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VIRAL AND VIROID DISEASES

Short communication

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The N-terminal 62 amino acid residues of the coat protein of *Tomato yellow leaf curl Thailand virus* are responsible for DNA binding

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Abstract The DNA-binding activity and DNA-binding domain of *Tomato yellow leaf curl Thailand virus* coat protein were investigated. A full-length coat protein (CP) and two truncated derivatives lacking the amino (CPΔ1-62) and carboxyl (CPΔ126-257) termini were produced in *Escherichia coli* as fusion proteins to glutathione-*S*-transferase (GST). Southwestern analysis showed that GST-CP bound both single-stranded (ss) and double-stranded (ds) DNA, while GST-CPΔ126-257 interacted only with ssDNA. Neither ss nor dsDNA bound to GST-CPΔ1-62. The results suggested that a putative DNA-binding domain is located at the N-terminal 1-62 amino residues.

Key words *Tomato yellow leaf curl Thailand virus* · Coat protein · DNA-binding domain · Geminivirus

Tomato yellow leaf curl Thailand virus (TYLCTHV) causes leaf curling and yellowing in tomato crops. The virus is transmitted by whitefly *Bemisia tabaci* and is classified into genus *Begomovirus*. Genome characterization revealed that TYLCTHV possesses two genomic components containing both DNA A and DNA B (Attathom et al. 1994; Rochester et al. 1990, 1994; Sawangjit et al. 2005). The DNA A encodes for six open reading frames including replication-associated protein and coat protein (CP), while the DNA B encodes for movement proteins. Although TYLCTHV is a bipartite geminivirus possessing DNA A and DNA B, agroinoculation with only DNA A causes systemic infection in *Nicotiana benthamiana* and tomato plants (Rochester et al. 1990). The ability of TYLCTHV DNA A to systemically infect plants suggested that it could act like its monopartite counterparts, for example, *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl virus* (Khey-Pour et al. 1991; Navot et al. 1991). The viruses contain a single genomic component and are causal agents of tomato yellow leaf curl.

To establish systemic infection in a plant, a geminivirus must move from the infection sites into the plant nuclei to replicate its genome, which is subsequently translocated to uninfected cells (Hanley-Bowdoin 1999). Recently, many lines of evidence have shown that geminiviral CPs play a vital role in directing viral nucleic acids into and out of the nucleus (Kunik et al. 1998; Liu et al. 1999; Kotlitzky et al. 2000; Rhee et al. 2000). Studies of *Tomato yellow leaf curl virus* (TYLCV-[IL]) CP revealed that the protein contains a nuclear localization signal (NLS) at the N-terminus (Kunik et al. 1998). The NLS was shown to be essential for translocation of the viral CP into plant nuclei. Furthermore, Palanichelvam and coworkers (1998) found that the CP binds cooperatively to single-stranded DNA in a sequence-nonspecific manner.

Because DNA A of TYLCTHV acts like its monopartite counterparts, we were interested in examining whether TYLCTHV CP could bind DNA, and, if it did, we would attempt to localize the DNA-binding domain. To do this, we expressed a full-length CP of *Tomato yellow leaf curl Thailand virus*-[2] (TYLCTHV-[2]) (Attathom et al. 1994) and two truncated derivatives lacking the N-terminal and C-terminal residues in *Escherichia coli.* The proteins were then used for Southwestern analysis to investigate their ability to bind nucleic acids.

The full-length *CP* gene was amplified by polymerase chain reaction (PCR) using primers CPF 5′-TAATTCGTGCGAAGAGGATCCTCGAAGCGTCCA-3′ and CPR

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The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under accession numbers AF141922 (TYLCTHV-[2]), AF220561 (RTBVCN), X01633 (MSV) and AF295401 (ToLCBV-[Ban5])

5′-TATAAAATTTAAAAGCTTTTAATTCGTCAC-3′ and a full-length clone of TYLCTHV-[2] DNA A as a template (accession no. AF141922). The two mutated genes were generated by PCR amplification. One of the mutated genes, designated *CPΔ1-62,* was amplified from nucleotide 624 to 1209 of TYLCTHV-[2] using primers CPΔ1-62F 5′-AGAATGTATAGAGGATCCGATGTCCCT-3′ and CPR; this resulted in a deletion of amino residues 1–62 at the N-terminus (CPΔ1-62). The other mutant, *CPΔ126-257*, was amplified using primers CPF and CPR, but the template was mutated by digesting the full length clone with *Bgl*II and filling in with a Klenow fragment (New England Biolabs, USA). The mutation caused frame shifting and introduced a premature stop codon in the *CP* gene whereby the mutant could produce a truncated CP lacking C-terminal amino residues 126–x257 (CPΔ126-257). The PCR products were then cloned into pGEX-2T (Amersham, Biosciences, USA) to generate pGEX-CP, pGEX-CPΔ1-62, and pGEX-CPΔ126-257. Insertion of the *CP* genes into pGEX-2T resulted in fusion of the genes to *GST* gene at their 5′ terminus. The plasmids were then used to transform *E. coli* strain DH5α. The bacteria containing each plasmid were separately grown overnight at 37°C. The culture was then diluted 1:100 in fresh Luria Bertani (LB) medium and shaken until the absorbance at 420 nm (OD_{420}) reached 0.5–0.7. Protein expression was induced by addition of isopropyl β-*d*-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, and the bacterial culture was further grown for another 3 h. The cell pellet was collected by centrifugation and resuspended in 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (100 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 10% SDS, 0.5% bromphenol blue, 20% glycerol). The samples were boiled for 10 min to denature the proteins. SDS-PAGE and Western blot analysis using a monoclonal antibody against TYLCTHV-[2] CP and anti-glutathione-*S*-transferase (anti-GST) antiserum (Amersham) revealed the presence of a wild-type fragment (GST-CP) of 54 kDa and two truncated derivatives (GST-CPΔ1-62 and GST-CPΔ126-257) of 47 and 40 kDa, respectively (Fig. 1 and data not shown). The fusion proteins were then purified. However, due to the poor solubility of the fusion proteins, we could not solubilize the proteins even in denaturing conditions using a high concentration of urea and guanidine hydrochloride. Therefore, crude extracts were used to investigate the DNA-binding ability.

Southwestern analysis using crude proteins was performed as described by Zhang et al. (2000). Two DNA probes were used in this study and generated by PCR amplification using a PCR high prime Digoxygenin labeling kit (Roche, Applied Science, Germany). A specific DNA probe contained the portion of TYLCTHV-[2] DNA A spanning nucleotide 138 to 352, which covers the common region and *AV2* gene. A nonspecific DNA probe was derived from *Rice tungro bacilliform virus*-Chainat isolate (RTBVCN) belonging to Caulimoviridae (accession no. AF220561). The RTBVCN probe consisted of nucleotides 6101 to 6367. The genomes of RTBVCN and TYLCTHV-[2] only have 16% identity at the nucleotide level. Southwestern analysis

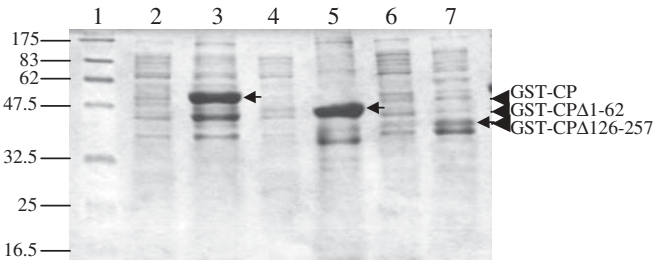

Fig. 1. Expression of TYLCTHV-[2] CP and its truncated derivatives in *Escherichia coli*. The cell pellets were collected and washed with 1.5% (w/v) *N*-laurylsarcosine to eliminate *E. coli* proteins before loading onto a 15% sodium dodecyl sulfate (SDS) polyacrylamide gel. Fragments of 54, 47, and 40 kDa were detected in extracts from cells containing pGEX-CP, pGEX-CPΔ1-62, and pGEX-CPΔ126-257, respectively. Lane *1*, prestained protein marker, broad range (New England Biolabs); lanes *2* and *3*, glutathione-*S*-transferase coat protein (GST-CP); lanes *4* and *5*, GST-CPΔ1-62; lanes *6* and *7*, GST-CPΔ126-257. Lanes *2*, *4*, and *6*, crude proteins extracted from noninduced cells; lanes *3*, *5*, and 7, crude proteins obtained from induced cells. *Arrows* and *arrowheads* indicate full-length and truncated TYLCTHV-CP proteins, respectively

was then performed by equally loading crude proteins on 15% SDS polyacrylamide gel. After electrophoresis was completed, the proteins were transferred to nitrocellulose membrane using electroblotting (semi-dry transfer cell, BioRad, USA). The membrane was then soaked in binding buffer [25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 25 mM HEPES, pH 7.9, 10% glycerol] for 1 h at room temperature before transferring to blocking solution containing 5% skim milk in binding buffer followed by 2.5% skim milk in binding buffer. The membrane was then incubated with a DNA probe either in single-stranded or double-stranded form in binding buffer containing 2.5% skim milk for 2 h at 4°C. For a dsDNA probe, a DIG-labeled probe was directly used for incubation with the membrane, while a single-stranded (ss) DNA probe was generated by boiling a double-stranded (ds) DNA probe for 5 min and chilling on ice for 3 min before adding to binding buffer. After incubation with a DNA probe, the membrane was washed and incubated with anti-DIG-AP followed by CSPD solution (DIG luminescent detection kit, Roche) using the manufacturer's protocol. The membrane was exposed to X-ray film. Western blot analysis was then performed using the same membrane to confirm presence of the proteins.

Southwestern analysis revealed that GST-CP bound both ssDNA and dsDNA probes derived from TYLCTHV-[2] DNA A (Fig. 2A, B, lane 4), but GST protein and bovine serum albumin (BSA) did not bind the DNA probes (Fig. 2A, B and E, F, lanes 2, 3, and 1). Therefore, the binding was the result of an interaction between TYLCTHV-[2] CP and the DNA probes, not with the GST protein. The appearance of two bands in lane 4 suggested degradation of GST-CP. Western blot analysis using anti-GST revealed that the top band was the size expected for GST-CP (54 kDa, Fig. 2E, F, lane 4) and the bottom one was assumed to be a degradation product. It is believed that the band with

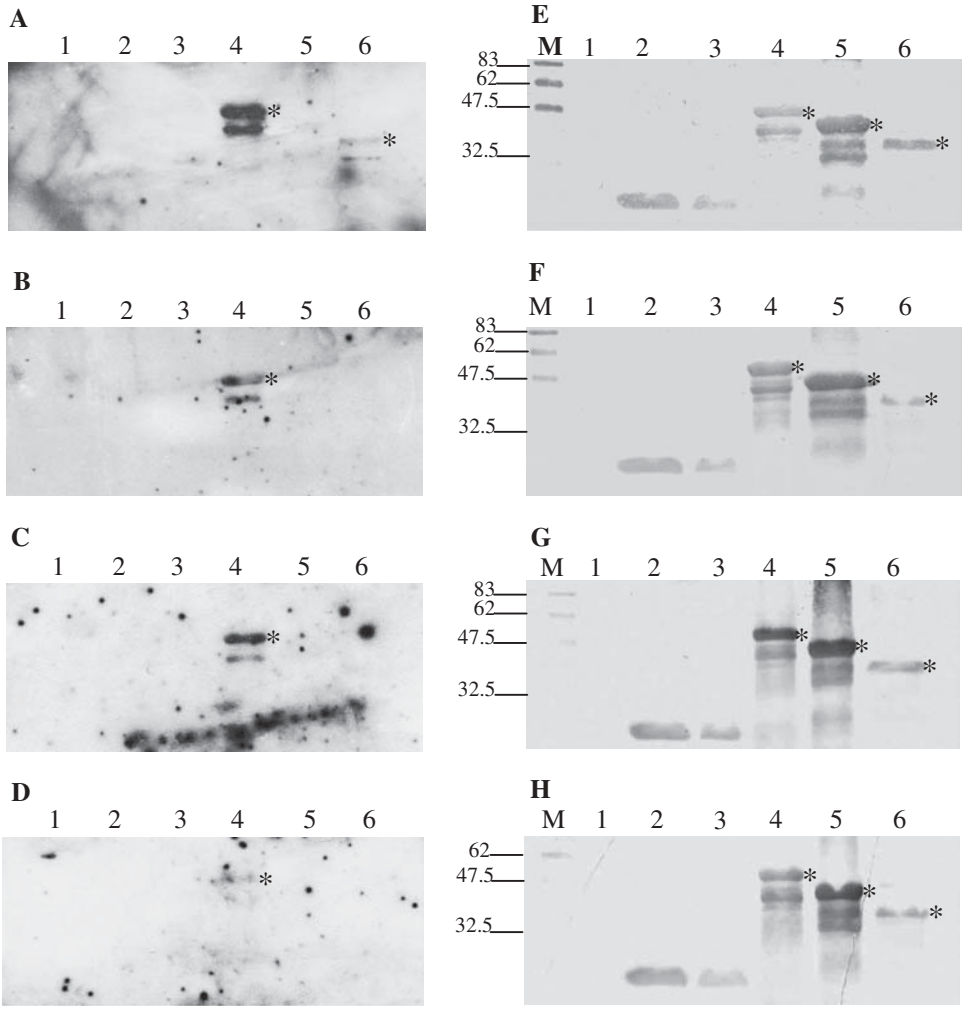

Fig. 2A–H. Ability of *Tomato yellow leaf curl Thailand virus* (TYLCTHV)-[2] coat protein (CP) and its derivative to bind related and unrelated DNA. Southwestern analysis of TYLCTHV-[2] CP and its derivative in binding TYLCTHV-[2] single-stranded (ss) DNA (**A**) and double-stranded (ds) DNA (**B**) or *Rice tungro bacilliform virus*-Chainat isolate (RTBVCN) ssDNA (**C**) and dsDNA (**D**) probes. After being exposed to the film, the membranes were probed with anti-glutathione-*S*-transferase (anti-GST) antibody (Amersham) to examine the presence of TYLCTHV-[2] CP and its derivatives. **A**, **B**, **C**, and **D** correlate with **E**, **F**, **G**, and **H**, respectively. *M*, Prestained protein marker (broad range, New England Biolabs); lane *1*, bovine serum albumin; lanes *2* and *3*, GST; lane *4*, GST-CP; lane *5*, GSTΔ1-62; lane *6*, GSTΔ126-257. *Asterisks* indicate major bands of TYLCTHV-[2] CP and its truncated derivatives with the expected sizes. Membranes **A** and **B** and membranes **C** and **D** were exposed simultaneously to X-ray film for 30 min

low molecular mass was a degradation product of GST-CP rather than a product of nonspecific binding between *E. coli* protein and the DNA probe; *E. coli* proteins in the crude protein of GST did not have any binding (Fig. 2A, B, lanes 2 and 3).

The ability of GST-CP to bind unrelated DNA was analyzed using RTBVCN DNA probe. It was found that the GST-CP bound both ssDNA and dsDNA although weaker binding was seen for the dsDNA probe (Fig. 2C, D, lane 4). The ability of TYLCTHV-[2] CP to bind both specific and nonspecific DNA probes indicated that the protein binds to DNA in a sequence-nonspecific manner. Our result was consistent with the finding reported by Palanichelvam et al. (1998) using TYLCV-[IL] CP.

DNA-binding activity of the two truncated CPs named GST-CPΔ1-62 and GST-CPΔ126-257 was further examined. GST-CPΔ1-62, with its 62 residues at the N-terminal deleted, had lost the ability to bind TYLCTHV-[2] ssDNA and dsDNA probes (Fig. 2A, B, lane 5). Loss of binding activity was not due to a protein deficiency because Western blot analysis revealed the presence of GST-CPΔ1-62, albeit the protein had some degradation (Fig. 2E, F, lane 5). In the case of the truncated protein lacking the C-terminus, GST-CPΔ126-257 was able to bind the TYLCTHV-[2] ssDNA probe, but not the dsDNA (Fig. 2A, B, lane 6). Like GST-CP, Southwestern analysis revealed two bands of the expected size (40 kDa) and presumably the degradation product of GST-CPΔ126-257. The ability of GST-CPΔ1-62 and GST-CPΔ126-257 to bind unrelated DNA was further examined using RTBVCN ssDNA and dsDNA probes. Southwestern analysis revealed that the proteins did not bind either probes (Fig. 2C, D, lanes 5 and 6).

Geminiviruses are known to replicate in plant nuclei. It is well documented that the coat protein of geminiviruses plays a role in directing the transport of the virus genome into the plant nuclei. Recently, studies of the CPs from *Maize streak virus* (MSV), TYLCV-[IL] and *Tomato leaf curl Bangalore virus* (ToLCBV-[Ban5]) showed that the CPs bind both ssDNA and dsDNA, with a preference for ssDNA (Liu et al. 1997; Palanichelvam et al. 1998; Kirthi and Savithri 2003). Liu and coworkers (1997) showed that MSV CP lacking either 20 or 80 N-terminal amino residues lost the ability to bind DNA. This finding and our result lent support to the DNA-binding domain of geminiviral CP being located at the N-terminus. However, a conserved motif between the two CPs was not found. Alignment of TYLCTHV-[2] and MSV CP showed some conserved basic amino acids around residues 1–40. It is tempting to presume

that electrostatic forces between cations of the basic residues and anions of phosphate groups on the DNA may be involved in protein–DNA interaction (William and Maher 2000).

Interestingly, a study of the DNA-binding domain in ToLCBV-[Ban5], a begomovirus, revealed a line of evidence that a zinc finger motif is responsible for DNA binding. Kirthi and Savithri (2003) showed that deletion of 24 or 50 amino acids from the N-terminus of ToLCBV-[Ban5] CP did not prevent the CP from binding DNA. A conserved putative zinc finger motif featuring C68, C72, H81, and H85 was found to account for DNA binding. The putative motif is also found in TYLCTHV-[2] CP at the same positions, but the H81 is replaced by N81. In our experiment, loss of 62 N-terminal amino residues abolished DNA binding; however, it cannot be ruled out that the motif may play a part in the DNA-binding ability.

Binding of geminiviral CP to DNA is shown to be sequence independent (Liu et al. 1997; Palanichelvam et al. 1998; Kirthi and Savithri 2003). It is worth noting that the C-terminal-deleted mutant (CPΔ126-257) was able to interact only with specific ssDNA probe. This raised a question of whether the N-terminal residues of TYLCTHV-[2] CP are specific for binding only to its own DNA. This requires further experimental investigation.

In our recent study, we inoculated tomato plants with *Agrobacterium* containing one and a-bit-mer of a CPΔ126-257 DNA A mutant clone and found that the mutant did not systemically infect the plants while the wild type (one and a-bit-mer of TYLCTHV-[2] DNA A) did. Inoculation of tobacco leaf discs with *Agrobacterium* containing the CPΔ126-257 mutant showed that the mutant could produce single-stranded DNA but to a lesser extent than the wild type did (C. Pitaksutheepong, unpublished data). This could explain why the CP is not essential for viral replication but is important for systemic infection in plants, perhaps to protect the viral genome from plant nucleases (Palanichelvam et al. 1998). We are now interested in examining an N-terminal-deleted mutant and whether it can replicate in tobacco leaf discs or systemically infect tomato plants.

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