Systemic infectivity and cysteine reactivity of Cys mutants**

We tested whether the Cys mutants were able to infect systemically. To this end, we inoculated Chinese cabbage, a natural

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**Figure 3.** Cysteine reactivity. Wildtype (WT) and Cys mutants were reacted with fluorescein-5-maleimide, and analyzed by SDS-PAGE. After viewing under a UV lamp (left panels), the gel was stained with Coomassie (right panels). Two different size markers were used, and some components (75 kDa and 25 kDa) of the marker used with Cys 1A and 5 were fluorescent.
host of TYMV, with the Cys mutants and examined young systemic leaves. Systemic mosaic symptom was observed in all the Chinese cabbage plants inoculated with Cys mutants at a similar timing as the one observed with wildtype (about 10 days after inoculation), although a slightly delayed symptom development was observed with Cys 1A mutant. The 5th leaf from the inoculated leaf was then analyzed for the presence of TYMV CP (see Fig. 2D). The results showed the presence of viral products in all cases, indicating that the CPs of all Cys mutants can assemble into intact virions and move systemically. However, CP levels of the Cys mutants were generally lower than those of the wildtype, especially in the case of Cys 1A mutant. This finding is not consistent with the result obtained in N. benthamiana (see Fig. 2B). Possible explanations for this discrepancy will be discussed in the Discussion section.

We then examined the cysteine reactivity of the Cys mutants. To this end, conjugation reaction with fluorescein-5-maleimide was performed. The Cys mutant viral particles were purified by two rounds of PEG precipitation. After purification, the viral particles were kept in 10~20 mM TCEP until conjugation reaction, since, as demonstrated by Wang et al. (4), TCEP provides stabilization of cysteine-exposed virus particles and does not inhibit the reaction with maleimides. The TYMV particles were made react with a 25-fold molar excess of fluorescein-5-maleimide, and the unreacted dyes were removed using a 7 K Zeba spin desalting column. Thereafter, the reaction products were examined by SDS-PAGE. UV light irradiation of the Cys mutant samples showed a bright green fluorescence, whereas a very weak fluorescence was observed in the case of wildtype (Fig. 3, left panels). The same gel stained with Coomassie showed that the fluorescent band corresponds to 20 kDa TYMV coat protein (Fig. 3, right panels). This suggests that the newly introduced cysteine residue was highly reactive to fluorescein-5-maleimide in all Cys mutants.

DISCUSSION

Native TYMV has four Cys residues, all of which are not very reactive. Since some cysteine residues are present inside the virus particles (14), they are not reactive. One cysteine residue is predicted to be on the surface. Obviously, however, this one too is nearly unreactive, probably because it is buried in the valley on the surface. The results obtained in the present study show that all tested Cys mutants are very reactive to fluorescein-5-maleimide. This clearly shows that the C-terminus of the CP subunit is exposed to solvent environment and can be freely accessed. It also suggests that the amino acid residues around the newly added cysteine are not of great importance in terms of the reactivity of the cysteine. In general, the C-terminal extension does not seem to negatively affect the CP assembly. Although Bransom et al. (16) reported that attempts to purify the mutant particles with five amino acid C-terminal extension by PEG precipitation were unsuccessful, the PEG precipitation worked well in our experiments, indicating that surface property of our mutants was not significantly changed with the extension.

The low CP level in Chinese cabbage systemic leaf and delayed symptom development observed with Cys 1A suggest that Cys 1A virions might be unstable, as in the case of the mutant reported by Bransom et al. (16) who showed that the C-terminal extension of five amino acids resulted in less stable virions and that the CP level in young systemically infected leaf was ca. 30% compared to that of wildtype. The overall decrease of the CP levels might also indicate a defect in the systemic spread of some Cys mutants. This could be due to the presence of reactive thiol groups on virus surface. Long-distance movement of plant viruses involves translocation from mesophyll cells to sieve elements via successive crossings of small openings like plasmodesmata (17). TYMV seems to move systemically in the form of virus particles, since, as demonstrated by Bransom et al. (16), systemic spread does not occur without CP synthesis. Therefore, the aggregation of viral particles by interviral cross-linking through disulfide linkages, even though it is not extensive, could hamper systemic spread. With regard to this hypothesis, it is noteworthy that Cys 1A sample showed a distinct ‘slowly moving’ band in agarose gel, indicating the presence of some cross-linked viral particles.

Alternatively, the low level of CP in Chinese cabbage could be ascribed to innate immunity elicited by the mutants. Previous research has demonstrated that insertion of a foreign sequence into TYMV genome severely restricts viral replication, and co-expression of an RNAi suppressor p19 relieves the inhibition (18). Although, in this study, p19 construct was co-introduced
along with the TYMV constructs into both *N. benthamiana* and Chinese cabbage, the p19 is not expressed in systemic leaves of Chinese cabbage. This difference could have resulted in the low level of CP in Chinese cabbage.

The Cys mutants obtained in the present study would be very useful in drug delivery. Drugs or fluorescent dyes have been conjugated to or loaded into nanoparticles derived from plant viruses in several different ways. For example, an anticancer drug doxorubicin was encapsulated into hibiscus chlorotic ringspot virus (19) by *in vitro* reconstitution in the presence of the drug or covalently bound to external surface carboxylates of the CPMV viral nanoparticle (20). The drug-loaded or drug-conjugated viral nanoparticles would be further modified for efficient drug delivery. Cancer-specific ligand would also be used for targeting. Finally, plant viral nanoparticles need to be decorated with cell penetrating peptide to make them enter mammalian cells. This requires the presence of as many diverse functional groups as possible on the surface of nanoparticles. The contribution of our results is that thiol groups are now available, which adds high-utility functional groups to TYMV delivery vectors.

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