The **Dahlia mosaic virus** gene VI product N-terminal region is involved in self-association

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The **Dahlia mosaic virus** (DMV) is an important pathogen infecting **Dahlia** spp. (Pahalawatta et al., 2008; Albouy et al., 1992; Brierley and Smith, 1950; Brunt, 1971). DMV is a member of the **Caulimoviridae** and as such, possesses a circular, double-stranded DNA genome of ∼8 kbp. The DMV genome encodes six putative open reading frames, which correspond to gene products P1–P6 (Hull, 2002; Richins and Shepherd, 1983). The functions of the DMV proteins are mostly inferred from what is known about the corresponding proteins for **Cauliflower mosaic virus** (CaMV) (Pahalawatta et al., 2008).

**Cauliflower mosaic virus** gene VI encodes the major viroplasm matrix protein, a multifunctional polypeptide essential for caulimovirus infection (Haas et al., 2005). Viroplasms are the likely site of CaMV genome replication, assembly and storage of newly formed virions (Himmelbach et al., 1996; Rothnie et al., 1994). In addition, CaMV gene VI is a major determinant for host specificity, symptom severity, virus systemic spread, translational transactivation and RNA silencing suppression (Agama et al., 2002; Bonneville et al., 1989; Daubert and Routh, 1990; Haas et al., 2008; De Tapia et al., 1993; Qiu et al., 1997; Schoelz et al., 1991). CaMV P6 self-associates and four regions have been implicated in this process (Haas et al., 2005; Li and Leisner, 2002). These interactions were suggested to play a role in viroplasm formation. While the CaMV P6 has been studied intensely, little is known regarding the interactions of equivalent proteins for the other caulimoviruses. Therefore, the current study was performed to determine: (1) whether the DMV P6 self-associates and (2) which putative domains play a role in self-association.

Full-length DMV gene VI (Supplementary Fig. 1) was amplified by polymerase chain reaction (PCR) using primers (given in Supplementary Table 1) designed based on the DMV sequence harbored by the cloned DMV genome (Richins and Shepherd, 1983) and inserted into the yeast two hybrid vectors pEG202 and pG4-5 (Gyuris et al., 1993). Details regarding the experimental methodology are available upon request.

Yeast transformants expressing both LexA DNA-binding domain (DBD)-P6 and B42 transcription activation domain (TAD)-P6 (Fig. 1A) showed both β-galactosidase activity (Fig. 1B) and leucine-independent growth (Fig. 1C and D). However, DMV P6 did not show interaction with either LexA DBD or the B42 TAD alone, based on lack of β-galactosidase activity and the inability of the co-transformants to grow on media lacking leucine. Self-interaction of DMV P6, was specific, as this protein did not interact with its CaMV counterpart (yeast two-hybrid vectors expressing the CaMV P6 were described in Li and Leisner, 2002). Expression of DMV P6 in yeast was confirmed through Western blotting (Fig. 1E) although the level of expression was low.

To confirm self-association biochemically, DMV P6 was inserted into Gateway compatible protein expression destination vectors (pMAL-c2X and pDEST15). The proteins were expressed in *E. coli*,...
Fig. 1. Self-interaction of DMV P6. (A) Schematic diagram of the constructs tested for leucine independent growth and β-galactosidase activity. Black box, LexA DBD in pEG202; hatched box, B42 TAD in pJG4-5; gray box, full-length DMV P6 (amino acids 1–504); white box, full length Cauliflower mosaic virus P6 (amino acids 1–520); numbers to the left of each pair of constructions correspond to the β-galactosidase assays shown in (B) and the plates in (C). (B) β-Galactosidase activity of yeast transformants expressing constructs as represented in (A). The bar graph shows average β-galactosidase units for three different experiments along with the standard deviation. Numbers at the bottom correspond to the construction pairs as presented in (A) and plates in (C). (C) Growth of yeast transformants on media with (+L) and without (−L) leucine. (D) Key for the plates in (C). (E) The protein gel blot analysis of protein levels expressed by LexA fused full-length DMV P6 in yeast. The sizes of the molecular weight markers (M) are given on left in kDa. Numbers at bottom correspond to the constructions shown in A; arrow at right indicates the location of the DMV P6 product. (F) Maltose-binding pull-down analysis of P6 self-association. Load represents the protein content initially loaded to the MBP column; wash, the protein not adhering to the column; elution, the material that attached to the column and was desorbed by the addition of maltose. Proteins were subjected to protein gel blot analysis and probed with the anti-GST primary antibody. (F.a) MBP-tagged P6 mixed with GST-tagged P6. (F.b) MBP alone mixed with GST alone. (F.c) MBP-P6 mixed with GST alone. (F.d) GST alone mixed with MBP alone. (F.e) The same combination as F.a, except that proteins were first subjected to RNase treatment.

and subjected to MBP pull-down assays as described by Hapiak et al. (2008), but using anti-GST antibodies (Invitrogen Corporation, Carlsbad, CA) instead of CaMV P6 antibodies. Approximately half of the GST-DMV P6 mixed with the MBP-DMV P6 resin bound and was eluted with 10 mM maltose in column buffer indicative of binding (Fig. 1F, part a). However, no cross-reacting bands were observed in the “elution” fraction of samples containing: GST-P6 added to the amylose resin expressing MBP alone (Fig. 1F, part b); empty pDEST expressing GST alone and MBP-P6 (Fig. 1F, part c) or when crude samples of both empty vectors were used for pull down analyses (Fig. 1F, part d). CaMV P6 contains two non-specific RNA-binding domains (De Tapia et al., 1993). Because the DMV P6 contains analogous sequences, it is possible that self-association could be mediated by RNA bridging the interactions. To rule out this possibility, the in vitro biochemical studies were performed in the presence of RNase A. RNase treatment had no effect on P6 self-interaction (Fig. 1F, part e).

Taken together, yeast two-hybrid and pull-down data indicate that the DMV P6 self-associates like its CaMV counterpart (Haas et al., 2005; Li and Leisner, 2002). This interaction is not affected by RNase, strongly suggesting that binding of DMV P6 to itself is an authentic protein–protein association. DMV P6 did not interact with the corresponding protein from CaMV showing that the interaction is specific. These data add further evidence that the DMV P6 self-association is mediated by authentic protein–protein interactions but not RNA bridging, since the CaMV protein is a known RNA-binding protein (De Tapia et al., 1993).

To identify the region responsible for self-association, DMV P6 was divided into 4 portions based on the sequence similarity with CaMV P6 self-association domains (Li and Leisner, 2002) (Supplemental Fig. 1). These regions were generated by PCR (using primers indicated in Supplemental Table 1 as described in Supplemental Fig. 1), inserted into the yeast two-hybrid vectors and tested for interaction with full-length P6 in yeast two-hybrid analyses (Fig. 2). The only yeast transformants showing leucine-independent growth and β-galactosidase activity were those expressing P6 fused to the TAD and expressing portions of P6 containing the 115 N-terminal amino acid residues connected to the DBD. None of the remaining co-transformants were able to grow on leucine-deficient media and all lacked β-galactosidase activity. To identify the portion(s) of DMV P6 that the N-terminal region interacted with, this part of the polypeptide was tested against the other P6 regions. Yeast two hybrid analyses, indicated that the N-terminal region bound efficiently only to itself. Hence, only the yeast transformants expressing the N-terminal region of P6 fused to both the LexA DBD and B42 TAD showed leucine-independent growth and β-galactosidase activity. Therefore, the DMV P6 N-terminal 115 amino acid region was the only segment capable of binding to the full-length protein. This region also was able to bind to itself, independent of the rest of P6 and none of the other
Fig. 2. Interaction of full-length DMV P6 with different regions. (A) Schematic diagram of the constructs tested for leucine independent growth and β-galactosidase activity. Black box, LexA DBD in pEG202; hatched box, B42 TAD in pJG4-5; gray box, full-length or regions of DMV P6; letters inside boxes indicate portions of P6 present within the polypeptide; FL, full length (amino acids 1-504); (A) amino acids 1–115; (B) 158–239; (C) 235–366; (D) 401–504. Numbers to the left of each pair of constructions correspond to the β-galactosidase assays shown in (B) and the plates in (C). (B) β-Galactosidase activity of yeast transformants expressing constructs as represented in (A). The bar graph shows average β-galactosidase units for three different experiments along with the standard deviation. Numbers at the bottom correspond to the construction pairs as presented in (A) and plates in (C). (C) Growth of yeast transformants on media with (+L) and without (−L) leucine. (D) Key for the plates in (C).

Fig. 3. Sequence alignment of the D1 regions for DMV and CaMV P6s. The DMV and CaMV D1 regions from the P6 sequences were aligned using the Clustal 2.0.12 program (with gap penalties at −12/−2) the sequences showed 26% identity and a global alignment score of 60. Underlined amino acids are the locations of the putative nuclear export signals as suggested by the NES predictor program.

DMV MEEELKALRFPKKKLEIELNLSCEKISLY----ETMLTDISVQTQKTEAPSQQTQDG 56
CaMV MENIEKLLMQEKILMLEDL
DMV KDGSLPLIDALEKSITVRAEESPQVDXPANTNTTSSPVANGSNGIISPLMAIT 114
CaMV TPSQVKAITQETAPKESTN-PLMANLPPDNVQTEIRPVPR

DMV P6 regions corresponding to self-association domains of its CaMV counterpart were capable of binding to either full-length P6 or to the N-terminus. The CaMV P6 N-terminal domain does self-associate (Haas et al., 2005), but the rest of the protein also plays a role in the process (Li and Leisner, 2002). Previous work implicated the central region (D3) of CaMV P6 as essential for self-association because it binds to the other domains but not itself (Li and Leisner, 2002; De Tapia et al., 1993), yet this does not appear to be the case for the DMV counterpart. This is true despite the fact that D3 equivalent region of DMV P6 shares a higher sequence identity (33% amino acid sequence identity based on the Clustal 2.0.12 program) with the corresponding region of the CaMV protein than the N-terminal domain (26% amino acid sequence identity). Hence, different caulimoviruses may share similar genome arrangements and protein organizations but the specifics of protein interaction are likely unique to each particular virus.

CaMV P6 enters and exits the nucleus and the nuclear export signal (NES) was localized to the CaMV P6 N-terminal region (Haas et al., 2005). Therefore, we examined the DMV P6 sequence for the presence of a predicted NES using the NES predictor program (Cour et al., 2004). A NES was predicted to span amino acid residues 10–20 of the N-terminal region of DMV P6 (Fig. 3). Because this region is analogous to the portion of CaMV P6 (amino acid residues 11–20) that empowers export from the nucleus in infected cells (Haas et al., 2005), it is likely that this portion of the DMV P6 protein also plays a similar role.

In summary, we have shown that: (1) DMV P6 protein specifically self-associates and (2) P6 self-association is likely mediated through N-terminal region. Finally, a putative nuclear exclusion signal was predicted for DMV P6 that was localized to the N-terminal region.

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Appendix A. Supplementary data


References


