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REVIEW PAPER

A model for intracellular movement of *Cauliflower mosaic virus*: the concept of the mobile virion factory

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Abstract

The genomes of many plant viruses have a coding capacity limited to <10 proteins, yet it is becoming increasingly clear that individual plant virus proteins may interact with several targets in the host for establishment of infection. As new functions are uncovered for individual viral proteins, virologists have realized that the apparent simplicity of the virus genome is an illusion that belies the true impact that plant viruses have on host physiology. In this review, we discuss our evolving understanding of the function of the P6 protein of *Cauliflower mosaic virus* (CaMV), a process that was initiated nearly 35 years ago when the CaMV P6 protein was first described as the 'major inclusion body protein' (IB) present in infected plants. P6 is now referred to in most articles as the transactivator (TAV)/viroplasmin protein, because the first viral function to be characterized for the *Caulimovirus* P6 protein beyond its role as an inclusion body protein (the viroplasmin) was its role in translational transactivation (the TAV function). This review will discuss the currently accepted functions for P6 and then present the evidence for an entirely new function for P6 in intracellular movement.

Key words: *Cauliflower mosaic virus*, cytoskeleton, inclusion bodies, intracellular movement, plasmodesmata, virion factory.

Introduction

The genomes of many plant viruses have a coding capacity limited to <10 proteins (Hull, 2002). This small number of viral proteins must interact with the host to complete extensive multicomponent functions necessary for viral gene expression, replication, and virion assembly. In addition, virus–host interactions are involved in suppression or elicitation of plant defenses, as well as symptom development (Culver and Padmanabhan, 2007). Recent findings make it increasingly clear that individual plant virus proteins interact with several targets in the host for establishment of infection (Hull, 2002). To understand fully the influence of viruses on their host, efforts now are focused on an exhaustive characterization of all interactions between viral and host proteins (e.g. Navratil *et al.*, 2009). Viruses are particularly attractive subjects for this type of analysis precisely because of the limited number of proteins they encode. In addition, as our knowledge of the plant virus interactome increases, it may ultimately lead to a re-evaluation of the known functions of some plant virus proteins.

The first complete plant virus genome sequence appeared in 1980 (Franck *et al.*, 1980). In the ensuing decade, the genomes of numerous plant viruses were determined and a race began to identify the functions of proteins encoded in their genome. Some of the most common functions for viral

proteins included replication, encapsidation, and movement, as it was recognized early on that all plant viruses must have at least one protein dedicated to each of these functions. For example, the genomic sequences of *Tobacco mosaic virus* and *Brome mosaic virus* each has the capacity to code for two proteins necessary for replication, one protein for cell to cell movement, and a capsid protein (Hull, 2002).

However, the simplicity of the genome structure of plant viruses belies the true impact that plant virus proteins have on the physiology of the plant. This impact may occur on multiple levels, through physical interactions between viral and host proteins necessary for the three originally identified activities as well as through the influence of viral silencing suppressor proteins or viral siRNAs on host protein and RNA metabolism. In this review, we discuss our evolving understanding of the function of the P6 protein of *Cauliflower mosaic virus* (CaMV) and its interaction with the host, a process that was initiated nearly 35 years ago when the P6 protein was first described as the 'major inclusion body protein' (IB) present in infected plants (Shockey *et al.*, 1980; Covey and Hull, 1981). P6 is now referred to in most articles as the transactivator (TAV)/viroplasmin protein, because the first function determined for the *Caulimovirus* P6 protein beyond its presence in the virus inclusion body (the viroplasmin) was as a translational transactivator (the TAV function) (Bonneville *et al.*, 1989; Gowda *et al.*, 1989). This review will discuss the currently accepted functions for P6 and then present additional evidence for a new function for P6 in intracellular movement, a function that has only recently been recognized (Harries *et al.*, 2009*a*).

Genome structure of CaMV

To understand the newly proposed role for CaMV P6 in movement, it is important to discuss briefly the genome structure of CaMV as well as the primary functions for the six proteins encoded in the CaMV genome (Hohn and Fütterer, 1997). CaMV is a pararetrovirus; the virus replicates through reverse transcription of a terminally redundant RNA template. The CaMV genome consists of circular, double-stranded DNA (dsDNA)~8 kb in size (Fig. 1A). Viral DNA isolated from virions is not covalently closed, as in many CaMV strains there are two single-stranded discontinuities in the coding strand and one single-stranded discontinuity in the non-coding DNA strand. Six proteins encoded by the virus have been characterized (proteins P1–P6); one other open reading frame (ORF) has been identified (P7; Wurch *et al.*, 1990), but to date a P7 protein has not been found in infected plants. Two RNAs are transcribed from the viral genome sequence. The 19S RNA is the mRNA for the P6 protein, whereas the 35S RNA serves as a polycistronic mRNA for all six viral proteins. The 35S RNA is also the template for reverse transcription and production of progeny viral genomes.

CaMV shares many features with other viruses, as it has one protein whose primary function is for cell to cell movement (P1), one capsid protein (P4) for formation of the icosahedral virions, and one protein for replication (P5; the reverse transcriptase). CaMV is different from most viruses in that it has one protein dedicated to aphid transmission (P2) and a second protein (P3) with a dual role in aphid transmission and cell to cell movement. The P3 protein associates with the virions (Leclerc *et al.*, 1998, 2001) and interacts with the P2 and P1 proteins to mediate aphid transmission and cell to cell spread of the virions, respectively (Leh *et al.*, 2000; Stavolone *et al.*, 2005). In contrast to other CaMV proteins, the function associated with the P6 protein is unique in nature; it is only found in certain species of the genus *Caulimovirus* and does not appear to have a homolog in other viruses or even other organisms (Hohn, 2013).

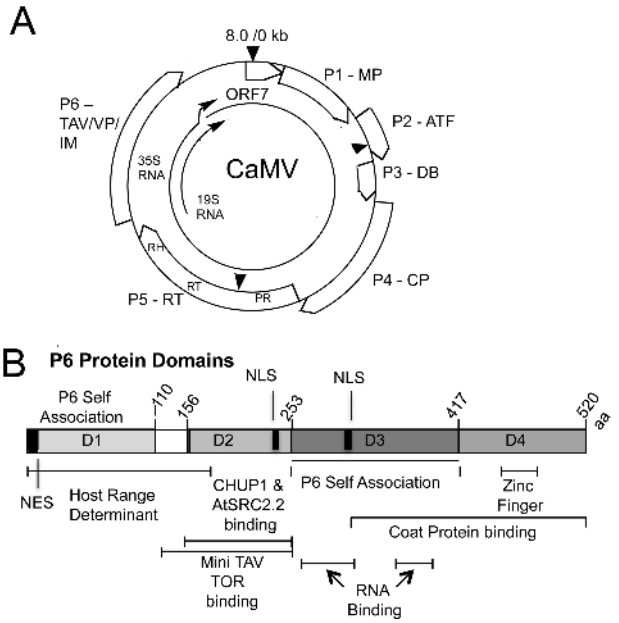

Fig. 1. Genome map of CaMV and P6 protein domains. (A) Genome map of CaMV. The single-stranded discontinuity in the negative-sense DNA strand is indicated by the triangle outside of the circle, whereas single-stranded discontinuities in the positive-sense DNA strand are indicated by triangles inside the circle. The two viral mRNAs are represented by the inner circles, and the 3' end of the RNA is indicated by the arrowhead. The functions for each of the ORFs are MP, cell-to-cell movement protein; ATF, aphid transmission factor; DB, DNA-binding protein—also has role in aphid transmission and cell-to-cell movement; CP, coat protein; RT, reverse transcriptase; TAV/VP/IM, translational transactivator, viroplasmin, intracellular movement. The domains within the reverse transcriptase are: PR, protease; RT, reverse transcriptase; and RH, ribonuclease H activity. (B) Domain map for the P6 protein. Domains D1–D4 refer to specific regions of P6 investigated for self-association (Li and Leisner, 2002; Haas *et al.*, 2005). The mini TAV is the minimal region for the translational transactivation function. The two NLS sequences indicate nuclear localization signals, whereas the NES sequence is a nuclear export signal.

The P6 effector protein of CaMV: a master switch in the virus infection cycle

The P6 protein of CaMV has roles in multiple steps of the viral replication cycle. In addition, it is a pathogen effector, an elicitor of various host responses during infection (Schoelz *et al.*, 2003). Figure 2 presents a variation of an interactome map that illustrates interactions within the virus and with the

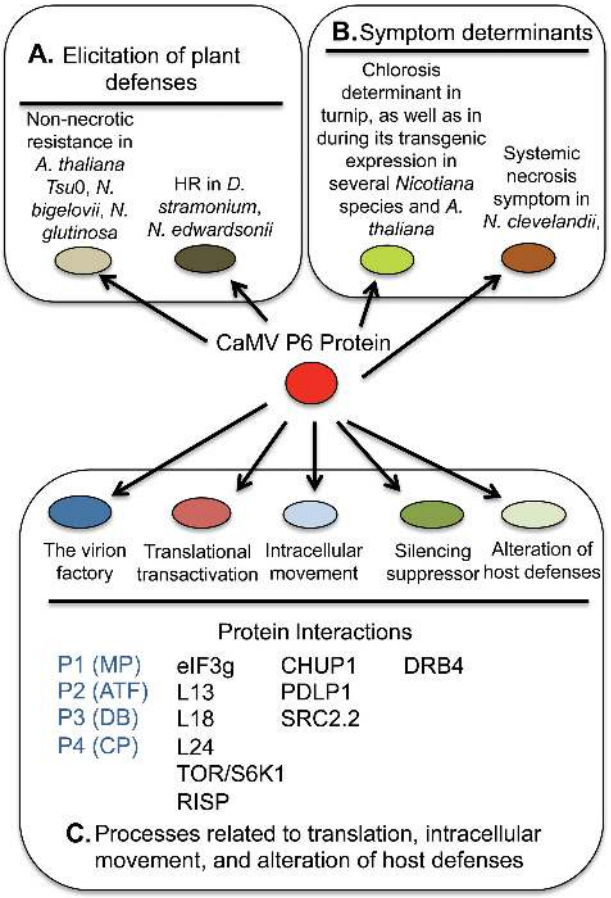

Fig. 2. The multifunctional effect of the CaMV P6 protein. (A) Role of P6 in elicitation of plant defenses and restriction of virus spread. (B) Role of P6 in symptom development. (C) Role of P6 in translation, intracellular movement, and alteration of host defenses. Interactions with host or viral proteins are listed below each of the functions associated with the interaction. Viral proteins are listed on the left.

host plant that have been characterized. In this section we will briefly trace the evolution of our knowledge of functions associated with the P6 protein to illustrate one of the major themes of this review, which is that even after its discovery decades ago there is still more to be learned about the role of the P6 protein in the CaMV infection process and its impact on host physiology.

The CaMV P6 protein was originally identified as the principal component of the amorphous, electron-dense IBs that form in CaMV-infected cells (Shockey *et al.*, 1980; Covey and Hull, 1981). In systemically infected leaves, the P6 IBs are large enough to be visible in a light microscope (Fig. 3A) and can attain a size comparable with a chloroplast. The P6 IBs of CaMV strain Cabbage-S have been observed in inoculated leaves as early as 3 d post-inoculation (DPI) as small clusters of virions embedded in a matrix of P6 protein (Xiong *et al.*, 1982). At 10 DPI, the IBs are surrounded by aggregates of P6 protein 'apparently devoid of virions', and these satellite protein masses associate with ribosomes on the endoplasmic reticulum (ER) (Xiong *et al.*, 1982). For many years, the P6 IBs were not thought to interact with any host membranes or organelles in the cytoplasm, but it was speculated as early as 1971 that they might be 'factories for virion assembly' (Martelli and Castellano, 1971). Although the P6 IBs do not have any discernible structure and are not bounded by a membrane (Fig. 3B) (Martelli and Castellano, 1971), the domains of the P6 protein responsible for self-assembly (Li and Leisner, 2002; Haas *et al.*, 2005) as well as domains for interaction with other proteins have been identified (Fig. 1B).

At approximately the time the sequence for the P6 inclusion body protein was mapped on the CaMV genome, it was discovered that the protein has a key role in eliciting plant defenses in resistant hosts and symptoms in susceptible hosts (Fig. 2A, B). In fact, CaMV P6 was the first plant viral gene to be shown by recombinant DNA techniques to trigger a hypersensitive response (HR) in resistant hosts and to elicit a specific symptom, chlorosis, in the susceptible host, turnip (Daubert *et al.*, 1984). P6 is responsible for triggering HR upon inoculation of virions to *Datura stramonium* and *Nicotiana edwardsonii* (Daubert *et al.*, 1984; Schoelz *et al.*, 1986) and a non-necrotic defense response in *Arabidopsis thaliana* ecotype Tsu0, *N. bigelovii*, and *N. glutinosa* (Fig. 2A) (Schoelz and Shepherd, 1988; Cole *et al.*, 2001). Studies with chimeric viruses have shown that sequences within the N-terminal third of the P6 protein determine whether the P6 protein will elicit or evade plant defenses (Fig. 1B).

CaMV P6 is also a prominent chlorosis determinant. CaMV strains typically cause a systemic mosaic or chlorosis in turnips; these symptoms were first associated with the P6 protein through the same set of chimeric viruses that were used to map its host range effects (Daubert *et al.*, 1984). Later on it was shown that the P6 protein elicits chlorosis independently of other viral proteins, as transgenic tobacco and Arabidopsis plants that express only P6 exhibit chlorosis and stunting (Baughman *et al.*, 1988; Goldberg *et al.*, 1991; Cecchini *et al.*, 1997; Yu *et al.*, 2003). Interestingly, not all versions of P6 elicit chlorosis. Chimeric viruses that contain P6 from the D4 strain are capable of replication and movement in turnip, but infected plants remain symptomless (Daubert *et al.*, 1984). Furthermore, transgenic Arabidopsis plants expressing only P6 protein from the D4 strain are also symptomless, in contrast to chlorosis observed in transgenic Arabidopsis that express P6 from strains CM1841, W260, or Cabb B-JI (Cecchini *et al.*, 1997; Yu *et al.*, 2003). Attempts to map the chlorosis determinant to specific domains within P6 were unsuccessful, as chimeric P6 constructs elicited intermediate levels of chlorosis (JES, unpublished). In addition to the chlorosis symptom, a systemic cell death symptom specific to *N. clevelandii* is also conditioned by P6 of CaMV strain W260 (Fig. 2B; Király *et al.*, 1999; Palanichelvam *et al.*, 2000).

Although early studies showed that P6 had a prominent role in host–pathogen interactions, it was not clear what role the protein might have in the virus infection cycle until 1989, when it was found to be essential for translation of the CaMV 35S RNA (Fig. 2C) (Bonneville *et al.*, 1989; Gowda *et al.*, 1989). The 35S RNA is different from that of most eukaryotic organisms because it is a polycistronic mRNA that encodes all six CaMV proteins. In contrast, most plant mRNAs are monocistronic, and plant ribosomes are incapable of efficient re-initiation of translation at a downstream cistron. The 35S RNA undergoes limited splicing within the CaMV genome

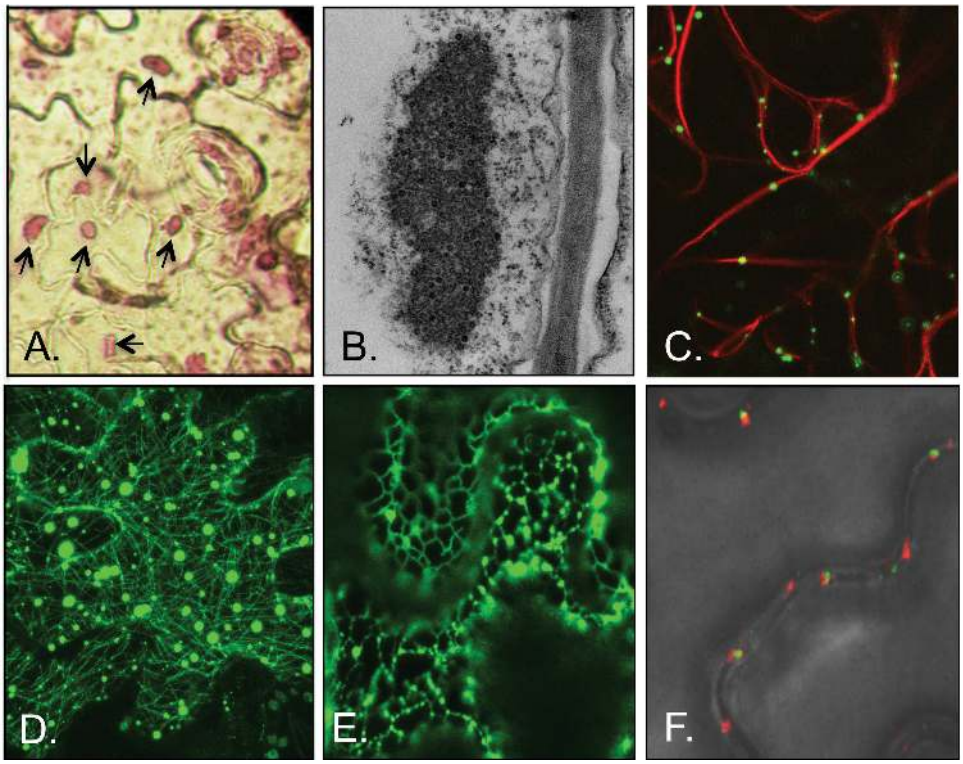

Fig. 3. Six views of P6 IBs. (A) A light micrograph of epidermal cells stained with phloxine B. The P6 IBs are stained pink and indicated by arrows. (B) An electron micrograph of a P6 IB. Individual CaMV virions can be seen embedded within the matrix and in the vacuolated regions of the IB. (C–F) Confocal micrographs of *N. benthamiana* leaves co-agroinfiltrated with a P6 construct and a subcellular marker. (C) P6 protein fused to GFP forms IBs that co-localize with actin microfilaments (labeled with dsRed–Talin). (D) P6–GFP co-expressed with the microtubule-binding domain (GFP–MDB) of mammalian MAP4. (E) P6–GFP expressed in *N. benthamiana* line 16C in which the ER is also labeled with GFP. (F) Localization of P6–RFP (red fluorescemnt protein) with the plasmodesmal protein PDLP1–GFP. (C) and (D) are reproduced with permission from Harries PA, Palanichelvam K, Yu W, Schoelz JE, Nelson RS. (2009) The *Cauliflower mosaic virus* protein P6 forms motile inclusion bodies that traffic along actin microfilaments and stabilize microtubules. Plant Physiology 149, 1005–1016, Copyright American Society of Plant Biologists (www.plantphysiol.org). (F) is reproduced with permission from Rodriguez A, Angel CA, Lutz L, Leisner SM, Nelson RS, Schoelz JE. (2014) Association of the P6 protein of Cauliflower mosaic virus with plasmodesmata and plasmodesmal proteins. Plant Physiology 166, 1–14. Copyright American Society of Plant Biologists (www.plantphysiol.org).

spanning the ORF7–P2 region (Fig. 1A), and these spliced versions have been identified in infected plants (Kiss-László *et al.*, 1995; Bouton *et al.*, 2015). However, even the mature, spliced derivatives of the 35S RNA contain more than one cistron, and it is generally accepted that most CaMV proteins may be synthesized only through re-initiation of translation of downstream cistrons. The translation of the complex, polycistronic 35S RNA presents a major problem for the success of CaMV infections.

Amazingly, the P6 protein essentially reprograms ribosomes for translation of a polycistronic message. The protein is unique in nature for this role. During initial experiments to characterize this function, P6 was co-introduced into protoplasts with a bicistronic reporter plasmid. Re-initiation of translation of the cistron in the second position only occurred when a functional copy of P6 was present in the cell. P6 was given the name of translational transactivator (or TAV) and the core region of the P6 protein responsible for translational transactivation was designated the mini TAV (Fig. 1B) (DeTapia *et al.*, 1993). The exact mechanism for the TAV function of P6 has not yet been identified—no enzymatic function for the P6 protein has been found—but P6 does physically interact with the host translational machinery. The P6 protein physically interacts with several ribosomal proteins including L13, L18, and L24, which together contribute to the large ribosomal subunit (Leh *et al.*, 2000; Park *et al.*, 2001). In addition, P6 protein interacts with a protein necessary for translation initiation (eIF3g) (Park *et al.*, 2001), a novel protein involved in re-initiation (RISP) (Thiébeauld *et al.*, 2009), and the protein kinase TOR (Target of Rapamycin), a protein that activates translation initiation in eukaryotes (Fig. 2) (Schepetilnikov *et al.*, 2011). Consequently, the TAV function of P6 may be physically to reshape eukaryotic ribosomes so they can reinitiate translation of a polycistronic message.

The P6 IBs are now considered to be 'virion factories' (Fig. 2C), specific pathogen organelles in which host and viral proteins are recruited for replication and virion assembly (de Castro *et al.*, 2013). In the case of the CaMV virion factory, the P6 protein forms the matrix for the synthesis and accumulation of the other CaMV proteins (Hohn, 2013). The matrix is believed to be held together through protein–protein interactions, as the P6 protein has been shown to interact physically with itself as well as with CaMV proteins P1, P2, P3, P4, and even P7, although P7 has not been observed during infection (Fig. 2C) (Himmelbach *et al.*, 1996; Li and Leisner, 2002; Haas *et al.*, 2005; Hapiak *et al.*, 2008; Lutz *et al.*, 2012). Lutz and co-workers found that mutations within domain D3 (Fig. 1B) that affected the self-association of P6 resulted in

smaller IBs, reduced propagation of viral DNA, and a slower rate of systemic infection (Lutz *et al.*, 2015). They speculated that mutations within domain D3 might hinder the maturation of virions or perhaps the movement of virions to plasmodesmata (PD).

P6 IBs also serve as a virion reservoir during aphid transmission (Bak *et al.*, 2013). Under stress conditions such as aphid feeding, virions are released from the P6 IBs and these virions accumulate with the P2 protein on the microtubule network (Martiniere *et al.*, 2009, 2013). At this location, the virions are more accessible for acquisition by the aphid vector. Moreover, the process is reversible; upon termination of the stress, the virions are released from the microtubules and return to the P6 IB (Bak *et al.*, 2013).

In addition to functioning as a chlorosis determinant in susceptible hosts, it is now well established that the P6 protein has a profound affect on plant defenses. This subject has been reviewed recently by Hohn (2013), but will be briefly discussed here. CaMV infection of the susceptible genus *Arabidopsis* induces alterations in the salicylic acid (SA) and jasmonic acid (JA) pathways, as well as in the accumulation of H_2O_2 (Love *et al.*, 2005), and the analysis of transgenic plants expressing P6 protein confirmed that P6 protein alone induces alterations in these pathways. Transgenic Arabidopsis plants that express P6 exhibit a suppression of SA-mediated defenses and an increased susceptibility to *Pseudomonas syringae*. The expression of NPR1, a key regulator of the SA pathway, was also increased in the transgenic plants and its subcellular localization was shifted towards the nucleus. In addition, JA-mediated defenses are enhanced in the transgenic plants, and consequently they have a reduced susceptibility to *Botrytis cinerea* (Love *et al.*, 2007*b*, 2012). The expression of P6 in transgenic Arabidopsis plants also alters the ethylene and auxin pathways (Geri *et al.*, 2004; Love *et al.*, 2007*b*). It is not clear how P6 alters host immunity; even in the case of NPR1 it is not known if P6 physically interacts with NPR1 to alter its subcellular location. However, an analysis of P6 deletion mutants showed that the N-terminal 110 amino acids are necessary but not sufficient to suppress SA-mediated defenses (Laird *et al.*, 2013).

The P6 protein has also been shown to function as a silencing suppressor (Love *et al.*, 2007*a*). A portion of the P6 protein is transported into the nucleus where it has been shown to interfere with production of siRNAs (Haas *et al.*, 2008; Shivaprasad *et al.*, 2008). P6 co-immunoprecipitates with DRB4, a host protein that is an accessory to processing of *trans*-acting siRNAs (tasiRNAs) by DCL4 (Haas *et al.*, 2008). It has been suggested that P6 interferes with the function of DRB4, perhaps by affecting the nuclear localization of DRB4 or through conformational changes in the protein (Haas *et al.*, 2008).

P6 proteins present concurrently in the cell may independently target different activities in a host (activities listed in Fig. 2), and this contention is supported by results from two independent experimental procedures. In the first procedure, deletion/mutagenesis was used to dissect P6 functions experimentally. Palanichelvam and Schoelz (2002) showed that mutations that abolish the elicitation of HR had no effect on TAV function. Similarly, mutations in the TAV domain that knocked out virus replication had no effect on the capacity of P6 to act as a silencing suppressor (Haas *et al.*, 2008; Laird *et al.*, 2013). In the second procedure, the P6 protein has been shown to interact physically with distinct host proteins necessary for translational transactivation, intracellular movement (see below), and silencing suppression (Fig. 2). Although no specific host proteins have been identified yet that interact with P6 to trigger the HR of *N. edwardsonii* or the non-necrotic defense response exemplified by *N. glutinosa*, it would not be surprising if these plant defense responses are mediated by host NBS-LRR (nucleotide-binding site-leucine-rich repeat) genes (Jones and Dangl, 2006).

A new role for P6 in intracellular movement for delivery of virions to plasmodesmata

Recently the subcellular localization of P6 has been re-evaluated due to the availability of intracellular fluorescent marker proteins and confocal microscopy. The ability to fuse proteins of interest with fluorescent marker proteins such as green fluorescent protein (GFP) allowed the tagging of the P6 protein and the visualization of its subcellular location when ectopically expressed. A P6 protein with GFP fused to its N-terminus (GFP–P6) was used to demonstrate that the P6 protein is shuttled between the nucleus and cytoplasm, and that the signals associated with import and export are essential for virus infectivity (Haas *et al.*, 2005). In a second study, GFP was fused to the C-terminus of the P6 protein (P6–GFP) and transiently expressed in *Nicotiana benthamiana*, revealing that P6 IBs formed associations with microfilaments (Fig. 3C), microtubules (Fig. 3D), and the ER (Fig. 3E), and that P6 IBs were capable of movement on microfilaments (Harries *et al.*, 2009*a*).

In any study in which a protein is tagged with a fluorescent protein such as GFP, it is important to show that the fusion does not affect the function of the protein. Harries and co-workers (2009*a*) showed that GFP fused to the C-terminus of GFP maintained TAV function comparable with that of the unmodified P6 protein. The assay for TAV function involved co-expression of CaMV P6 protein, alone or fused with GFP, with a bicistronic reporter plasmid consisting of the CaMV ORF7 in the first cistron and *GUS* (β-glucuronidase) in the second. A significant level of GUS protein was detected only when P6 or P6–GFP were agroinfiltrated with the bicistronic reporter plasmid. Complementation studies have now been completed to show that P6–GFP is able to support the expression of all viral proteins, resulting in replication and encapsidation of CaMV DNA into virions in *N. benthamiana* leaves (JES, unpublished). In this assay, the P6–GFP plasmid was co-agroinfiltrated with a full-length CaMV clone that contained a defect in its P6 gene, thus forcing the P6–GFP functionality. CaMV virions can be recovered from leaves at 7 d post-infiltration, demonstrating that P6–GFP is fully capable of supporting the development of the virion factory.

The studies with P6–GFP (Harries *et al.*, 2009*a*) also suggested a radically different view of P6 IB dynamics, both size

and motion, from most earlier research that was centered on results from the electron microscope. Most electron micrographs of the P6 inclusion bodies had been taken of mature infections in systemically infected leaves, and these plant cells typically contained one very large IB. In contrast, Harries *et al.* (2009*a*) found that at 2–4 d post-infiltration, the plant cell contains dozens of P6 IBs of varying size, some which were stationary and some which were in motion. This suggested that the P6 protein initially forms small aggregates in the cell that eventually coalesce, possibly after transport, as the infection matures. On careful analysis of some older electron microscopy data the presence of smaller IBs could be observed in plant cells during early stages of infection after inoculation with CaMV virions (Fujisawa *et al.*, 1967; Xiong *et al.*, 1982).

To investigate the nature of movement of the P6–GFP IBs, *N. benthamiana* leaves agroinfiltrated with P6–GFP were infiltrated with latrunculin B, a pharmacological agent that disrupts microfilaments. This treatment abolished the movement of ectopically expressed P6–GFP IBs and blocked the development of CaMV local lesions in the CaMV host *N. edwardsonii*. Harries *et al.* (2009*a*) suggested that P6 IBs have a key role in intracellular movement of the virus by utilizing the host cytoskeleton to deliver the CaMV virions to PD. In contrast, treatment of leaves with oryzalin had no effect on movement of P6–GFP IBs. Oryzalin is a pharmacological agent that destabilizes microtubules. Interestingly, the presence of P6–GFP IBs in a cell stabilized microtubules against the effect of oryzalin. Based on these experiments, Harries and co-workers (2009*a*) proposed that P6 IBs move on microfilaments and are responsible for delivery of virions to PD. Since the association with microtubules involves only very large IBs, it is possible that the host cell is performing an aggresome-type response in an attempt to clear the cell of the massive aggregates (Kopito, 2000).

Two subsequent studies provided important confirmation for elements of the model. Angel *et al.* (2013) showed that the P6 protein interacted with CHUP1 (for Chloroplast Unusual Positioning protein). The interaction was originally discovered in a yeast two-hybrid screen and subsequently confirmed by co-immunoprecipitation and co-localization of the P6 protein and CHUP fused with fluorescent markers. Significantly, CHUP1 anchors chloroplasts to microfilaments and is essential for movement of chloroplasts on microfilaments in response to changes in light intensity (Oikawa *et al.*, 2003, 2008). Angel and co-workers (2013) found that a truncated version of CHUP1 that blocked movement of chloroplasts (Oikawa *et al.*, 2008) also blocked the movement of P6 IBs in a transient expression assay. They suggested that the interaction of CHUP1 with CaMV P6 may explain the association of P6 with and movement on microfilaments, and the aggregation of small IBs into larger ones. However, they noted that silencing of CHUP1 in *N. benthamiana* through the use of virus-induced gene silencing only slowed the rate of CaMV lesion development rather than abolishing it, an indication that functional redundancy for intracellular movement of P6 IBs may exist.

What cellular element could be functioning in place of CHUP1 to aid P6 IB movement? In healthy plants, microfilaments work in conjunction with motor proteins (myosins) for the movement of several types of organelles within the cell. Microfilaments can be thought of as a highway, and myosins can be considered a vehicle for intracellular movement of cargo (e.g. plant organelles), anchoring organelles as they transiently bind microfilaments during their transport function. Myosins, specifically myosins VIII, XI-K, and XI-2, have been implicated in intracellular movement of viruses such as *Beet yellows virus* (Avisar *et al.*, 2008), *Turnip mosaic virus* (Agbeci *et al.*, 2013), *Grapevine fanleaf virus* (GFLV; Amari *et al.*, 2011), *Tobacco mosaic virus* (Kawakami *et al.*, 2004; Harries *et al.*, 2009*b*; Amari *et al.*, 2014), *Tomato spotted wilt virus* (Feng *et al.*, 2013), *Rice stripe virus* (Yuan *et al.*, 2011), and *Tomato bushy stunt virus* (Harries *et al.*, 2009*b*). However, none of these studies showed that inhibition of a single myosin, or even a combination of myosins, can completely block the movement of any virus. Thus, like the actin-binding CHUP1, the potential for redundant function after silencing any one myosin appears to exist for transport of these viruses.

Recently we crossed Arabidopsis T-DNA knockout lines for myosins XI-K and XI-2 with the knockout line for CHUP1 to create double and triple knockout combinations. Significant delays in infection were observed for CaMV infections of the double and triple knockout lines relative to any of the single knockout lines or the wild type Col-0 (JES, unpublished), indicating that CHUP1 and several vegetatively expressed myosins may be functionally redundant for intracellular movement of CaMV.

More recently we have determined that P6 IBs were observed adjacent to PD (Rodriguez *et al.*, 2014), and P6 physically interacted with two host proteins localized to PD, PDLP1 (for Plasmodesmatal-Located Protein 1) and AtSRC2.2 (for Soybean Response to Cold), as well as the CaMV movement protein (MP) (Hapiak *et al.*, 2008). The MPs of CaMV and GFLV are responsible for the formation of tubules that permit transport of virions to adjacent cells (Schoelz *et al.*, 2011). PDLP1 has been implicated in the cell to cell movement of CaMV and GFLV (Amari *et al.*, 2010). Both PDLP1 and AtSRC2.2 have been localized to the tubules formed by the MP in addition to interacting with the CaMV P6 protein. Interestingly the P6 protein was found adjacent to PD (Fig. 3F), suggesting that the protein itself was not transited through tubules to adjoining cells. Rodriguez and co-workers (2014) suggested that P6 IBs might form a complex with PDLP1, AtSRC2.2, and CaMV MP for delivery of virions into the tubules formed from the CaMV MP.

A model for intracellular movement: the concept of the mobile virion factory

A model for intracellular movement of CaMV was proposed by Harries and co-workers (2009*a*), and an updated version is presented in Fig. 4 based on results since that publication. One key element of the model is the linkage between replication

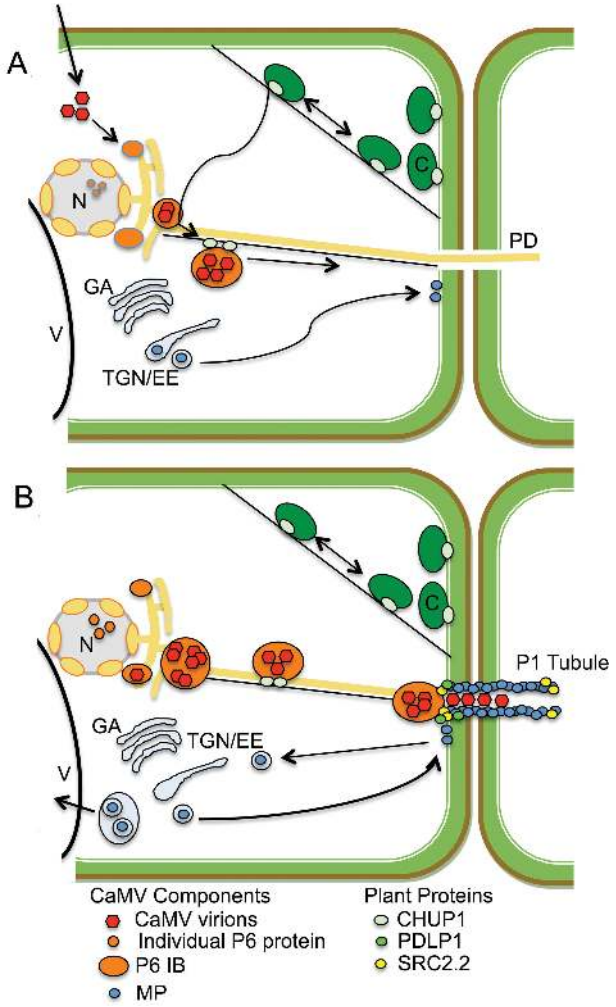

Fig. 4. Model for intracellular movement of CaMV. (A) Early events include entry of virions into the cell through either an aphid vector or mechanical inoculation, followed by synthesis of the CaMV proteins, formation of the virion factory, incorporation of CHUP1 into the virion factor, and vesicular transport of the MP to the plasma membrane. N, nucleus; C, chloroplast, PD, plasmodesmata; v, vacuole; GA, Golgi apparatus; TGN/EE, *trans*-Golgi network/early endosome. (B) Later events include formation of the tubule by the CaMV MP and docking of the virion factory for delivery of virions to the tubule.

and intracellular movement, an element also introduced for plant RNA viruses discussed in recent reviews (Schoelz *et al.*, 2011; Tilsner *et al.*, 2012, 2013; Heinlein, 2015). The model also incorporates the known strategy that CaMV uses for cell to cell movement, involving the formation of tubules composed of the CaMV MP (Perbal *et al.*, 1993; Kasteel *et al.*, 1996). The tubule strategy is also used by tospoviruses, comoviruses, nepoviruses, and bromoviruses for their intercellular movement (Schoelz *et al.*, 2011). The MPs of viruses such as CaMV dramatically restructure the PD for movement of its virions, deleting the desmotubule and increasing the diameter to allow for movement of the CaMV icosahedral virion, which has a diameter of 50 nm. In contrast, the MPs of many other viruses increase the size exclusion limit of the PD without removing the desmotubule to allow movement of an MP–RNA aggregate rather than the virion. Consequently, a model for intracellular movement of viruses transported through tubules should take into account the form in which a virus moves through PD, in this instance as a virion.

The first portion of the model is focused on the role of P6 IBs as a site for translation of the 35S RNA and for virion assembly (Fig. 4A). Harries *et al.* (2009*a*) showed that P6 IBs are associated with the ER, and, given the role of the ER in protein synthesis, it is possible that this location could facilitate the recruitment of ribosomes into the P6 IBs. As CaMV proteins accumulate within P6 there is a shift towards virion assembly; that is, the formation of the virion factory. It is likely that reverse transcription of the viral 35S RNA template into dsDNA occurs in association with virion formation (Marsh and Guilfoyle, 1987; Takatsuji *et al.*, 1992). The proper formation of the virion factory may be necessary for normal virus accumulation and spread as prevention of specific interactions between P6 protein monomers is correlated with a hinderance in the growth of the P6 IBs, reduced accumulation of CaMV DNA in inoculated leaves, and slowed systemic movement of the virus (Lutz *et al.*, 2015). Harries and co-workers (2009*a*) also noted that P6 IBs form an association with microtubules. Although P6 IBs did not appear to move on the microtubule network, they did stabilize microtubules in the presence of oryzalin (Harries *et al.*, 2009*a*). It was suggested that the microtubule network might act as a scaffold for the assembly of IBs. Alternatively, this may also represent a triggering of the host aggresome response. More research is clearly necessary to decipher what role, if any, microtubules have for intracellular movement of CaMV. In addition, P6 monomers are transported into the nucleus (Fig. 4) for suppression of the host silencing machinery (Haas *et al.*, 2008; Shivaprasad *et al.*, 2008; Hohn, 2013). It may be that this activity represents an early event in the infection cycle, when concentrations of P6 are very low and the virus uses this suppression to allow initial accumulation.

At some point in the infection cycle in our model, the P6 protein redirects CHUP1 away from its role in transport of chloroplasts to transport of the P6 IBs on microfilaments (Fig. 4A, B) (Angel *et al.*, 2013). Since silencing of CHUP1 failed to block CaMV infections, P6 IBs probably utilize alternative host proteins such as myosins for intracellular transport. However, the P6–CHUP1 interaction is significant because it is the first finding that a plant virus protein directly interacts with a host protein known to attach physically to microfilaments. Such an interaction has not been demonstrated between a plant virus protein and myosins, which are known to be important for intercellular movement of many viruses.

It is also intriguing to note that there are overlapping P6 domains for interaction with CHUP1, AtSRC2.2, and TOR (Fig. 1B). This suggests that the P6 region corresponding to Domain 2 may have the capacity to interact with a broad range of proteins to facilitate a variety of functions. On the other hand, the P6 domain responsible for interacting with the coat protein (CP) is distinct from the binding domain for CHUP1, AtSRC2.2, and TOR (Fig. 1B). This suggests that the P6 protein can serve as a link between the host proteins involved in transport and localization and the virion complex: a concept incorporated into our model (Fig. 4). Specifically, two host

proteins, PDLP1 and AtSRC2.2, were shown to interact with P6 and also to be incorporated into the tubule formed with the CaMV MP (Amari *et al.*, 2010; Rodriguez *et al.*, 2014) (Fig. 4B). P6 has also been shown to interact directly with the MP (Hapiak *et al.*, 2008). It is possible that PDLP1 and AtSRC2.2 have key roles in forming a complex at the inner opening of the tubule for delivery of virions from the P6 IBs to the tubule. The discovery that P6 IBs were found adjacent to PD (Rodriguez *et al.*, 2014) provided further evidence that the P6 IB could have a role in delivering CaMV virions to PD.

A unique element of the model for intracellular movement of CaMV is that virions are directly delivered to PD by P6 IBs, independently of MP movement (Fig. 4). This distinguishes the CaMV model from those of most other viruses. In those models, either the MP is postulated to move the viral nucleic acid, or the replication complex and MP function co-operatively in some manner to transport the viral nucleic acid to the PD (Heinlein, 2015). In contrast, the available evidence suggests that the CaMV MP moves to the PD independently of the P6 IBs. In fact, the CaMV MP is thought first to encounter CaMV virions at the PD (Stavolone *et al.*, 2005). Furthermore, Carluccio *et al.* (2014) have shown that the CaMV MP is shuttled from the *trans*-Golgi network to the plasma membrane and PD through a vesicular transport pathway and that excess MP may be recycled back to the central vacuole (Fig. 4B).

The close proximity of CaMV proteins in the virion factory to the PD may help load the particles into the P1 tubules. The CaMV CP binds to the C-terminus of P6 (Himmelbach *et al.*, 1996) as well as to the C-terminus of the P3 protein. The N-terminus of the P3 protein binds to the C-terminus of the CaMV MP, which lines the interior of the tubule. It is possible that the interactions between CaMV proteins facilitate the transfer of virions from the virion factory to the tubule. Indeed, electron micrographs have suggested that MP only co-localizes with the P3–virion complex at the entrance to or within the PD (Stavolone *et al.*, 2005). However, what triggers the release, and how the virions exit the IB is unknown. Once inside the tubule, several mechanisms could promote virion transport through the tubule to the adjacent cells. For example, the association of a P3–virion complex with MP, PDLP1, or AtSRC2.2 within the tubule could support a treadmilling mechanism for movement through the tubule.

Future directions

The P6 protein was first identified as the major constituent of the CaMV amorphous IBs and to be a host range determinant over 30 years ago (Shockey *et al.*, 1980; Covey and Hull, 1981; Daubert *et al.*, 1984). A few years later, its role in translation was revealed (Bonneville *et al.*, 1989; Gowda *et al.*, 1989) and it appeared that its function was settled as a viroplasmin/transactivator. However, in the past 10 years P6 has been shown to have a profound effect in modulating host defenses and now has been shown to have a central role in virus movement. It is clear that there is much more to be learned about the effect of P6 on host physiology, as well as more to be learned about the structure of the P6 IBs. For example, what other host proteins may be associated with the P6 virion factory? It is likely that the composition of the P6 IBs is dynamic, with the host protein composition changing as the P6 IB shifts from a role in translation and virion assembly, to suppression of host defenses, to intracellular movement, and, lastly, to docking at PD. At each step it is likely that only a fraction of the host proteins have been identified. For example, P6 has been shown to interact physically with CHUP1; however it is clear that intracellular movement is governed by host interactions that extend beyond CHUP1. Furthermore, more research is needed to understand the significance of P6-mediated interactions with subcellular components such as the ER and microtubules.

All of the research to date underscores that the CaMV P6 protein is capable of interacting with a variety of host proteins to facilitate multiple steps in the viral infection process. As a consequence of these interactions, P6 also is capable of disrupting a number of physiological processes in its host. P6 is unusual as it does not appear to have a counterpart in other plant or animal viruses (Hohn *et al.*, 2013). However, other types of plant virus proteins also have been shown to be multifunctional, with interactions with multiple host proteins. Deciphering the role of these functions and interactions involving P6 over time in the physiology of CaMV will be the central goal of future research with this virus.

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