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Short communication

Mutational Analysis of Cucumber Mosaic Virus Movement Protein Gene

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The movement protein of cucumber mosaic virus (CMV) is required for cell-to-cell movement of viral RNA. The movement of viral RNA occurs through the plant intercellular connection, the plasmodesmata. The viral movement protein was known to be multi-functional. In this work, a series of deletion mutants of CMV movement protein gene were created to identify the functional domains. The mutated movement proteins were produced as inclusion body in *E. coli*, and purified and renatured. A polyclonal antibody was raised against the CMV-Kor strain (Korean isolate) movement protein expressed in *E. coli*. The ability of the truncated proteins to bind to ssRNA was assayed by UV cross-linking and gel retardation analyses. The results indicate that the domain between amino acids 118 and 160 of CMV movement protein is essential for ssRNA binding.

Keywords: CMV-Kor, Movement protein, ssRNA binding.

Introduction

Plant virus-encoded movement proteins are required for the active transport of the viral genome from primary infected cells to neighboring healthy cells and also for symptom development in plants. Viral movement protein is also known to be relevant to host range determination. In many cases, the success or failure of viral spread from the primary infection site determines the actual host specificity of the plant virus (Mise *et al.*, 1993; De Jong *et al.*, 1995). Well-characterized viral movement proteins have several common features such as cooperative binding to nucleic acids, cell wall localization in the infected plant, modification of the size exclusion limit of plasmodesmata, and symptom development. (Berna *et al.*, 1991; Citovsky *et al.*, 1990; 1992; Giesman-Cookneyer and Lommel, 1993; Osman *et al.*, 1993; Schoumacher *et al.*, 1994; Vaquero *et al.*, 1994; 1997; Cooper and Dodds, 1995; Ding *et al.*, 1995). However, there is no evidence that RNA transport mechanisms are identical for all plant viruses. Unlike the tobacco mosaic virus (TMV), CMV and alfalfa mosaic virus (AlMV) require coat protein for cell-to-cell movement in addition to movement protein (van der Kuyl *et al.*, 1991; Canto *et al.*, 1997). When CMV movement protein that was expressed and purified from *E. coli* was microinjected into tobacco mesophyll cell, CMV movement protein was able to move from cell to cell and the size exclusion limit of plasmodesmata was increased (Ding *et al.*, 1995).

Protein binding to nucleic acids often involves positively-charged amino acids and correct folding of the protein. Most of the viral movement proteins have a single highly positively charged domain for nucleic acid binding. The TMV movement protein has two independent single-stranded binding domains. One is a highly positively-charged domain and the other is located in a predominantly hydrophobic region (Citovsky *et al.*, 1992). CMV-Kor (Korean isolate) movement protein has 280 amino acids, distinct from other CMV movement proteins containing 279 amino acids (Kim *et al.*, 1996). In this work, we have purified the CMV-Kor movement protein from *E. coli* and characterized its binding properties to nucleic acid.

Materials and Methods

cDNA cloning of CMV-Kor movement protein gene and construction of deletion mutants The plasmid pBSKSMP containing cDNA of CMV-Kor movement protein gene was constructed as follows: A 1 kb DNA fragment of movement protein gene was prepared by PCR from CMV-Kor cDNA. The primers used for PCR were the start primer, 5′-ggatccatggctttc

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caaggtacc-3′ and stop primer, 5′-ctggatcctcaaaactatgatgttggc-3′. The start primer had a *Bam*HI linker and start codon and the stop primer had a *Bam*HI linker and complement sequences of the 3′-flanking region of the movement protein gene. The PCR products were phosphorylated with polynucleotide kinase (PNK) (Boehringer Mannheim, Germany) and were subcloned into the *Eco*RV site of pBluescript KS+ (Stratagene, La Jolla, La Jolla, USA). Serial deletion mutants were created by inverse-PCR using the supercoiled plasmid pBSKSMP as a template and *pfu* DNA polymerase (Stratagene, La Jolla, USA) (Atreya *et al.*, 1992). PCR products were phosphorylated with PNK and self-ligated. The primers for dM1 and dM7 had start and stop codons, respectively.

Expression of recombinant proteins Each pBluescript clone was digested with *Bam*HI and then cloned into the *Bam*HI site of pQE30, an *E. coli* expression vector (Qiagen, Dusseldorf, Germany). Each resulting plasmid was transformed into *E. coli* M15/pRep4 by standard methods and spread on plates containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin, Expression of recombinant proteins was induced by 1 mM isopropyl-β-D-thiogalactoside (IPTG). The proteins were produced as inclusion body in *E. coli*, and purified and renatured. The inclusion bodies were solubilized by resuspending in denaturing buffer (8 M urea, 10 mM Tris·HCl, pH 8.0, 1 mM EDTA, 500 mM NaCl, 10% glycerol) and were renatured by dialysis against binding buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA, 200 mM NaCl, 10% glycerol) containing decreasing amounts of urea until its complete removal and then analyzed by SDS-PAGE (Laemmli, 1970).

Preparation of antibody Antiserum against the CMV movement protein was raised by injecting 500 μg of purified movement protein into a rabbit and booster injecting the same amount of the protein 14 d later. The antiserum was purified as previously described (Jang *et al.*, 1998). Fifty μg of the purified movement protein was applied onto a PVDF membrane (5 mm × 5 mm). The piece of membrane on which the protein had been concentrated was treated with a 5% skim milk solution at 4°C for 1 h. The strip of membrane was incubated in 1 ml of antiserum solution (500 μl of anti-CMV movement protein and 500 μl of 5% skim milk in 10 mM Tris·Cl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, and 1% NaN_3) at 4°C for 2 h with gentle rocking. The strip was rinsed well and the specific antibody was extracted with 200 μl of 100 mM glycine (pH2.5). The extract was then immediately neutralized with 20 μl of 1 M Tris·Cl (pH 8.0).

UV-crosslinking and gel retardation One ng of ^{32}P-labeled 350 nucleotide (3′UTR of CMV-Kor RNA3) ssRNA probe was incubated with 20 to 40 ng of the movement protein or its derivatives for 30 min at 4°C. After incubation, the reaction mixtures were irradiated at room temperature in open microtubes with 3.6 J of UV light in a FLUO-LINK apparatus (Vilber Lourmat, Marne La Vallee, France). Unprotected RNA was fully digested for 30 min at 37°C by 500 ng of RNase A treatment, and the cross-linked RNA-protein complex was analyzed on 12% SDS-PAGE (Laemmli, 1970). The gel was dried and exposed to X-ray film for 48 h at –80°C. For the gel retardation assay, each protein was incubated with a ^{32}P-labeled ssRNA probe as above and the samples were loaded on a 5% native polyacrylamide gel and electrophoresed in 20 mM Tris·Cl, 3 mM sodium acetate, and 1 mM EDTA at pH 7.5. The gel was run at 4°C, dried, and autoradiographed.

Results and Discussion

To identify the functional domains of CMV-Kor movement protein, a series of deletion mutants of the CMV movement protein gene were created (Fig. 1). The movement protein gene was isolated by RT-PCR using specific primers corresponding to the nucleotides 120–1168 of CMV-Kor RNA3. The deletion mutants were created by inverse-PCR using pBSKSMP containing the CMV-Kor movement protein gene as a template, specific oligonucleotide primers, and *pfu* polymerase. A polyclonal antibody was raised using the CMV-Kor movement protein expressed in *E. coli*. The purified proteins were analyzed by SDS-PAGE and Western blotting with an anti-CMV 3a antiserum (Fig. 2). The results showed that each protein gave a single band of the expected size.

To identify the RNA binding domain, photochemical cross-linking analysis was carried out as described previously (Citovsky *et al.*, 1990). Each protein was incubated with a ^{32}P-labeled 350 nucleotide ssRNA, produced by *in vitro* transcription of a cDNA clone of CMV-Kor RNA3 3′UTR, and irradiated with UV light. The RNA-protein complexes were then incubated with RNase A and the products were analyzed by SDS-PAGE and autoradiography. The results showed that all the deletion mutant proteins, except dM4, could bind to ssRNA, as did wild-type movement protein (Fig. 3). The ability of mutant proteins to bind ssRNA was further tested by gel retardation analysis. Protein-RNA complexes were prepared as above and were subjected to native polyacrylamide gel analysis. The dM4 was also unable to retard the ssRNA probe (Fig. 4). The ability of all other mutant proteins to bind ssRNA was similar to wild-type movement

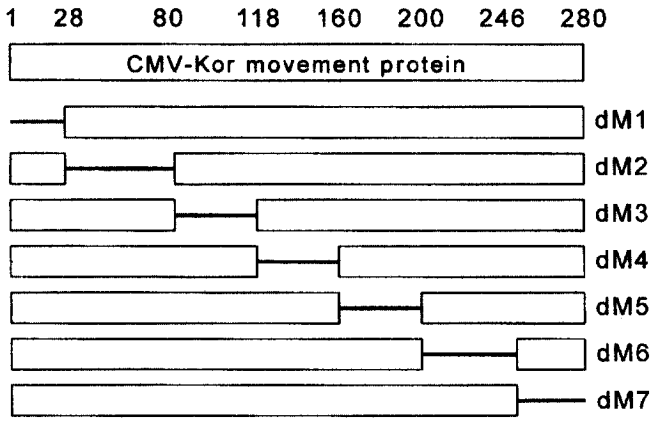

Fig. 1. Schematic presentation of deletion mutants of CMV-Kor movement protein gene. Bold lines indicate the deleted region in each mutant. The numbers above the physical map refer to the amino acids of CMV-Kor movement protein.

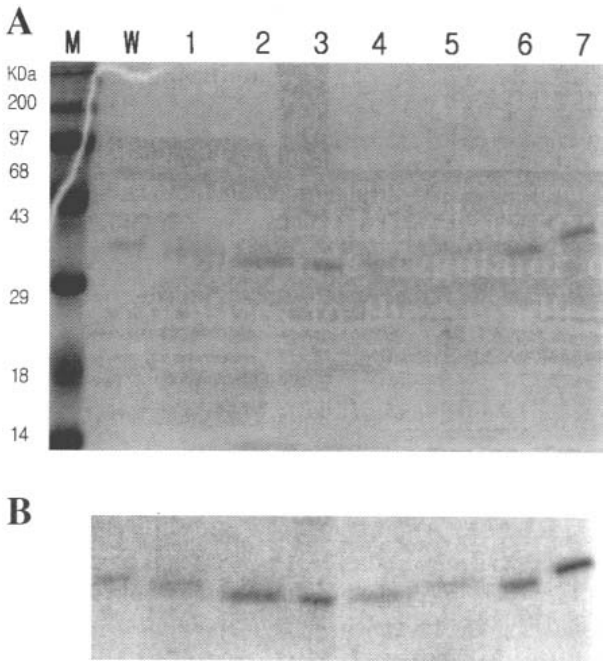

Fig. 2. Analysis of the CMV-Kor movement protein and its derivatives purified from recombinant *E. coli*. A. Each protein was run on 12% SDS polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Lane W, wild-type movement protein; Lane 1, dM1; Lane 2, dM2; Lane 3, dM3; Lane 4, dM4; Lane 5, dM5; Lane 6, dM6; Lane 7, dM7; M, Marker proteins (MW values are indicated on the left side). B. Immunoblot analysis of the CMV-Kor movement protein and its derivatives. The anti-CMV MP rabbit IgG was used as a primary antibody and peroxidase-conjugated anti-rabbit goat IgG as a secondary antibody. The substrate for peroxidase was DAB.

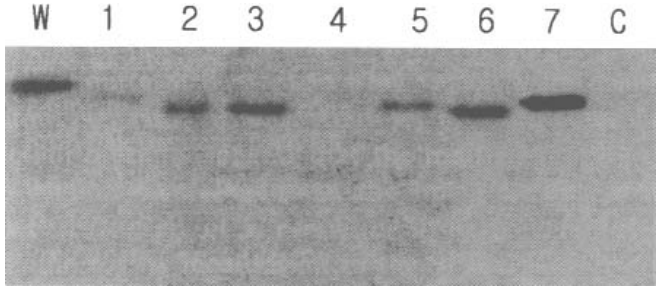

Fig. 3. Analysis of the RNA binding of the CMV-Kor movement protein and its derivatives by UV cross-linking. One ng of ^{32}P-labeled 350 nucleotide (3′ UTR of CMV-Kor RNA3) ssRNA probe was incubated with 20 to 40 ng of the movement protein or its derivatives for 30 min at 4°C. After incubation, the reaction mixtures were irradiated at room temperature in open microtubes with 3.6J of UV light in a FLUO-LINK apparatus (Vilber Lourmat). Unprotected RNA was fully digested for 30 min at 37°C by 500 ng of RNase A treatment, and the cross-linked RNA-protein complex was analyzed by 12% SDS-PAGE. The gel was dried and exposed to X-ray film for 48 h at –80°C. Lane W, wild-type movement protein; Lane 1, dM1; Lane 2, dM2; Lane 3, dM3; Lane 4, dM4; Lane 5, dM5; Lane 6, dM6; Lane 7, dM7; Lane C, RNA only.

protein. These results show that the domain between amino acids 118 and 160 of CMV-Kor movement protein is essential for ssRNA binding. Mutant movement proteins expressed in transgenic plants may be capable of blocking the function of the wild-type movement protein (Doem *et al.*, 1992; Lapidot *et al.*, 1993). The transgenic plant containing the mutant movement protein from which the RNA-binding domain was eliminated may be able to block viral movement by binding or masking the host factor(s) before wild-type movement protein production in the plant. In fact, when the dM4 gene was introduced into *Nicotiana tabacum*, the transgenic tobacco plants showed high resistance against CMV-Kor (You *et al.*, manuscript in preparation).

This domain has the ribokinase domain and a few positive-charged R-groups in their amino acids (118-LS^PID$^-$GQCVS^LH$^+$NH$^+$D$^-$LPALVS^FQPT^YD$^-$CPME$^-$T^VGN R$^+$K$^+$R$^+$CFAV$^-$160: +, basic; –, acid; ^, polar). According to the secondary structure prediction of this region by the algorithm of Garnier *et al.* (1975), turn structures are between 121 and 145 amino acids and the α-helix structure is between 150 and 156 amino acids. From this analysis, the binding does not seem to be particularly sequence dependent. This region may contribute to the correct folding of CMV-Kor movement protein rather than direct interaction with viral RNA. There is no sequence homology between this region and the RNA-binding domain of the other virus movement protein so far. The RNA binding activity of the TMV movement protein is presumed to be relevant to the movement function. The nucleoprotein complex becomes shaped into a suitable form for transport through the plasmodesmata (Citovsky *et al.*, 1990; 1992). The CMV movement protein may have an unfoldase function like the TMV movement protein, since the CMV movement protein is able to bind to tRNA-like-structured RNA which corresponds to the 3′ UTR of

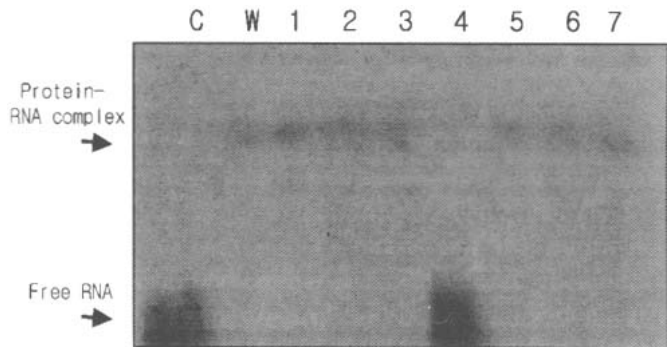

Fig. 4. Gel retardation assay. The incubation condition is the same as that in Fig. 3. The samples were loaded on a 5% native polyacrylamide gel and electrophoresed in 20 mM Tris-Cl, 3 mM sodium acetate and 1mM EDTA at pH 7.5. The gel was run at 4°C, dried, and autoradiographed. Lane C, RNA only; Lane W, wild-type movement protein; Lane 1, dM1; Lane 2, dM2; Lane 3, dM3; Lane 4, dM4; Lane 5, dM5; Lane 6, dM6; Lane 7, dM7.

CMV RNA3 used as the RNA probe in our experiment. Further *in vitro* mutagenesis and binding studies are needed to identify the exact amino acids which make direct contact with viral RNA.

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