Aphid transmission of cauliflower mosaic virus requires the viral PIII protein

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The open reading frame (ORF) III product (PIII) of cauliflower mosaic virus is necessary for the infection cycle but its role is poorly understood. We have used in vitro protein binding (‘far Western’) assays to demonstrate that PIII interacts with the cauliflower mosaic virus (CaMV) ORF II product (PII), a known aphid transmission factor. Aphid transmission of purified virions of the PII-defective strain CM4-184 was dependent upon added PII, but complementation was efficient only in the presence of PIII, demonstrating the requirement of PIII for transmission. Deletion mutagenesis mapped the interaction domains of PIII and PII to the 30 N-terminal and 61 C-terminal residues of PIII and PII, respectively. A model for interaction between PIII and PII is proposed on the basis of secondary structure predictions. Finally, a direct correlation between the ability of PIII and PII to interact and aphid transmissibility of the virus was demonstrated by using mutagenized PIII proteins. Taken together, these data argue strongly that PIII is a second ‘helper’ factor required for CaMV transmission by aphids.

Keywords: aphid transmission factor/cauliflower mosaic virus/far Western/ORF III product/transmission

Introduction

Cauliflower mosaic virus (CaMV) is a member of the caulimoviruses, a genus of plant viruses with a circular double-stranded DNA genome encapsidated in an icosahedral particle of 50 nm diameter (for reviews, see Rothnie et al., 1994; Jacquot et al., 1997). Caulimoviruses are classified among the pararetroviruses since they replicate their genome via reverse transcription of a pre-genomic RNA. The CaMV genome (8 kbp) contains six major open reading frames (ORFs). The ORF I product (40 kDa) is involved in cell–cell movement of the virus (Thomas et al., 1993). ORF IV codes for the coat protein component (57 kDa) (Daubert et al., 1982) and ORF V for a protein of 78 kDa, which possesses aspartate proteinase (Torruela et al., 1989) and reverse transcriptase activities (Takatsuji et al., 1986). The ORF VI product (62 kDa) is a multifunctional protein involved in host range symptomatology (Schoelz and Shepherd, 1988), translational transactivation of other CaMV ORFs (Bonneville et al., 1989) and formation of electron-dense viroplasms, which are the site of genome replication and particle morphogenesis (Mazzolini et al., 1989). ORF II codes for an 18 kDa protein (PII), which is dispensable for infection but which is required for aphid transmission of the virus (Woolston et al., 1983). Thus, when acquired first by aphids, PII (also known as the aphid transmission factor or ATF) can assist plant–plant transmission of a non-transmissible CaMV isolate from crude extracts of infected plants (Blanc et al., 1993). PII is not sufficient to transmit purified CaMV particles, however, suggesting either that the purification procedure alters the surface of the viral particle or that additional viral and/or cellular factor(s) that are lost during the purification are required for virus transmission (Blanc et al., 1993).

The ORF III product (PIII) is the only CaMV protein (15 kDa) to which no function has been previously assigned. It is known to possess non-sequence-specific nucleic acid-binding activity, which maps to a C-terminal basic domain located between amino acids 112 and 126 (Mougeot et al., 1993; Jacquot et al., 1996). Recently, in vitro experiments have shown that the 32 N-terminal residues are involved in tetramerization of PIII via leucine zipper motifs (Leclerc et al., 1998). Furthermore, it has been demonstrated that PIII is needed for the infection cycle of CaMV, but that amino acids 61–80 and the four C-terminal residues are dispensable for this purpose (Jacquot et al., 1998). Finally, a chimeric PIII protein consisting of the N-terminus of figwort mosaic caulimovirus and the C-terminus of CaMV cannot replace CaMV PIII in the infection cycle, suggesting that specific interactions occur either between the two functional regions or between the N-terminal region and another viral or cellular factor (Jacquot et al., 1998).

In this study, we tested the capacity of CaMV PIII to interact with proteins from crude extracts of healthy or CaMV-infected turnip leaves. We show that PIII is able to interact specifically with the aphid transmission factor (ORF II product) and that it is involved in CaMV transmission. The interaction domains in both proteins have been mapped, and a hypothesis for the mode of
action of PIII is proposed. This represents the first biological function to be assigned to this protein.

**Results**

**CaMV PIII protein interacts with PII**

To determine if PIII interacts with plant and/or virus proteins, crude extracts from healthy and CaMV-infected plants were separated by SDS–PAGE and transferred onto nitrocellulose membranes. PII, produced and partially purified from *Escherichia coli*, was the overlay protein and antibodies raised against PII were used to detect interactions. In our experimental conditions, two bands were observed specifically for extracts from CaMV-infected plants (Figure 1B, lane i), but not for healthy plant extract (Figure 1B, lane h), suggesting that the corresponding proteins may be of viral origin. The 15 kDa protein corresponded to PIII itself, as demonstrated by control experiments using mock bacterial extracts as overlay (Figure 1C, lane i). The second band, which appeared only when PIII was present in the overlay solution, is a protein with an apparent molecular mass of ~18 kDa (Figure 1B, lane i). The only viral protein of this molecular mass is the product of ORF II. Furthermore, the 18 kDa band was also detected with antibodies raised against PII (Figure 1D, lane i). We conclude that PIII is able to interact specifically with PII.

Interaction between PIII and PII was confirmed by performing similar far Western experiments with PII produced in Sf9 insect cells infected with a baculovirus recombinant and using PIII as overlay protein. As shown in Figure 2, PIII interacts strongly with PII (Figure 2A, lane +), whereas no band was detected at the position of PII when the blot was incubated with an extract from control bacteria (Figure 2A, lane +). Interaction between PIII and PII was similarly observed when PIII produced and partially purified from *E.coli* was blotted onto the nitrocellulose membrane and PII was used as overlay protein (Figure 2B, 2, lane +). A weak band was also observed at a lower molecular mass; it corresponds to a cross-reaction of bacterial proteins with antibodies raised against PII, as it was still detected when no PIII was present on the membrane (Figure 2B, 2 and 3, lane +) and when PII was not present in the overlay (Figure 2B, 3).

**PII is required for aphid transmission of purified CaMV**

Interaction between PIII and PII suggests that PIII might play a role in plant–plant spread of CaMV by aphids. To test this hypothesis, transmission tests were performed with purified virions of strain CM4-184, a CaMV strain carrying a deletion in ORF II. In a first feed, aphids were allowed to acquire PII produced in insect cells and, in a second feed, they were offered highly purified CM4-184 virions mixed with either an *E.coli* extract containing PIII or a control extract. They were then transferred onto turnip plants and the transmission efficiency was determined 3 weeks later (3 w.p.i.). As shown in Table I, 39% of the tested plants were infected when transmission assays were done in the presence of PIII, whereas no infection was observed when PII was absent. Similarly, highly purified virions of Cabb B-JI, a CaMV-transmissible strain without any deletion, can be transmitted at an equivalent rate only if both PII and PIII are added. These results demonstrate that PII is necessary for CaMV transmission, in combination with PIII.

**Mapping a PII domain involved in both PII binding and aphid transmission**

PII mutants corresponding to deletions of 10, 18 or 20 amino acid residues and spanning the complete sequence (Figure 3A, 1) were tested for their capacity to bind PII (Figure 3A, 2). Deletions at the N-terminus (mutants...
PIIIΔ1/20 and PIIIΔ21/40 abolished the interaction with PII whereas the other deletions did not. Shorter deletions of five amino acids each were then introduced within the first 40 amino acids of PIII (Figure 3B, 1). Only the two mutants deleted from positions 31–35 and 36–40, respectively, were able to bind PII (Figure 3B, 2), indicating that the minimal domain of interaction is restricted to the 30 N-terminal amino acid residues of PII. A faint band, sometimes observed at a lower molecular mass in Western assays (Figure 3A, 2 and B, 2, top panels), may represent N-terminal degradation products of recombinant PIII proteins, since these products were not detected in the far Western experiments (Figure 3A, 2 and B, 2, bottom panels). The faint band observed for all the mutants in the far Western assays (Figure 3B, 2) corresponds to the cross-reaction between a bacterial protein and the anti-PII antibodies discussed above.

All mutants harbouring deletions of five amino acids were tested in aphid transmission assays (Table I). Comparison of the results in Figure 3B and Table I shows that the ability of PIII to interact with PII correlated with its activity in aphid transmission. Thus, all the deletions within the 25 N-terminal residues of PIII totally abolished both PII binding (Figure 3B, 2, bottom panel) and transmission (Table I), while PII carrying deletions between amino acids 30 and 40 assisted transmission but less efficiently than wild-type PIII. The mutant PIIIΔ26/30, in which the deletion lies near the border of the PIII domain characterized here, has intermediate properties since it did not interact detectably with PII but could still mediate aphid transmission, although with low efficiency (8%).

Mapping of a PII domain responsible for interaction with PIII

Far Western experiments were also performed to identify the PII domain responsible for interaction with PIII. GST–PII fusions (Schmidt et al., 1994) were expressed in E.coli and submitted to the PIII binding assay. As shown in Figure 4B, fusion of GST to the N-terminus of PII did not affect its ability to bind to PIII. An interaction was also observed with a protein of ~31 kDa, which probably arises from cleavage in the GST moiety of the fusion protein or from internal initiation during the translation process. GST–PIIΔ1/55 and GST–PIIΔ1/98 still bind to PIII as efficiently as full-length GST–PII. Hence, a large N-terminal portion of PII (residues 1–98) is not involved in the interaction. When the deletion extended up to amino acid 118 or 128 (proteins GST–PIIΔ1/118 and GST–PIIΔ1/128), weaker signals indicate that the corresponding region (residues 99–128) affects the efficiency of the PIII–PII interaction. Finally, GST–PIIΔ1/134 and GST–PIIΔ1/145 were no longer able to bind PIII, as was also the case for GST–PIIΔ1/147/159, in which the C-terminal proximal 13 amino acids have been deleted. Taken together, these results suggest that although the 31 C-terminal residues of PII are sufficient to undergo an interaction with PIII, the complete domain may be larger and extend between amino acids 99 and 159. Interestingly, the same C-terminal region of PII has previously been reported to interact with viral particles (Schmidt et al., 1994). The latter were transmitted efficiently by aphids, suggesting that significant amounts of PII were still present in the less extensively purified virus preparation used in these experiments.

Model of interaction between PIII and PII

Secondary structure predictions using the program SOPMA (significant improvement in protein secondary structure prediction from multiple alignments; Geourjon and Deleage, 1995) suggests that the interaction domains in both proteins are located in α-helical regions. In PIII,
Table I. Efficiency of CaMV transmission by aphids in the presence of different versions of PIII proteins

<table>
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<th>First feed</th>
<th>Second feed</th>
<th>Transmission efficiency</th>
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<tr>
<td>+ PII</td>
<td>40/80</td>
<td>39</td>
</tr>
<tr>
<td>+ PII/A1/5</td>
<td>34/80</td>
<td>0/160</td>
</tr>
<tr>
<td>+ PII/A6/10</td>
<td>33/80</td>
<td>0/160</td>
</tr>
<tr>
<td>+ PII/A1/15</td>
<td>31/80</td>
<td>0/160</td>
</tr>
<tr>
<td>+ PII/A6/20</td>
<td>26/80</td>
<td>0/160</td>
</tr>
<tr>
<td>+ PII/A2/25</td>
<td>22/80</td>
<td>17/32</td>
</tr>
<tr>
<td>+ PII/A2/30</td>
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<td>17/32</td>
</tr>
<tr>
<td>+ PII/A3/35</td>
<td>14/160</td>
<td>8/34</td>
</tr>
<tr>
<td>+ PII/A3/40</td>
<td>8/35</td>
<td>24</td>
</tr>
<tr>
<td>– PII</td>
<td>0/80</td>
<td>23</td>
</tr>
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</table>

The first feed consisted of PII expressed by recombinant baculovirus in insect cells.

The second feed was a suspension of purified CaMV particles mixed with a bacterial extract either containing (+) or not containing (−) wild-type PIII or a deleted version of PIII (PII/A1/5 to PII/A3/40).

The transmission efficiency was determined from the number of plants developing symptoms at 3 w.p.i.

**Fig. 4.** Mapping of the domain on PII that interacts with PIII. Full-length and truncated versions of PII were fused at their N-terminus to GST as illustrated in (A). The hatched box represents the GST protein (not to scale) and empty boxes the PII sequences. Thin broken lines correspond to in-frame deletions of ORF II. (B) The fusion proteins produced in E.coli were separated by SDS–PAGE, transferred onto nitrocellulose membrane, incubated with PIII and PII–PIII interactions were detected with antibodies raised against PIII. Molecular masses of marker proteins are indicated to the left.

<table>
<thead>
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<th>A</th>
<th>B</th>
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<tr>
<td><img src="image.png" alt="Graph" /></td>
<td><img src="image.png" alt="Graph" /></td>
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an α-helix from residue 4 to 32 is predicted, whereas in PII two α-helices (α1 and α2) are predicted between amino acids 101 and 128, and 137 and 158 (Figure 5A). Sequence analysis and three-dimensional homology modelling of the three α-helices (Krüger et al., 1998) reveal the presence of leucine zipper motifs in the α-helix of PIII and the α1-helix of PII (Figure 5B), suggesting that the two α-helices could interact in a coiled-coil structure. The leucine zipper motifs of PIII and PII consist of three true consecutive heptads (residues in each heptad are denoted ‘a’–‘g’ delimited at each ‘a’ position by a hydrophobic residue (Ile7, Ile14 and Met21 for PIII, and Leu102, Leu109 and Ile116 for PII), allowing formation of three intermolecular hydrophobic interactions between the two helices (Figure 5B). A fourth hydrophobic bond could form between Ile28 of PIII and Ile123 of PII. The location of the latter corresponds to position ‘a’ of a potential heptad. These interactions appear to be stabilized by ionic bridges between acidic residues at position ‘d’ of the first and third heptads of PIII (Glu10 and Asp24), and basic residues at positions ‘b’ and ‘d’ of the first heptad (Lys103 and Lys106) and at position ‘b’ of the third heptad (Lys117) of PII, respectively. These ionic bridges appear to be exposed to solvent (Figure 5B, three-dimensional representation). Therefore, the two α-helices could form a parallel coiled-coil structure as illustrated by the three-dimensional model (Figure 5B). Sequence analysis of the α2-helix of PII shows that it contains an unusual surface polarity pattern. By projecting the sequence on an α-helix, three distinct hydrophobic, acidic and basic domains can be identified, suggesting that the α2-helix may be involved in three types of interaction (Figure 5C). Furthermore, α2 is separated from the α1-helix by a short peptide containing two prolines (Figure 5B), which could be important for its positioning.

**Effect of point mutations in the interaction domains of PIII and PII**

Proteins with point mutations in each of the aforementioned α-helices were analysed for their capacity to interact with their respective partner. PIII-Lys corresponds to a PIII protein in which Ile14 and Met21 at the ‘a’ positions of the leucine zipper motifs were substituted by lysines. This mutant was no longer able to interact with PII (Figure 6A, bottom panel), indicating that the hydrophobic bonds involving these two amino acids are crucial for the formation of the protein complex. Point mutations were also introduced into the α1-helix of PII expressed in E.coli as a fusion protein with GST. Residues Leu102, Leu109 and Ile116 at the ‘a’ position of the leucine zipper motifs and Ile105 at position ‘d’ of the first heptad were replaced by aspartic acid residues. The resulting PIIAsp mutant binds PIII, but only with very low efficiency compared with GST–PII (compare the strength of the signals in
Figure 5. Secondary structure predictions for the N-terminus of PIII and the C-terminus of PII. (A) The interaction domains of PIII and PII identified by far Western experiments are represented by blue and pink boxes, respectively. Regions of each domain predicted to fold into α-helices are indicated by open boxes. (B) Model of the interaction between the PIII α-helix and the PII α₁-helix. The α-helices contain a series of typical leucine zipper heptad motifs (top). The heptad residues at positions a, b, d and e involved in interactions are indicated below each sequence. Hydrophobic amino acid residues represented in yellow correspond to the first position (‘a’) in each heptad of the leucine zipper repeat. Amino acids in red and blue are acidic and basic residues, respectively, involved in the interaction between PIII and PII. Predicted hydrophobic and ionic bonds between residues of PIII and PII are also represented. To the right is a perspective view of the PIII–PII interaction showing the interhelical coiling. (C) Structural organization of the PII α₂-helix. Basic (blue), acidic (red) and hydrophobic amino acids (green) are arranged in three distinct domains. The position of amino acids within the primary sequence is shown at the top. To the right is a wheel arrangement of the amino acid residues viewed down the axis of the PII α₂-helix. K1 is the first amino acid of the α₂-helix.

Figure 6B, bottom panel). We conclude that these residues are also important for PII–PIII interaction. PII proteins mutated in the C-terminal sequence Ile–Ile–Gly (amino acids 157–159) were used previously for interaction studies between PII and semi-purified virions (Schmidt et al., 1994). The same mutants were also tested for their ability to bind PIII. Substitution of residue Ile157 by Asn (mutant 157m) and Gly159 by Ser (mutant 159m) abolished or strongly reduced the PII–PIII interaction, respectively. Replacement of Ile158 by Ser (mutant 158m) had a lesser effect (Figure 6C, bottom panel). These results suggest that the C-terminus of the α₂-helix, and in particular Ile157, is important for the PII–PIII interaction.

**Discussion**

In contrast to other CaMV proteins, no direct information was available previously concerning the function(s) of PIII during the viral multiplication cycle, although indirect evidence from previous studies suggested that PIII could...
be involved in interactions with viral and/or cellular proteins. By in vitro protein–protein binding experiments, we show here that PIII specifically interacts with the CaMV aphid transmission factor (ATF or PII). Moreover, by complementing aphid transmission of purified virions with both PII and PIII, we demonstrate that PIII plays a crucial role in the molecular mechanism of aphid transmission and hence represents a second transmission factor.

CaMV is transmitted by aphids in a non-circulative manner, and the success of transmission depends on the presence of ATFs. ATFs are generally believed to interact, via two distinct domains, with both the virus particle and a putative receptor in the aphid stylet, forming a reversible bridge between the two. This reversible bridging would be responsible for both retention of infectious virus in the vector’s mouthparts and its subsequent release in a new host plant (for a review, see Pirone and Blanc, 1996). Such a scenario appears to hold true for the genus Potyvirus (a genus for which aphid transmission has been studied extensively), where the purified ATF can assist aphid transmission of purified particles (Thornbury et al., 1993). In the case of caulimoviruses, the CaMV ATF PII expressed in insect cells is able to assist the transmission of a naturally non-transmissible isolate when the latter is acquired by aphids from infected plants but not when it is supplied as a purified virus preparation (Blanc et al., 1993). This result was given two possible interpretations: (i) the purification procedure altered the coat protein, hence rendering the virions non-aphid-transmissible; or (ii) an additional plant or virus component was required for the aphid transmission process and was eliminated upon virus purification. The results described herein demonstrate that the latter hypothesis is correct. Thus, not one but two viral ATFs are required for aphid transmission of caulimoviruses, a unique situation among known non-circulatively transmitted plant viruses.

The domain by which PIII interacts with PII was mapped to the 30 N-terminal amino acids. Deletions introduced in this region abolished CaMV transmission, demonstrating that there is a direct correlation between the property of PIII to bind PII and its function in transmission. The domain of PII that interacts with PIII was mapped to the 61 C-terminal residues (positions 99–159) and appears to consist of two subdomains, both of which are important for interaction as shown by the results of far Western experiments. This PII region corresponds precisely to the domain previously reported to mediate an interaction between PII and less extensively purified virus particles (Schmidt et al., 1994). At present, the question is open as to whether PII binds independently to the surface of the virion or whether the intervention of PIII is required. Preliminary far Western experiments favour the latter idea since highly purified CaMV virions, which do not contain a detectable amount of PII, do not bind efficiently to membrane-immobilized PII but the efficiency of binding can be greatly improved by inclusion of purified PIII along with the virions in the overlay solution (data not shown). PIII might intervene in binding of PII to the virion either directly, by serving as a bridge between PII and the virus capsid, or indirectly, by inducing structural changes in PII that would permit it to bind to the capsid itself (the two models are not mutually exclusive). Whether PIII is involved in other steps of the virus transmission process remains to be discovered.

Secondary structure predictions for the PIII and PII interaction domains indicate that they can form α-helices: one α-helix in PIII (residues 4–32), and two (α₁ and α₂) in PII (residues 101–128 and 137–158, respectively) separated by a small peptide containing two prolines. The secondary structure prediction for PII is in agreement with a bipartite organization of its interaction domain, as suggested by far Western results. Sequence analysis shows that the α₁-helix of PIII and the α₂-helix of PII contain leucine zipper motifs, which could form a coiled-coil structure (Cohen and Parry, 1990), where the nature and the position of residues exposed to solvent are similar to those found in homodimers of the yeast transcription factor GCN4 (O’Shea et al., 1991; Ellenberger et al., 1992). However, contrary to classical leucine zipper motifs, position ‘d’ in PIII is occupied by a polar residue. Such a model is in accordance with the alternative motifs described by Tropsha et al. (1991), in which the fourth amino acid of each leucine zipper is polar, suggesting formation of interhelical hydrogen bonds. Polar residues are also present at this position in the coiled-coil between Max and c-Myc proteins (Lavigne et al., 1995).

Our model is confirmed by the results of far Western assays showing that the interaction between both proteins is lost or highly reduced if key hydrophobic amino acids...
of the leucine zipper motifs (present at position ‘a’) of PII and PIII are substituted by polar residues. Nevertheless the α2-helix is not sufficient for formation of the PIII–PII complex since a deletion in the α2-helix also impairs the interaction between these two proteins. The C-terminal triplet Ile157–Ile158–Gly159 of the α2-helix, which is highly conserved among caulimoviruses, seems to play an important role in this interaction since substitution of Ile157 or Gly159 by polar amino acids abolishes or strongly reduces PIII–PII interaction. We conclude that both the C-terminal α-helices of PII are probably involved in interactions with the α-helix at the N-terminus of PIII. Further structure analysis of the transmissible complex should determine the specific role of each region of the different partners.

In addition to its capacity to interact with PII, PIII has also been shown to form in vitro homotetramers through an N-terminal coiled-coil (Leclerc et al., 1998). This latter interaction involves classical leucine zipper motifs, in contrast to the hetero-oligomer formed between PIII and PII, which is required not only for aphid transmission but also for infectivity in plants (Jacquot et al., 1998).

### Materials and methods

#### Plasmid constructions

CaMV ORF III mutants derived from Cabb-S isolate DNA were cloned into the NdeI–CelI sites of pET-3a vector and expressed in E.coli BL21/DE3(physS). Plasmids pETCa3 containing the complete ORF III sequence and pETCa3Δ18, used for the synthesis of a PIII protein deleted of its C-terminal 18 residues (PIIIΔ12/129; numbers refer to the first and last deleted amino acid residues), have already been described (Jacquot et al., 1998). Plasmids pETCa3Δ1-20, pETCa3Δ21-40, pETCa3Δ41-60, pETCa3Δ61-80, pETCa3Δ81-100 and pETCa3Δ100-115 were obtained by PCR amplification of ORF III previously cloned into plasmid pCA (Jacquot et al., 1998), using the primer fpΔ1-20 (for pETCa3Δ1-20) or fpNdeI (for the other plasmids) as forward primer and rpCelI as reverse primer (Table II). Plasmids pETCa3Δ1-20, pETCa3Δ21-25, pETCa3Δ26-30, pETCa3Δ31-35, pETCa3Δ36-40 and pETCa3Δ41-60 were constructed by overlap extension PCR (Stappert, 1994) using the primers shown in Table II. All the resulting plasmids were confirmed to be error-free by sequencing.

CaMV ORF II mutants were derived from CaMV Cabb-B-JI isolate. Two heterologous expression systems were used to produce PII and its truncated or mutated derivatives. Full-length ORF II and 5’ or 3’ terminally deleted versions of the ORF were cloned into pGEX-3X vector (Amersham Pharmacia Biotech) to produce fusion proteins between GST (at the N-terminus) and PII (see Table II). Plasmids pETCa3Δ16/20, pETCa3Δ21/25, pETCa3Δ26/30, pETCa3Δ31/35, pETCa3Δ36/40 and pETCa3Δ41/60 were constructed by overlap extension PCR (Stappert, 1994) using the primers shown in Table II. All the resulting plasmids were confirmed to be error-free by sequencing.

#### Table II. Oligonucleotides used as primers to generate PCR products for truncated and mutated CaMV PIII proteins

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<thead>
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<th>Name</th>
<th>Sequence*</th>
<th>Name of recombinant vectors</th>
<th>Protein encoded by PCR-derived product</th>
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<td>fmpΔ36/40</td>
<td>5’tcttaagattattgattccacagagttaaaagtttagggc3’</td>
<td>pETCa3Δ36/40</td>
<td>—</td>
</tr>
<tr>
<td>fmpPIIIlys</td>
<td>5’gaagttctagttgaattctagtaacaggtgatggatctac3’</td>
<td>pETCa3LyS</td>
<td>—</td>
</tr>
</tbody>
</table>

*Oligonucleotide primers include NdeI or CelII sites (underlined). Nucleotides corresponding to the wild-type sequences are in bold upper case letters. Positions of deletions in forward primers (fp) and megaprimer (fmp) and of substitutions are indicated by a transverse bar and in italics, respectively. Non-viral nucleotide sequences are in lower case letters.
Preparation of plant crude extracts and production of viral proteins

Three weeks after inoculation of turnip plants (Brassica campestris L. var. rapa cv. Just Right) with CaMV CabB-JI, leaves (10 g) were crushed in 30 ml of HMK buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 12 mM MgCl2) and filtered through two layers of gauze. Proteins (40 µg) in the resulting crude extract were fractionated by SDS–PAGE and transferred onto nitrocellulose membrane for Western and far Western experiments. Wild-type and mutant PIII were produced in the E.coli BL21/DE3(pLySs) expression system and partially purified (Jacquot et al., 1996). GST–PIII fusion proteins were expressed in E.coli and PIII in insect cells (Schmidt et al., 1994).

Western blotting assays

PIII and PII proteins, and proteins in plant crude extracts were separated by SDS–PAGE and transferred onto nitrocellulose membranes (Schleicher and Schuell). The rabbit polyclonal antibodies used in Western blot assays were raised against PIII (diluted 1/30 000) or against the C-terminal region of PII (diluted 1/5000). Secondary antibodies were goat anti-rabbit IgG conjugated either to peroxidase or to alkaline phosphatase for detection of immune complexes with PII and with PIII or coat protein, respectively. They were diluted and used according to the manufacturer’s instructions (Sigma).

Far Western assays

A protein blotting protein overlay technique was used to detect interactions between proteins. PIII and PII (2 µg, estimated after staining with Coomassie Blue for PII or with Poncet Red for PII) were used in the test. Proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes. Membranes were washed three times at 4°C in buffer HB (10 mM Tris–HCl pH 7.5, 75 mM KCl, 2.5 mM MgCl2, 300 mM NaCl) containing 5% milk and incubated for 2 h in the presence of overlay components in the same mixture. After additional washes, membranes were incubated with antibodies raised against the overlay component, in buffer HB supplemented with milk and then with the secondary antibodies. Interactions were detected as described above.

CaMV purification

CaMV virions (isolate CM4-184, which contains a 421 bp deletion in ORF II) were purified according to Hull et al. (1976) but with a second centrifugation step through a 10–40% sucrose gradient.

Aphid transmission experiments

CaMV CM4-184 used for transmission tests with Myzus persicae was propagated in turnip plants grown under glass-house conditions (20–23°C, 16 h photoperiod). Transmission assays were performed as previously described (Blanc et al., 1993). In several tests, the aphids were allowed to acquire PII (0.5 µg/ml) during a first feed through paraffin membrane, and purified virions (0.1 µg/ml) mixed with a recombinant bacterial extract containing wild-type PIII or a derivative (0.2 µg/ml) in a second feed. Aphids were then transferred onto healthy plants (10 aphids per plant) and symptom appearance was noted (3 w.p.i.). Control assays with or without the complete PIII were generally carried out on 40 plants. Transmission with most of the PIII mutants was tested in two independent experiments with 80 plants each. For mutants PHIIA31/35 and PHIIA36/40, the transmission assay was performed once with 34 and 35 plants, respectively, using 32 plants for the control with PIII.

Sequence analysis and three-dimensional homology modelling

Sequence analysis and three-dimensional homology modelling of proteins were performed as described previously (Krüger et al., 1998).

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References


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