Requirement of Cauliflower Mosaic Virus Open Reading Frame VI Product for Viral Gene Expression and Multiplication in Turnip Protoplasts

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Abstract: Cauliflower mosaic virus (CaMV) open reading frame (ORF) VI product (P6) has been shown to be the major constituent of viral inclusion body, to function as a post-transcriptional transactivator, and to be essential for infectivity on whole plants. Although these findings suggest that P6 has an important role in viral multiplication, it is unknown whether P6 is required for viral multiplication in a single cell. To address this question, we transfected turnip protoplasts with an ORF VI frame-shift (4 bp deletion) mutant (pCaFS6) of an infectious CaMV DNA clone (pCa122). The mutant was uninfected. Co-transfection of plasmids expressing P6 complemented the mutant. Overexpression of P6 elevated the infection rate in co-transfection experiments with either pCa122 or pCaFS6. This would have been achieved by elevating the level of pregenomic 35S RNA, a putative polycistronic mRNA for ORFs I, II, III, IV and V, and by enhancing the accumulation of these five viral gene products. When CaMV ORFs I, II, III, IV and V were expressed from monocistronic constructs in which each of the ORFs was placed just downstream of the 35S promoter, the accumulation of ORF III, IV and V products depended on the co-expression of P6. The accumulation of ORF I and II products was not detected, even in the presence of P6. These results suggest that P6 is involved in the stabilization of other viral gene products as well as in the activation of viral gene expression, and thus, is a prerequisite for CaMV multiplication.

Key words: Cauliflower mosaic virus, ORF VI, Protein stabilization, Viral multiplication

Cauliflower mosaic virus (CaMV), a member of the caulimovirus group, has circular double-stranded DNA of about 8 kilobase pairs (2, 10, 16) that codes for a cell-to-cell movement factor (open reading frame (ORF) I) (47, 48), an aphid transmission factor (ORF II) (1, 49), a DNA binding protein with unknown biological function (ORF III) (17), a capsid protein (ORF IV) (6), reverse transcriptase (ORF V) (46) and the major constituent of the virus inclusion body matrix (ORF VI) (5, 39, 53). Protein products specified by ORFs I (28, 31, 55), II (20, 50), III (52), IV (6, 26), V (41, 56) and VI (19, 45) (P1, P2, P3, P4, P5 and P6, respectively) were readily detected in CaMV-infected plants. P3 and P5 as well as P4 (capsid protein) were detected in purified virions (8, 17, 18, 56). ORF VII and VIII products have not been detected in infected plants (51). ORFs II, VII and VIII are dispensable for viral infectivity (7, 9, 43).

CaMV infects cruciferous plants, multiplying to about 10^4 particles per cell (32). Progeny viruses are surrounded by a virus-specified matrix forming a virus inclusion body, also called a viroplasm (11). Viroplasms become large subcellular structures which are observable under low-magnification light microscopy after staining (44). As the viroplasm contains the majority of virions in CaMV-infected cells, it is believed to be the site of viral DNA replication and virus assembly. This idea has been strongly supported by biochemical and ultrastructural studies on CaMV DNA replication in the viroplasm (33, 34, 36) and the analysis of CaMV DNA

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Abbreviations: CaMV, cauliflower mosaic virus; FITC, fluorescein isothiocyanate; ORF, open reading frame; RITC, rhodamine B isothiocyanate.
replication-competent virion-like particles (29, 30, 35). A recent study that demonstrates specific interaction between P4 and P6 further supports the idea that the viroplasm is the site of viral assembly (21).

In vitro mutagenesis of cloned CaMV DNA has revealed that ORF VI is required for CaMV to infect whole plants (7, 9), in which every cell harboring the virus forms viroplasms. By contrast, in turnip protoplasts infected with CaMV, viroplasms have not been detected (13). Moreover, we have previously reported that CaMV multiplies to a larger extent at 24 °C than at 30 °C, while viroplasms grow much larger at the higher temperature (54). These observations indicate that the morphological growth of viroplasms does not correlate with virus multiplication. This may possibly be explained as follows: 1) a small amount of viroplasm matrix is sufficient for virus assembly and viral DNA replication in CaMV-infected cells, 2) viroplasm growth is neither necessary for nor the result of the accumulation of a large number of virions, and 3) a large viroplasm is necessary for virion accumulation at high temperatures or is a result of the high level expression of viroplasm matrix protein encoded by ORF VI at high temperatures.

Until Hohn et al demonstrated that P6 activates the expression of other viral ORFs in a post-transcriptional manner (3, 23), the expression of CaMV ORFs I, II, III, IV and V was a mystery. P6 enables the translation of polycistronic mRNA (14) and allows ribosomes to shunt the stem-loop structure in the long leader sequence of 35S RNA (15). P6, through these and possibly other functions, activates the expression of all CaMV genes (22). However, since P6 was characterized in the transient expression assay system, it remains uncertain whether ORF VI is required for viral gene expression during the CaMV multiplication cycle.

In vitro mutagenesis and transient assays for transactivator function studies strongly suggest that P6 is probably necessary for CaMV multiplication in single plant cells. However, some plant viral genes, such as the movement protein gene, are essential for infecting the whole plant but not necessary for multiplication in a single cell (e.g., CaMV ORF I (47, 48)). CaMV P6 has also been implied to have a function in the systemic spread of the virus (42). Therefore, we want to determine whether P6 is required for viral multiplication in protoplasts in order to elucidate the function of P6 during the viral multiplication cycle. Previously, the analysis of the function of P6 in CaMV-infected protoplasts has been prevented for two reasons. The first is that a conditionally defective mutant in the ORF VI function (e.g., ts mutants) had not been isolated. The second is that it had been impossible to analyze lethal in vitro mutants of CaMV due to the low DNA transfection efficiency of plant protoplasts. These problems have been overcome by using a highly efficient turnip protoplast transfection system (48). In this system, protoplast transfection with cloned CaMV DNA results in mature virion formation in a manner very similar to virus infection in vivo.

In this study, we have examined the function of P6 in the CaMV multiplication cycle in turnip protoplasts. Successful transfection of turnip protoplasts with an ORF VI-defective CaMV DNA clone would demonstrate that P6 is necessary for CaMV multiplication. We have further examined the role of P6 in viral gene expression and the stable accumulation of viral gene products.

Materials and Methods

Construction of plasmids. Recombinant plasmid pCaMV10 made by inserting infectious CaMV CM1841 DNA into the SalI site of pBR322 (16) was a kind gift from Drs. R. J. Shepherd and C. Matsui. An infectious CaMV clone, pCa122, which contains 1.2 copies of CaMV CM1841 DNA, was described previously (48) (Fig. 1A). Plasmid vectors, pUC118 and pBluescript were purchased from Takara Shuzo (Kyoto, Japan) and Toyobo (Osaka, Japan), respectively. Restriction endonucleases and modifying enzymes were purchased from Takara Shuzo or New England Biolabs (Beverly, Mass., U.S.A.).

ORF VI-defective mutant viral DNA, pCaFS6, was obtained by digesting pCa122 with SacI (Fig. 1A), followed by T4 DNA polymerase treatment and recircularization. Expression vector pMCT was described previously (48). The plasmids p35S-P1, p35S-P2, p35S-P3, p35S-P4 and p35S-P5 were generated by inserting fragments containing the coding regions of ORFs I, II, III, IV and V, respectively (see Fig. 1B and its legends), between the SalI and SacI sites in pMCT. The plasmid p35S-P6, which expresses ORF VI under the control of the 35S promoter, was described previously as pEXP6 (48). Plasmid p35S-P6 was digested with HindIII, filled in with Klenow enzyme, and recircularized to obtain a frame shift mutant of p35S-P6 called p35S-FS6. A PstI-ClaI fragment (#5383-7987, nucleotide number refers to ref. 16) was cloned into pBluescript to obtain p195P6, which expresses ORF VI under the control of the 19S promoter. A SacI-EcoRI fragment (#5822-8031/0-409) was cloned into pBluescript SK−, resulting in pSE, a plasmid for generating a Northern blot probe.

Plant growth, preparation of protoplasts and transfection of protoplasts with plasmid DNA. Turnip protoplasts were prepared (12) and transfected with plasmid DNA (48) as described. Briefly, DNA (50 μg of pCa122 or pCaFS6; 20 μg each of p35S-P1, 2, 3, 4, 5 and 6,
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p35S-FS6 and p19S-P6; plus heterogeneous salmon testes DNA to even out the total DNA amount) dissolved in 0.5 ml of 10 mM MES buffer (pH 5.8) containing 0.5 mM mannitol and 40 mM CaCl₂ was added to a protoplast pellet (1 X 10⁶ cells) and immediately treated with 24% (w/v, final concentration) polyethylene glycol (average M.W. of 4,000, Sigma, St. Louis, Mo., U.S.A.) for 30 min on ice. After being diluted with 0.5 M mannitol containing 40 mM CaCl₂, incubated for 30 min on ice and washed three times with 0.5 M mannitol containing 50 mM glycine and 50 mM CaCl₂ (pH 8.5), the protoplasts were cultured as described previously (48).

Isolation of RNA from protoplasts and Northern blotting. Total RNA was isolated from transfected protoplasts as described previously (4). RNA was fractionated on 1.1% agarose gels containing 0.66 M formaldehyde and transferred to Biodyne A nylon membrane (Pall, Glen Cove, N.Y., U.S.A.) by a capillary procedure. A probe was prepared by transcribing antisense RNA in vitro using T3 RNA polymerase (GIBCO/BRL, Grand Island, N.Y., U.S.A.) from pSE in the presence of DIG-UTP (Boehringer-Mannheim, Mannheim, Germany). Hybridization and visualization of signals were performed as described in the DIG-ELISA kit (Boehringer-Mannheim).

Results and Discussion

Requirement of ORF VI for CaMV Multiplication in Turnip Protoplasts

The mutant CaMV plasmid, pCaFS6, was transfected into turnip protoplasts in fifteen or more independent experiments. In pCaFS6-transfected protoplasts, neither virion-specific nor P6-specific immunofluorescence was observed (Fig. 2, A and D), while fluorescent specks were observed in protoplasts transfected with wild-type pCal22 and stained with antibodies (data not shown). This result indicates that the viral DNA mutation is lethal in transfected turnip protoplasts, as it is in whole plants.

When pCaFS6 was co-transfected with p19S-P6, a plasmid expressing ORF VI under the control of the 19S promoter, virion-specific and P6-specific immunofluorescence was observed (Fig. 2, A and D), while fluorescent specks were observed in protoplasts transfected with wild-type pCal22 and stained with antibodies (data not shown). This result indicates that the viral DNA mutation is lethal in transfected turnip protoplasts, as it is in whole plants.

During the course of the experiments, we noticed that transfection of turnip protoplasts with pCaFS6 plus p35S-P6, a plasmid expressing P6 under the control of protein A as described previously (27, 37, 38, 48). Antibody to P5 (41) was a generous gift of Dr. Thomas Hohn. Immunofluorescent staining for CaMV virion protein was performed 3 days post-transfection as described previously (13). Rhodamin B isothiocyanate (RITC)-labeled anti-P6 antibody was prepared and used in the same manner as FITC-labeled anti-CaMV antibody. A Western blot analysis was performed when the pCa122 infection rate was greater than 25% as determined by immunofluorescent staining, as described previously (37).

Immunofluorescent staining and Western blotting. Antibodies to P1, P2, P3, P4 and P6 were raised against the fusion proteins of viral gene products and bacterial
Fig. 2. Microphotographs of immunostained transfected-turnip protoplasts. Protoplasts were transfected with pCaFS6 (A and D), p19S-P6 (B and E) and pCaFS6 + p19S-P6 (C and F). Transfected protoplasts were cultured for 72 hr and double-stained with RITC-labeled anti-P6 antibody (A, B and C) and FITC-labeled anti-purified CaMV particle antibody (D, E and F). Upward arrows indicate a protoplast infected with CaMV. Rightward arrows indicate protoplasts expressing P6 but not virion proteins.
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Two ORF VI-expressing plasmids, p19S-P6 and p35S-P6, were constructed with very similar 5' leader sequences. We found that P6 production from p35S-P6 was much greater than from p19S-P6 (Fig. 3B, VI, lanes 1, 2 and 3), as would be expected from previous reports demonstrating that the 35S promoter is stronger than the 19S promoter (40).

p19S-P6, and to a greater extent p35S-P6, complemented pCaFS6 and enhanced the infection rate of pCa122, although the degree of stimulation differed in each experiment (Table 1). In a few experiments, transfected cells were double-stained with RITC-labeled anti-P6 antibody and FITC-labeled anti-CaMV antibody. In Fig. 2, C and F, the upward arrows indicate a protoplast positive for both virion protein and P6, and the rightward arrows indicate a protoplast positive for P6 but negative for virion protein. Cell counting revealed that some P6-positive cells were negative for virion protein but that

Fig. 3. A: Complementation of CaMV ORF VI-defective mutant by ORF VI-expressing plasmid. Turnip protoplasts were transfected as indicated at the top of the panel (+, included; -, not included in the transfection) and cultured for 72 hr. Total protein from the protoplasts was analyzed by Western blotting. Numbers of ORFs are shown on the left and the molecular weight of the detected polypeptide on the right. B: Effect of P6 overexpression on the accumulation of P1, P2, P3 and P5. Turnip protoplasts were transfected, cultured and analyzed as in A. The signals for ORF II product are hardly visible in lanes 2, 4 and 5, but these signals are clearly observed in the original blot.

Table 1. Efficiency of successful infection with CaMV of protoplasts transfected with pCa122 or pCaFS6 with or without P6-expressing plasmids

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>% Protoplasts positive for virion protein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Exp. 4</th>
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<tr>
<td>pCa122+p35S-FS6</td>
<td>7.2</td>
<td></td>
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<tr>
<td>pCa122+p19S-P6</td>
<td>-&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.0</td>
<td>14.7</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>pCa122+p35S-P6</td>
<td>-</td>
<td>36.0</td>
<td>15.6</td>
<td>17.9</td>
<td></td>
</tr>
<tr>
<td>pCaFS6+p35S-FS6</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pCaFS6+p19S-P6</td>
<td>3.7</td>
<td>20.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pCaFS6+p35S-P6</td>
<td>9.4</td>
<td>32.6</td>
<td>-</td>
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<sup>a</sup> Efficiency of successful infection is shown as a percentage of virion protein-positive protoplasts among those examined under a fluorescent microscope. The protoplasts which had died before fixation emitted intense yellow auto-fluorescence (see Fig. 2) and were not counted.

<sup>b</sup> Not examined.
all cells positive for virion protein were positive for P6 (Fig. 4), suggesting that P6 accumulation is a prerequisite for the multiplication of CaMV.

The pCaFS6 mutant was complemented by co-transfection with plasmids expressing ORF VI. However, it is possible that the complementation was achieved by recombination between two plasmid DNA molecules (24). In a few of the co-transfection experiments of pCaFS6 and p35S-P6, we observed virion-specific immunofluorescence in about 10% of the transfected protoplasts within 24 hr (data not shown). It is very unlikely that DNA recombination occurred at such high efficiency in such a short period. Moreover, the strength of the promoter driving the expression of P6 correlated well with the enhancement in infection rate (Table 1). Therefore, pCaFS6 is most likely complemented in trans by the P6-expressing plasmids, and not by recombination. This is also supported by the Northern blot analysis of RNA from transfected protoplasts as described below, which demonstrated that pCaFS6 could transcribe 35S RNA to a similar extent with pCa122 (Fig. 5, lanes 1 and 2). This result indicates that the mutation in pCaFS6 does not affect cis elements acting on the viral genome, suggesting that intermolecular recombination is not required for the rescue of CaMV DNA from pCaFS6.

The overexpression of P6 greatly enhanced the transfection rate of turnip protoplasts with CaMV DNA (Table 1), although the mechanism of enhancement remains unclear. This experimental system is useful for analyzing CaMV gene expression as well as gene function. We used this system in the analysis of an ORF I-defective mutant, revealing the ORF is dispensable for viral replication in protoplasts (48).

Transcription of ORF VI-Deficient CaMV Genome and the Effects of P6 on 35S RNA Level

To elucidate how P6 enables CaMV to multiply in turnip protoplasts, we examined the effect of P6 on 35S RNA level in transfected protoplasts. Differing levels of 35S RNA were detected in protoplasts transfected with pCa122+p35S-FS6, pCaFS6+p35S-FS6, pCaFS6+p 19S-P6 and pCaFS6+p35S-P6 at 6 and 26 hr post-transfection (hpt) (Fig. 5). 35S RNA levels in pCaFS6+p35S-FS6 transfected protoplasts were unaltered from 6 to 26 hpt (Fig. 5, lanes 2 and 9), while 35S RNA levels increased in protoplasts transfected with pCa122+p35S-FS6, pCaFS6+ p19S-P6 and pCaFS6+p35S-P6 (Fig. 5, lanes 1, 3, 4, 8, 10 and 11). Since P6 does not affect 35S promoter activity (3), 35S RNA is very likely transcribed at the same efficiency in these transfected protoplasts. Therefore, the above results suggest that 35S RNA stably accumulates in protoplasts expressing P6, and less stably in protoplasts not expressing P6.

Also, in a transient assay system, Bonneville et al have reported an elevated 35S-like RNA level in the presence of P6 (3), and concluded that P6 increases the utilization of RNA and thereby enhances 35S RNA levels. This is based on the observation that, in the presence of P6, reporter enzyme activity was enhanced to a much larger extent than the RNA levels (3). In this study, 35S RNA was readily detected irrespective of the presence of P6 (Fig. 5, lanes 1, 2, 3, 4, 8, 9, 10 and 11), while CaMV multiplication was detected only in the presence of P6 (Table 1 and Fig. 2). These observations are consistent with previous transient assay results (3), demonstrating that P6 functions in the transient assay system in the same manner as in CaMV multiplication, at least for interaction with 35S RNA. Bonneville’s conclusion (3) is further supported by the Northern blotting of RNA from a monocistronic construct expressing P6, p35S-P6 and its frame shift mutant p35S-FS6 (Fig. 5, lanes 5, 6, 12 and 13). Although both the plasmids have the same promoter, p35S-P6 gave much greater RNA levels...
than p35S-FS6, suggesting that the more actively RNA is translated, the more stably RNA accumulates.

**Effect of P6 on the Accumulation of CaMV Gene Products in Transfected Turnip Protoplasts**

The above results indicate that, although the RNA levels are lower than in the presence of P6, 35S RNA can be transcribed in the absence of P6. We therefore examined the effect of P6 on CaMV gene product accumulation in transfected turnip protoplasts. Turnip protoplasts were transfected with pCaFS6, pCaFS6 + p19S-P6, pCaFS6 + p35S-P6, pCa122, pCa122 + p19S-P6 or pCa122 + p35S-P6 (Fig. 3B, lanes 1, 2, 3, 4, 5 and 6, respectively). As expected from reported promoter strength (40), p35S-P6 caused a greater accumulation of P6 than p19S-P6 did (Fig. 3B). The accumulation of P1, P2, P3 and P5 correlated with the accumulation of P6 (Fig. 3B). This result demonstrates that P6 enhances accumulation of not only viral capsid protein but of other structural and non-structural proteins.

The above result suggests that the increased accumulation of P6 further activates translation from 35S RNA and/or facilitates the stable accumulation of other viral gene products. In a transient expression assay system, co-transfection with 35S promoter-driven ORF VI-expression plasmid reportedly activates the translation of the ORF I-fused chloramphenicol acetyltransferase gene to a larger extent than that the 19S promoter-driven plasmid (3). It is also possible, in our experimental system, that the overexpressed P6 further activated the translation of other CaMV gene products from 35S RNA.

**Stabilization of Viral Structural Proteins Expressed Independently of Viral Genome by P6**

An ORF VI mutant, pCaFS6-transfected protoplasts, did not exhibit CaMV-specific immunofluorescence or viral gene product accumulation. This suggests that P6 is involved in the synthesis and/or stabilization of other viral gene products. To further examine P6’s role, P1, P2, P3, P4 and P5 were expressed from monocistronic constructs and the effect of P6 on the accumulation of each protein was examined. Plasmids monocistronically expressing CaMV gene products were transfected with or without p35S-P6. The accumulation of P1 and P2 was not detected in the protoplasts transfected with the monocistronic constructs in the presence or absence of p35S-P6 (Fig. 6, lanes 3, 4 and 5). However, an ORF III-encoded 14 kDa polypeptide, an ORF IV-encoded 58 kDa polypeptide and an ORF V-encoded 75 kDa polypeptide were detected when P6 was co-expressed (Fig. 6, lane 4). Since these three gene products are detected in the purified virion (8, 17, 18, 56), it appears that P6 stabilizes CaMV structural proteins but not non-structural proteins encoded by ORFs I and II. P4 accumulated as 58 kDa polypeptide in transfected turnip protoplasts when monocistronic constructs, including p35S-P4, were co-transfected with p35S-P6 (Fig. 6, lane 4), while P4 accumulated as a 54 kDa polypeptide in pro-
toplasts transfected with pCaFS6+p35S-P6 (Fig. 6, lane 1). Although both 54 and 58 kDa polypeptides are apparently processed products of full-length P4 (18, 26), it is not known why they have differing sizes.

Although we have not confirmed the difference in stability of P3, P4 and P5 in the presence or absence of P6 in a pulse-chase experiment, the above results strongly suggest that these CaMV structural proteins are stabilized by P6. This is supported by the fact that P6 does not affect either 35S promoter activity or translation from monocistronic mRNA (3).

Transient assay has suggested that P6 activates the expression of ORFs I, II, III, IV and V (22). The above results suggest that P6 has a role in the stabilization of P3, P4 and P5. This stabilization function makes it unclear whether P6 is also involved in transactivating P3, P4 and P5 synthesis in the protoplasts transfected with pCaFS6+p35S-P6 in our experimental system. By contrast, P1 and P2 did not accumulate in the presence of P6 when expressed monocistronically (Fig. 6, lane 4), although they did in the protoplasts transfected with pCa122 or pCaFS6+P6-expressing plasmids (Fig. 3, A and B). These results suggest that the stable accumulation of P1 and P2 might require some other factor(s), which exist in the protoplasts transfected with the CaMV genome.

P6 stabilized P3, P4 and P5, but not P1 and P2 when all six proteins were expressed monocistronically. Although it is unknown why only structural (P3, P4 and P5) and not non-structural (P1 and P2) proteins accumulated, the present results suggest P6 is involved in the virion-protecting function of the viroplasm. In addition, P6 stabilized the structural proteins in the absence of 35S RNA, which is believed to be a template for viral DNA replication (25), and progeny viral DNA. Recently, ORF VI product was reported to specifically interact with capsid protein (P4) and was implied to be involved in nucleocapsid assembly (21). The P6-aided accumulation of monocistronically expressed structural proteins observed in this study may be from the assembly of structural proteins in the absence of a viral genome. Himmelbach et al speculated that P6 may act as i) a type of chaperone, ii) a nucleation site for assembly and iii) a scaffold protein in assembly (21). Our study suggests that P6 may stabilize and concentrate structural proteins which may then spontaneously assemble. Further analysis of CaMV assembly requires an in vivo system in addition to an in vitro assembly system. The present experimental system, using monocistronic constructs expressing CaMV gene products, could provide a system for assembly studies.

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