Self-interaction of ORF II protein through the leucine zipper is essential for Soybean chlorotic mottle virus infectivity

Yutaka Takemoto*, Tadaaki Hibi

Department of Agricultural and Environmental Biology, Laboratory of Plant Pathology, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

Received 10 September 2004; returned to author for revision 15 October 2004; accepted 19 November 2004

Abstract

The ORF II protein (PII) of Soybean chlorotic mottle virus (SbCMV) is essential for the virus life cycle. We investigated the interactions of SbCMV PII with itself and with other essential virus proteins using a Gal4-based yeast two-hybrid system. PII interacted only with itself and not with any other virus proteins. The PII–PII interaction was confirmed by a Sos-based yeast two-hybrid system and a far-western analysis. Deletion mutagenesis mapped the self-interacting domain to the C-terminal 48 amino acids (amino acids 154–201), which contain two putative leucine zipper motifs. Introduction of amino acid substitutions to leucine/isoleucine in zipper sequences prevented the PII–PII interaction and abolished the infectivity of SbCMV. These results revealed that the self-interaction of PII through a leucine zipper is necessary for virus infection.

© 2004 Elsevier Inc. All rights reserved.

Keywords: Soybean chlorotic mottle virus; ORF II protein; Self-interaction; Leucine zipper; Infectivity

Introduction

Soybean chlorotic mottle virus (SbCMV) is the type species of the genus “Soybean chlorotic mottle-like viruses” in the family Caulimoviridae (Hibi, 2001; Hull et al., 2000). The double-stranded DNA genome of SbCMV (8178 bp) contains eight ORFs and one large noncoding region (NCR) (Conci et al., 1993; Hasegawa et al., 1989; Hibi et al., 1986; Verver et al., 1987). The members of the genus “Soybean chlorotic mottle-like viruses” SbCMV, Peanuts chlorotic streak virus (PCISV), Cestrum yellow leaf curling virus (CmYLCV), and Blueberry red ringspot virus (BRRV), are distinguished from viruses belonging to the genus Caulimovirus by the occurrence of three ORFs between ORF I and ORF IV instead of two ORFs (ORF II and III) (Glasheen et al., 2002; Hasegawa et al., 1989; Mushegian et al., 1995; Stavolone et al., 2003) (Fig. 1). These three ORFs, designated either Ib, II, and III in SbCMV or A, B, and C in PCISV, CmYLCV, and BRRV, show little or no similarity to the two ORFs of the caulimoviruses. In SbCMV, the ORF Ib product is dispensable, whereas ORFs II and III are essential for systemic infection (Takemoto and Hibi, 2001). ORF III of SbCMV presumably corresponds to ORF III of the caulimoviruses as its product contains a basic/proline-rich domain in the C-terminal region and a putative leucine zipper sequence in the N-terminal region. On the other hand, the ORF II product is unique to SbCMV-like viruses, and its role in the virus life cycle is not well understood, although it has been shown to be required for virus replication or assembly (Takemoto and Hibi, 2001).

In this study, we investigated possible interactions between SbCMV ORF II protein (PII) and other essential...
proteins using the Gal4-based yeast two-hybrid system. PII showed no binding to any other SbCMV proteins, but interacted with itself via the domain located in the C-terminal 48 residues containing two putative leucine zipper motifs. The PII–PII interaction was also detected by the Sos-based yeast two-hybrid system and confirmed by far-western blot analysis. Moreover, amino acid substitutions of leucine/isoleucine in the motifs led to a noninfectious virus, suggesting that the leucine zipper-mediated interaction between PIIs is important for its function.

Results

Self-interaction of ORF II protein

Interactions of the PII with itself and with other essential virus proteins were examined by the yeast two-hybrid system based on the Gal4 transcription activator. As shown in Fig. 2A, when AH109 yeast cells were transformed with pGBK/II that contains the ORF II fused with the Gal4 DNA binding domain (BD) and one of the pGAD derivatives expressing a SbCMV protein fused with the Gal4 activation domain (AD), only the combination of pGBK/II and pGAD/II resulted in growth of cells on the -LWAH plate. Similarly, when pGAD/II was introduced into AH109 cells with one of the pGBK derivatives expressing a SbCMV protein fused with BD, no cell growth was observed on the -LWAH plate, except for cotransformants with pGAD/II and pGBK/II. These reciprocal experiments show that PII domains in AD and BD fusion proteins interacted in the yeast nucleus.
Interactions of the PII with itself and with other essential virus proteins were further examined in yeast cytoplasm by the Sos recruitment-mediated yeast two-hybrid system. In this system, cell growth at restrictive temperature (37 °C) can be observed only when Sos fusion protein is localized to the plasma membrane through interaction with membrane-anchored Myr fusion protein. Cotransformants with pSos/II and pMyr/II formed colonies on the -UL/Gal plate at 37 °C, indicating that the PII–PII interaction also occurs in yeast cytoplasm (Fig. 2B). Although cotransformants with pSos/Ia and pMyr/II also grew at 37 °C, cell growth was observed even on the -UL/Glu plate, which is a repressing condition for expression of Myr fusion protein (Fig. 2B). Since the Ia protein is involved in cell-to-cell movement (Takemoto and Hibi, 2001), this galactose-independent growth at restrictive temperature is thought to arise from localization of the Sos/Ia fusion protein to the plasma membrane without binding to the Myr/II fusion protein. Indeed, no interaction was observed in the combination of pSos/II and pMyr/Ia (Fig. 2B).

The PII–PII interaction was further examined in vitro by far-western blot analysis. Escherichia coli-expressed and -purified hexa-histidine S-tagged PII protein (His6·S-PII) and hexa-histidine S tag (His6·S tag) were electrophoresed through a 17% SDS-polyacrylamide gel and blotted onto a PVDF membrane. The membranes were incubated with purified maltose binding protein-tagged PII protein (MBP-PII) or maltose binding protein (MBP). Binding of the probe protein was detected using anti-MBP antibody. As shown in Fig. 2C, the MBP-PII probe clearly bound to the His6·S-PII while the His6·S tag band was not recognized (central panel, lanes 1 and 2). Moreover, no band appeared at the position of His6·S-PII when the membrane was probed with MBP alone (right panel, lane 1). The results indicate that the PII–PII binding occurs in vitro as well as in yeast and mediation of other proteins is not necessary for the interaction as the binding could be observed between purified recombinant proteins.

Mapping of the domain mediating the PII–PII interaction

To map the portion of the PII self-interaction, ORF II was divided into three fragments (PII-N, amino acids 1–62; PII-M, amino acids 63–130; PII-C, amino acids 131–201), and these fragments were tested for interactions with the full-length PII and with themselves by the Gal4-based yeast two-hybrid system. As summarized in Fig. 3, the interaction of PII-C either with full-length PII or with itself was observed, and no interaction could be detected in any other combination. This result shows that the PII-C fragment is sufficient for binding and other regions are not involved in the interaction. To determine the interacting domain more precisely, additional analysis was carried out with the N-terminal deletion series of the PII-C fragment (Fig. 3: PII141-201, PII154-201, PII175-201). Interaction was detected when the deletion extended up to amino acid 140 or 153 (PII141-201, PII154-201), but PII175-201 was no longer able to bind to full-length PII. The data demonstrate that sequences involved in self-interaction are present within the C-terminal 48 amino acids.

Computer analysis based on the COILS algorithm (Lupas, 1996) suggested that the interacting domain (C-terminal 48 amino acids) contains two putative leucine zipper motifs that are composed of leucine/isoleucine heptad repeat (Fig. 4A). Leucine zippers are known to engage in protein–protein interaction, and hydrophobic bonds caused by leucine/isoleucine are the major driving force for intermolecular binding through this motif (Harbury et al., 1993). To investigate their contributions to self-interaction, double or triple replacements of leucine/isoleucine by lysine (similarly sized hydrophilic residue) were introduced into these zipper motifs, and the interaction ability of these mutants was examined by the Gal4-based yeast two-hybrid system. No mutants harboring substitutions in the zipper motifs interacted with wild type PII (Fig. 4B: LysZ1-A, LysZ1-B, LysZ1-C, and LysZ2), whereas mutations just outside zipper sequences had no effect on the interaction (Fig. 4B: LysC3). These results suggest that both leucine zipper sequences are necessary for the PII–PII interaction.

Effect of amino acid substitutions in PII leucine zipper on the infectivity of SbCMV

To examine the biological significance of the PII–PII interaction, the same triple or double amino acid substitutions were introduced into the infectious clone pSbCMV1.3 (Takemoto and Hibi, 2001), and the mutants were inoculated to Phaseolus vulgaris. No progeny DNA was detectable in the upper leaves by dot blot hybridization assay in the case of any mutant harboring the substitutions.
in leucine zipper sequences (Fig. 4C: pSb1.3/LysZ1-A, LysZ1-B, LysZ1-C, and LysZ2), while a mutant harboring the substitutions outside the zipper motifs was fully infectious (Fig. 4C: pSb1.3/LysC3). Similarly, the DNA of the leucine zipper mutants could not be detected in the inoculated leaves (data not shown). These results strongly suggest that the PII–PII interaction through leucine zipper sequences is essential for virus infectivity.

Discussion

The amino acid sequence of SbCMV ORF II shows some identity with ORF B in other SbCMV-like viruses (18.4–24.9%), and the C-terminal leucine zipper motif is conserved among SbCMV-like viruses (Glasheen et al., 2002; Stavolone et al., 2003; Y. Takemoto, unpublished data). These features suggest that the ORF II protein of SbCMV and ORF B proteins of other SbCMV-like viruses may play a similar role in the virus life cycle. However, in PCiSV, it has been shown that more than half of the ORF B coding sequence is dispensable for infection (Mushegian et al., 1995). Therefore, it is necessary to investigate whether each ORF B product is essential or nonessential for infectivity in individual viruses.

Although the existence of a leucine zipper in the SbCMV ORF III protein (PIII) has been mentioned (Leclerc et al., 1998; Stavolone et al., 2001), no PII–PIII interaction was found using both Gal4- and Sos-based yeast two-hybrid systems. In Cauliflower mosaic virus (CaMV), which is the type member of the genus Caulimovirus, the interaction between ORF II protein (aphid transmission factor: ATF) and ORF III protein (virion-associated protein) via leucine zippers was shown to be necessary for aphid transmissibility (Leh et al., 1999). In this study, the possibility of a PII–PIII interaction could not be excluded, as false-negative results may occur in yeast two-hybrid systems and a host protein may mediate an interaction between these two proteins in planta. However, SbCMV ORF II showed no significant sequence homology with ATF (Hasegawa et al., 1989), and no virus transmission was detected using several species of aphids (Iwakie et al., 1984). Therefore, the absence of an interaction between PII and PIII would not contradict the results found for CaMV.

In the genus Caulimovirus, ORF III is indispensable for infection (Daubert et al., 1983; Dixon et al., 1983; Jacquot et al., 1998) and its product interacts with itself through a leucine zipper (Leclerc et al., 1998; Stavolone et al., 2001). The features found here for SbCMV ORF II are similar to those of caulimovirus ORF III. However, SbCMV ORF III, which is also indispensable and contains a leucine zipper, shows more similarity than SbCMV ORF II to those of caulimovirus ORF III as it has a basic/proline-rich domain.
in the C-terminal region (Takemoto and Hibi, 2001). Therefore, it is not likely that SbCMV ORF II is a substitute for ORF III of the caulimoviruses.

In this study, we found that PII interacts with itself via the C-terminal leucine zipper sequences, and this self-interaction is essential for virus infectivity. We have shown previously that PII is required for virus replication or assembly, however, its function is still unidentified (Takemoto and Hibi, 2001).

Recently, Kobayashi and Hohn (2003) established a reporter-targeted PCR assay to study CaMV DNA replication in turnip protoplasts. A similar assay system would be most useful to assess the replication of the mutants and would certainly provide important information on the function of the PII protein. However, no transfection of P. vulgaris or Glycine max protoplasts with viral DNA has been reported yet and all our attempts to set up such a system have been unsuccessful so far. Further experiments to screen for host proteins interacting with PII and observations of the subcellular localization of PII in SbCMV-infected cells are also in progress. We believe these results should further clarify its role in the virus life cycle.

Materials and methods

Yeast two-hybrid analysis

In the Gal4 transcription activator-based yeast two-hybrid system (MATCHMAKER GAL4 Two-Hybrid System 3; Clontech), ORFs Ia, II, III, IV, V, VI and the truncated form of ORF V (trV; amino acids 153–692) lacking the aspartic protease domain were amplified by PCR from an infectious 1.3-mer DNA clone pSbCMV1.3 (Takemoto and Hibi, 2001). The resulting PCR products were ligated into the NdeI–XhoI site of pGAD-T7 and the NdeI–SalI site of pGBK-T7 vectors. Saccharomyces cerevisiae strain AH109 was cotransformed with pGAD and pGBK recombinant DNAs by the lithium acetate method (Gietz et al., 1992). Cotransformants were plated on synthetic medium lacking leucine and tryptophan (-LW plate) or lacking leucine, tryptophan, adenine, and histidine (-LWAH plate). Interactions were detected by nutritional requirements, namely colony formation of the transformants (-LWAH plate) or lacking leucine, tryptophan, adenine, and histidine (-LW plate). Interactions were also detected by nutritional requirements, namely colony formation of the transformants (-LWAH plate). Interactions were detected by nutritional requirements, namely colony formation of the transformants (-LWAH plate). Interactions were detected by nutritional requirements, namely colony formation of the transformants (-LWAH plate). Interactions were detected by nutritional requirements, namely colony formation of the transformants (-LWAH plate). Interactions were detected by nutritional requirements, namely colony formation of the transformants (-LWAH plate).

Far-western analysis

The PII coding region was ligated into the BamHI–XhoI site of pET30a (Novagen) for the expression of hexahistidine S-tagged PII protein (His6-S-PII) and the BamHI–SalI site of pMalc2x (New England Biolabs) for the expression of maltose binding protein-tagged PII protein (MBP-PII). Each construct was introduced into E. coli strain BL21CodonPlus-(DE3)-RIL (Stratagene), and expressed proteins were purified using Ni-NTA resin (Qiagen) and amylose resin (New England Biolabs), respectively. Purified His6-S-PII (ca. 30 kDa) and His6-S tag (ca. 12 kDa) were run on 17% SDS-polyacrylamide gel and blotted onto Immobilon P membrane (Millipore). The membranes were probed with MBP-PII or MBP at 0.5 mg/ml in phosphate-buffered saline at 4 °C overnight. Binding of probe protein was revealed by immunodetection using anti-MBP antibody (New England Biolabs) and alkaline phosphatase-labeled goat anti-rabbit IgG secondary antibody (BIOSOURCE).

Construction of the PII mutants

Deletion mutants of the PII were generated by PCR using the respective primer pairs (II-F and II-N-R for PII-N, II-M-F and II-M-R for PII-M, II-C-F and II-R for PII-C, II-F and II141-201-R for PII141-201, II-F and II154-201-R for PII154-201, and II-F and II175-201-R for PII175-201; Table 1). Introductions of double or triple replacements of leucine/isoleucine were performed by the megaprimer PCR method (Sarkar and Sommer, 1990) using II-F, II-R, and appropriate mutagenic primers (LysZ1-A-R for LysZ1, LysZ1-B-R for LysZ1-B, LysZ1-C-R for LysZ1-C, LysZ2-F for LysZ2, and LysC3-F for LysC3; Table 1). To construct the mutants of pSbCMV1.3 infectious clone, NdeI and XhoI sites were introduced into the MluI–NcoI fragment of pSbCMV1.3 at the ORF II start codon and just upstream of the ORF III start codon, respectively, by overlap extension PCR (Ho et al., 1989) using six primers (IISalA/F for LysZ1, IISalB/F for LysZ2, and IIXhoI/R for LysC3; Table 1). The fragment obtained was cloned into pGEM5Zf(+) (Promega) and the resulting plasmid was designated pPII cassette. The mutated PII fragments were cloned into the NdeI–XhoI site of the pPII cassette and then the wild-type MluI–NcoI fragment of pSbCMV1.3 was replaced with the MluI–NcoI fragments of the mutant cassette vectors. All constructs were verified by DNA sequencing using an ABI 3100 genetic analyzer (Applied Biosystems).

Infectivity assay and dot blot hybridization assay

Kidney bean (P. vulgaris cv. Honkintoki) was grown from seeds in a greenhouse. Two primordial leaves were dusted with Celite abrasive and inoculated with 25 μl DNA (500 μg/ml) in 0.05 M phosphate buffer (pH 7.0) by rubbing with a fingerstall. The inoculated leaves were rinsed
Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-F</td>
<td>5'-CACACAGGATCCCATATGTTCTAGATTAGAAAAGATCTAC-3'</td>
</tr>
<tr>
<td>II-N-R</td>
<td>5'-GTCGACCTCGAGTCATATTATATAGTAGTTTCTGTAGG-3'</td>
</tr>
<tr>
<td>II-M-F</td>
<td>5'-CACACAGGATCCCATATGGGAGTCCCGATAGGAAAACAACTG-3'</td>
</tr>
<tr>
<td>II-M-R</td>
<td>5'-GTCGACCTCGAGTCATAACGTCTCTGTTATCTTAGTATG-3'</td>
</tr>
<tr>
<td>II-C-F</td>
<td>5'-CACACAGGATCCCATATGGAAATAAATAGGAAAATCAATATAAAGATGCTTTAC-3'</td>
</tr>
<tr>
<td>II-R</td>
<td>5'-GTCGACCTCGAGTCATGATACATAAGTTTTATTTTC-3'</td>
</tr>
<tr>
<td>II141-201-R</td>
<td>5'-CACACAGGATCCCATATGGGAACAGATACACAAAACAGGAAATGATCCCTTAAC-3'</td>
</tr>
<tr>
<td>II154-201-R</td>
<td>5'-CACACAGGATCCCATATGGGAACAGATACACAAAACAGGAAATGATCCCTTAAC-3'</td>
</tr>
<tr>
<td>II175-201-R</td>
<td>5'-CACACAGGATCCCATATGGGAACAGATACACAAAACAGGAAATGATCCCTTAAC-3'</td>
</tr>
<tr>
<td>LysZ1-A-R</td>
<td>5'-GTCGACCTCGAGTCATGAAACGAAATAGGAAATCAATATAAAGATGCTTTAC-3'</td>
</tr>
<tr>
<td>LysZ1-B-R</td>
<td>5'-GTCGACCTCGAGTCATGAAACGAAATAGGAAATCAATATAAAGATGCTTTAC-3'</td>
</tr>
<tr>
<td>LysZ1-C-R</td>
<td>5'-GTCGACCTCGAGTCATGAAACGAAATAGGAAATCAATATAAAGATGCTTTAC-3'</td>
</tr>
<tr>
<td>LysZ2-F</td>
<td>5'-CAATAGCAACGAGATGCAAAACAGGAAATCAATATAAAGATGCTTTAC-3'</td>
</tr>
<tr>
<td>LysC3-F</td>
<td>5'-GTCGACCTCGAGTCATGAAACGAAATAGGAAATCAATATAAAGATGCTTTAC-3'</td>
</tr>
<tr>
<td>llSacI/MluI-F</td>
<td>5'-CACACAGGATCCCATATGGGAACAGATACACAAAACAGGAATGATCCCTTAAC-3'</td>
</tr>
<tr>
<td>llNdeI-F</td>
<td>5'-GATCTATTAGAAGAGAAAACTAATATGCTCGAGATTAGAAAGAGATCTAC-3'</td>
</tr>
<tr>
<td>llNdeI-R</td>
<td>5'-GTCGACCTCGAGTCATGAAACGAAATAGGAAATCAATATAAAGATGCTTTAC-3'</td>
</tr>
<tr>
<td>llXhoI-F</td>
<td>5'-GTCGACCTCGAGTCATGAAACGAAATAGGAAATCAATATAAAGATGCTTTAC-3'</td>
</tr>
<tr>
<td>llXhoI-R</td>
<td>5'-GTCGACCTCGAGTCATGAAACGAAATAGGAAATCAATATAAAGATGCTTTAC-3'</td>
</tr>
<tr>
<td>III-M-R</td>
<td>5'-GTCGACCTCGAGTCATGAAACGAAATAGGAAATCAATATAAAGATGCTTTAC-3'</td>
</tr>
</tbody>
</table>

*Positions of base substitution causing amino acid replacements are indicated in italics. Restriction enzyme sites used for cloning are underlined. BamHI, GGATGG; MluI, ACGCGT; NcoI, CCATGG; NdeI, CATATG; SacI, GAGCTC; XhoI, CTCGAG.*
immediately with water and plants were transferred to a growth chamber at 70% relative humidity at 32 °C during the 14-h light period (ca. 20,000 lx) and at 28 °C during the 10-h dark period. Symptoms were scored visually at various periods after inoculation. The accumulation of viral DNA in the infected leaves was verified by a dot blot hybridization assay (Maule et al., 1983). In brief, 0.1 g of infected tissue (3 weeks (upper leaves) or 1 week (inoculated leaves) after inoculation) was homogenized in 200 μl of TE buffer (pH 8.0) and an equivalent volume of 1 M NaOH was added. The samples were incubated at room temperature for 10 min. Each sample (5 μl) was spotted onto a BIODYNE PLUS membrane (Pall) and hybridized with full-length SbCMV DNA (Takemoto and Hibi, 2001).

Acknowledgments

We deeply appreciate Dr. François J. Kraus of the University of Tokyo, Dr. Alexander V. Karasev of Thomas Jefferson University, and Dr. William O. Dawson of the University of Florida for critical reading of the manuscript and fruitful discussions.

References


