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Characterization of a specific interaction between IP-L, a tobacco protein localized in the thylakoid membranes, and *Tomato mosaic virus* coat protein

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ABSTRACT

We previously demonstrated a specific interaction between *Tomato mosaic virus* (ToMV) coat protein (CP) and a tobacco protein designated IP-L that may be involved in the long-distance movement of ToMV. Here, using the yeast two-hybrid system and GST pull-down assay, we demonstrated that the N-terminal helical region (residues 3–18) of IP-L is required for the interaction, while two α-helical domains (residues 21–31 and 142–147) of ToMV CP are involved. Furthermore, using immunoblotting, we showed that both of the IP-L and the majority of ToMV CP are co-localized in the chloroplast thylakoid membranes. These results provide further evidence for the association between tobamovirus CPs and thylakoid membrane components, which has been shown to be involved in chlorosis formation during viral infection, and indicate that the interaction between ToMV CP and IP-L may affect chloroplast function and stability and thus leading to chlorosis.

The ability of plant viruses to invade host plants and cause diseases is determined by molecular interactions between host and virus factors. These interactions directly affect virus replication, cell-to-cell movement, systemic movement, and symptom development, as well as the elicitation of host defense responses [1]. To date, many host factors have been identified to interact specifically with some viral gene products, including coat protein (CP), movement protein (MP) and replicase protein [2–6]. However, the explicit roles of these host factors and their interactions with virus factors in viral pathogenicity and host plant resistance remain to be investigated.

Tomato mosaic virus (ToMV), a member of the *Tobamovirus* genus, has a positive-sense single-stranded RNA genome that encodes at least four proteins [7]. The 130- and 180-kDa replicase proteins are translated directly from the genomic RNA using the same first initiation codon, and the 180-kDa protein is synthesized by read-through of the amber termination codon of the 130-kDa protein gene. Although both the replicase proteins are involved in viral RNA replication, they exhibit different functions. The 180-kDa protein is necessary for viral RNA replication and performs the known functions associated with the replication, whereas the 130-kDa protein appears to enhance the efficiency of the replication [8]. The 30-kDa MP and 17-kDa CP are translated from the respective subgenomic mRNAs, which are synthesized during the replication cycle. It has been shown that MP is essential for cell-to-cell movement [9], and CP mainly plays a role in long-distance movement of tobamoviruses in plants [10,11].

We previously identified a tobacco protein designated IP-L that interacts with ToMV CP using a Gal4-based yeast two-hybrid system [12]. The initial functional analysis of IP-L using virus-induced gene silencing (VIGS) showed that it may be involved in the long-distance movement of ToMV [12]. However, the molecular basis of the interaction between ToMV CP and IP-L, as well as the roles of the interaction in viral infection, is not well understood. In this study, we characterized the interaction between IP-L and ToMV CP, and identified the domains required for the interaction on both proteins using the yeast two-hybrid system and GST pull-down assay. Furthermore, we investigated the subcellular localization of IP-L using immunoblotting, and showed that IP-L is localized in the thylakoid membranes in tobacco leaf tissue, which suggests that its interaction with ToMV CP may be involved in chlorosis formation during viral infection.

Materials and methods

Plants, virus source and cell lines. Nicotiana benthamiana was used as the host plant and grown on soil in growth chamber with 70% relative humidity and a 12-h light/12-h dark photoperiod at 22

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to 26 °C. Leaves of 4-week-old plants were mechanically inoculated with ToMV and then harvested 7 days after inoculation. *Escherichia coli* strains DH5α and BL21 (DE3) (Novagen), kept in our laboratory, were used as a cloning strain and an expression strain, respectively. *Saccharomyces cerevisiae* strain AH109 (Clontech) was used in yeast two-hybrid assay to characterize the interaction between ToMV CP and IP-L.

Chemicals. The polyclonal antisera against IP-L and ToMV CP were raised by injecting rabbits with IP-L and ToMV CP cleaved from purified GST-fusion proteins, respectively, according to the standard immunization protocol. HRP labeled goat anti-rabbit IgG was purchased from Amersham Biosciences. Other chemicals were purchased from either Sigma (St. Louis, MO) or TAKARA Shuzo (Otsu, Japan), unless otherwise noted.

Plasmid constructs. The constructs used in the yeast two-hybrid assay were generated by inserting the deletion mutants derived from the IP-L or ToMV CP gene into vectors pGADT7 and pGBKT7 (Clontech). Polymerase chain reaction (PCR) was used to generate the deletion mutants that were flanked by appropriate restriction sites. Primers used for PCR are listed in Table 1. pGADT7-IP-L harboring the full-length IP-L gene and pGBKT7-ToCP harboring the entire ToMV CP gene were reported previously [12] and used as templates in PCR. DNA fragments encoding residues 1–52 (primers IP-L-F and IP-L-RΔC103), 1-104 (primers IP-L-F and IP-L-RΔC51), 20–155 (primers IP-L-FΔN19 and IP-L-R), 36–155 (primers IP-L-FΔN35 and IP-L-R), 53–155 (primers IP-L-FΔN52 and IP-L-R) and 105–155 (primers IP-L-FΔN104 and IP-L-R) of IP-L, were amplified and cloned between the EcoRI and BamHI sites of pGADT7 to generate pGADT7-IP-LΔC103, pGADT7-IP-LΔC51, pGADT7-IP-LΔN19, pGADT7-IP-LΔN35, pGADT7-IP-LΔN52, pGADT7-IP-LΔN104. DNA fragments encoding residues 1–54 (primers CP-F and CP-RΔC105), 1–106 (primers CP-F and CP-RΔC53), 1–137 (primers CP-F and CP-RΔC22), 1–149 (primers CP-F and CP-RΔC10), 16–54 (primers CP-FΔN15 and CP-RΔC105), 16–149 (primers CP-FΔN15 and CP-RΔC10), 16–159 (primers CP-FΔN15 and CP-R), 32–54 (primers CP-FΔN31 and CP-RΔC105), 32–137 (primers CP-FΔN31and CP-RΔC22), 32–159 (primers CP-FΔN31 and CP-R), 55–106 (primers CP-FΔN54 and CP-RΔC53), 55–159 (primers CP-FΔN54 and CP-R), 107–137 (primers CP-FΔN106 and CP-RΔC22), 107–149 (primers CP-FΔN106 and CP-RΔC10) and 107–159 (primers CP-FΔN106 and CP-R) of ToMV CP, were amplified and cloned into pGBKT7 to generate pGBKT7-CPΔC105, pGBKT7-CPΔC53, pGBKT7-CPΔC22, pGBKT7-CPΔC10, pGBKT7-CPΔN15ΔC105, pGBKT7-CPΔN15ΔC10, pGBKT7-CPΔN15, pGBKT7-CPΔN31ΔC105, pGBKT7-CPΔN31ΔC22, pGBKT7-CPΔN31, pGBKT7-CPΔN54ΔC53, pGBKT7-CPΔN54, pGBKT7-CPΔN106ΔC22, pGBKT7-CPΔN106ΔC10 and pGBKT7-CPΔN106, respectively. To obtain sufficient amounts of recombinant proteins for *in vitro* binding assays, the full-length IP-L and ToMV CP gene, excised from the plasmids pGEX-IP-L and pGEX-CP [12], were cloned between the EcoRI and XhoI sites of pET30a (+) vector (Novagen) to generate pET-IP-L and pET-ToMV-CP, respectively. The deletion mutants of IP-L gene were cut from the yeast two-hybrid constructs described above, and cloned between the EcoRI and XhoI sites of pGEX-6P-1 vector (Amersham Biosciences) in frame with the glutathione-*S*-transferase (GST) gene. Similarly, the mutants of ToMV CP gene were cloned between the EcoRI and SalI sites of pGEX-6P-1 vector. The sequences of all constructs were confirmed by DNA sequencing.

Table 1
Primers used for the construction of the IP-L and ToMV CP mutants

Gene	Primer	Sequence (5′ → 3′) [a]
IP-L	IP-L-F	GGCGAATTCATGGTTCTCCAAACTCAA
	IP-L-FΔN19	GAGAATTCAACTACTATTCCGATAGTAGC
	IP-L-FΔN35	GAGAATTCCCTTCGGGCTATTCCACCAT
	IP-L-FΔN52	GCAGAATTCATGATGGGTCATGGTGGC
	IP-L-FΔN104	GCCGAATTCGGCTATGGTAGTGGAATG
	IP-L-RΔC51	AGCGGATCCACCATCACTATGGATCATAC
	IP-L-RΔC103	GAAGGATCCCATGTGATTGTGAGTGGAC
	IP-L-R	GTCGGATCCTTATTCCTCCAAATCCTTA
The CP gene	CP-F	GCGAATTCATGTCTTACTCAATCACTTCTCC
	CP-FΔN15	GCGAATTCTCTGTATGGGCTGACCCTAT
	CP-FΔN31	GCGAATTCTTAGGTAACCAGTTTCAA
	CP-FΔN54	GCGAATTCCCTTTCCCTCAGAGCACC
	CP-FΔN106	GCGAATTCGAAACGTTAGATGCTACCCG
	CP-RΔC10	TCGGATCCAGACATACTTTCAAAAGTATT
	CP-RΔC22	GCGGATCCAGTACCTCTTACTAGTTCATT
	CP-RΔC105	GAGGATCCTTTCCACACCTCGCTGAAC
	CP-RΔC53	GCGGATCCAGCTGTTGTCGGACTCTGC
	CP-R	GCGGATCCTTAAGATGCAGGTGCAGAG

[a] Restriction enzyme sites (EcoRI and BamHI) used for cloning were underlined. Primers were synthesized by Invitrogen.

Yeast two-hybrid assay and β-galactosidase assays. Yeast two-hybrid assay was carried out using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech) according to the manufacturer's protocols. *S. cerevisiae* strain AH109 was transformed with the yeast two-hybrid constructs described above using the small-scale lithium acetate (LiAc) method (Clontech), and subsequently plated on SD minimal medium lacking adenine, histidine, leucine and tryptophan (SD/-Ade/-His/-Leu/-Trp). The β-galactosidase colony-lift filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a substrate was then carried out to assess the interactions according to the manufacturer's protocols (Clontech).

GST pull-down assay. His-tagged ToMV CP and IP-L were expressed and then purified by Nickel-affinity chromatography according to the instructions of the manufacturer (Novagen), respectively. The deletion mutants of ToMV CP and IP-L, fused to GST, were expressed and purified using glutathione sepharose 4B affinity resin as described previously [12]. Concentrations of the purified proteins were determined using the BCA protein assay (Pierce Biochemicals). GST pull-down assay was performed as described previously [13]. Briefly, 1.5 μg of GST control and GST-fusion proteins were incubated with glutathione sepharose 4B beads for 1 h at 4 °C, respectively. The beads were washed to remove unbound proteins and then incubated with 1.5 μg of His-tagged proteins for 3 h at 4 °C. After extensive washing, bound proteins were eluted by boiling in SDS sample buffer and analyzed by immunoblotting using rabbit anti-His-tag Polyclonal antibody and HRP labeled goat anti-rabbit IgG as the primary and secondary antibodies, respectively.

Chloroplast isolation and fractionation, and immunoblotting. Chloroplasts, isolated from healthy and systemically infected tobacco leaves, were subfractionated as described previously [14]. Intact thylakoids were further fractionated by sonication on ice. The thylakoid membranes were separated from the lumen extract by ultracentrifugation and resuspended in 10 mM Hepes–KOH (pH 8.0). Proteins in each fraction were subsequently analyzed by immunoblotting using rabbit anti-serum against IP-L and ToMV CP, respectively.

Results and discussion

The N-terminal helical region of IP-L is required for its interaction with ToMV CP

To identify the regions of IP-L that mediate its interaction with ToMV CP, four IP-L deletion mutants (IP-LΔN52, IP-LΔN104, IP-LΔC51 and IP-LΔC103) were constructed and analyzed with the yeast two-hybrid system. In addition to the full-length IP-L, mutants IP-LΔC51 and IP-LΔC103 were able to interact with ToMV CP, whereas the other two mutants IP-LΔN52 and IP-LΔN104 lacking 52 and 104 amino acids (aa) from the N terminus, respectively, abolished their interaction (Fig. 1A). This indicated that the N-terminal 52 aa of IP-L are sufficient for the interaction.