The Cauliflower Mosaic Virus Protein P6 Forms Motile Inclusions That Traffic along Actin Microfilaments and Stabilize Microtubules

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The gene VI product (P6) of Cauliflower mosaic virus (CaMV) is a multifunctional protein known to be a major component of cytoplasmic inclusion bodies formed during CaMV infection. Although these inclusions are known to contain virions and are thought to be sites of translation from the CaMV 35S polycistronic RNA intermediate, the precise role of these bodies in the CaMV infection cycle remains unclear. Here, we examine the functionality and intracellular location of a fusion between P6 and GFP (P6-GFP). We initially show that the ability of P6-GFP to transactivate translation is comparable to unmodified P6. Consequently, our work has direct application for the large body of literature in which P6 has been expressed ectopically and its functions characterized. We subsequently found that P6-GFP forms highly motile cytoplasmic inclusion bodies and revealed through fluorescence colocalization studies that these P6-GFP bodies associate with the actin/endoplasmic reticulum network as well as microtubules. We demonstrate that while P6-GFP inclusions traffic along microfilaments, those associated with microtubules appear stationary. Additionally, inhibitor studies reveal that the intracellular movement of P6-GFP inclusions is sensitive to the actin inhibitor, latrunculin B, which also inhibits the formation of local lesions by CaMV in Nicotiana edwardsonii leaves. The motility of P6 along microfilaments represents an entirely new property for this protein, and these results imply a role for P6 in intracellular and cell-to-cell movement of CaMV.

Cauliflower mosaic virus (CaMV), the type member of the genus Caulimovirus, has a circular double-stranded DNA genome known to encode six open reading frames (ORFs). The gene product of ORF VI (P6) is a multifunctional protein whose ascribed functions have increased in number since its initial characterization over 20 years ago. P6 was originally described as the most abundant CaMV protein in infected plants (Odell and Howell, 1980) and was later shown to be the major constituent of amorphous, electron-dense inclusion bodies that are thought to be the sites of virion assembly (Fujisawa et al., 1967; Rubio-Huertos et al., 1968; Himmelbach et al., 1996; Cecchini et al., 1997). Indeed, despite the detection of other viral proteins in CaMV inclusions, the P6 protein on its own is capable of forming inclusion bodies (Cecchini et al., 1997; Li and Leisner, 2002; Haas et al., 2005).

P6 is the major pathogenicity determinant for CaMV (Daubert et al., 1984; Baughman et al., 1988; Stratford and Covey, 1989; Zijlstra and Hohn, 1992) and was recently shown to be a suppressor of RNA silencing (Love et al., 2007). In addition, P6 also functions as an avirulence determinant, as it has been shown to be responsible for eliciting a hypersensitive response in Nicotiana edwardsonii and Datura stramonium, as well as nonnecrotic resistance in Nicotiana bigelovii and Arabidopsis (Arabidopsis thaliana) ectotype Tsu-O (Daubert et al., 1984; Schoelz et al., 1986; Wintemantel et al., 1993; Agama et al., 2002). The portion of the P6 protein recognized by plants is localized to the N-terminal third of the protein (Wintemantel et al., 1993; Palanichelvam et al., 2000; Agama et al., 2002). P6 also has a significant effect on plant metabolism, as it is responsible for down-regulating or inducing expression of several plant genes (Geri et al., 1999), including genes involved in ethylene signaling (Geri et al., 2004).

Replication of CaMV involves the production of a polycistronic RNA intermediate, the 35S RNA, and P6
acts as a translational transactivator (TAV) by modifying the host translational machinery to allow for reinitiation of translation on this RNA (Ryabova et al., 2002). To carry out this function, the P6 protein physically interacts with the initiation factor eIF3 (Park et al., 2001), as well as ribosomal proteins L13 (Bureau et al., 2004), L18 (Leh et al., 2000), and L24 (Park et al., 2001). Finally, P6 is also a nucleocytoplastic shuttle protein whose nuclear export is dependent upon a Leu-rich sequence near its N terminus, a region that is also involved in inclusion body formation (Li and Leisner, 2002; Haas et al., 2005). Although the precise role of the P6 protein’s nucleocytoplastic shuttle function during infection remains to be elucidated, P6 does have the capacity to bind RNA (De Tapia et al., 1993; Cerritelli et al., 1998) and as such may act to control export of the 35S RNA from the nucleus to the cytoplasm, drawing the 35S RNA into the nascent P6 inclusion bodies where viral proteins are translated.

Despite the recognized intracellular movement of P6 from cytoplasm to nucleus and the disparate cytoplasmic functions of this protein, factors controlling intracellular transport of P6 remain unknown. The cytoskeleton has been implicated in the intracellular trafficking of a number of plant viral proteins. For example, proteins encoded by several viruses have been found to colocalize with actin microfilaments, including the TGBp2 movement protein from Potato virus X (PVX), TGBp2 and TGBp3 from Potato mop-top virus, the Hsp70 homolog from Beet yellow virus, as well as both the movement (MP) and 126-kD proteins from Tobacco mosaic virus (TMV; McLean et al., 1995; Haupt et al., 2005; Ju et al., 2005; Liu et al., 2005; Prokhnevsky et al., 2005). In addition, inhibitor studies recently demonstrated that the intracellular trafficking of potato leafroll virus MP to the plasmodesmata (PD) is dependent upon an intact actin cytoskeleton (Vogel et al., 2007). Together, these studies suggest that the trafficking of viral proteins along actin filaments is a mechanism utilized by highly divergent RNA viruses.

The only documented example of a plant viral protein found to colocalize with both microfilaments and microtubules in cells is the TMV MP (McLean et al., 1995; reviewed in Beachy and Heinlein, 2000; Lucas, 2006), which has been shown to associate with and stabilize microtubules and contains a motif thought to mimic the region of tubulin responsible for lateral junctions between microtubules (Boyko et al., 2000; Ashby et al., 2006). Interestingly, the CaMV gene II product (P2), an aphid transmission factor, was previously shown by immunoelectron microscopy to associate with microtubules in both insect and plant cells, although the significance of this interaction remains unclear (Blanc et al., 1996). In addition to these two viral proteins found to colocalize with microtubules in planta, the Hsp70 homolog from Beet yellow virus and the coat protein from PVX have both been shown to interact with microtubules in vitro (Karasev et al., 1992; Serazev et al., 2003). Evidence that the intracellular localization of grapevine fanleaf virus MP is disturbed by oryzalin, as well as the finding that the geminivirus replication protein AL1 interacts with a kinesin by yeast two-hybrid assay, may also indicate a potential association of these proteins with microtubules (Kong and Hanley-Bowdoin, 2002; Laporte et al., 2003).

In this study, we utilize a fusion between the C terminus of P6 and GFP to visualize P6 inclusions in live cells. We demonstrate that the fusion of P6 with GFP does not interfere with its ability to act as a TAV. We further demonstrate that P6-GFP inclusion bodies move intracellularly and are associated with microtubules, actin microfilaments, and the endoplasmic reticulum (ER). Although P6-GFP inclusion bodies associated with microtubules appear stationary, we show that P6-GFP bodies can traffic along microfilaments and that this movement is severely reduced by treatment with the actin inhibitor latrunculin B (LatB). LatB treatment of N. edwardsonii leaves inhibits the formation of local lesions by CaMV, indicating the potential that P6 trafficking on microfilaments is necessary for CaMV cell-to-cell movement. Additionally, the association of P6-GFP inclusion bodies with microtubules prevents the disruption of microtubules by oryzalin, denoting a tight association between these two proteins. We discuss the potential role of P6 movement and cytoskeletal association in CaMV infection.

RESULTS

P6-GFP Retains Its Ability to Function as a TAV

Previous studies have shown that CaMV P6 protein expressed transgenically is able to form the amorphous inclusion bodies characteristic of CaMV infections (Cecchini et al., 1997). Furthermore, numerous studies involving ectopic expression of P6 have characterized the role of P6 in translational transactivation (Ryabova et al., 2002), as well as its role in nuclear import and export (Haas et al., 2005). To probe the subcellular localization of P6 inclusions, we fused GFP to its C terminus, and to ensure that the GFP fusion did not alter the properties of P6, we evaluated the capacity of the P6-GFP fusion to transactivate the expression of GUS from the bicistronic GUS construct, p71-GUS (Palanichelvam and Schoelz, 2002; Fig. 1A). P6-GFP was co-agroinfiltrated with the bicistronic p71-GUS plasmid into N. edwardsonii leaves, and GUS expression was analyzed at 4 d postinfiltration (dpi). GUS activity was measured in relation to a positive control plasmid (pMonoGUS) whose GUS expression was driven by the 35S promoter. We found that co-infiltration of P6-GFP significantly enhanced GUS expression compared with expression by p71-GUS alone. In fact, the TAV function of the P6-GFP protein was indistinguishable from the unmodified P6 protein (Fig. 1B). Consequently, we show in a commonly used assay involving ectopic expression of P6 that fusion of GFP to its C terminus had no effect on its TAV function.
The P6-GFP Fusion Protein Forms Motile Cytoplasmic Inclusion Bodies

To determine whether a fusion of GFP to the C terminus of P6 would affect its capacity to form inclusions, a P6-GFP construct (Fig. 2A) was agroinfiltrated into *N. benthamiana* leaves, and the agroinfiltrated leaf areas were examined by fluorescence microscopy at 3 dpi. Compared with free GFP (Fig. 2A, pCH32) that produced a diffuse GFP signal that was evenly distributed in the cytoplasm (Fig. 2B), inclusion bodies tagged with GFP were visible at the periphery of the epidermal cells in P6-GFP-infiltrated leaves (Fig. 2C, arrows) and were clearly distinguishable from chloroplasts in the underlying parenchyma cells (Fig. 2, B and C, red bodies). As these epidermal cells are highly vacuolated, this finding is consistent with the cytoplasmic localization of P6 inclusion bodies previously observed by electron microscopy (Cecchini et al., 1997). The size of P6-GFP inclusion bodies was highly variable, ranging between approximately 0.5 and 20 μm in diameter.

Time-lapse video microscopy revealed P6-GFP inclusion bodies to be highly motile (Supplemental Video S1) with an average velocity of 2 μm/s (maximum of approximately 8 μm/s). Inclusion bodies appeared to be restricted to the cytoplasm of a single cell, as we failed to observe movement of bodies between cells.

P6 Inclusion Bodies Associate with Actin Filaments and ER

Given the observed motility of P6 inclusion bodies and the demonstrated role for the cytoskeleton and its associated motor proteins in the intracellular movement of organelles and vesicles (for review, see Lee and Liu, 2004), we wanted to examine the possibility that P6 inclusion bodies could be associated with the cytoskeleton. To this end, we coexpressed P6-GFP with fluorescent markers for the actin cytoskeleton in *N. benthamiana* leaves via agroinfiltration (Fig. 3). When P6-GFP (Fig. 3A) was co-infiltrated with a fusion between DsRed and the actin binding protein, talin (DsRed2-talin; Fig. 3B), we observed colocalization of many P6 inclusion bodies with actin microfilaments (Fig. 3C, arrows). In contrast to GFP, DsRed forms a large tetramer (Baird et al., 2000) that may prevent

Figure 1. Analysis of TAV function of P6-GFP. A, Structure of the GUS reporter constructs. The bicistronic construct p71-GUS consists of two ORFs from CaMV strain CM1841 included in the nucleotide sequence from 12 to 413. This sequence contains a complete copy of ORF VII and the start codon for P1, with the GUS gene fused in-frame to the CaMV P1 gene. Black boxes represent the left and right borders of the T-DNA (TL and TR). White boxes illustrate CaMV sequences including the 35S promoter present in the Agrobacterium vector pKYLX7, hatched boxes illustrate the reporter gene GUS, and the stippled boxes illustrate the *rbcS* transcript termination signals. B, GUS activity is expressed as a percentage of MonoGUS at 4 dpi with the indicated constructs.

Figure 2. P6-GFP fusion protein forms cytoplasmic inclusion bodies upon agroinfiltration into *Nicotiana* leaves. A, Structure of constructs in pKYLX7 plant expression vector containing either free GFP (pCH32) or a C-terminal fusion of CaMV P6 with GFP (P6-GFP). Black boxes indicate the left and right borders of the T-DNA (TL and TR, respectively). Stippled boxes denote the *rbcS* transcript termination signal. B and C, GFP fluorescence in leaves of *N. benthamiana* agroinfiltrated with pCH32 (B) or P6-GFP (C) at 3 dpi. Arrows mark the position of selected individual inclusion bodies in the cytoplasm of highly vacuolate leaf epidermal cells. Asterisks mark large inclusions. The outline of individual *N. benthamiana* cells is clearly visible due to cytoplasmic GFP fluorescence. Red bodies in B and C are chloroplasts. Bars = 25 μm.
access of talin to the network of fine microfilaments within the cell and account for the fact that the DsRed2-talin construct primarily labels thick actin cables and bundles (Blancaflor, 2002; Liu et al., 2005).

To further assess the association of P6 bodies with microfilaments, we utilized a construct expressing the actin-binding domain 2 of Arabidopsis fimbrin 1 with GFP fused to both the N and C termini (GFP-ABD2-GFP; Wang et al., 2008). This construct labels a fine network of actin microfilaments compared with DsRed2-talin (compare Fig. 3E with Fig. 3C). Co-infiltration of GFP-ABD2-GFP with P6-GFP resulted in colocalization between P6 inclusion bodies and actin filaments. G, ER labeled with GFP in N. benthamiana line 16C. H and I, Colocalization of P6-GFP inclusion bodies with the ER. Arrows mark examples of P6-GFP inclusions associated with the ER. Image in D is a projection of 24 optical sections taken at 0.5-μm intervals. Images in A to C and E to I are single optical sections. Bars = 25 μm (A–D) or 10 μm (E–I).

P6 Bodies Can Traffic along Microfilaments

To determine whether any of the motile P6 inclusion bodies were associated with microfilaments, we utilized time-lapse confocal microscopy to view movement of P6 inclusion bodies in the presence of GFP-ABD2-GFP-labeled actin. We observed that the movement of P6 bodies was partially inhibited in many cells, likely due to the inhibitory effect of high concentrations of actin label on myosin-dependent movement (data not shown; Holweg, 2007). However, despite this inhibition, we found that P6 inclusion bodies are indeed capable of trafficking along GFP-ABD2-GFP-labeled actin filaments (Fig. 4; Supplemental Video S2). Although dynamic actin filaments are clearly visible in the presence of the GFP-ABD2-GFP label (Sheahan et al., 2004; Wang et al., 2008), movement of P6 inclusion bodies was predominantly observed along existing microfilaments that appeared stable for the...
duration of body movement. Figure 4 shows examples of unidirectional movement of P6-GFP inclusion bodies along microfilaments over 25- to 35-s time periods. Arrows mark the progression of inclusion body movement in each sequence. We also observed movement of P6-GFP inclusions along DsRed-talin-labeled microfilaments, but at a reduced number (data not shown).

To evaluate the importance of the actin cytoskeleton in P6-GFP body movement, we treated tissue with the actin inhibitor, LatB. We found that infiltration of 5 μM LatB resulted in complete disruption of GFP-ABD2-GFP-labeled microfilaments at 2 h postinfiltration (data not shown). When we observed P6 inclusion bodies by time-lapse confocal microscopy 2 h after treatment with 5 μM LatB, we consistently found that body movement was virtually eliminated (Supplemental Video S3).

CaMV Infection Is Inhibited by LatB Treatment

Given the observation that CaMV P6 inclusion bodies traffic along microfilaments and that this movement is inhibited by LatB, we wanted to determine the effect of LatB on CaMV infection. To this end, we infiltrated half leaves, bounded at the midrib, of the CaMV local lesion host, *N. edwardsonii*, with either 5 μM LatB or a dimethyl sulfoxide (DMSO) buffer control. Infiltrations were performed 3 h before the entire leaf surface was inoculated with CaMV (strain W260) and were repeated at 7 dpi. In total, we observed 21 lesions across four leaves on the buffer-treated tissue and no lesions on an equal number of half-leaves treated with LatB. Figure 5A shows necrotic lesions (arrows) formed on the buffer-infiltrated half of a leaf at 14 dpi. To confirm that the lesions on the buffer infiltrated half leaves were indeed caused by CaMV and to check for the presence of CaMV in the LatB-treated tissue, we isolated leaf panels (100 × 200 mm) with and without lesions from the buffer-treated half leaves and panels without lesions (because none was observed) from the LatB-treated half leaves. We then isolated genomic DNA from individual panels and attempted to amplify a portion of the P6 gene by PCR. We detected strong P6 signal in all (4/4) leaf panels that contained a visible lesion (Fig. 5B) and little (Fig. 5B, leaves 2 and 4) or no (Fig. 5B, leaves 1 and 3) signal from the LatB-treated leaf tissue. We also detected a low level of P6 in the majority (3/4) of the samples from the buffer-treated areas lacking visible lesions (Fig. 5B, leaves 2, 3, and 4). This suggests that CaMV infection was widespread on the buffer-infiltrated half leaves, because it was detectable by PCR, even at levels insufficient to cause lesion formation. To ensure that all of our genomic DNA preps were of similar quality, we amplified EF1α from each DNA sample as a control (Fig. 5B).

Although the LatB-treated tissue looked healthy, we wanted to confirm that the observed reduction of CaMV accumulation in the presence of LatB was not due to the LatB treatment simply killing the cells in the infiltrated area. To test this, we performed semiquantitative reverse transcription (RT)-PCR to determine transcript levels of the housekeeping gene, EF1α, in either the control or LatB-treated leaf tissue. We found that EF1α transcript levels were not noticeably affected by LatB treatment (Fig. 5C).

P6 Inclusion Bodies Also Colocalize with Microtubules

To examine whether P6-GFP was associated with microtubules, we coexpressed P6-GFP with GFP-
MBD, which consists of a fusion between GFP and the microtubule-binding domain of mammalian MAP4 (Marc et al., 1998). Two days following agroinfiltration, GFP-MBD labeled a dense meshwork of cortical microtubules in the epidermal cells of *N. benthamiana* (Fig. 6A). When coexpressed with P6-GFP, we observed colocalization between P6 inclusion bodies and cortical microtubules in all cells surveyed (Fig. 6, B–D). P6 inclusions were found along the length of microtubules (Fig. 6C, arrowheads) and also at branch points between microtubules (Fig. 6C, arrows). When we observed P6 bodies by time-lapse confocal microscopy, we found that the movement of P6 inclusions appeared unaffected by the presence of the microtubule label (data not shown). Close observation of P6-GFP inclusion bodies in the cortical microtubule network revealed that despite the movement of some inclusion bodies at the cortex, the vast majority of the bodies associated with microtubules did not move (Supplemental Video S4).

### P6 Inclusions Stabilize Microtubules

To further assess the potential role of microtubules in P6 intracellular movement, we infiltrated leaf tissue with 20, 50, or 100 μM oryzalin, a potent microtubule depolymerizing agent. At 2 h postinfiltration, we observed severe fragmentation of GFP-MBD-labeled cortical microtubules in the absence of P6 (Fig. 7, −P6). In the presence of 100 μM oryzalin, the microtubules were almost completely abolished. Surprisingly, we found that coexpression of P6-GFP prevented the disruption of microtubules by 20 and 50 μM oryzalin (Fig. 7, + P6). Even in the presence of 100 μM oryzalin, microtubules were still present during P6-GFP expression, although they were more fragmented. We imaged approximately eight cells per treatment and found the observed microtubule phenotypes to be consistent for each treatment. Interestingly, when we checked P6-GFP inclusion body movement following oryzalin treatment, we found that it was consistently indistinguishable from the inclusion body movement in untreated tissue, even in the presence of 100 μM oryzalin (Supplemental Video S5).

**Figure 5.** The actin inhibitor LatB inhibits CaMV infection. A, Representative image showing necrotic lesions (arrows) on a half leaf of *N. edwardsonii* infiltrated with a buffer control (− LatB) and no lesions on the other half leaf infiltrated with 5 μM LatB (+ LatB). Half leaves were infiltrated with LatB or buffer control 3 h prior to inoculation of the entire leaf surface with CaMV (strain W260) and again at 7 dpi. Lesions were imaged at 14 dpi. B, The presence of CaMV was determined by PCR detection of P6 from leaf panels (100 × 200 mm) either with (lesion +) or without (lesion −) visible lesions from the indicated halves (+ or − LatB) of four leaves. PCR of EF1α from genomic DNA is shown as a loading control. C, Semiquantitative RT-PCR analysis of EF1α transcript from leaf halves described in A and B treated with LatB (LatB +) or buffer control (LatB −). The color of the gel images in B and C was inverted to enhance sensitivity.

**Figure 6.** P6 inclusion bodies colocalize with microtubules. Observation of microtubules and P6 inclusion bodies in *N. benthamiana* leaf epidermal cells. A, A dense network of cortical microtubules is labeled with GFP-MBD. B to D, Coexpression of P6-GFP with GFP-MBD reveals colocalization of P6 inclusion bodies with microtubules. Arrows in C mark examples of P6 inclusions at the junctions of microtubules. Arrowheads mark examples of P6 bodies along the length of a microtubule. Images in A and B are projections of 20 to 30 optical sections taken at 0.5-μm intervals. Images in C and D are single optical sections. Bars = 25 μm (A, B, and D) or 10 μm (C).
DISCUSSION

In this study, we reveal new properties for the CaMV P6 protein: intracellular movement along actin microfilaments and association with the ER and microtubules. In addition, although the details of the intracellular movement of a number of plant RNA viruses have begun to be elucidated, there is currently no information available regarding the intracellular trafficking of plant DNA virus proteins along the cytoskeleton. Here, we demonstrate an association of P6 inclusions with microtubules as well as the actin/ER network and further show that while microtubules are dispensable for movement, actin microfilaments are required for P6 body trafficking and CaMV accumulation. We discuss the potential implications of these findings below.

P6-GFP Functionality

We have shown that fusion of GFP to the C terminus of P6 had no affect on TAV function; the TAV activity of P6-GFP was comparable to wild type P6. Although the P6-GFP construct has not yet been tested in an infectious CaMV clone, our work has direct application for the large body of literature in which P6 has been expressed ectopically and its functions characterized. For example, P6 of CaMV and Figwort mosaic virus was first expressed ectopically in plant cells nearly 20 years ago (Bonneville et al., 1989; Gowda et al., 1989); these articles established that P6 was necessary for post-transcriptional expression of genes on the 35S RNA, and later work showed that P6 physically interacts with ribosomal proteins (Leh et al., 2000; Park et al., 2001; Bureau et al., 2004), suggesting that this interaction facilitates translation of the polycistronic 35S RNA (Ryabova et al., 2002). Recently, a P6-GFP fusion protein was expressed ectopically to reveal a new property for P6 as a nucleocytoplasmic shuttle protein (Haas et al., 2005). In addition, ectopically expressed P6 triggers a hypersensitive response in N. edwardsonii (Palanichelvam et al., 2000) and transgenically expressed P6 causes disease-like symptoms in Nicotiana species and Arabidopsis (Baughman et al., 1988; Goldberg et al., 1991; Zijlstra and Hohn, 1992; Geri et al., 1999; Yu et al., 2003). The results from studies involving P6 ectopic expression have mimicked results from studies with infectious chimeric CaMV isolates regarding the pathogenic properties of P6 (Daubert et al., 1984; Schoelz et al. 1986; Wintermantel et al., 1993). Consequently, our discovery that ectopically expressed P6-GFP inclusion bodies associate with and traffic along microfilaments likely sheds new light on the localization of P6 after its ectopic introduction into plant cells and during virus infection.

P6-GFP Associates with the ER/Actin Network and Traffics along Microfilaments

Although a movement function for P6 has not been previously demonstrated, Király et al. (1998) alluded to this possibility when they showed that the P6 gene product of CaMV strain D4 enhanced the systemic movement of CaMV strain CM1841 in N. bigelovii. Our finding that P6 inclusion bodies are motile and traffic along actin filaments supports a role for P6 in virus intracellular and later intercellular movement. This conclusion is further supported by the fact that LatB treatment strongly inhibits both P6 body movement (Supplemental Video S3) and CaMV accumulation (Fig. 5).

Although a number of plant viral proteins, including TGBp2 from PVX and the TMV 126-kD protein, have been shown to traffic along actin filaments (Ju et al., 2005; Liu et al., 2005), this is the first report, to our knowledge, of an interaction between a protein from a plant DNA virus and the actin cytoskeleton. Indeed, despite vast differences between CaMV (a DNA virus) and TMV (an RNA virus), striking similarities exist between CaMV P6 and the 126-kD protein from TMV. Like P6, the 126-kD protein is a multifunc-

Figure 7. P6 inclusion bodies stabilize microtubules against depolymerization. GFP-MBD-labeled microtubules in the presence (+ P6) or absence (− P6) of CaMV P6-GFP at 2 h following infiltration with the indicated concentration of the microtubule inhibitor oryzalin. Images are projections of 20 to 25 optical sections taken at 0.5-μm intervals. Bars = 25 μm.
tional protein that forms cytoplasmic bodies, can function as both an avirulence determinant and a silencing suppressor, and traffics along actin microfilaments (Shintaku et al., 1996; Padgett et al., 1997; Abbink et al., 1998; Erickson et al., 1999; Ding et al., 2004; Liu et al., 2005). The 126-kD protein is a major constituent of virus replication complexes and has been proposed to shuttle these complexes and the viral RNA contained within to the PD for intercellular transport (Kawakami et al., 2004; Liu et al., 2005). Given the similarities between CaMV and TMV noted above, the fact that TMV cell-to-cell movement is inhibited by LatB treatment (Kawakami et al., 2004; Liu et al., 2005) may imply that our observed lack of CaMV accumulation in LatB-treated tissue (Fig. 5) represents a similar inhibition of CaMV intercellular spread. In the case of CaMV, cell-to-cell movement is primarily thought to occur through tubules in the form of virions (Kitajima and Lauritis, 1969; Stavolone et al., 2005). The fact that P6-containing inclusions are thought to be sites of virion assembly raises the possibility that P6 motility could deliver these inclusions and the virions they contain to the PD for intercellular transport. It is also conceivable that the disruption of actin-dependent intracellular movement of CaMV inclusions could inhibit virion assembly and thus in turn prevent cell-to-cell spread of the virus. In addition, although our results clearly do not rule out the possibility that LatB treatment may inhibit CaMV translation or replication, our RT-PCR analysis demonstrates that cells are indeed viable following LatB treatment (Fig. 5C). Also, we did detect a low level of CaMV DNA sequence by PCR in some LatB-infiltrated tissues (Fig. 5B), consistent with a defect in cell-to-cell movement rather than a complete inhibition of single cell virus multiplication. Thus, for CaMV, it seems plausible that its decreased accumulation in leaves after LatB treatment is due to lack of transport or encapsidation rather than an inhibition of virus replication. However, additional studies, potentially similar to those that examined TMV accumulation in protoplasts (Liu et al., 2005), will be necessary to verify such a conclusion. In the TMV work, LatB treatment inhibited virus accumulation in single cells but not to a level that would inhibit virus cell-to-cell movement in the plant, based on comparison with a mutant TMV that accumulated to the same low level in protoplasts but moved normally cell to cell (Liu et al., 2005). A similar conclusion was made in studies with the TMV MP where TMV mutants, expressing MP at only 6% of the level observed during infection by the wild-type virus, showed no defect in intercellular spread (Arce-Johnson et al., 1995).

It is interesting to note that 126-kD protein and CaMV P6 are not considered the MPs of their respective viruses. However, previous studies have shown that both proteins interact or colocalize with their respective cell-to-cell MPs. The 126-kD protein colocalized with MP-GFP in virus replication complexes during virus infections in both N. benthamiana leaves and BY-2 tobacco protoplasts (Liu et al., 2005). The P6 protein has been shown to physically interact with its MP (P1) in a yeast two-hybrid screen (Hapiak et al., 2008). The fact that both of these proteins have been shown to traffic along actin microfilaments demonstrates that association with the host cytoskeleton is a strategy employed by diverse viruses and further reveals that viruses often rely upon more than just the classically defined MPs to facilitate movement (for review, see Lucas, 2006; Harries and Nelson, 2008). In fact, a number of plant viruses such as the potexvirus, PVX, rely upon three proteins termed the triple gene block for intra-/intercellular movement (for review, see Verchot-Lubicz et al., 2007).

Aside from a potential role in cell-to-cell movement, it is also possible that P6 body movement along microfilaments may target virus to other subcellular locations such as the ER or the nucleus, both of which maintain a close association with the actin cytoskeleton. Indeed, it is interesting to note that P6 inclusions appear to localize to the ER (Fig. 3, H and I, arrows), although we cannot rule out the possibility that P6-GFP expression may induce enriched ER rearrangements separate and in addition to the P6 bodies. In addition to its role as a site of protein translation, this endomembrane system spans the cytoplasm establishing connections among the plasma membrane, nucleus, and PD (Grabski et al., 1993; Staehelin, 1997; Cantrill et al., 1999). It is possible that the ER may play a critical role in the intra-/intercellular movement of P6 and other CaMV proteins.

Although further work will be required to clarify the molecular basis of actin-dependent P6 inclusion body movement, several pieces of evidence suggest a role for myosin in this process. First, the average rate of body movement that we observed (2 μm/s) was within the range expected for myosin-dependent transport of plant organelles (Nebenführ et al., 1999; Avisar et al., 2008), and second, the fact that P6 bodies were observed traveling along existing filaments is also characteristic of motor-driven movement. We cannot rule out the possibility that some component of P6 movement may result from cytoplasmic streaming, because this activity itself is a myosin-dependent process (Kachar and Reese, 1988).

**P6 Associates with and Stabilizes Microtubules**

The discovery that CaMV P6 inclusion bodies colocalize with microtubules is of particular interest given the very limited number of plant viral proteins that have been shown to share this localization. Indeed, aside from the well-studied TMV MP, the only other example of a plant viral protein that colocalizes with microtubules is CaMV P2. It is particularly interesting that both P2 and P6 CaMV proteins share microtubule localization, because there is currently no known evidence for a physical interaction between these viral components. While P6 is involved in many aspects of...
the viral lifecycle, P2 is thought to serve as a simple regulator of aphid transmission, because deletion of this gene does not affect virus replication or cell-to-cell spread (Howarth et al., 1981). However, a point mutation in the P2 ORF was previously found to affect the stability of P6 inclusion bodies (Qiu et al., 1997), and our finding that both proteins colocalize with microtubules raises the possibility that P2 may be a minor component of the P6 inclusions. In reference to this possibility, it is pertinent to note that like P6, the P2 protein alone can form inclusions, although these bodies are electron-lucent and readily distinguishable from the electron-dense P6 inclusions (Espinoza et al., 1991).

Given the motility of P6 inclusion bodies observed in leaf epidermal cells (Supplemental Video S1) as well as the documented role of microtubule motors in intracellular transport (for review, see Reddy, 2001), we were surprised to find that P6 bodies associated with microtubules appeared stationary. Although we did observe the frequent movement of bodies in the vicinity of microtubules, these motile inclusions did not appear to be physically associated with microtubules (Supplemental Video S4), and their movement was inhibited by the actin depolymerizing drug, LatB (Supplemental Video S3) but not the microtubule antagonist oryzalin (Supplemental Video S5).

Our inability to assign the obvious function of intracellular movement to the P6-microtubule association leaves the significance of the interaction between P6 inclusions and microtubules to be elucidated. Our finding that association of P6 bodies with microtubules protects filaments from fragmentation by oryzalin (Fig. 7) indicates a strong binding between P6 and microtubules. Although we do not yet know whether this is a direct interaction, a similarly strong binding was demonstrated for CaMV P2 and TMV MP, which were both also shown to stabilize microtubules (Blanc et al., 1996; Ashby et al., 2006). One possible explanation for the association is that microtubules may serve as a framework for inclusion body assembly. It seems plausible to suggest that individual P6 proteins or small aggregates could traffic along microtubules to stationary sites of assembly. Indeed, a similar model has been proposed for the formation of aggresomes similar to P6. In addition, the association of P6 protein with microtubules could facilitate inclusion body degradation. The degradation of potato leafroll virus MP, for example, was recently found to require intact microtubules, and a similar model has been proposed for the microtubule associated degradation of TMV MP (Curin et al., 2007; Vogel et al., 2007). Together, these results may encourage a reassessment of the roles of host proteins whose association with microtubules may have been assumed to denote a major function in intracellular movement.

The Potential Role of Intracellular Movement in P6’s Multiple Functions

The finding that the P6-GFP fusion utilized in these studies is still functional for translational transactivation (Fig. 1), a process that requires interaction with host ribosomes, indicates that the fusion is capable of normal physiological activities and opens the door for future studies utilizing this construct to examine the potential role of intracellular movement in P6’s many other functions. For example, P6 may traffic along microtubules to the nucleus where it is known to act as a nucleocyttoplasmic shuttle protein (Haas et al., 2005), potentially controlling the export of 35S polycistronic viral RNA into the cytoplasm. Alternatively, P6 could utilize the microfilament network to shuttle 35S RNA away from the nucleus. Because P6 inclusions are thought to be sites of translation from the 35S RNA, P6 might facilitate translation by shuttling RNA or ribosomes to the inclusions. However, because P6 inclusions are motile, it is also possible that the viral inclusions may themselves move to meet the 35S RNA. The role of the ER in this process remains unknown, but given the presence of ribosomes in the ER network as well as the known association of the ER network with nuclear pores (Staehelin, 1997) and the actin cytoskeleton (Cowin and Burke, 1996; Boevink et al., 1998), it seems likely that the ER may serve to bring the diverse components required for viral RNA movement and translation together.

Although a potential role for intracellular movement in P6’s suppression of silencing activity may not be obvious, a number of other plant viral suppressors of silencing also have demonstrated movement functions (Kasschau and Carrington, 2001; Ding et al., 2004; Bayne et al., 2005). For example, the suppressor activity of both P25 from PVX and potyviral HC-Pro has actually been tied to the movement function by mutant analysis (Kasschau and Carrington, 2001; Bayne et al., 2005). It is possible that movement of P6 to or from the nucleus could facilitate its action as a silencing suppressor, and it will be interesting to examine whether there is any relationship between the movement and suppressor functions in CaMV P6. The further investigation of the role movement plays in the multiple functions attributed to CaMV P6 may help reassess the potential role of intracellular movement for other viral proteins or host factors that may perform functions similar to P6. In addition, the association of P6 protein with multiple cytoskeletal elements indicates the presence of a complex system regulating the accumulation and movement of both CaMV and host factors.

MATERIALS AND METHODS

Plasmids

The Agrobacterium tumefaciens binary vector pKYLX7 (Schardl et al., 1987) was used for all constructs involving CaMV gene VI and for the GUS reporter plasmids. The plasmids p71-GUS, plasmid pMonoGUS, and pW260VI (P6 CaMV P6 Traffics along Microfilaments and Binds Microtubules
without GFP) were made previously (Palanichamy and Schoelz, 2002). The gene VI-GFP fusion was created by recombinant PCR. The coding sequence for W260 gene VI was amplified from pW260VI using the forward primer 5'-GGGCTCGAGATGAGAATAGGTTTAACTC-3' and the reverse primer 5'-AAGTCGCTTCCATATGCTTCTGTTGAAGACT-3', whereas the GFP coding sequence was amplified from pCH2 (Chalfie et al., 1994; Voinnet et al., 1998) with the forward primer 5'-ACGTCGCTTAAAGGTTTTACTTCTTCTTCGTTCTGTT-3' and the reverse primer 5'-GGGGTACCTTATTTGTATAGTTCATCCATGCC-3'. The PCR products were gel purified and subsequently combined into a single PCR reaction with the W260 gene VI forward primer and the GFP reverse primer. The PCR product of the gene VI-GFP fusion DNA product was initially cloned into the plasmid pGEM7zf- (Promega) at the unique XhoI and KpnI sites. The gene VI-GFP coding sequence was determined to verify that no point mutations had been incorporated due to PCR, and subsequently this fusion sequence was ligated into the Agrobacterium vector pRK7X7 at the XhoI and KpnI sites to create pRK7GFP. All plasmids were mobilized into A. tumefaciens strain CS8 (GV2260) with the helper plasmid pRK2013 by triparental mating (Ditta et al., 1980). Agrobacterium with pRK7X7 and its derivatives were selected on Luria-Bertani medium supplemented with 50 µg/mL kanamycin and 100 µg/mL carbenicillin. DsRed-talin, GFP-ABD2-GFP, and GFP-MBD constructs were described elsewhere (Marc et al., 1998; Blancatlor, 2002; Wang et al., 2008).

Plant Growth and Virus Infections

CaMV virion inoculum was purified from W260-infected turnip (Brassica rapa subsp. rapa) leaves as described (Schoelz et al., 1986). Equal volumes of CaMV prep were used to inoculate leaves of Nicotiana edwardsonii previously infiltrated with LatB or DMSO buffer control, as described below. N. edwardsonii was germinated and grown according to procedures described for other Nicotiana species (Ding et al., 1995). Following inoculation, plants were placed in a growth chamber (Conviron model E7/2) at 20°C, 12-h light cycles (150 µmol m⁻² s⁻¹).

PCR and Semiquantitative RT-PCR

For detection of CaMV DNA in inoculated leaves, genomic DNA was prepared from leaf panels (100 × 200 mm). Individual panels were ground with plastic pestles in 1.5-mL microfuge tubes containing 500 µL of extraction buffer (0.2 mM Tris-HCl, pH 9.0, 0.4 mM LiCl, 25 mM EDTA, 1% SDS). Following centrifugation for 5 min (13,000g) at room temperature, 350 µL of supernatant was transferred to a new tube containing an equal amount of isopropanol and mixed by inversion. DNA was pelleted by centrifugation for 10 min (13,000g), air dried, and resuspended in 400 µL TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). Then 3 µL of genomic DNA was used as template for PCR. For CaMV detection, a 1.2-kb fragment of P6 was amplified using primers 6426F, 5’-CAAAGCTTAAAGGCGCTGTT-3’ and 7551R, 5’-ACCCTGA-GCAGAAACCTAT-3’. EFlA was amplified with primers 12009F, 5’-TGGTGTCTCCAAACGGCTGTTT-3’ and 13070F, 5’-ACGCTGTA-GATCTTAAACGGCAAACTT-3’. Thirty cycles of PCR were carried out with both primer sets. For semiquantitative RT-PCR analysis, leaf panels were ground in liquid nitrogen and RNA was isolated using the RNeasy plant mini kit (Qiagen). DNase-treated RNA (1 µg) and Moloney murine leukemia virus (Promega) reverse transcriptase were used for cDNA synthesis with 15- to 18-base oligo(dT) primers (Invitrogen). EFlA was amplified from a 1/50 dilution of each cDNA reaction with primers described above for either 16 or 24 cycles. Ex-Taq enzyme mix (Takara) was used to perform all PCR reactions.

Fluorescence Colocalization

Agrobacterium containing CaMV P6-GFP, and fluorescent markers for the cytoskeleton were grown under selection and infiltrated into Nicotiana benthamiana at a final OD₆₀₀ of 0.5 as described previously (Voinnet et al., 1998). N. benthamiana line 16C containing ER-GFP labeling was described previously (Ruiz et al., 1998). Agroinfiltrated leaves were examined for fluorescence expression between 2 and 3 dpi as described below.

Confocal Microscopy

N. benthamiana leaf tissue was mounted in water under a coverslip between 2 and 3 of following infiltration with Agrobacterium containing the P6-GFP or actin labeling constructs. Images were acquired on Bio-Rad 1024ES (Bio-Rad Laboratories) or a Leica TCS SP2 (Leica Microsystems) confocal imaging system. DsRed2-talin was excited at 568 nm and images were captured at 598 nm. GFP constructs were excited at 488 nm and captured at 522 nm. For double labeling experiments with P6-GFP and DsRed2-talin, green and red channels were imaged separately and then superimposed. For time-lapse microscopy, images were obtained every 5 s from a single optical plane. Images were processed using Adobe Photoshop (Adobe) and ImageJ software (version 1.38e).

Treatment with Microtubule and Microfilament Destabilizing Drugs

For treatment of N. benthamiana, stock solutions of LatB (VWR International) and oryzalin (Chem Service) were made in DMSO at 10 mM and 2 mM concentrations, respectively. Immediately prior to infiltration, stocks were diluted to 5 µM for LatB and 20, 50, or 100 µM for oryzalin. Equivalent dilutions of DMSO were used as a control. For LatB treatment of N. edwardsonii, a LatB stock solution was prepared at 20 mM in DMSO and diluted to 5 µM in water prior to infiltration. An equivalent dilution of DMSO (1:4,000; 3.2 mM final concentration) was used as a buffer control. Half leaves (divided at the midrib) were infiltrated with either LatB or buffer control 3 h before virus inoculation and again at 7 dpi.

Transactivation Assays

Agrobacterium containing the appropriate constructs were grown to an OD₆₀₀ of 2.0, resuspended in an equal volume of AB minimal medium (Chilton et al., 1974) plus acetosyringone (2.0 mM), and mixed in equal volumes prior to agroinfiltration. N. edwardsonii leaves were harvested at 4 dpi for the measurement of GUS by the chemiluminescent GUS-light kit (Tropix). The relative GUS expression level of each sample was calculated and the mean of three replicates presented as a percentage of the pMonGUS control.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Video S1. Movement of P6 inclusion bodies in N. benthamiana leaf epidermal cells agroinfiltrated with P6-GFP. Images were taken every 5 s for 4 min.

Supplemental Video S2. Time-lapse movie depicting the movement of P6-GFP inclusion bodies along GFP-ABD2-GFP labeled actin filaments. Images were taken every 5 s for 2 min.

Supplemental Video S3. P6-GFP body movement is disrupted 2 h following treatment with 5 µM LatB. Images were taken every 5 s for 3 min.

Supplemental Video S4. P6-GFP inclusion bodies colocalized with GFP-MBD labeled microtubules. Although body movement is readily apparent, P6 inclusions associated with microtubules appear stationary. Although the motile bodies appear larger in this particular movie, we did not observe any consistent size difference between stationary and motile bodies. Images were taken every 5 s for 3 min.

Supplemental Video S5. The movement of P6-GFP inclusion bodies is unaffected 2 h following infiltration with 100 µM oryzalin. Images were taken every 5 s for 5 min.

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