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Novel protein kinase interacts with the *Cucumber mosaic virus* 1a methyltransferase domain ☆,☆☆

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Abstract

The *Cucumber mosaic virus* (CMV)-encoded 1a protein has been implicated to play a role in replication of the viral genome along with 2a and one or more host factors. To identify the host cell factors interacting with CMV 1a, we used the yeast two-hybrid system using tobacco cDNA library. One of the cDNA clones encoded a protein homologous to the *Arabidopsis* putative protein kinase and was designated as Tcoi2 (Tobacco CMV 1a interacting protein 2). Tcoi2 specifically interacted with methyltransferase (MT) domain of CMV 1a protein in yeast cell. In vitro analyses using recombinant proteins showed that Tcoi2 also specifically interacted with CMV 1a MT domain. Tcoi2 did not have autophosphorylation activity but phosphorylated CMV 1a MT domain. Analysis of the subcellular localization of the Tcoi2 fused to GFP demonstrated that it is targeted to the endoplasmic reticulum. These results suggest Tcoi2 as a novel host factor that is capable of interacting and phosphorylating MT domain of CMV 1a protein.

Keywords: Tcoi2; CMV 1a protein; Replication complex; Kinase

Phosphorylation/dephosphorylation is the most common posttranslational modification and affects the protein functions and stability of many cellular proteins, such as those involved in the control of gene expression, signal transduction, and cell cycle regulation [1]. Phosphorylation of serine/threonine residues is also known to regulate the formation of protein complexes [2], and it can also be used as a signal to trigger ubiquitination and degradation of proteins [3].

Phosphorylation of viral proteins is also common. For example, phosphorylation of the tobamovirus or polerovirus movement protein (MP) regulates MP function in plants [4,5] and phosphorylation of coat protein (CP) can inhibit binding with viral RNA [6]. Protein phosphorylation can also regulate intracellular distribution of viral proteins [7] and regulate virus replication. For example, protein:RNA interaction and replicase assembly of animal *Vesicular stomatitis virus* (VSV) are controlled by phosphorylation [8] and phosphorylation of replication proteins could regulate interaction between the replication proteins [9,10]. However, the role of phosphorylation/dephosphorylation of replication proteins in *Cucumber mosaic virus* (CMV) is limited so far [10].

CMV belongs to the cucumovirus group and infects more than 1000 plant species [11]. It contains three individual segments of capped messenger-sense RNAs in its genome: RNAs 1, 2, and 3 [12]. RNAs 1 and 2 encode proteins 1a and 2a, respectively. Both 1a and 2a are necessary for viral RNA replication in a single cell [13,14], and RNA 2 also encodes a protein that has been designated as 2b [15]. The 2b has been tentatively implicated in host-specific long-distance movement of CMV [16] and as a suppressor of virally induced gene silencing [17]. RNA 3 encodes two proteins, the

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☆☆ *Abbreviations:* CMV, *Cucumber mosaic virus*; GST, glutathione-*S*-transferase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tcoi2, tobacco CMV 1a interacting protein 2.

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movement protein (MP) and the coat protein (CP). MP is directly translated from RNA 3, whereas CP is translated from subgenomic RNA 4 [18]. Both the MP and the CP are necessary for the efficient cell-to-cell movement of CMV [16,19–21]. The CMV-encoded 1a protein has been implicated not only in replication, but also in the regulation of systemic infection [22]. It features two functional domains: an N-terminal domain that is a putative methyltransferase domain (MT), and is involved in the capping of genomic and subgenomic RNAs, and a C-terminal domain that is a putative helicase domain (Hel) [23–26]. These two domains are known to interact with each other in the yeast two-hybrid system [27]. Also, the CMV 1a protein has been shown to interact with the N-terminal region of the 2a protein in the yeast two-hybrid system [10].

As the CMV 1a protein has been implicated not only in replication, but also in the regulation of systemic infection, identification of cellular proteins that are able to interact and regulate the CMV 1a protein may constitute the first step toward understanding the mechanisms underlying the CMV intracellular propagation or other functions of this protein. To identify host factors which interact with CMV 1a protein, we applied the yeast two-hybrid system. In this study, we isolated one protein that belongs to a member of protein kinase. It was designated as Tcoi2, and shown to specifically interact with and phosphorylate the MT domain of CMV 1a.

Materials and methods

Virus strain and RNA extraction. The propagation and purification of CMV-Kor have been described previously [28,29]. CMV-Kor RNAs were extracted with TNE (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 10 mM Na_2-EDTA) from virus particles, and then precipitated by ethanol. The resulting RNAs were dissolved in diethylpyrocarbonate-treated water and quantitated by measuring the absorbance at 259 nm.

Plant material and virus inoculation. Tobacco (*Nicotiana tabacum* cv. NC82) plants were grown at 25 °C, with a photoperiod of 16 h light/8 h dark. Plant sap containing the CMV-Kor particles was prepared by grinding infected leaves in phosphate buffer (0.25 M Na_2HPO_4, 5 mM EDTA) and rubbing them onto the surface of 2 or 3 fully expanded young leaves of tobacco seedlings (8–10 leaf stage) with Carborundum (mesh 500) (Hayashi Chemical, Japan). CMV-inoculated leaves were harvested 48 h after inoculation and used as an RNA source for cDNA library construction.

Yeast two-hybrid analysis. The MatchMaker II GAL4 two-hybrid system was purchased from Clontech Laboratories. The pACT2 activation domain (AD) expression vector was utilized in the construction of a cDNA library, with the RNA extracted from the CMV-infected tobacco leaves. The library was then screened, using the pAS2-1/CMV 1a DNA-binding domain (DB) fusion vector as bait. To construct the pAS2-1/CMV 1a, a CMV 1a cDNA fragment was prepared by PCR, and then cloned into the *Bam*HI site of pAS2-1 vector. The primer set which was used for this amplication was *CMV 1a-5′* (5′-AGGATCCAAATGGCG ACGTCCTCGTTC-3′) and *CMV 1a-3′* (5′-AGGATCCACTAAGCACG AGCAATACA-3′). The two fusion vectors were co-transformed into *Saccharomyces cerevisiae* strain Y190 (*MATa*, *HIS3*, *lacZ*, *trp1*, *leu2*, and *cyh*^{r}*2*) or HF7c (*MATa*, *HIS3*, *lacZ*, *trp1*, and *leu2*), as recommended by the manufacturer. The transformed yeast cells were selected on minimal synthetic dropout (SD) medium, lacking Leu, Trp, and His. In order to help eliminate false positive clones, the SD medium plates were supplemented with 50 mM (Y190) or 5 mM (HF7c) 3-amino-1,2,4-triazole. Yeast cells were permitted to grow at 30 °C for 8–12 days and then subjected to colony-lift filter assays, which monitored β-galactosidase activity using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as substrate. The putative positive clones (Leu^+, Trp^+, and His^+) identified by this assay were selected by re-transformation. Plasmids were isolated from each Leu^+, Trp^+, His^+, and $LacZ^+$ transformant, using the Yeast Plasmid Isolation Kit (Clontech, USA). The plasmids were then transformed into *Escherichia coli* XL1-Blue cells (Stratagene, USA) via electrophoration. The bacterially propagated pACT2 fusion plasmids were isolated and analyzed.

Isolation of full-length cDNA clones. To isolate the full-length cDNA clone of Tcoi2, approximately 5×10^4 clones were screened from the cDNA library using the random-primed ^{32}P-labeled cDNA fragment obtained from yeast two-hybrid screening as a probe. Hybridization was carried out according to a published protocol [30]. After hybridization, the membranes were washed with 2× SSC at room temperature for 10 min twice, 0.1× SSC containing 0.1% SDS at room temperature for 10 min and at 65 °C for 5 min. Filters were exposed to X-ray film with an intensifying screen at −70 °C for 48 h. Positive clones were excised with the helper phage and recircularized to generate a subclone in the pBluescript SK-phagemid vector (Stratagene, USA).

In vitro transcription/translation analysis. The pGADT7 plasmids, harboring the coding sequences of CMV 1a, CMV 1a MT, CMV 1a Hel or CMV 2a under the control of the T7 promoter, were used as templates in coupled in vitro transcription/translation reactions with the TNT wheat germ system (Promega, USA). Proteins were synthesized for 1 h with [^{35}S]Met (Amersham–Pharmacia Biotech, UK) used as a radiolabel.

In vitro binding assay. Ten micrograms of GST-Tcoi2 fusion protein was mixed with 10 μl of in vitro translation mixture containing radiolabeled CMV 1a, CMV 1a MT, CMV 1a Hel or CMV 2a polypeptide. To this mixture 10 μl of glutathione–agarose beads and 65 μl of binding buffer (20 mM Tris–HCl, pH 7.6, 100 mM KCl, 2 mM $CaCl_2$, 5 mM dithiothreitol, and 0.5% glycerol) were added. As a control, the CMV 1a, CMV 1a MT or CMV 1a Hel was incubated with GST protein. The binding mixtures were incubated at 4 °C for 90 min and centrifuged at 13,000 rpm. Protein pellets were then resuspended in sample loading buffer, boiled, and electrophoresed in 12% SDS–polyacrylamide gel. After electrophoresis, the gel was dried and exposed to X-ray film for autoradiography.

Kinase assays. GST-fused recombinant proteins; GST-Tcoi2, GST-CMV 1a MT, GST-CMV 1a Hel, and GST-CMV 2a, were used for kinase assays [31]. The proteins were mixed in kinase buffer (40 mM Hepes, 10 mM $MgCl_2$, and 3 mM $MnCl_2$, pH 7.4) with [γ-^{32}P]ATP and incubated for 30 min at 25 °C. The reaction was terminated by heat denaturation. The reaction products were subjected to SDS–PAGE and analyzed by autoradiography on BAS-2500 system (Fuji).

Polyethylene glycol-mediated protoplast transformation and localization test. For the in vivo targeting of Tcoi2, a fragment with a signal peptide was generated by PCR, using the primers Tcoi2-5′ (5′-GGGATCCAA TGCTGACTTATGAAAGATTTTG-3′) and *Tcoi2Nonstop* (5′-AAGGA TCCATAAAAGAAGCATCATAATTG-3′). The Tcoi2-green fluorescent protein (GFP) fusion construct was generated by positioning the coding region of Tcoi2 cDNA without the termination codon in-frame to the N terminus of the soluble-modified GFP (smGFP)-containing plasmid. The plasmid contains a subcloned product of the CaMV 35S-mGFP4-NosT region from pBIN 35S-mGFP4 into the high copy pUC118 plasmid [32,33]. The fusion constructs were then introduced into *Arabidopsis* protoplasts, prepared from whole seedlings by the polyethylene glycol-mediated transformation procedure [34]. The expression of this fusion construct was monitored at various time points after transformation using a Zeiss (Jena) Axioplan fluorescence microscope, and the images were captured with an Axiocam (Jena) digital camera.

Results

Isolation of Tcoi2 by yeast two-hybrid screening

A tobacco cDNA library was constructed in the yeast GAL4 activation domain (AD) expression vector with

RNA extracted from leaves that were infected by CMV. To isolate a plant host factor interacting with CMV 1a protein, the library was screened using the full-length CMV 1a as bait. From 1.5×10^6 independent clones screened, we identified 368 positive clones. When these 368 clones were re-transformed and subjected to colony-lift filter assay, 14 clones were observed to exhibit β-galactosidase activity (data not shown). Sequence analysis of these 14 clones revealed that one of the clones was an uncharacterized gene predicted to encode a novel putative kinase protein. This cDNA insert was designated as Tcoi2 (tobacco CMV 1a interacting protein 2) and was characterized further.

The interaction between CMV 1a and Tcoi2 was confirmed using additional plasmid combinations and constructs to test for autonomous activation of the *HIS3* and *lacZ* reporter genes, for possible direct interactions with the GAL4 DNA-binding domain (BD) or GAL4 AD, and for potential artifacts caused by high expression of the encoded GAL4 fusion protein (Table 1). Neither pAS2-1::CMV 1a (bait) nor pACT2::Tcoi2 (prey) alone activated the *HIS3* or *lacZ* reporter gene. Tcoi2 (expressed from pACT2::Tcoi2) did not interact with the GAL4 BD itself, nor CMV 1a (expressed from pAS2-1::CMV 1a) was observed to interact with the GAL4 AD, when the plasmid was co-transformed with the parent plasmid carrying either the GAL4 BD or the GAL4 AD. The CMV 1a protein has two functional domains: a putative methyltransferase domain (MT) and a putative helicase domain (Hel). To test which domain of CMV 1a interacts with Tcoi2, each domain was fused to the GAL4 BD. Tcoi2 was observed to interact with CMV MT (aa 1–456) but not with the CMV Hel (aa 646–993). And next, the interaction of Tcoi2 with other CMV-encoded proteins that are 2a, MP, and CP was tested. The Tcoi2 did not interact with any other CMV-encoded proteins (data not shown). These results indicate that Tcoi2 specifically interacted with CMV 1a through its MT.

Table 1
Interaction test of Tcoi2 and CMV 1a in yeast cells

GAL4 BD[a] vector	GAL4 AD[b] vector	His	β-Galactosidase activity[f]
pAS2-1::CMV 1a	–[c]	–	–
–[c]	pACT2::Tcoi2	–	–
pAS2-1::CMV 1a	pACT2	–	–
pAS2-1	pACT2::Tcoi2	–	–
pAS2-1::CMV 1a	pACT2::Tcoi2	+	+
pAS2-1::CMV 1a-MT[d]	pACT2::Tcoi2	+	+
pAS2-1::CMV 1a-Hel[e]	pACT2::Tcoi2	–	–

[a] BD, binding domain.
[b] AD, activation domain.
[c] No coexpressed vector.
[d] Methyltransferase domain of CMV 1a protein.
[e] Helicase domain of CMV 1a protein.
[f] The colony-lift filter assay and liquid culture assay using *o*-nitrophenyl-β-D-galactopyranoside as substrate.

Sequence analysis of Tcoi2 and genomic DNA gel blot analysis

To isolate the full-length cDNA clones, 5×10^4 clones were screened from the cDNA library using the random-primed ^{32}P-labeled cDNA fragment obtained from yeast two-hybrid screening as a probe. The complete nucleotide sequence of Tcoi2 revealed a 1995 bp cDNA 571 amino acid residue protein. A position-specific iterative Basic Local Alignment Search Tool (PSI-BLAST) search predicted residues 165–435 of Tcoi2 containing a kinase domain belonging to the tyrosine protein kinases (Fig. 1A). Homology searches revealed that its deduced amino acid sequence was highly similar to putative kinase of *Arabidopsis thaliana* (71% identity) and putative receptor kinase PERK1 of *Oryza sativa* (57% identity, Fig. 1B). To verify Tcoi2 as a tobacco gene, total genomic DNA was isolated and digested with three restriction endonucleases, *Eco*RI, *Hin*dIII, and *Xba*I. The DNA was separated in agarose gel and the blot was hybridized with a radiolabeled probe specific for the Tcoi2 ORF under medium stringency. As shown in Fig. 1C, two DNA fragments hybridized with Tcoi2, indicating the presence of duplicated Tcoi2-like sequences in the tobacco genome.

In vitro binding analysis

To verify protein–protein interaction between CMV 1a and Tcoi2, a binding assay was performed in vitro. First, the coding region for Tcoi2 was subcloned into a bacterial expression plasmid to express the Tcoi2 proteins as glutathione-*S*-transferase (GST) fusion proteins and then the recombinant protein was purified by using glutathione–agarose chromatography. As shown in Fig. 2A, an approximately 90 kDa proteins, an estimated size of the GST-Tcoi2 fusion protein, was obtained. For the binding assay, [^{35}S]Met-labeled CMV 1a and CMV 1a MT were synthesized in vitro using a cell-free translation system. [^{35}S]Met-labeled CMV 1a Hel and CMV 2a were also synthesized in vitro for a negative control as they did not interact with Tcoi2 in yeast cell. Each of the [^{35}S]Met-labeled polypeptide synthesis was confirmed and then incubated with the GST-Tcoi2 fusion protein. The mixture was allowed to interact with glutathione–agarose beads at low temperature, and the eluate from the beads was analyzed by SDS–PAGE. The eluted GST-Tcoi2 fusion protein trapped to the glutathione–agarose beads turned out to have formed complexes with the CMV 1a (Fig. 2B-(i), lane 2) or CMV 1a MT polypeptide (Fig. 2B-(ii) lane 5), as shown by the band resolved in an SDS-gel. In contrast, no significant interaction was observed when the CMV 1a (Fig. 2B-(i), lane 1), CMV 1a MT or CMV 1a Hel polypeptide (Fig. 2B-(ii), lanes 2 and 3) was incubated with the GST control protein. The very faint binding was not beyond the background level of CMV 1a MT binding to glutathione–agarose beads only (Fig. 2B-(ii), lane 1). Moreover, no significant interaction was observed in the

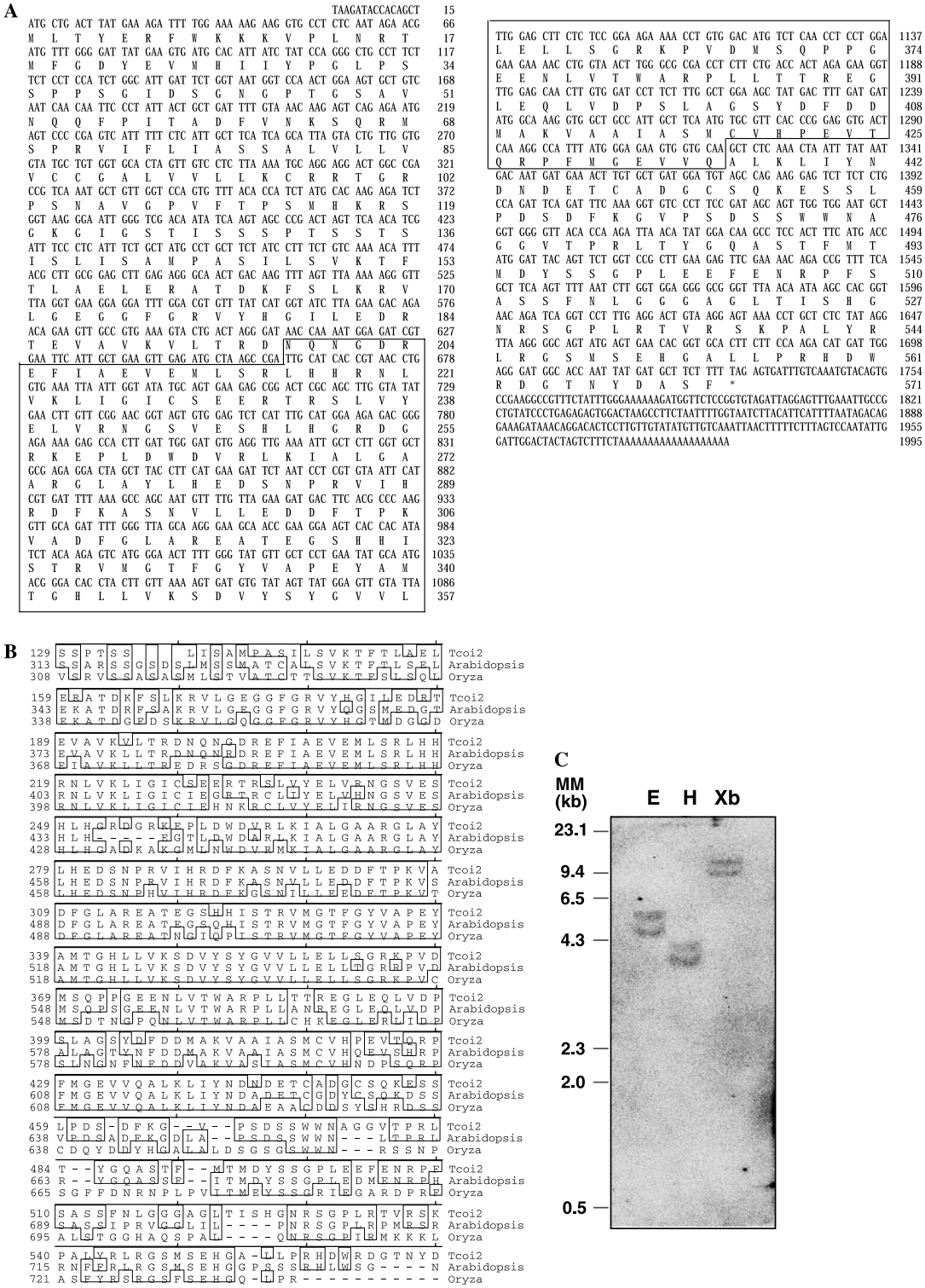

Fig. 1. Sequence analysis of the Tcoi2 cDNA clone and genomic DNA gel blot analysis. (A) Nucleotide and predicted amino acid sequences of Tcoi2 (GenBank Accession No. DQ202472). Asterisk shows the termination codon. The region corresponding to the kinase domain is boxed. (B) Comparison of the predicted amino acid sequence of Tcoi2 with a putative kinase of *Arabidopsis thaliana* (GenBank Accession No. AAM91792) and PERK1 of *Oryza sativa* (GenBank Accession No. XP468388). Identical amino acids (consensus from a minimum of three sequences) are boxed. (C) Genomic DNA gel blot analysis. Tobacco genomic DNA was digested with *Eco*RI (E), *Hin*dIII (H), or *Xba*I (Xb), and separated on 0.8% agarose gel. After transferred to a Nytran Plus membrane, the blot was hybridized with ^{32}P-labeled full-length Tcoi2 cDNA probe under medium stringency. Autoradiograms were visualized with a Fuji-BAS 2500 phosphor image analyzer. DNA size standards (MM) are shown on the left.

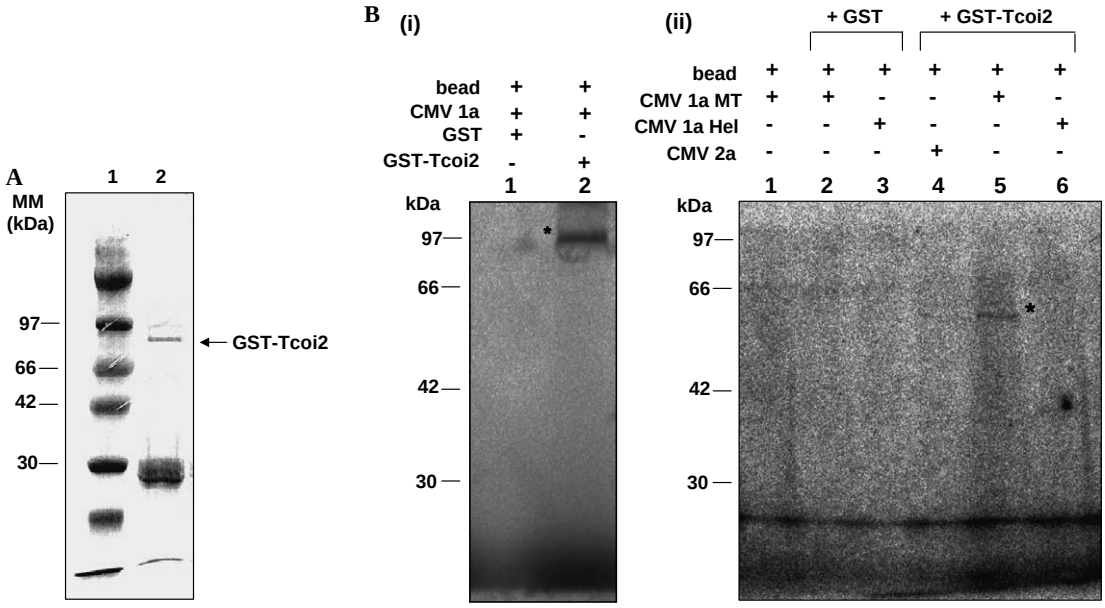

Fig. 2. In vitro interaction test of recombinant Tcoi2 protein and CMV protein. (A) The purification of recombinant GST-Tcoi2 protein via affinity chromatography. Protein expression was induced in *E. coli* that was transformed with the GST expression plasmid. Recombinant GST-Tcoi2 protein, purified on glutathione–agarose beads, was run on SDS–PAGE, and the gel was stained with Coomassie brilliant blue. Lane 1, protein molecular weight maker; lane 2, purified GST-Tcoi2 fusion protein (5 μg). Arrowhead indicates the position of purified recombinant GST-Tcoi2 proteins (~90 kDa). (B) In vitro binding assay of Tcoi2 and the CMV 1a protein (i) or CMV 1a MT protein (ii). GST-Tcoi2 protein was incubated with in vitro translated, [^{35}S]Met-labeled CMV 1a, CMV 1a MT, CMV 1a Hel or CMV 2a in the presence of glutathione–agarose beads. As a control, GST was incubated with [^{35}S]Met-labeled CMV 1a, CMV 1a MT or CMV 1a Hel. Precipitates from the binding mixture were subjected to SDS–PAGE and proteins were visualized by autoradiography. Asterisks on the autoradiogram indicate the position of the [^{35}S]Met-labeled CMV 1a (97 kDa) or CMV 1a MT (50 kDa).

mixtures of GST-Tcoi2 and CMV 1a Hel polypeptide (Fig. 2B-(ii), lane 4) or CMV 2a polypeptide (Fig. 2B-(ii), lane 6). These results clearly indicate that the intermolecular association between Tcoi2 and CMV 1a or CMV 1a MT occurs effectively in vitro.

Kinase activity of Tcoi2

To test whether Tcoi2 has protein kinase activity, we performed in vitro kinase assays using purified GST-Tcoi2 fusion protein. For this assay, GST-CMV 1a MT, GST-CMV 1a Hel, and GST-CMV 2a proteins were purified by glutathione–agarose chromatography and were used as a substrate for the phosphorylation reaction (Fig. 3A). As shown in Fig. 3B, GST-Tcoi2, synthesized in *E. coli* and purified, did not have autophosphorylation activity (Fig. 3B, lane 1) and it did not phosphorylate GST control protein (lane 2), GST-CMV 1a Hel (lane 5) or GST-CMV 2a (lane 6). But GST-Tcoi2 phosphorylated recombinant GST-CMV 1a MT very efficiently (lane 4). These data suggest that Tcoi2 was able to specifically phosphorylate CMV 1a MT protein.

Subcellular localization of Tcoi2

In order to investigate the subcellular localization of Tcoi2, the empty plasmid carrying green fluorescence protein (GFP) alone as control or the plasmid with the fusion gene Tcoi2::*GFP* was transfected into protoplasts prepared from *Arabidopsis* by polyethylene glycol-mediated transformation. The fluorescent signals were visualized by microscopy after 24 h incubation. As shown in Fig. 4A, Tcoi2::GFP was primarily localized in faint fluorescent vesicle-like structures surrounding the nucleus. The fluorescent signal was also observed in small, discrete cytoplasmic patches of uniform size, and dispersed randomly throughout the cell whereas the GFP control was distributed uniformly in the cytosol.

As noted above, the reticulated pattern of fluorescence that represents the localization of Tcoi2 resembled the distribution of the endoplasmic reticulum (ER). To determine if Tcoi2 is associated with the ER, subcellular co-localization test was performed by using ER luminal binding protein (Bip) as an ER marker. For this assay, *Bip* was tagged with *red fluorescent protein* (*RFP*) and then the plasmid was co-transfected with the one carrying Tcoi2::GFP into *Arabidopsis* protoplasts. Bip was localized to perinuclear areas and shown as small aggregates surrounding the nucleus and scattered in the cytoplasm and in fluorescent bodies similar to the previously reported pattern [35] and Tcoi2::GFP completely overlapped with Bip::RFP (Fig. 4B). Thus, we concluded that at least a portion of the Tcoi2::GFP detected is associated with the ER (yellow in merged image).

Discussion

In this work, we isolated a novel tobacco plant cDNA, Tcoi2, whose putative translation product was capable of

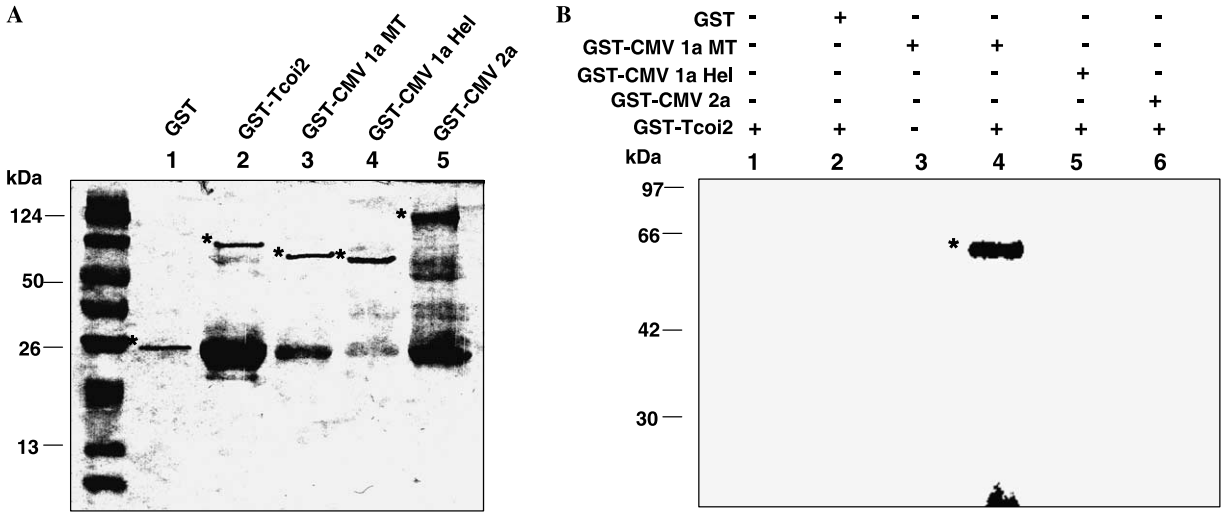

Fig. 3. Assay of CMV 1a MT protein phosphorylation by Tcoi2. (A) The purified proteins were separated on SDS–PAGE and visualized by Coomassie brilliant blue staining. Positions of molecular mass markers (kDa) are shown on the left. Asterisks denote the positions of the purified proteins. Lane 1, GST (27 kDa); lane 2, GST-Tcoi2 (90 kDa); lane 3, GST-CMV 1a MT (77 kDa); lane 4, GST-CMV 1a Hel (65 kDa); lane 5, GST-CMV 2a (124 kDa). (B) Purified GST, GST-CMV 1a MT, and GST-CMV 1a Hel were used for kinase assays with GST-Tcoi2. Reaction products were subjected to 10% SDS–PAGE and analyzed by autoradiography. The combinations of proteins used for each assay are indicated above the lanes with + (presence) and − (absence). Asterisk on the autoradiogram indicates the position of recombinant protein detected by autoradiography.

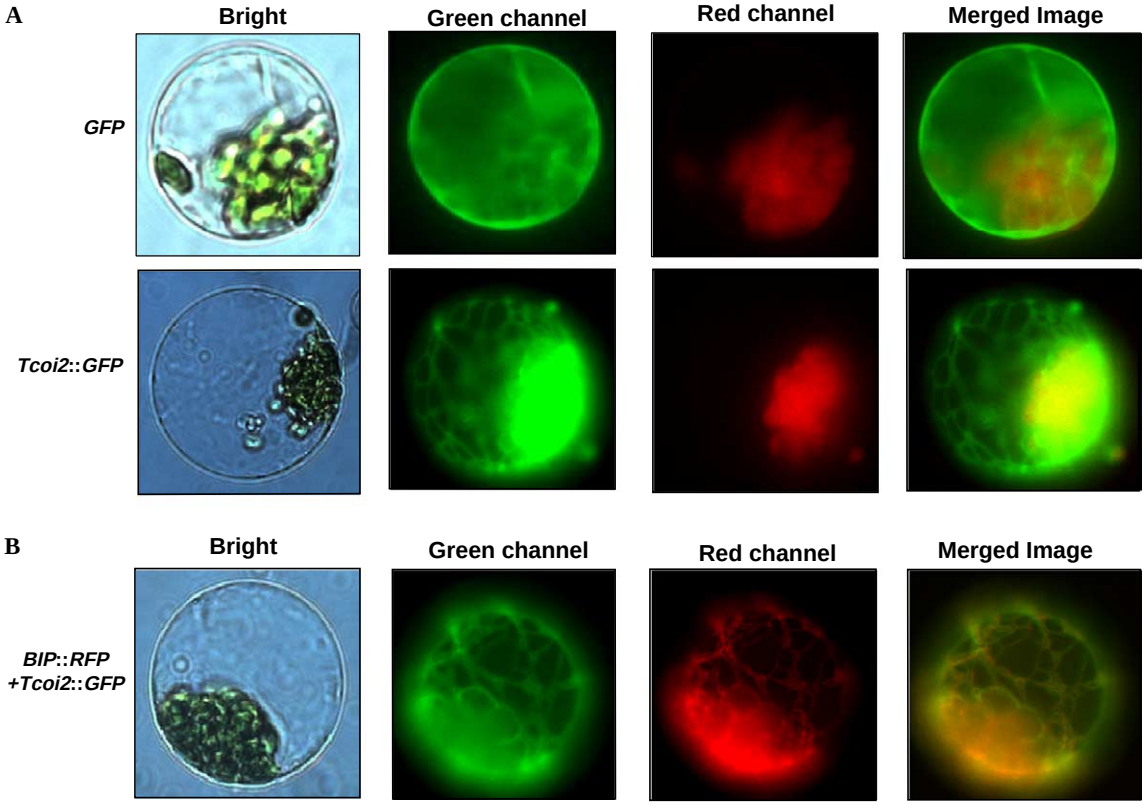

Fig. 4. Subcellular localization of Tcoi2::GFP in *Arabidopsis*. (A) Subcellular localization of GFP control and GFP-fused Tcoi2 in *Arabidopsis* protoplasts. The first panel shows the bright field image. The second panel shows the GFP images. The third panel shows the chlorophyll (CH) and the last panel shows overlap of GFP (green) and CH (red). (B) Subcellular co-localization of Bip::RFP with Tcoi2::GFP in *Arabidopsis* protoplasts. Expressions of the introduced genes were examined at 24 h after transformation. The first panel shows the bright field image. The second panel shows the GFP images. The third panel shows the RFP images and the merged images in fourth panel represent digital superimpositions of red and green signals in which areas of fluorescence co-localization appear yellow.

interacting with *Cucumber mosaic virus*-encoded 1a protein by using yeast two-hybrid system. Tcoi2 encoded a protein highly homologous to the putative kinase of *A. thaliana* that contains a kinase domain belonging to the tyrosine protein kinases [36]. Although the functions of these two kinases have not been known, the highly conserved sequences in Tcoi2 and putative kinase of *A. thaliana* reflect a similar role in the two plants. The highly conserved kinase domain could be responsible for the interaction with CMV 1a protein. To verify the specific binding sites for CMV 1a protein, Tcoi2 deletion study should be performed. On the other hand, Tcoi2 specifically interacted with full-length and MT domain of CMV 1a protein in yeast cell and in vitro (Table 1 and Fig. 2).

The phosphorylation of viral proteins has been examined in several plant viruses. In *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV), phosphorylation of movement protein (MP) was shown in vivo and appeared to be important for viral pathogenesis [5]. CMV MP and CMV replicase, protein 2a, were phosphorylated and phosphorylation of the 2a protein inhibited the interaction between 1a and 2a proteins [10,37]; however, phosphorylation has never been reported for CMV 1a protein. Our in vitro kinase assay has shown that MT domain of CMV 1a protein is phosphorylated by Tcoi2, though the site and the effect of phosphorylation of this domain remain unknown at this point (Fig. 3). Actually, Tcoi2 was isolated as an interactor with full-length CMV 1a protein in yeast cell and Tcoi2 interacted with full-length CMV 1a in vitro. Unfortunately, however, we could not confirm the phosphorylation of the full-length CMV 1a protein as we were unable to express the full-length CMV 1a protein in *E. coli*.

Previous work has shown that the phosphorylation of the 2a protein prevents the formation of 1a and 2a protein complexes that is essential for the replication [10] and suggested that other functions of phosphorylation of the 2a protein may be associated with virus movement or in activating host defense responses. Indeed, CMV1a protein is not only known to interact with the 2a protein and but also interacts each other in the yeast two-hybrid system [27]. Thus, phosphorylation of CMV 1a protein may change the interactions between CMV viral proteins or with other host proteins, leading to either the increase or the decrease of CMV infection.

Cellular kinases involved in MP phosphorylation were reported to be associated with the cell wall [38] or ER [39]. Replication of *Tobacco mosaic virus* (TMV) is connected with ER-associated membranes at early stages of infection and TMV-MP specific protein kinases (PKs) associated with the ER of tobacco were capable of phosphorylating TMV MP. Similar to MP-specific PKs, Tcoi2 is associated with the ER (Fig. 4), though CMV replication is known to occur in close association with tonoplast [40]. Thus, it would be possible that CMV 1a protein phosphorylation occurs before tonoplast targeting.

So far phosphorylation has been demonstrated for several viral gene products, but this is the first characterization about the cellular kinase involved in CMV 1a protein phosphorylation. Its roles in viral pathogenesis and propagation in plant cells need to be clarified in order to understand its regulatory role in virus–host interactions.

Acknowledgments

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