ASSOCIATION OF THE P6 PROTEIN OF *Cauliflower mosaic virus* WITH PLASMODESMATA AND PLASMODESMAL PROTEINS

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By

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The undersigned, appointed by the dean of the Graduate School,

have examined the Dissertation entitled

ASSOCIATION OF THE P6 PROTEIN OF *Cauliflower mosaic virus* WITH PLASMODESMATA AND PLASMODESMAL PROTEINS

Presented by Andres Rodriguez

A candidate for the degree of

PhD in Plant, Insect and Microbial Sciences

And hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

I dedicate this work to my wife Sandra Valdes and my son Tomas Rodriguez-Valdes who have been there to encourage me to never give up. Valdecita, there are not enough words to thank you for your support and love all these years. Thanks for all the great moments together. “Tommy” you are more than anything my reason to never give up and keep on working to give you a better future. I love you more than anything my baby. This manuscript is all dedicated to you. Love having you in my life. I love you my family.

I also want to dedicate my dissertation to my parents Leonor Gonzalez de Rodriguez and Fernando Rodriguez Lopez because they provided me with education and values that I needed to get this far in my career. I am what I am now because of all the efforts and sacrifices you both have been through to give me the best education. I had the opportunity to give me the best caring, loving, parents I could have ever got.

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ABSTRACT

The P6 protein of Cauliflower mosaic virus (CaMV) is responsible for the formation of inclusion bodies (IBs), which are the site for viral gene expression, replication and virion assembly. Moreover, recent evidence indicates that ectopically expressed P6 IBs move in association with actin microfilaments. Since CaMV virions accumulate preferentially in P6 IBs, we hypothesized that P6 IBs have a role in delivering CaMV virions to the plasmodesmata. We recently discovered that the P6 protein interacted with a C2 calcium-dependent membrane targeting protein (designated AtSRC2-2) in a yeast two-hybrid screen and confirmed this interaction through co-immunoprecipitation and co-localization assays in the CaMV host, *Nicotiana benthamiana*. An AtSRC2-2 protein fused to RFP was localized to the plasma membrane and specifically associated with plasmodesmata. The AtSRC2-2-RFP fusion also co-localized with two proteins previously shown to associate with plasmodesmata: the host protein PDLP1 and the CaMV movement protein (MP). Since P6 IBs were found to co-localize with AtSCR2-2 and had previously been shown to interact with CaMV MP, we investigated whether a portion of the P6 IBs might also be associated with plasmodesmata. We examined the co-localization of P6-GFP IBs with PDLP1, the CaMV MP, and with aniline blue, a chemical stain for callose, and found that P6-GFP IBs were associated with each of these markers. Furthermore, a P6-RFP protein was co-immunoprecipitated with PDLP1-GFP. Our evidence that a portion of P6-GFP IBs associate with AtSRC2-2, PDLP1, and CaMV MP at plasmodesmata supports a model in which P6 IBs function to transfer CaMV virions directly to plasmodesmata.

CHAPTER I

LITERATURE REVIEW

GENOMIC STRUCTURE, REPLICATION AND EXPRESSION OF *Cauliflower mosaic virus* (CaMV)

Identification and host range of CaMV.

Cauliflower mosaic virus (CaMV) was first reported in November 1932 in a cauliflower (*Brassica aleracea* L. var. *botrytis* L.) field near Alvarado in the San Francisco Bay in California and by the mid 30's the average incidence of the disease was determined to be more than 30% in the cauliflower fields in the coastal valleys of central and southern California (Tompkins, 1937). The symptoms caused by CaMV in cauliflower were described by Tompkins, 1937. He indicated that the first symptom in an infected plant was vein clearing in the leaves, which usually started at the base of the leaf and then spread to the whole leaf. This symptom lasted for about 20 days when a "vein banding" symptom appears. It consisted of "narrow, continuous, dark-green areas parallel with and adjoining the midrib and lateral veins". Mottling could also occur in the chlorotic areas between the veins in which subsequent small and necrotic lesions could

develop. Such lesions were mostly accompanied with a slight bending of the midrib and leaf shape alteration. In cauliflower, stunted plants were observed when infection occurred in young plants. Tompkins (1937) was also responsible for identification of the vector, which he showed were aphids that reproduced on crucifers in nature. These aphids include cabbage aphid (*Brevicoryne brassicae* (L.)), false cabbage or turnip aphid (*Rhopalosiphum pseudobrassicae* (Davis)). In addition, he noted that other aphids that do not reproduce on cauliflower under normal conditions, such as the green peach aphid (*Myzus persicae* (Sulzer)) were able to transmit the virus.

The host range of CaMV was initially reported to be limited to crucifers (Tompkins, 1937; Walker *et al*, 1945). However, the different strains of CaMV can be distinguished from one another based on the capacity to move systemically in solanaceous hosts such as *Datura stramonium*, *Nicotiana bigelovii*, *N. clevelandii*, *N, edwardsonii*, *N. glutinosa* and *N. benthamiana* (Schoelz *et al*, 1986; Schoelz & Wintermantel, 1993).

Genomic Structure of CaMV.

CaMV, one of the top ten viruses studied in molecular plant pathology (Scholthof *et al*, 2011), was the first virus shown to encapsidate DNA rather than RNA (Shepherd *et al*, 1968) and the first plant virus to be completely sequenced (Franck *et al*, 1980). CaMV is the type member of the Caulimoviruses, a group of plant viruses whose genome is

composed of circular, double-stranded (ds) DNA of about 8Kb in length, which is encapsidaded into icosahedral virions approximately 50 nm in diameter (Shepherd, 1981). Its genome replicates by reverse transcription of a terminally redundant transcript and codes for six major proteins (ORFs I-VI) (Fig. 1.1; Harries *et al*, 2009a; Hass *et al*, 2002; Schoelz and Wintermantel, 1993). The ORFs in the viral genomic DNA are very compact; most of the ORFs are separated or overlap only by a small number of nucleotides. The one exception is ORF VI, which is separated by the two intergenic regions (~ 150 bp and 700 bp) that contain the promoter sequences for the 19S and 35S transcripts (Hass *et al*, 2002).

The viral genomic DNA that is encapsidated exists in an open circular form, caused by three single-stranded discontinuities (Fig. 1.1. Δ1-3) that are outcome of reverse transcription. One discontinuity (Δ1) is found in the minus strand, whereas the other (Δ2 and Δ3) are found in the plus strand (Hull and Howell, 1978; Volovitch *et al*, 1978). These discontinuities create a triple-stranded structure with an overlapping sequence of 8-20 bp between the 5' and 3' termini of the single stranded DNA.

The host RNA polymerase II is responsible for transcription of the viral DNA from the alpha strand (minus strand) into two main capped and polyadenylated transcripts that share the same 3' terminus and are responsible for translation of the viral genome (Fig. 1.1. Covey *et al*, 1981; Odell *et al*, 1981; Pfeiffer and Hohn, 1983). The 35S RNA is larger than the full-length DNA genome, with a terminal redundancy of 180 nucleotides. It serves as a replication intermediate during replication of the viral genome by reverse transcription (Guilley *et al*, 1982; Hull and Covey, 1983; Pfeiffer and Hohn, 1983). In addition, since the 35S RNA is polycistronic, it is also used as the template for translation

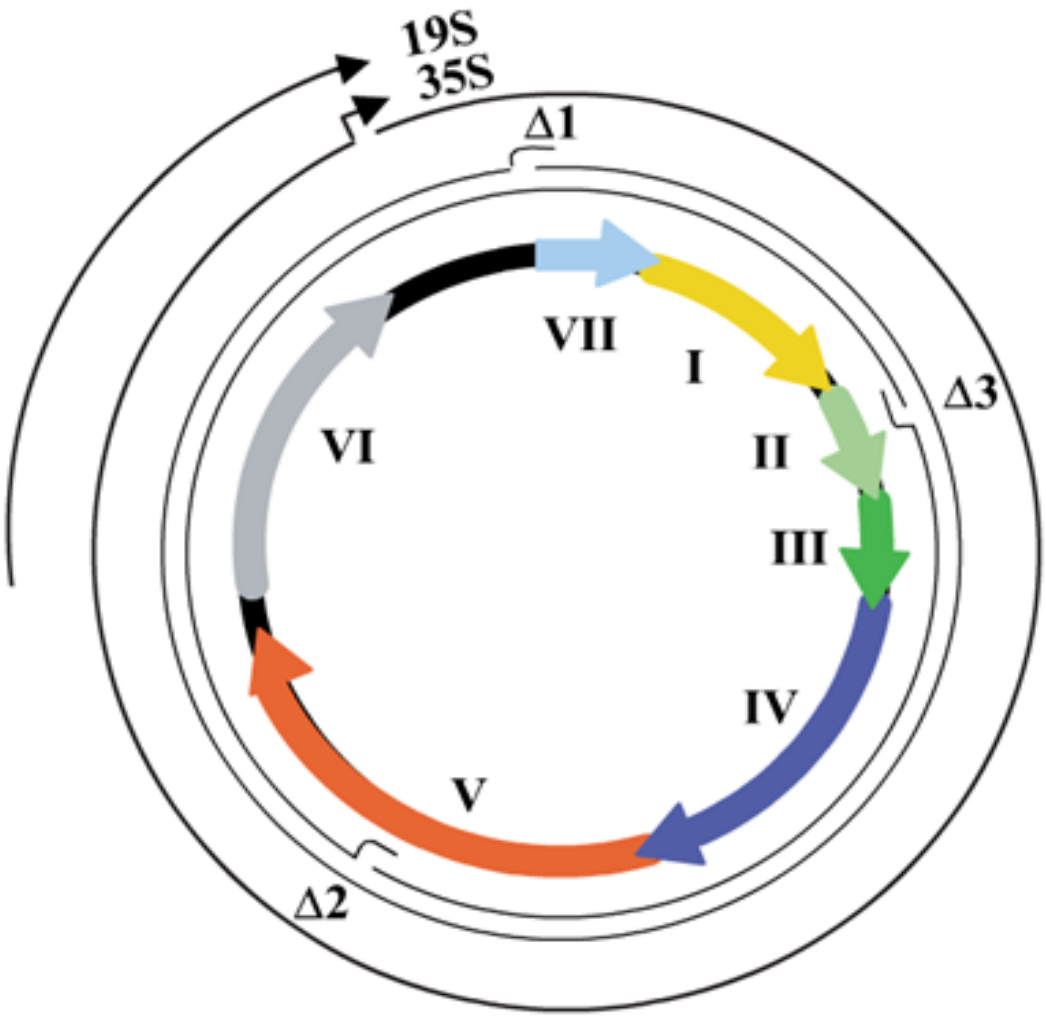

Figure 1.1. Genomic structure of CaMV. Thin lanes show the dsDNA with the respective discontinuities (Δ1-3). Colored arrows indicate ORFs I (cell-to-cell movement), II and III (aphid transmission), IV (Coat Protein), V (Precursor of proteinase, reverse transcriptase and RNAse H) and VI (Inclusion body and translational transactivator protein). Length of RNAs 19S and 35S is also shown on the outside of the circular dsDNA. Regulatory sequences for the 19S and 35S is illustrated by the black intergenic regions. (Taken from Hass *et al*, 2002). *Figure release approved by John Wiley and Sons Publishers*.

of ORFs I-V (Futterer and Hohn, 1991; Scholthof, 1992). The 19S RNA is monocistronic and its main role is to express the gene VI (Fig.1.1. Covey and Hull, 1981; Guilley *et al*, 1982; Odell and Howell, 1980).

Replication of CaMV.

The replication strategy of CaMV is similar to that of animal viruses that replicate through reverse transcription such as the retroviruses and Hepatitis B-type viruses. The model that has been proposed for the replication of CaMV (Guilfoyle *et al*, 1987; Hull and Covery, 1983; Pfeiffer and Hohn, 1983; Shoelz and Wintermantel, 1993) is illustrated in figure 1.2.

Unencapsidation of CaMV virions occurs right after their entrance into the plant cell by either mechanical inoculation or vector transmission. During the start of the infection process, the viral DNA is transported into the host nucleus (Ansa *et al*, 1982; Guilfoyle *et al*, 1983a) and the terminal repeats at the three discontinuities (Fig, 1.1. Δ1-3 and Fig. 1.2A) are digested by host endonucleases and ligated to yield a covalently closed, circular DNA that associates with histones, forming a supercoiled minichromosome that harbors approximately 42±1 nucleosomes (Hass *et al*, 2002; Menissier *et al*, 1982; Olszewski *et al*, 1982). In the next step, a host RNA polymerase II transcribes the 1.9 kb 19S and 8.2 kb 35S RNAs using the minus strand of the minichromosome as template (Fig. 1.2B. Guilfoyle *et al*, 1983a,b; Olszewski *et al*, 1982).

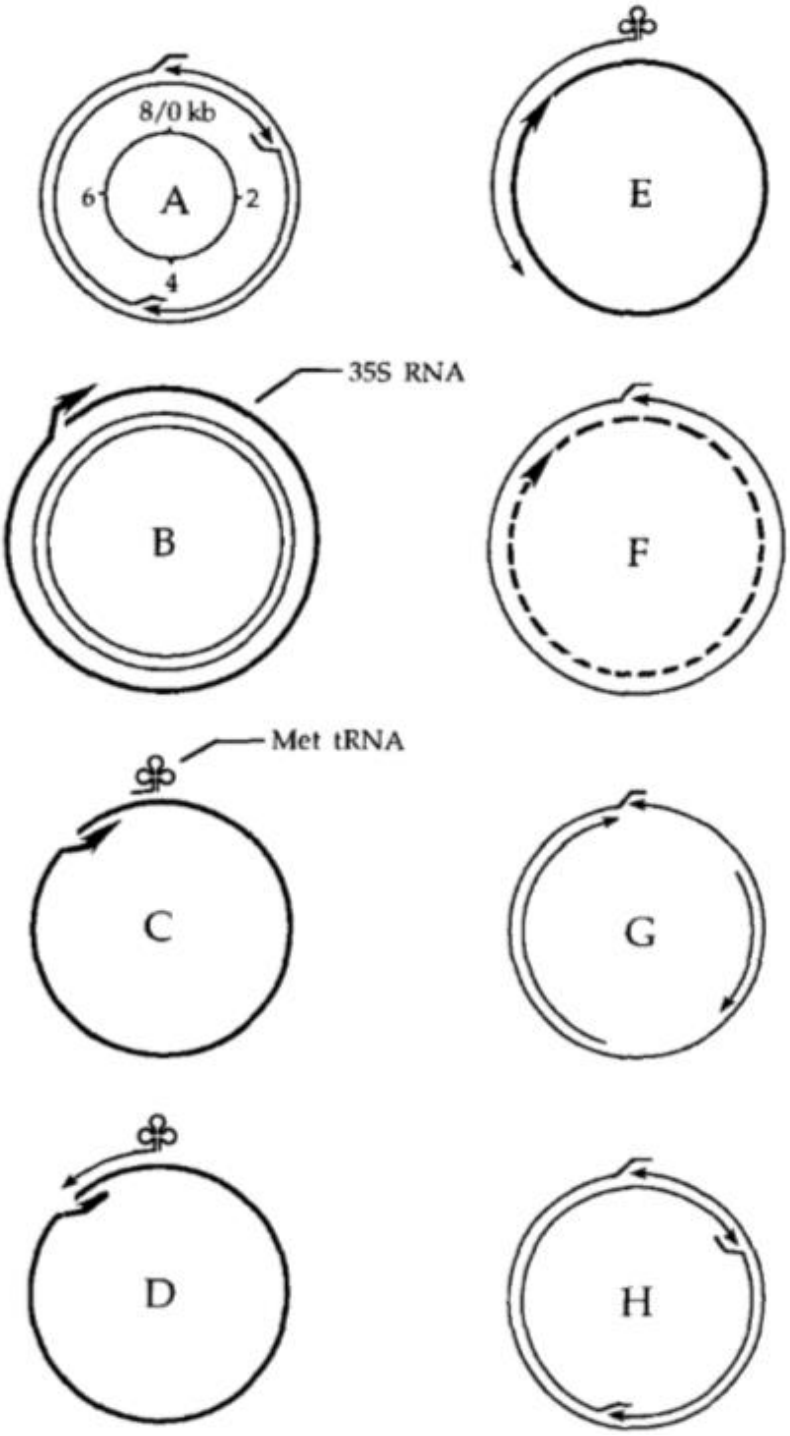

Figure 1.2. Replication of CaMV genomic material through reverse transcription (Schoelz and Wintermantel, 1993). Arrowheads indicate the 3' termini of the nucleotide sequences. The innermost circle shows the size of CaMV DNA in Kilobases. *Figure release approved by the American Society of Plant Physiologists.*

The 19S and 35s transcripts are transported from the nucleus to the cytoplasm where translation and reverse transcription occurs. The 19S RNA is translated to produce the 66kDa protein P6, the major component of the non-membrane-limited, cytoplasmic, amorphous and electron dense inclusion bodies (IBs). P6 IBs are considered to be "virion factories" where viral genome replication, protein synthesis and virus assembly/accumulation occur (Cecchini *et al*, 1997; Favali *et al*, 1973; Hohn and Fütterer, 1997; Kamei *et al*, 1969; Odell and Howell, 1980; Shockey *et al*, 1980).

Reverse transcription of the 35S RNA is believed to occur within the IBs when the 3' terminus of a host initiator $tRNA^{met}$ binds to a 15 ribonucleotide complementary sequence located 600 bp from the 5'-terminus of the 35S RNA (Fig. 1.2C. Guilley *et al*, 1983; Pfeiffer and Hohn, 1983). The $tRNA^{met}$ serves as primer for the synthesis of the minus strand of DNA. This process, carried out by the reverse transcriptase encoded by CaMV gene V, continues for the first 600 nucleotides (nt), until it reaches the 5'end of the 35S RNA (Fig. 1.2D). At this point, the ribonuclease H (RNAse H) component of the reverse transcriptase digests the 180 nt terminal repeat at the 5' terminus of the 35S RNA. The 3'end of the 35S RNA, which contains the same 180 nt terminal repeat, hybridizes with the newly formed minus DNA strand. DNA synthesis resumes and the entire minus strand is produced (Fig. 1.2F). DNA synthesis ends up at the discontinuity Δ1 with a short redundancy of the 15 nt of the $tRNA^{met}$ priming sequence (Guilfoyle, 1987; Schoelz and Wintermantel, 1993). RNAse H degrades the 35S RNA (Fig. 1.2F) and small RNA fragments bind to G-rich polypurine regions of the minus DNA strand (close to nt positions 1632 and 4218).These small RNAs serve as primers for the synthesis of the plus or beta DNA strand (Fig. 1.2G. Guilfoyle, 1987; Schoelz and Wintermantel, 1993) and

they give rise to discontinuities Δ2 and Δ3. DNA synthesis from discontinuity Δ2 is interrupted at Δ1, where a template switch occurs due to the 14 nucleotide redundancy in the minus strand. Afterwards, DNA synthesis resumes until reached Δ3, where it terminates. Similarly, DNA synthesis from Δ3 continues until it reaches Δ2 (Fig. 1.2H). The newly synthesized DNA is either encapsidated into virions or transferred into the nucleus to reinitiate the replication cycle.

Functions of CaMV Proteins I-V.

Analysis of the nucleotide sequence of different CaMV strains has shown the potential to encode for eight different ORFs (Balaza *et al*, 1982; Chenault *et al*, 1992; Franck *et al*, 1980; Gardner *et al*, 1981). However, only the protein products for ORFs I-VI have been identified and characterized *in vivo*. The protein products for ORFs VII and VIII have not been detected *in vivo*, and it is generally considered that they do not code for proteins necessary for infection (Schultze *et al*, 1990; Wurch *et al*, 1990). The 35S RNA serves as the mRNA for genes I – V. Translation of the 35S RNA is thought to take place on the surface of the P6 IBs, where polysomes have been shown to concentrate. All the viral gene products except P2 accumulate in the P6 IBs (Druker *et al*, 2002; Martinez-Izquierdo *et al*, 1987).

The P1 protein encoded by ORF I is a 40 kDa protein that facilitates cell-to-cell movement of CaMV virions. It has the capacity to bind nucleic acids and has homology

to the well-characterized 30kDa cell-to-cell movement protein of *Tobacco mosaic virus* (TMV) (Citovsky *et al*, 1991; Hull and Covey, 1985). P1 has been detected in cell wall-enriched fractions of infected turnip leaves (Albrecht *et al*, 1988) and shown to be localized to plasmodesmata of infected mesophyll cells (Linstead *et al*, 1988). P1 also has an essential role in tubule formation across the cell wall and through plasmodesmata to allow for intercellular movement of CaMV virions (Conti *et al*, 1972; Linstead *et al*, 1988; Perbal *et al*, 1993).

The P1 protein has been shown to produce long tubular structures on the surface of protoplasts infected with CaMV (Perbal *et al*, 1993; Thomas and Maule, 1995; Thomas *et al*, 1993). The protein contains a central domain that is responsible for targeting to the periphery of the cell (Huang *et al*, 2001). In addition, the N-terminus is localized at the outer side of the tubule, whereas the C-terminus is found at the inner face (Thomas & Maule 1995). Tubule formation occurs at the plasmodesma through dramatic redistribution of the desmotubule or appressed ER within plasmodesmata, leading to an increase in the pore size of the channel and allowing the passage of virions with diameters up to 50 μm (Schoelz *et al*, 2011). Nonetheless, P1 does not directly interact with CaMV coat protein. This interaction is mediated by the P3 protein of CaMV, a small 15 kDa protein necessary for intracellular movement and aphid transmission (Stavolone *et al*, 2005). The P1 protein also interacts with a protein involved in vesicle trafficking, designated as the movement protein-interacting 7 (MPI7) protein. MPI7 is a Rab acceptor homolog which belongs to the PRA1 gene family in *Arabidopsis* involved in vesicular trafficking between different cell organelles and compartments such as ER, Golgi and endosomes (Kamei *et al*, 2008; Huang *et al*, 2001). Transgenic *A. thaliana*

expressing a MPI7-GFP fusion showed cytoplasmic localization, concentrated in punctate spots. However, when MPI7-YFP was co-expressed with CFP-MP in protoplasts, a portion of CFP-MP co-localized with MPI7-YFP (Huang *et al*, 2001).

More recently, CaMV P1 has been shown to physically interact with the plasmodesmata-located protein 1 (PDLP1, Fernandez-Calvino *et al*, 2011; Amari *et al*, 2010). PDLP1, characterized initially by Thomas *et al*, 2008, is found at the plasmodesmal-associated membrane with its N-terminus in the apoplast and its C-terminus in the symplast. It is targeted to plasmodesmata via the secretory pathway in a COPII-dependent manner. Plasmodesmal localization of PDLP1 was inhibited by addition of Brefeldin A, which inhibits protein trafficking from the ER to the Golgi apparatus (Thomas *et al*, 2008; Klausner *et al*, 1992). Interestingly, Brefeldin A and cytoskeletal assembly inhibitors (i.e. oryzalin, propyzamide, cytochalasin B and taxol) were also used in *A. thaliana* protoplasts to study the localization of CaMV P1. Only treatment with Brefeldin A inhibited tubule formation by P1, but did not affect foci development (Huang *et al*, 2000).

Furthermore, confocal microscopy analyses indicate that PDLPs interact with the MPs of CaMV and *Grapevine fanleaf virus* (GFLV), at the base of tubules. Additionally, inhibition of GFLV tubule formation by its MP appears to be due to a lack of targeting of PDLP1 into plasmodesmata, when cells are treated with Brefeldin A (Amari *et al*, 2010). The authors suggest that PDLP1 provides a plasmodesma docking platform that causes the assembly of the MP into tubules (Amari *et al*, 2010). The proteins in the PDLP family are thought to be functionally redundant. For example, the authors needed to develop an *Arabidopsis pdlp1*, *pdlp2* and *pdlp3* triple mutant to obtain a significant reduction in

plants with CaMV systemic infection at 21 dpi. Furthermore, they confirmed that the reduction was caused by an impairment of intercellular movement rather than a replication problem because CaMV viral replication in the triple mutant was similar to that in the wild-type protoplasts (Amari *et al*, 2010).

The P2 protein is encoded by ORF II, has a size of 18 kDa and is the major component of the electron-lucent IBs (Espinoza *et al*, 1991). It is crucial for aphid transmission of CaMV but dispensable for virus replication (Armour *et al*, 1983). Its role in aphid transmission was discovered with the characterization of the non-aphid transmissible strain CM4-184, which has a deletion of 421 bp within the ORF II (Howarth *et al*, 1981). Furthermore, a 105 bp deletion of gene II in CaMV NY8153 also was shown to be responsible for its inability to be transmitted by aphids (Armour *et al*, 1983).

Although P2 is considered the aphid transmission factor, research performed by Leh *et al*, (1999) showed that a second CaMV protein P3, encoded by ORF III is also necessary for transmission. P3 is a 15 kDa protein that has been detected in nuclei and IBs of infected turnip (Xiong *et al*, 1984). Additionally, P3 is found intimately associated with purified CaMV virions (Dautel *et al*, 1994; Giband *et al*, 1986) and in fact, the region of P3 that interacts with virions has been mapped to amino acids 60-110 in a C-terminal domain of P3 (Leh *et al*, 2001). It had been shown that purified CaMV virions could only be transmitted by aphids that had previously acquired the P2 protein (Blanc *et al*, 1993). Leh and coworkers (1999) showed that the P3 was also associated with the "purified" CaMV virions. In fact, if the P3 protein was stripped off the virions, then they could no longer be transmitted by the P2 protein. To facilitate transmission of CaMV

virions, P3 also associates with the second alpha helix of the P2 protein. For successful transmission, P3 binds initially to virions in order to interact with P2, and this triple association is formed within the aphids (Drucker *et al*, 2002).

The precursor of the coat protein (P4) has been mapped to ORF IV. The 57 kDa protein is post-translationally processed by the viral-encoded aspartic proteinase P5, which eliminates the acidic N- and C- termini of the precursor producing three CP subspecies: p44, p39 and p37 (Torruella *et al*, 1989). The subspecies of the CP that assembles into virions is the fully processed form of 37 kDa (p37; Champagne *et al*, 2007). Previous studies have shown that the CP in virions is glycosylated (Du Pleiss and Smith, 1981) and phosphorylated (Hahn and Shepherd, 1980). In addition, phosphorylation of the precapsid protein (p57) by a host casein kinase II (CKII) at the C-terminus of the protein has been shown to be required for virus infection (Champagne *et al*, 2007). Cryo-electron microscopy and image examination analysis showed that the CP of CaMV assembles approximately 420 copies into icosahedral virions of 50 nm in diameter with a triangulation number T=7 (Chapdelaine and Hohn, 1998; Cheng *et al*, 1992; Gong *et al*, 1990).

Gene V codes for the 79 kDa P5 protein and it is a reverse transcriptase (RT). The CaMV RT has homology to the *pol* polyprotein of retroviruses, since it contains an aspartic proteinase and a RT/RNAse H domain (Toh *et al*, 1983). However, the P5 protein does not contain an integrase domain, which distinguishes it from the RTs of the retroviruses. The lack of integrase function in the CaMV RT also means that CaMV does not integrate into the chromosome of its hosts as an essential step in its replication, and for this reason CaMV is considered a pararetrovirus (Toh *et al*, 1983). In 1985, Hohn and

collaborators isolated the 79 KDa polyprotein from CaMV-infected plants, and confirmed its reverse transcriptase activity (Hohn *et al,* 1985). It was later shown that the aspartate proteinase domain is released through self-cleavage of the P5 polyprotein (Torruella *et al,* 1989).

Functions of CaMV Protein VI.

The product of ORF VI (P6) is a 62 kDa protein that is the most abundant viral protein in CaMV-infected plants (Odell and Howell, 1980). It accumulates in plant cells in the form of electron-dense, amorphous IBs that are not surrounded by a membrane (Cecchini *et al*, 1997; Fujisawa *et al*, 1967; Odell and Howell, 1980). This multifunctional protein is quite unique because it shares no significant homology with proteins from any host proteins or with proteins in other virus families. Although other CaMV and host proteins accumulate in P6 IBs, only the P6 protein is necessary for IB formation, as electron microscopic analysis of transgenic *Arabidopsis thaliana* expressing the P6 protein have IBs that are structurally comparable to those present in CaMV-infected non-transgenic *A. thaliana* cells (Cecchini *et al*, 1997).

The P6 gene was the first viral gene to be shown to be responsible for triggering a hypersensitive response (HR), in the solanaceous host *Datura stramonium* (Daubert *et al*., 1984). The role of P6 as an HR determinant was revealed through the construction of chimeric viruses between CaMV strain D4, a virus that infected *D. stramonium* systemically, and strain CM1841, a CaMV strain that triggered HR. A subsequent paper showed that P6 was also responsible for triggering HR in *Nicotiana edwardsonii*, and

additionally that the portion of the protein responsible for HR elicitation in both *D. stramonium* and *N. edwardsonii* mapped to the N-terminal third of the protein (Schoelz *et al*., 1986; Wintermantel *et al*. 1993). Some CaMV strains elicit non-necrotic resistance in such diverse hosts as *N. bigelovii* and *A. thaliana* ecotype Tsu-0; additional studies showed that the N-terminal third of P6 also determined this non-necrotic resistance response, suggesting a commonality between non-necrotic resistance and HR (Agama *et al*, 2002; Wintermantel *et al*., 1993). Ectopic expression of P6 through agroinfiltration confirmed that P6 of CaMV strain W260 functioned as an avirulence determinant in *N. edwardsonii*, whereas P6 of CaMV strain D4 did not elicit any host response (Palanichelvam *et al*, 2000).

Moreover, the chimeric viruses between strain D4 and CM1841 established that P6 is a symptom determinant in the susceptible host, turnip (*Brassica campestris*) (Daubert *et al*, 1984). The role of P6 as a symptom determinant was confirmed when it was shown that transgenic tobacco plants that express P6 exhibit a chlorotic mottling phenotype (Baughman *et al*. 1988). Transgenic *Arabidopsis thaliana* plants that express P6 exhibit chlorosis and stunting (Zilstra and Hohn, 1992). Yu and coworkers (2003) made transgenic *Arabidopsis* plants that expressed P6 from CM1841, W260, and D4 to show that the symptoms in transgenic plants are related to the symptoms in virus-infected plants. The P6 of the D4 strain does not induce any symptoms in virus infected turnips and transgenic Arabidopsis that expressed D4 P6 also were symptomless. By contrast, CM1841 and W260 both induce chlorosis in virus-infected turnips, and transgenic Arabidopsis that expressed these versions of P6 developed chlorosis and were stunted.

The P6 protein of CaMV has also been shown to be a suppressor of gene silencing and suppressor of plant defenses. CaMV was inoculated to an amplicon-silenced 35S-GFP (GxA) Arabidopsis plant and strong GFP fluorescence was observed in CaMV-systemically infected Arabidopsis. In addition, an increase in GFP expression in GxA plants was observed when they were crossed with P6-transgenic plants (Love *et al*, 2007). Furthermore, P6 alters the metabolic profile of its host by inducing or down-regulating the expression of many plant genes, including genes related to ethylene signaling (Geri *et al*, 2004; Geri *et al*, 1999; Laird *et al*., 2013; Love *et al*., 2012).

P6 also has an additional function in the cell as a nuclear shuttle protein. Transient expression of full-length and deletion mutants of P6-eGFP in *N. tabacum* BY-2 cells showed that P6 IBs formed near the nucleus and that the N-terminus of the protein, specifically a leucine-rich region, was essential for its nuclear export (Hass *et al*, 2005). Although the biological importance of P6 as a nuclear shuttle protein has yet to be elucidated, the fact the P6 also binds to RNA suggests that it might bind to the 35S RNA to export it out of the nucleus and to the nascent P6 IBs, where the other viral proteins are translated (Cerritelli *et al*, 1998; De Tapia *et al*, 1993; Harries *et al*, 2009a).

P6 also has an essential role as a translational transactivator (TAV). In this capacity, it modifies the host machinery to reinitiate the translation of ORFs I-V present on the polycistronic 35S RNA (Ryabova *et al*, 2002). De Tapia *et al* (1993) showed that a minimal portion of the P6 protein, a region of approximately 100 amino acids located near the N-terminal third of P6, contains the minimal domain required for *in vivo* translational transactivation of proteins from dicistronic RNAs. This domain is called the mini-TAV. Nonetheless, deletion of the C-terminal half of P6 impairs the TAV function

of P6, suggesting that RNA-binding domains and zinc-finger motifs found in this region might also be important (De Tapia *et al*, 1993; Palanichelvam and Schoelz, 2002). P6 associates with the host polysomes and physically interacts with the subunit g of the eukaryotic initiation factor eIF3 (Angel *et al*, 2013; Park *et al*, 2001). Likewise, the miniTAV domain of P6 interacts with the 60S ribosomal subunit proteins, L13 (Bureau *et al*, 2004) and L18 (Leh *et al*, 2000). Moreover, the ribosomal proteins L24 and eIF3 compete with each other in order to interact with a region immediately downstream of the miniTAV domain (Park *et al*, 2001).

THE ROLE OF P6 IN INTRACELLULAR TRANSPORT OF *Cauliflower mosaic virus*

Intracellular movement of plant viruses.

To infect a host, plant viruses must transport their genomes from the initial site of replication within the cell to the plasmodesmata for movement to adjacent and remote cells. Although plant viruses may contribute key proteins to this process, it is generally accepted that plant viruses need to intimately interact with a repertoire of host factors and organelles for intracellular trafficking to plasmodesmata (Nelson and Citovsky, 2005; Schoelz *et al*, 2011;Tilsner and Oparka, 2012). Initial efforts to determine the subcellular

structures involved in plant virus intracellular movement suggested the cytoskeleton and the endomembrane transport system as active players in trafficking of viral proteins and viral genomes (Boevink and Oparka, 2005; Harries *et al*, 2009a,b; Heinlein *et al*, 1995; McLean *et al*, 1995). For example, several viral movement proteins (MPs) use the endoplasmic reticulum (ER) for delivery to plasmodesmata (Ju *et al*, 2005; Haupt *et al*, 2005; Heinlein *et al*, 1998). Once the MP gets to plasmodesmata, it plays a key role in the movement of viral particles or genomic nucleic acids to neighboring cells.

Plant viruses can be classified into two broad categories according to the structural changes their MPs induce in plasmodesmata. Some viral MPs, such as the MP of TMV, increases the size exclusion limit (SEL) of plasmodesmata, therefore allowing the transfer of a MP-RNA complex to the neighboring cell. Despite the increase of the plasmodesmal SEL, only subtle structural changes are caused by the MP (Benitez-Alfonso *et al*, 2010; Niehl and Heinlein, 2011; Scholthof, 2005). In contrast, other MPs such as those of CaMV and GFLV, generate a dramatic restructuring inside of plasmodesmata by removing the desmotule, which then leads to the formation of tubules. These MPs increase the SEL to allow for the movement of virions with a diameter of up to 50 nm (Lazarowitz and Beachy, 1999; Ritzenthaler and Hofmann, 2007; van Lent and Schmitt-Keichinger, 2006).

The initial studies for plant virus cell-to-cell movement suggested that the MP is responsible for intracellular movement, as well as intercellular movement. Indeed, many models for intracellular movement have focused exclusively on the role of the MP to this step in the infection process (Ashby *et al*, 2006; Boyko *et al*, 2007; Brandner *et al*, 2008; Gillespie *et al*, 2002; Harries *et al*, 2009; Kawakami *et al*, 2004; Sambade *et al*, 2008).

However, as other studies have examined viral replication, they have shown that plant viruses replicate in association with cell membranes or in the case of CaMV, viral replication occurs within IBs. Therefore, there must be an active mechanism for transport of the viral genome from the replication site to plasmodesmata. Moreover, recent studies indicate that other viral proteins, including proteins associated with replication or gene expression may also have an important function in intracellular transport of the newly replicated viral nucleic acid to plasmodesmata.

Intracellular movement of TMV, a non-tubule-forming plant virus.

Of the viruses that do not convert plasmodesmata to tubules, TMV is probably the most intensively studied. TMV replicates within virus replication complexes (VRCs) consisting of virus-encoded 126 and 183-KDa proteins, MP, and viral RNA as well as host cellular components such as polysomes, endoplasmic reticulum (ER), β-tubulin and the elongation factor EF1-α (dos Reis Figueira *et al*, 2002; Esau and Cronshaw, 1967; Heinlein *et al*, 1998; Mas and Beachy, 1999; Shalla, 1964). VRCs appear to be the site of viral replication, gene expression and assembly (Heinlein *et al*, 1998; Mas and Beachy, 1999). Furthermore, VRC size has been related to infection severity (Liu *et al*, 2005). During TMV infection, the tubular ER is converted into large aggregates, which revert to a tubular structure as the infection matures (Reichel and Beachy, 1998). In addition,

Reichel and Beachy (1998) showed that expression of MP-GFP in absence of the virus also leads to the formation of aggregates in the ER with similar form and size.

There are several competing hypotheses for how the TMV viral RNA is trafficked within the cell to the plasmodesmata. For example, several studies have emphasized the role of microfilaments in the intracellular movement of TMV. TMV lesion size was reduced in tissues treated with the pharmacological agent Latrunculin B, a chemical that interrupts actin microfilament formation (Harries *et al*, 2009b; Kawakami *et al*, 2004; Liu *et al*, 2005). Likewise, silencing of the microfilament-associated motor protein myosin XI-2 also yielded a reduction of lesion size (Harries *et al*, 2009b).

Since other studies have shown that TMV VRCs and 126 kDa IBs move on microfilaments, it has been suggested that myosins may mediate their movement on microfilaments (Harries *et al*, 2009b; Kawakami *et al*, 2004; Liu *et al*, 2005). The 126 kDa protein was originally characterized as a replicase (Ishikawa *et al*, 1986). It is a major component of the VRCs, and in fact is able to form IBs when expressed ectopically (Ding *et al*, 2004). It is also known as a symptom determinant (Shintaku *et al*, 1996), and is essential for cell-to-cell movement (Hirashima and Watabane, 2001). Both the VRCs in virus infected plants and the IBs formed through ectopic expression of the 126 kDa associate with the ER and microtubules. In addition, they are both capable of trafficking along actin microfilaments (Heinlein *et al*, 1998; Kawakami *et al*, 2004; Liu *et al*, 2005; McLean *et al*, 1995). Association of VRC, viral RNA and MP with the ER is thought to facilitate virus cell-to-cell movement (Guenoune-Gelbart *et al*, 2008). Furthermore, the membrane-bound host protein TOM1 contributes to the association of the VRCs to the ER (Hagiwara *et al*, 2003; Yamanaka *et al*, 2000).

On the other hand, studies carried out by Lewis and Lazarowitz (2010) identified an *Arabidopsis* synaptotagmin (SYTA) that binds to the MPs of TMV and *Cabbage leaf curl virus* (CaLCuV). In this study, SYTA knockdown plants caused an inhibition in cell-to-cell spread of the TMV and CaLCuV MPs and a delay in the systemic infection of CaLCuV in Arabidopsis. The authors speculate that these MPs pirate SYTA and the early endosome machinery for virus cell-to-cell movement.

Other studies have shown that TMV does not require an association with intact microfilaments early in infection (less than 24 h), but does need them for constant virus intercellular trafficking at about 2-6 days post infection (Hofmann *et al*, 2009). Hofmann and coworkers (2009) suggest that TMV might use membranes early in the infection process and then traffic along microfilaments for intercellular movement. Furthermore, Epel *et al* (2009) suggested that TMV replicase proteins may act as a shuttle to move the viral RNA along membranes and cytoskeleton and for delivery to plasmodesmata. At this point, the MP would form a ribonucleoprotein complex that moves to neighboring cells. Another alternative is that a membrane-mediated transport system with a complex formed by the TMV MP and replicase shuttles the viral RNA to plasmodesmata and that the cytoskeleton and the replicase will transfer the VRC from the plasmodesmata back to the interior of the cell for degradation or virus replication (Schoelz, *et al*, 2011).

Intracellular movement of CaMV, a tubule-forming plant virus.

In contrast to TMV, several classes of plant viruses move intercellularly by triggering a dramatic modification of plasmodesmata into tubules. These changes include removing the desmotubule and increasing the plasmodesmal size exclusion limit (SEL) from 5 to up to 50 nm (Benitez-Alfonso *et al*, 2010; Kitajima and Lauritis, 1969; Lucas *et al*, 1993; Niehl and Heinlein, 2011). Plant viruses that use this strategy for intercellular movement include the genera *Caulimovirus, Comovirus, Tospovirus, Bromovirus,* and *Nepovirus* (Kasteel *et al*, 1997; Kitajima and Lauritis, 1969; Melchner, 2000; Perbal *et al*, 1993; Ritzenthaler *et al*, 1995; Storms *et al*, 1995; van Lent *et al*, 1991). Early studies using electron microscopy images of ultrathin sections of Chinese cabbage leaves infected with CaMV, showed the formation of tubule-like structures at plasmodesmata (Conti *et al*, 1972). Likewise, electron micrographs also showed that virions of the comovirus *Cowpea mosaic virus* (CPMV) and nepovirus *Strawberry latent ringspot virus* (SLRSV), as well as the caulimovirus, *Dahlia mosaic virus* (DMV), were found lined up within these tubule-like structures (Kitajima and Lauritis, 1969; Roberts and Harrison, 1970; van Lent *et al*, 1991). Several studies established that MPs of CaMV, GFLV, *Tomato spotted wilt virus* (TSWV) and CPMV are essential and sufficient for tubule development (Perbal *et al*, 1993; Ritzenthaler *et al*, 1995; Storms *et al*, 1995; van Lent *et al*, 1991). In fact, studies performed on protoplast expressing MPs of different viruses showed the formation of tubules on the surface of the protoplasts. In regard to CaMV, this phenomenon was demonstrated in *A. thaliana* protoplasts expressing a fusion of the green fluorescent protein (GFP) and MP (Huang *et al*, 2000). In addition, tubules were

also induced to form in insect cells that expressed the MPs of either CaMV or CPMV (Kasteel *et al*, 1996).

Although the MP of CaMV is responsible for tubule formation, the evidence in the literature suggests that it does not directly interact with CaMV virions. Instead, studies suggest that the CaMV P3 protein provides a bridge to facilitate an indirect interaction between the MP and virions (Leh *et al*, 1999; Stavolone *et al*, 2005). P3 forms a rod-shaped tetramer structure with a N-terminal coiled-coil domain that associates with a trimer of the MP and a C-terminus anchored to the virions (Leclerc *et al*, 1998; Leh *et al*, 1999; Stavolone *et al*, 2005). Stavolone and coworkers (2005) have suggested that the interaction between MP and P3 occurs only within plasmodesmata and that the virion/ P3 complex utilizes an independent pathway in the cell from the one utilized by the MP to travel to plasmodesmata (Stavolone *et al*, 2005). Consequently, there is no need for a second CaMV protein to fulfill the role for intracellular transport of the virions to plasmodesmata.

Since it is generally accepted that P6 IBs are the site of viral genome replication, translation, virion assembly and accumulation (Cecchini *et al*, 1997; Favali *et al*, 1973; Hohn and Fütterer, 1997; Kamei *et al*, 1969; Odell and Howell, 1980; Shockey *et al*, 1980), we have hypothesized that they may also play a key role in delivery of CaMV virions to plasmodesmata.

Recent studies have shown that the P6 protein exhibits many of the characteristics that would be necessary for a role in trafficking of virions to the plasmodesmata. The subcellular localization of P6 IBs was determined for the first time by Harries *et al*, (2009a), in which they showed that ectopically expressed P6-GFP formed IBs that

associated with ER, microtubules and microfilaments. They further showed that P6-GFP IBs were capable of movement on microfilaments. To investigate the role of microfilaments in CaMV infection, *N. edwardsonii* half leaves were infiltrated with the actin polymerization inhibitor latrunculin B, followed by inoculation with CaMV. Harries *et al* (2009a) found that necrotic local lesions induced by CaMV developed on the untreated half leaf, but no lesions developed on the half leaf treated with latrunculin B. This experiment suggested that the movement of P6 IBs on actin microfilaments is essential for CaMV infection (Harries *et al*, 2009a). On the contrary, pretreatment of leaves with oryzalin had no inhibitory effect on movement of P6 IBs on microtubules (Harries *et al*, 2009a).

To further investigate the role of P6 protein in intracellular movement, Angel *et al* (2013) sought to identify host proteins that physically interact with CaMV P6 through the use of a yeast two-hybrid screen. The yeast two-hybrid screen was conducted with a cDNA library of transcripts representing one-week old Arabidopsis seedlings and full-lenth P6 as a bait. One protein identified in this screen was CHUP1 (Chloroplast Unusual Positioning 1), a protein encoded by a single gene in Arabidopsis that is located to the outer membrane of chloroplasts. CHUP1 is responsible for chloroplast movement on microfilaments in response to light intensity (Oikawa *et al*, 2003; 2008). A P6-Venus protein fusion was shown to co-localize with a CHUP1-ECFP (Enchanced Cyan Fluorescent Protein) fusion *in vivo* and the interaction of these two proteins was confirmed by Co-immuniprecipitation (Co-IP). Furthermore, expression of truncated CHUP1-ECFP, which is unable to traffic chloroplasts, inhibited P6-Venus IB movement. Finally, silencing of CHUP1 in *N. edwardsonii* caused a delay in CaMV lesion formation,

suggesting that CHUP1 is able to support CaMV infection through the interaction with P6 (Angel *et al*, 2013). Collectively, these experiments suggested that CHUP1 may function in the role of moving P6 IBs on microfilaments.

Arabidopsis thaliana PROTEINS WITH POTENTIAL ROLES IN SUPPORTING THE INTRACELLULAR MOVEMENT OF *Cauliflower mosaic virus* via P6

Several host proteins have been hypothesized to have a direct or indirect association with CaMV P6. Arabidopsis proteins such as AtSRC2.2 and CHUP1 were identified in a yeast two-hybrid screen of an Arabidopsis cDNA library with CaMV P6. The PDLP proteins were initially implicated in movement of CaMV through an interaction with the CaMV MP (Amari *et al*, 2011). The myosins XI-2 and XI-K have been suggested to contribute to the intracellular movement of several RNA viruses including TMV (Harries *et al*, 2009b).

AtSRC2.2, C2-Calcium-dependent membrane targeting protein.

One of the most prominent features of AtSRC2.2 (AT3G16510) is its C2 domain. The C2 domain was first identified as one of four conserved domains of the mammalian protein kinase C (Nalefski and Falke, 1996). Many proteins with C2 domains have since been characterized, and an important fraction of them are involved in signal transduction, membrane trafficking, and the formation of Ca^{2+}-dependent phospholipid complexes (Cho and Stahelin, 2006; Shin *et al*, 2005). In regard to the structure of the C2 domain, analysis of several C2 domain-containing proteins revealed that all C2 domains share an eight-antiparallel β-sandwich connected by poorly conserved loops and calcium-binding sites at one side of the domain (Cho, 2001).

Several C2-containing proteins have been shown to associate with membranes in a calcium-dependent manner (Hurley and Misra, 2000). In plants, a large fraction of proteins that harbor the C2 domain are involved in development and defense responses (Hua *et al,*2001; Kim *et al*, 2003; 2008; Takahashi and Shimosaka, 1997; Yang *et al*, 2006; 2007).

In *A. thaliana*, the 1080 bp coding region of the gene AT3G16510 consists of one exon that encodes a 39.2 kDa protein composed of 360 amino acid residues. This protein possesses a C2 Ca2+-dependent membrane targeting domain at the N-terminus and a proline-rich region toward the C-terminus. Interestingly, it shows highest homology to the Soybean Regulated by Cold 2 (SRC2) protein characterized by Takahashi and Shimosaka (Kim *et al*, 2008). SRC2 contains a C2 domain, seven repeats rich in proline, glycine, tyrosine and glutamine and also a hydrophobic C- terminus that might bind to

membranes. SRC2 transcript levels increase up to 48h under cold stress (5°C) in cold tolerant soybean plants, whereas it peaks at 12 h in cold sensitive plants (Takahashi & Shimosaka, 1997). A pepper version of the SRC2 protein (CaSRC2-1) was shown to be upregulated under abiotic and biotic stress, but unaltered upon treatment with plant defense chemicals such as salicylic acid, jasmonic acid, ethephon and abscisic acid (Kim *et al*, 2008). Kim and collaborators (2008) showed that in the Arabidopsis proteome, two other proteins are closely related to AT3G16510 (AtSRC2.2) and are clustered within the Arabidopsis SRC2 family. These proteins are AtSRC2.1 (At1g09070) and AtSRC2.3 (At4g15755). Research performed by Oufattole and co-workers (2005) showed that AtSRC2.1 binds to the motif PIEPPPHH of a membrane protein that transits from the ER to vacuoles.

A large number of plant proteins that contain a C2- calcium dependent domain have been characterized to date. For instance, Arabidopsis BONZAI1 (BON1) and BON1-associated protein (BAP1) are C2 domain-containing proteins with a direct role in controlling cell expansion and division at low temperatures. Since BON1 is localized to the plasma membrane, it was suggested that the role of this protein in plant development could involve membrane trafficking in response to external conditions (Hua *et al*, 2001). BAP1 and BAP2 have also been shown to be inhibitors of programmed cell death triggered by biotic and abiotic stress (Yang *et al*, 2006; 2007). Furthermore, rice OsERG1a and OsERG1b are two C2 domain-containing small proteins that bind to phospholipid vesicles in a calcium-dependent manner and are translocated to the plasma membrane upon treatment with a fungal elicitor or Ca^{2+} ions. OsERG1 is suggested to have a role in plant defense signaling (Kim *et al*, 2003). SS52 is another plant protein

that contains a C2 domain and a proline-rich region, just like AtSRC2.2. These two features are present in many plant and animal membrane proteins with unknown biological function. SS52 has been shown to be induced upon wounding and when overexpressed, SS52 caused a reduction in HR local lesion size and restricted the spread of virus in ToMV-infected leaves. Since autophagy is required to restrict HR spread during plant immune response (Liu *et al*, 2005; Patel and Dinesh-Kumar, 2008), Sakamoto and coworkers (2009) suggested that SS52 restricts cell death and virus spread most likely by modulating the autophagy process. As noted above in the intracellular movement of non-tubule-forming plant viruses section, *Arabidopsis* synaptotagmin SYTA, also contains two C2 domains, C_2A and C_2B (Lewis and Lazarowitz, 2010). SYTA binds to the MPs of TMV and CaLCuV at the plasma membrane. It has been suggested that the complex is directed onto endosomes that traffic these proteins at plasmodesmata via a recapture pathway (Lewis and Lazarowitz, 2010).

CHUP1, Chloroplast Unusual Positioning 1.

In the efforts to determine the components involved in chloroplast movement in Arabidopsis, Oikawa and co-workers (2003) identified one T-DNA line and five ethyl methanesulfonate-mutagenized plants that were defective in their capacity to move choloroplasts under high-intensity white light conditions. Analysis of the full-length cDNA clone revealed a single ORF of 3015 bp that encodes for a 112 kDa protein with 1004 amino acid residues. The CHUP1 (AT3G25690) gene contains nine exons and eight

introns that encode for a protein with multiple functional domains including a small hydrophobic domain at the N-terminus that associates with the chloroplast outer envelope, followed by a coiled-coil responsible for anchoring the chloroplast to the plasma membrane. The protein also contains a leucine zipper and actin-binding domains in the N-terminal half of the protein and towards the C-terminus there is a proline-rich and a second leucine zipper. CHUP1 has been shown to bind F-actin (Oikawa *et al*, 2003; 2008). Analysis of the different domains showed that a functional CHUP1 protein is localized on chloroplasts with the N-terminus embedded in the chloroplast outer membrane and the C-terminus facing the cytoplasm. Furthermore, the coiled-coil region of CHUP1 avoids chloroplast aggregation and assists in chloroplast movement (Oikawa *et al*, 2008). Recently, CHUP1 was shown to be one host factor used by the CaMV protein P6 to traffic P6-IBs on actin microfilaments (Angel *et al*, 2013).

AT5G43900 (Myosin XI-2) and AT5G20490 (Myosin XI-K).

Myosins are motor proteins that traffic along actin microfilaments using the energy from ATP hydrolysis. Most of the myosins characterized so far have an N-terminal motor domain with ATP-hydrolysis and actin-binding sites, a neck domain with binding sites for the myosin light chains and a C-terminal tail that differs in primary structure and sizes between the different myosin classes. The neck contains the light chains that serve as a lever arm in the motor function. Based on sequence similarity, myosins are clustered into 24 different types (Foth *et al*, 2006). However, only three of

them, VIII, XI, and XIII, are specific to plants (Reddy, 2001; Yakamoto, 2007). *A. thaliana* encodes for seventeen myosins: thirteen of them belong to the myosin type XI and the remaining four are found within the myosin VIII class (Harries *et al*, 2009b; Reddy and Day, 2001). Furthermore, myosins XI (XI-A to XI-E and XI-J) and VIII (VIIIB and ATM2) have been known for being uniquely expressed in pollen, whereas myosins XI-1, XI-2, XI-I, XI-K and ATM1 are highly expressed in vegetative organs (Avisar *et al*, 2009). Plant myosins are involved in several cell processes such as cytoplasmic streaming, organelle, vesicle, and nuclear transport, membrane trafficking, cytokinesis, signal transduction and intercellular communication through plasmodesma (Avisar *et al*, 2008b; Yokota and Shimmen, 2011). In recent years, several studies have suggested the role of plant myosins in trafficking plant viruses inter- and intracellularly (Avisar *et al*, 2008a; Harries *et al*, 2009b; Yuan *et al*, 2011). Furthermore, both myosins XI-K and XI-2 have been shown to be responsible for trafficking plasmodesmal-located proteins such as PDLP1, through the secretory pathway (Amari *et al*, 2011). However, it is important to note that Arabidopsis knockout lines of individual myosins remain susceptible to viruses. Consequently, if myosins collectively are essential for the intracellular movement of viruses, there might be functional redundancies among the myosin and related microfilament-associated cargo trafficking proteins such as CHUP1.

Plasmodesmata-located proteins. PDLPs.

PDLPs were characterized almost a decade ago through a bioinformatics analysis of a partial *Arabidopsis* cell wall proteome (Bayer *et al*, 2006; Thomas *et al*, 2008). In *Arabidopsis* the PDLP family is composed of eight type-I membrane proteins with molecular masses that vary in size from 30.2 to 35.3 kDa. The N-terminus contains a signal peptide followed by a large region that includes two similar regions annotated as domains of unknown function 26 (DUF26), a transmembrane domain and a C-terminal tail. The N-terminal portion of the PDLPs faces the apoplast (Thomas *et al*, 2008). Furthermore, the use of chemical inhibitors showed that PDLP1-GFP is delivered to plasmodesmata via the secretory pathway (Thomas *et al*, 2008).

In 2010, Amari and coworkers (2010) showed that PDLPs also act as receptors for MPs of tubule-forming plant viruses such as GFLV and CaMV. It was hypothesized that the PDLPs aid in the assembly of plant virus tubules and promote viral cell-to-cell movement (Amari *et al*, 2010; Ritzenthaler, 2011). Similar to the myosins, knock out lines of individual PDLPs did not appear to have a negative effect on tubule formation by the viral MPs. The authors created a triple knockout line composed of *pdlp1*, *pdlp2*, and *pdlp3* and found that this line was less susceptible to CaMV infection, as assessed through a reduction in the number of systemic infected plants that developed systemic symptoms relative to the wild-type Col-0. However, even with this triple mutant, CaMV systemic infection was not completely abolished (Amari *et al*, 2010).

Recently, it was shown that PDLP5, also known as HOPW1-1-Induced Gene1, is localized to the central region of the plasmodesma channel and inhibits symplast

trafficking at plasmodesmata through plasmodesmata closure (Lee *et al*, 2008). Furthermore, PDLP5 bridges the crosstalk between salicylic acid-dependent defense responses and plasmodesmata regulation. Overexpression of PDLP5 leads to cell death and chlorosis, as a consequence of salicylic acid production. (Lee *et al*, 2011). However, it is hard to say whether this is a direct or indirect effect caused by the overexpression of PDLP5. PDLP5 expression was shown to increase dramatically upon infection with *Pseudomonas syringae* pv. *maculicola* (Lee *et al*, 2008; Lee *et al*, 2011). Furthermore, bacterial growth in a *pdlp5* mutant plant was increased more than 10-fold at 3 days after inoculation, suggesting a role of PDLP5 in increasing innate immunity against *Pseudomonas syringae* pv. *maculicola* in a salicylic acid-dependent manner (Lee *et al*, 2011).

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CHAPTER II

ASSOCIATION OF THE P6 PROTEIN OF *Cauliflower mosaic virus* WITH PLASMODESMATA AND PLASMODESMAL PROTEINS.*

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INTRODUCTION

Through the years numerous studies have focused on the characterization of viral replication sites within the cell, as well as how plant virus movement proteins modify the plasmodesmata to facilitate cell-to-cell movement (reviewed in Benitez-Alfonso *et al*., 2011; Laliberté and Sanfaçon, 2010; Niehl and Heinlein, 2011; Ueki and Citovsky, 2011; Verchot *et al*., 2012). It is accepted that plant virus replication is associated with host membranes, and at some point, the viral genomic nucleic acid must be transferred from the site of replication in the cell to the plasmodesmata. This step could involve transport from a distant site within the cell, or alternatively, it may be that replication is coupled with transport at the entrance of the plasmodesmata (Tilsner *et al*., 2013). However even with the latter model, there is ample evidence that the viral proteins necessary for replication or cell-to-cell movement utilize intracellular trafficking pathways within the cell to become positioned at the plasmodesma. These pathways may involve microfilaments, microtubules, or specific endomembranes that participate in

macromolecular transport pathways, or combinations of these elements (Harries *et al*., 2010; Liu and Nelson, 2013; Patarroyo *et al*., 2013; Peña and Heinlein 2013; Schoelz *et al*., 2011; Tilsner and Oparka 2012).

The P6 protein of *Cauliflower mosaic virus* (CaMV) is one viral protein that had not been considered to play a role in viral movement until recently. P6 is the most abundant protein component of the amorphous, electron dense IBs present during virus infection (Odell and Howell, 1980; Shockey *et al*., 1980). Ectopic expression of P6 in *Nicotiana benthamiana* leaves resulted in the formation of inclusion-like bodies (I-LBs) that were capable of intracellular movement along actin microfilaments. Furthermore, treatment of *N. edwardsonii* leaves with latrunculin B abolished the formation of CaMV local lesions, suggesting that intact microfilaments are required for CaMV infection (Harries *et al*., 2009a). A subsequent paper showed that P6 physically interacts with CHUP1, a plant protein localized to the chloroplast outer membrane that contributes to movement of chloroplasts on microfilaments in response to changes in light intensity (Angel *et al*., 2013; Oikawa *et al*., 2003; 2008). The implication was that P6 might hijack CHUP1 to facilitate movement of the P6 IBs on microfilaments. Silencing of CHUP1 in *N. edwardsonii*, a host for CaMV, slowed the rate of local lesion formation, suggesting that CHUP1 contributes to intracellular movement of CaMV (Angel *et al*., 2013).

In addition to its role in intracellular trafficking, the P6 protein has been shown to have at least four distinct functions in the viral infection cycle. P6-containing IBs induced during virus infection are likely "virion factories", as they are the primary site for CaMV protein synthesis, genome replication, and assembly of virions (Hohn and

Fütterer, 1997). Second, P6 interacts with host ribosomes to facilitate re-initiation of translation of genes on the polycistronic 35S viral RNA, a process called translational transactivation (TAV) (Bonneville *et al*., 1989; Park *et al*., 2001; Ryabova *et al*., 2002). The miniTAV region of P6 (Fig. 2.1) defines the essential sequences required for translational transactivation (DeTapia *et al*., 1993). Third, P6 is an important pathogenicity determinant. P6 functions as an avirulence determinant in some solanaceous and cruciferous species (Daubert *et al*., 1984; Hapiak *et al*., 2008; Schoelz *et al*., 1986), and is a chlorosis symptom determinant in susceptible hosts (Baughman *et al*., 1988; Cecchini *et al*., 1997; Daubert *et al*., 1984; Goldberg *et al*., 1991). Finally, P6 has the capacity to compromise host defenses, as it is a suppressor of RNA silencing and cell death (Haas *et al*., 2008; Love *et al*., 2007), and it modulates signaling by salicylic acid, jasmonic acid, ethylene and auxin (Geri *et al*., 2004; Laird *et al*., 2013; Love *et al*., 2012). Domain D1 of P6 has been shown to be necessary but not sufficient for suppression of silencing and SA-mediated defenses (Laird *et al*., 2013).

Since P6-containing IBs are the site for virion accumulation and they are capable of movement, they may be responsible for delivering virions to the CaMV movement protein (MP) located at the plasmodesmata (reviewed in Schoelz *et al*., 2011). The vast majority of CaMV virions accumulate in association with P6-containing IBs. Furthermore, P6 physically interacts with the CaMV capsid and MP, as well as the two

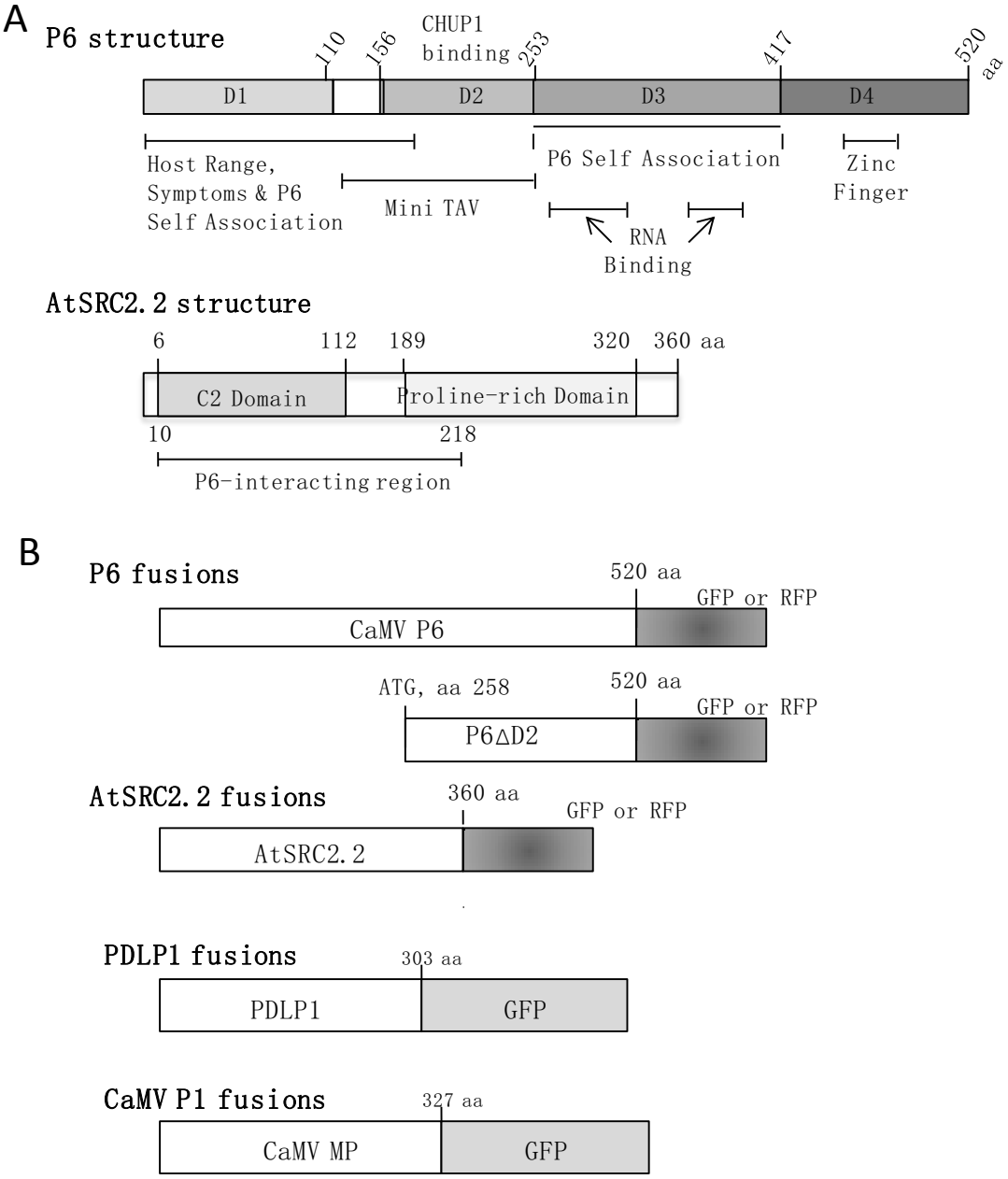

Figure 2.1 CaMV and host constructs used for confocal microscopy or co-immunoprecipitation. (A) Structure of CaMV P6 and AtSRC2.2 proteins. The functions of P6 domains D1-D4 tested for interaction with AtSCR2.2 are indicated by the shaded boxes. The mini TAV is the minimal region for the translational transactivation function. (B) Structure of P6 (Angel *et al*. 2013), AtSRC2.2, PDLP (Thomas *et al*., 2008), and CaMV MP fusions developed for confocal microscopy and/or co-immunoprecipitation.

proteins necessary for aphid transmission, P2 and P3 (Hapiak *et al*., 2008; Himmelbach *et al*., 1996; Lutz *et al*., 2012; Ryabova *et al*., 2002). Recent studies have indicated that P6 IBs serve as a reservoir for virions, in which the virions may be rapidly transferred to P2 electron lucent inclusion bodies for acquisition by aphids (Bak *et al*., 2013). It stands to reason that P6 IBs may also serve as a reservoir for CaMV virions to be transferred to the CaMV MP in the plasmodesmata.

CaMV virions move from cell to cell through plasmodesmata modified into tubules through the function of its MP (Kasteel *et al*., 1996; Perbal *et al*., 1993). However, studies have suggested that CaMV virions do not appear to directly interact with the MP. Instead, the MP interacts with the CaMV P3 protein (also known as the virion-associated protein or VAP), which forms a trimeric structure that is anchored into the virions (Leclerc *et al*., 1998; Leclerc *et al*., 2001). Electron microscopy studies have indicated that MP and VAP colocalize with virions only at the entrance to or within the plasmodesmata, and it has been suggested that the VAP/virion complex travels to the plasmodesmata independently from the MP (Stavalone *et al*., 2005). Consequently, there is a need for a second CaMV protein such as P6 to fulfill the role of delivery of virions to the plasmodesmata (Schoelz *et al*., 2011).

Additional studies have shown that the CaMV MP interacts with a family of plasmodesmal proteins, the PDLP proteins (Amari *et al*., 2010), as well as MPI7, an Arabidopsis protein that has been suggested to have a role in vesicular trafficking (Huang *et al*., 2001). The PDLPs comprise a family of eight proteins that are associated with plasmodesmata (Amari *et al*., 2010). In addition to its interaction with CaMV MP, PDLP1 interacts with the 2B protein of GFLV at the base of tubules formed by the 2B

protein. Furthermore, an Arabidopsis T-DNA mutant line in which three *PDLP* genes had been knocked out (*pdlp1-pdlp2-pdlp3*) responded to GFLV and CaMV inoculation with a delayed infection (Amari *et al*., 2010). This has led to the suggestion that the PDLPs might act as receptors for the MPs of the tubule forming viruses such as GFLV and CaMV (Amari *et al*., 2010; 2011).

To better understand the function of the P6 protein during CaMV intracellular movement, we have utilized a yeast two-hybrid assay to identify host proteins that interact with CaMV P6. We show that P6 physically interacts with a C2-calcium dependent protein (designated AtSRC2.2). AtSRC2.2 is a membrane-bound protein that is capable of forming punctate spots associated with plasmodesmata. The localization of AtSRC2.2 with plasmodesmata led to an analysis of interactions between P6 I-LBs, AtSRC2.2, PDLP1 and the CaMV MP, and also revealed that a portion of P6 I-LBs are found adjacent to plasmodesmata. These results provide further evidence for a model in which P6 IBs are capable of delivery of virions to plasmodesmata for their transit to other host cells.

RESULTS

CaMV P6 protein interacts with a C2-Calcium dependent membrane targeting protein (AtSRC2.2)

We previously utilized a yeast two-hybrid screen developed by Hybrigenics Services (Paris, France) to identify Arabidopsis proteins that interact with CaMV P6. The bait consisted of the full-length sequence of CaMV P6 from strain W260 (Wintermantel *et al*., 1993), and the prey consisted of proteins produced from an *A. thaliana* cDNA library representing transcripts from one-week old seedlings. Of the 85 Arabidopsis clones that interacted with P6, one was identified as CHUP1, and we were subsequently able to confirm the interaction between this protein and P6 by co-immunoprecipitation (co-IP) and co-localization *in vivo* (Angel *et al*., 2013).

An additional 17 clones were categorized in the Hybrigenics screen as encoding a C2-calcium dependent protein (AT3G16510). The C2-domain is found in proteins that form Ca^{2+}-dependent phospholipid complexes and are involved in signal transduction and membrane trafficking (Zhang and Aravind, 2010). AT3G16510 encodes a protein of 360 amino acids (Fig. 2.2A) that was previously reported to be homologous to SRC2 (for Soybean Genes Regulated by Cold (Takahashi and Shimosaka, 1997), and was designated AtSRC2.2 in earlier publications (Kim *et al*., 2008; Zhang *et al*. 2013). Previous analysis of AtSRC2.2 indicated that it belongs to a small gene family in Arabidopsis consisting of at least three members (Table 2.1; Fig. 2.2B) (Kim *et al*., 2008;

A

MANLTLELNVYSAKDLENVNLITKMDVYAVVWITGDDSRKNHKEKTPIDR 50
TGESEPTWNHTVKFSVDQRLAHEGRLTLVVKLVCDRIFGDKDLGEVQVPV 100
LELLHGSSSPSSNGDGQGMMRFVTYQVRTPFGKGQGSLTFSYRFDSPTFK 150
PDQPVSSHVFHQDPPVSSSHVYTNPMDIPSDFSSATTNYPPPQSSEANFY 200
PPLSSIGYPPSSPPQHYSSPPYPYPNPYQYHSHYPEQPVAVYPPPPPSAS 250
NLYPPPYYSTSPPQHQSYPPPPGHSFHQTQPSQSFHGFAPSSPQNQHGYG 300
YPPPTSPGYGYGCPTTQVPPKNNNNKPGLGLGLGVGAGLLGGALGGLLIS 350
DIVSDIGFDF 360

B

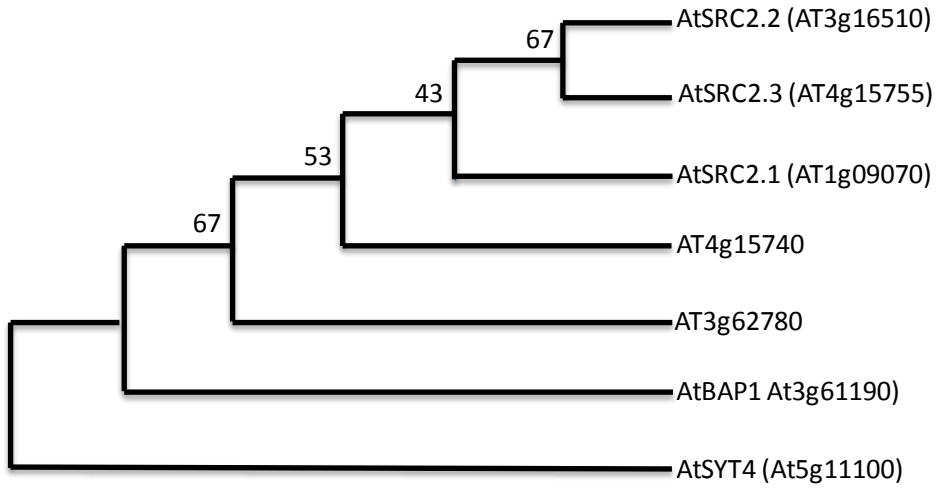

Figure 2.2 Primary amino acid sequence of AtSRC2.2 and structure of the AtSRC2 gene family. (A) Primary amino acid sequence of AtSRC2.2 protein. The sequence in red was the portion shown to interact with CaMV P6 in the initial two hybrid screen. (B) Phylogenetic analysis of the AtSRC2.2 gene family, as well as other, more distantly related Arabidopsis genes. The sequence in common to all of these genes is the C2 domain (underlined). Phylogenetic tree is based on protein sequence.

Table 2.1 Arabidopsis proteins that share homology with AtSRC2.2

Description[a]	Coverage	Identity	E value	Accession
AtSRC2.2 (At3g16510)	**100%**	**100%**	**0.0**	**NP188272.1**
AtSRC2.3 (At4g15755)	**50%**	**56%**	**8e-51**	**NP680701.1**
AtSRC2.1 (At1g09070)	**46%**	**43%**	**2e-32**	**CAA07573.1**
C2 domain containing (At4g15740)	**40%**	**45%**	**2e-29**	**NP193309.1**
C2 domain containing (At3g62780)	**48%**	**38%**	**3e-24**	**NP191837.1**
C2 domain containing (At2g33320)	37%	35%	1e-12	NP180890.1
C2 domain containing (At2g13350)	37%	32%	6e-11	NP178968.1
C2 domain containing (At1g04540)	37%	32%	6e-11	NP171948.1
C2 domain containing (At3g04360)	37%	31%	8e-09	NP566225.4
C2 domain containing (At3g05440)	27%	29%	1e-06	NP187195.1
C2 domain containing (At4g01200)	38%	27%	5e-04	NP192029.1
Bon Association Protein 1 (At3g61190) (BAP1)	**39%**	**27%**	**5e-04**	**NP567111.1**
C2 domain containing (At1g63220)	26%	31%	0.005	NP176511.4
C2 domain containing (At5g23950)	16%	35%	0.020	NP197783.1
C2 domain containing (At3g55470)	29%	28%	0.033	NP001078296.1
PTPA family protein (At4g08960)	36%	30%	0.094	NP567342.1
Synatotagmin-4 (AtSYT4) (At5g11100)	**28%**	**36%**	**0.23**	**NP196671.2**

[a]Genes in bold are included in the phylogenetic tree in Fig. 2.2B)

Zhang *et al*. 2013), and has plant and animal orthologs (Kim *et al*., 2008; Zhang *et al*., 2013). In the Hybrigenics yeast two-hybrid assay, the portion of AtSRC2.2 that interacted with P6 contained the C2 domain, the N-terminal region of the proline-rich domain and the intervening sequence between these two domains (Fig. 2.1, Fig. 2.2A).

To identify the specific regions of P6 that interacted with AtSRC2.2 in a second yeast two-hybrid assay, we utilized a series of deletion mutants in which P6 was divided into four domains (Fig. 2.1A; Li and Leisner, 2002). Yeast cells co-transformed with the full length P6 fused to the LexA DNA-binding domain and full-length AtSRC2.2 fused to the B42 activation domain grew on media lacking leucine and they expressed a low but detectable level of β-galactosidase (Fig. 2.3A). When the four domains of P6 were screened, yeast co-transformed with either domains D2 or D4 and AtSRC2.2 grew on media lacking leucine. However, the highest level of β-galactosidase activity was observed with the D2-AtSRC2.2 combination (Fig. 2.3B). Interestingly, no interaction was observed between domain D3 and AtSRC2.2. Since domain D3 was shown previously to interact with both the eukaryotic initiation factor 3 subunit g (eIF3g) and large ribosomal subunit protein L24 (Park *et al*., 2001), this result suggests that the TAV function of P6 differs from the domain responsible for the interaction with AtSRC2.2.

P6-GFP co-localizes and co-immunoprecipitates with AtSRC2.2-RFP.

To investigate whether P6 and AtSRC2.2 co-localized *in vivo*, the full-length cDNA of AtSRC2.2 from *A. thaliana* was fused at its 3' end with the red fluorescent

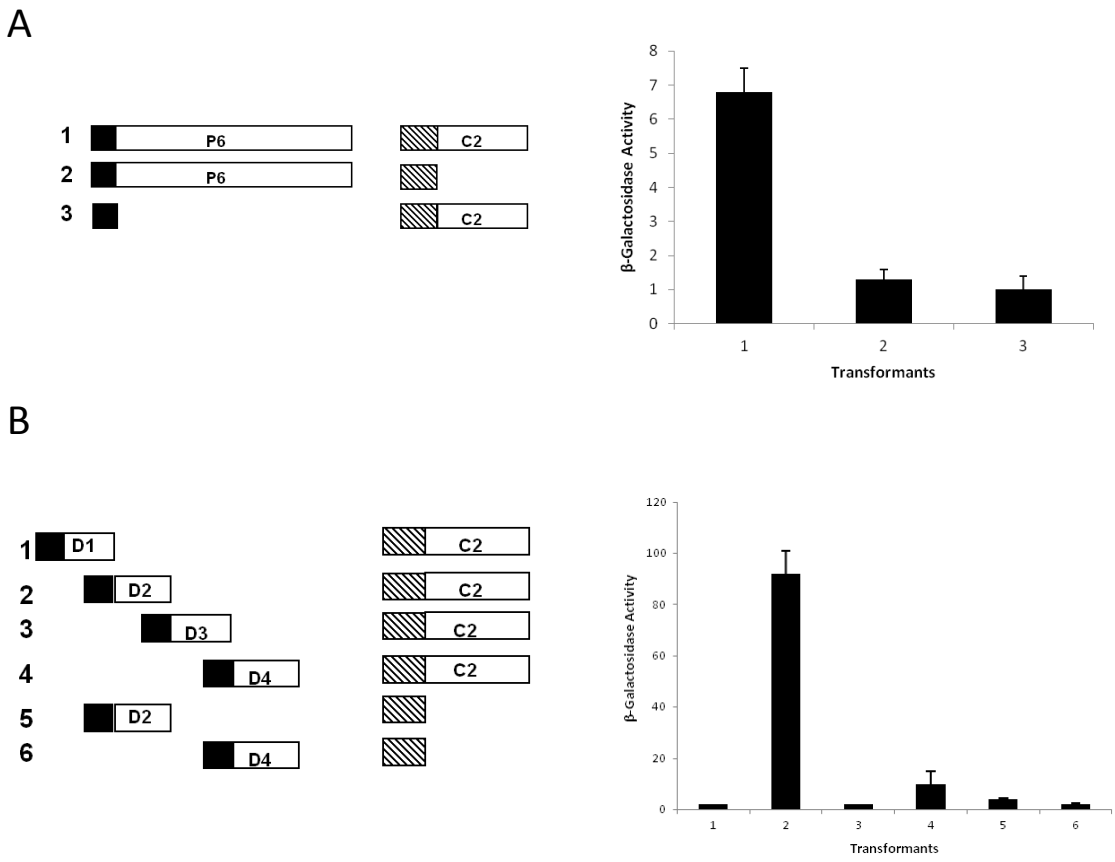

Figure 2.3 AtSRC2.2 preferentially interacts with D2 of P6 in a yeast two-hybrid analysis. (A) Interaction of full-length AtSRC2.2 with the full-length CaMV P6. (B) Interaction of full-length AtSRC2.2 with CaMV P6 domains. Numbers in the x-axis of each bar graph represent the different transformant combinations illustrated in the schematic diagrams at the left. In the schematic diagrams, striped box represent the transcriptional activator domain and black boxes show the DNA-binding domain.

protein (RFP) coding sequence and cloned into an *A. tumefaciens* expression pSITE vector (Fig. 2.1B, Chakrabarty *et al*., 2007, Martin *et al*., 2009). To visualize P6 I-LBs, we utilized P6-GFP, a construct in which the C-terminus of the full-length P6 coding sequence of CaMV strain W260 was fused with GFP (Fig. 2.1B, Angel *et al*., 2013; Harries *et al*., 2009a). Upon co-agroinfiltration of AtSRC2.2-RFP with P6-GFP into leaf panels of *Nicotiana benthamiana*, the AtSRC2.2-RFP protein was widely distributed throughout the cell, but often could be visualized as punctate spots (Fig. 2.4A). We also observed well-defined P6-GFP I-LBs of varying sizes (Fig. 2.4B), in agreement with previous studies (Angel *et al*., 2013; Harries *et al*., 2009a). We found that of 725 P6-GFP I-LBs examined, 32.7% also contained AtSRC2.2 (Fig. 2.4C). This study showed that AtSRC2.2-RFP could be present within P6 I-LBs.

To further examine whether P6 interacts with AtSRC2.2, we utilized a co-IP assay. *N. benthamiana* plant tissues were agroinfiltrated with P6-GFP, AtSRC2.2-RFP, or the combination of P6-GFP with AtSRC2.2-RFP, and protein extracts from infiltrated tissues were either used immediately for western blots or incubated with antibody against GFP immobilized onto sepharose beads. For the western blots, both P6-GFP and AtSRC2.2-RFP were readily detected when expressed individually or when co-expressed (Fig. 2.5A and B). Furthermore, antibodies to GFP and RFP did not cross react with each other at levels that would influence the co-IP results (Fig. 2.5A, B and D). For the co-IP, the sepharose beads were washed extensively to remove any unbound proteins, and then the bound proteins were eluted from the beads and analyzed by western blot with antibodies to RFP or GFP. A protein of about 70 kDa corresponding to AtSRC2.2-RFP was detected upon co-immunoprecipitation with P6-GFP (Fig. 2.5C Lane 4), but was not

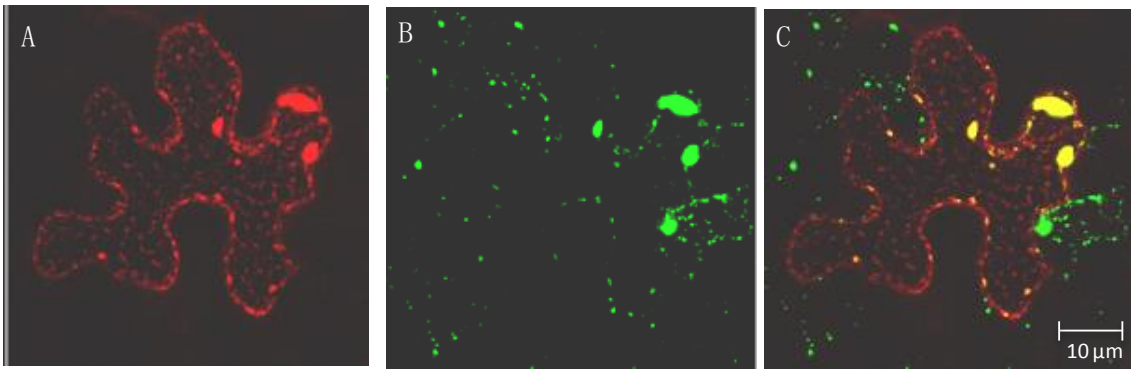

Figure 2.4 AtSRC2.2-RFP is co-localized with P6-GFP upon co-agroinfiltration. (A) Expression of AtSRC2.2-RFP. (B) Expression of P6-GFP in the same cell as A. (C) Overlay of panels A and B. Picture was taken at 3 dpi.

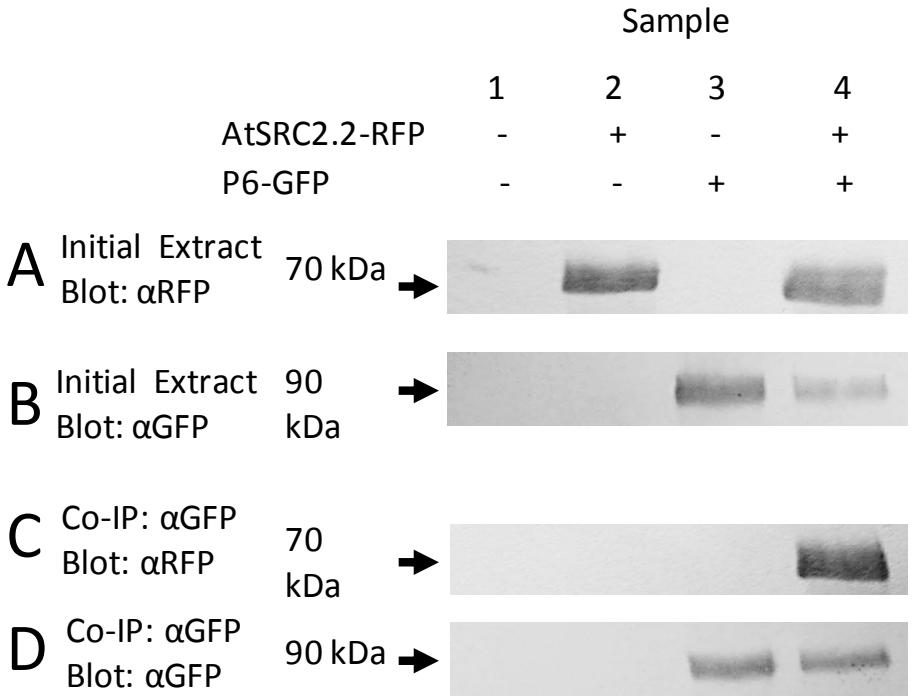

Figure 2.5 Co-immunoprecipitation of AtSRC2.2-RFP with P6-GFP after co-agroinfiltration of *N. benthamiana* leaves. Lane 1, Mock-inoculated control leaf; Lane 2, Expression of AtSRC2.2-RFP; Lane 3, Expression of P6-GFP; Lane 4, Co-expression of AtSRC2.2-RFP and P6-GFP. (A) Western blot for total proteins input probed against RFP antibodies. (B) Western blot for total protein input probed against GFP antibodies. (C) Western blot for proteins immunoprecipitated using GFP antibodies and probed against RFP antibodies. (D) Western blot for proteins immunoprecipitated using GFP antibodies and probed against GFP antibodies.

detected when P6-GFP was omitted from the co-immunoprecipitation assay (Fig. 2.5C Lane 2). With these results, we concluded that the co-immunoprecipitation of AtSRC2.2-RFP is dependent on its association with P6-GFP.

To examine the domain within P6 that interacts with AtSRC2.2 during co-IP analysis, the first 253 amino acid residues of P6 were deleted, a region corresponding to domains D1 and D2, and this construct was fused at its C-terminus to GFP (Fig. 2.1B, P6ΔD2-GFP). Agroinfiltration of P6ΔD2-GFP into *N. benthamiana* leaves resulted in the production of a 55 KDa protein that was detected in a western blot using anti-GFP antibodies. In contrast to the full-length P6-GFP protein, a co-IP showed that the P6ΔD2-GFP protein was unable to associate with the AtSRC2.2-RFP fusion protein (Fig. 2.6). These results are consistent with those from the yeast two-hybrid screen, which showed that the interaction of AtSRC2.2 with P6 is primarily dependent on the D2 domain of P6 (Fig. 2.3).

AtSRC2.2 localizes to the plasma membrane, and co-localizes with the plasmodesmata markers PDLP1 and CaMV MP.

The annotated sequence of AtSRC2.2 indicated that it might be associated with membranes. To investigate whether AtSRC2.2 was localized to the plasma membrane or the cytoplasm, AtSRC2.2-RFP was co-agroinfiltrated with free GFP in *N. benthamiana* leaves and cells were plasmolyzed. In this experiment, free GFP could be observed in the

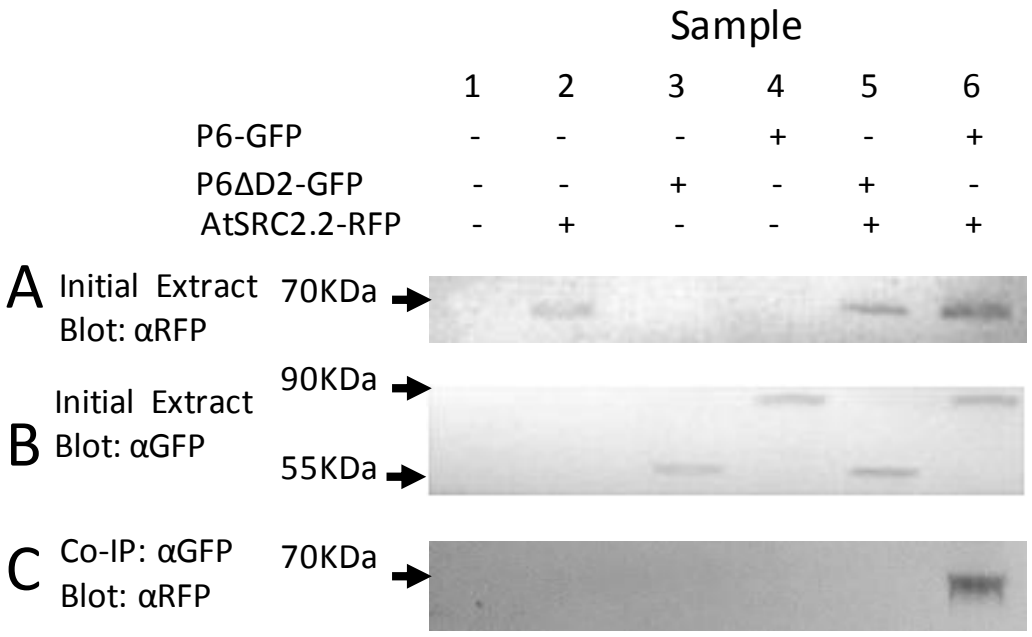

Figure 2.6 Co-Immunoprecipitation of AtSRC2.2-RFP with P6ΔD2-GFP and P6-GFP after co-agroinfiltration of *N. benthamiana* leaves. Lane 1, Mock-inoculated control leaf; Lane 2, Expression of AtSRC2.2-RFP; Lane 3, Expression of P6ΔD2-GFP; Lane 4, Expression of P6-GFP; Lane 5, Co-expression of P6ΔD2-GFP with AtSRC2.2-RFP; Lane 6, Co-expression of P6-GFP with AtSRC2.2-RFP. (A) Western blot for total proteins input probed against RFP antibodies. (B) Western blot for total protein input probed against GFP antibodies. (C) Western blot for proteins immunoprecipitated using GFP antibodies and probed against RFP antibodies.

interior of the cell, whereas AtSRC2.2-RFP remained primarily at the periphery (Fig. 2.7A). We concluded that AtSRC2.2 is present at the plasma membrane, as predicted from its amino acid sequence. This result is also in agreement with the subcellular localization of CaSRC2-1, an ortholog in *Capsicum annuum* that was shown to be associated with the plasma membrane (Kim *et al*. 2008).

The observation that AtSRC2.2-RFP protein formed numerous punctate spots (Fig. 2.4A) indicated that it might be associated with plasmodesmata. To determine whether a portion of the AtSRC2.2 proteins expressed in a cell might be localized to plasmodesmata, AtSRC2.2-RFP was co-agroinfiltrated with PDLP1-GFP or CaMV MP-GFP, two proteins that serve as plasmodesmal markers (Amari *et al*., 2010; Thomas *et al*., 2008). The MP-GFP protein formed punctate spots (Fig. 2.7B), consistent with earlier subcellular localization studies that placed the CaMV MP at plasmodesmata (Amari *et al*., 2010). The AtSRC2.2-RFP protein was widely distributed in the cell (Fig. 2.7C), but also formed punctate spots that co-localized with the MP-GFP protein (Fig. 2.7D). We observed 1028 P1-GFP foci in 20 different fields, and observed co-localization with AtSRC2.2-RFP in 443 foci (43.1%).

In experiments involving co-agroinfiltration of AtSRC2.2-RFP with PDLP1-GFP, we observed the association of AtSRC2.2-GFP with this marker for plasmodesmata, both in plasmolyzed cells (Fig. 2.7E-G) and unplasmolyzed cells (data not shown). Observation of plasmolyzed cells showed that 259 PDLP1-GFP foci out of 822 total (31.5%) exhibited co-localization with AtSRC2.2-RFP. Plasmolysis of the cell indicated that AtSRC2.2 was associated with PDLP1 in the plasma membrane (Fig. 2.7G, white arrows), but both PDLP1 and AtSRC2.2 were retained in the area of the cell wall where

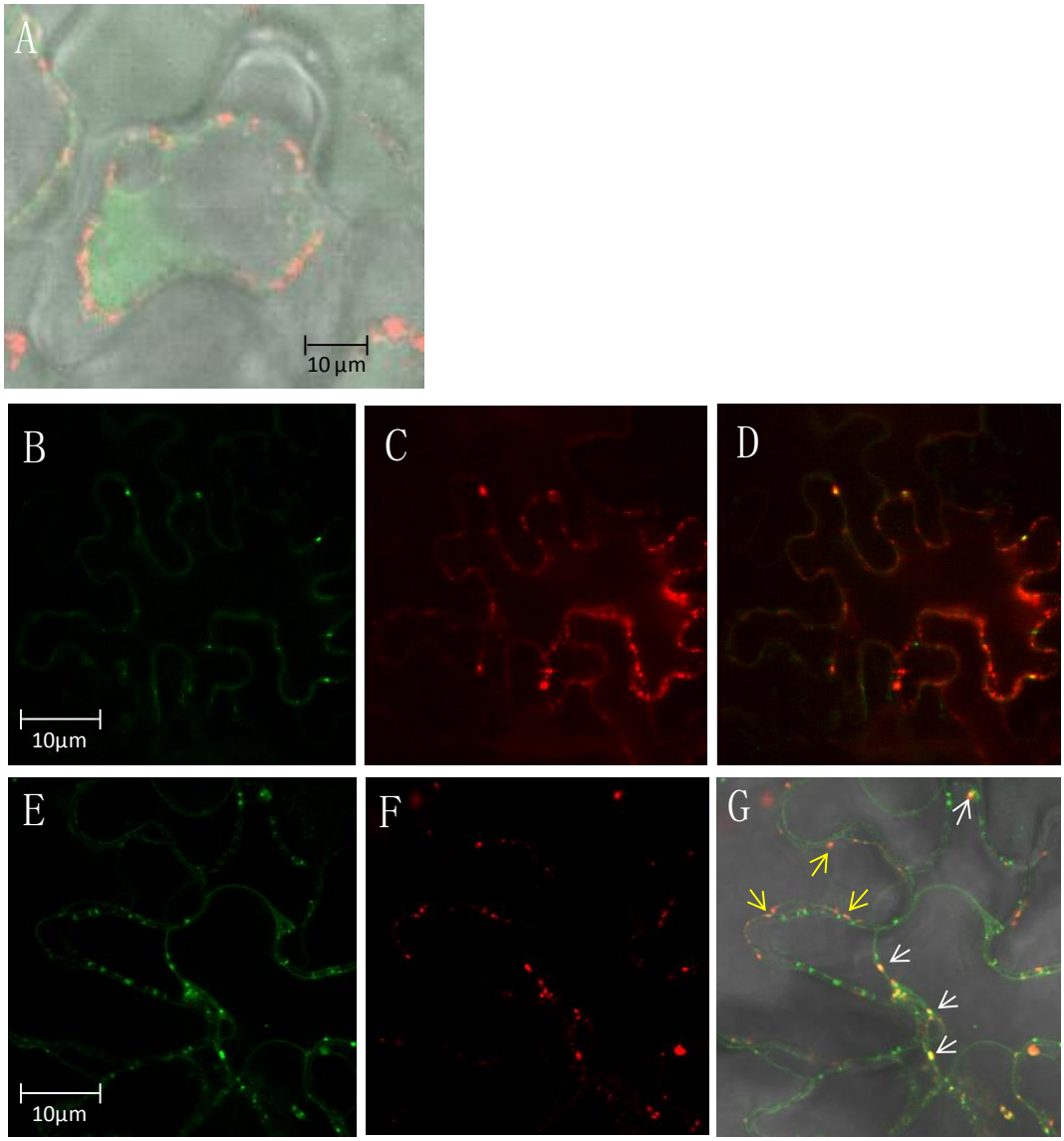

Figure 2.7 Association of AtSRC2.2 with membranes and with plasmodesmatal marker proteins PDLP1 and CaMV MP. (A) *N. benthamiana* leaf tissue coagroinfiltrated with AtSRC2.2-RFP and free GFP. Panels B-D illustrate colocalization of CaMV MP-GFP with AtSRC2.2-RFP in *N. benthamiana* leaf tissue. (B) MP-GFP. (C) AtSRC2.2-RFP. (D) overlay of B and C. Panels E-G illustrate colocalization of CaMV AtSRC2.2-RFP with PDLP1-GFP in plasmolyzed *N. benthamiana* cells. (E) PDLP1-GFP. (F) AtSRC2.2-RFP. (G) overlay of E and F.

they also co-localized (Fig. 2.7G, yellow arrows). In unplasmolyzed cells, we observed 1136 PDLP1-GFP foci in 18 different fields and found evidence for co-localization with AtSRC2.2 in 365 foci (32.1%). The experiments with PDLP1 and CaMV MP indicated that AtSRC2.2 was associated with proteins known to localize to the plasmodesmata, in addition to its association with P6 protein.

AtSRC2.2 is associated with tubules formed from the CaMV MP.

The CaMV MP forms tubules that project through plasmodesmata, which are then used for transport of the icosahedral virions from cell to cell (Benitez-Alfonso *et al*., 2010; Kasteel *et al*., 1996; Perbal *et al*., 1993). Tubules were reported in plant tissues infected with CaMV in early studies (Conti *et al*., 1972). Similar tubule structures are produced in plant protoplasts and insect cells after the introduction and expression of the CaMV MP (Huang *et al*., 2000; Kasteel *et al*., 1996). However, the fusion of GFP to either the N- or C-terminus of MP inhibited tubule formation in insect cells (Thomas and Maule, 2000) as well as in *N. benthamiana* leaves (Amari *et al*., 2010). Thomas and Maule (2000) found that co-expression of wild type MP with MP-GFP restored the formation of fluorescent tubules in insect cells, indicating that the fusion of GFP to MP inhibited the development of tubule structures in insect cells.

Similar to findings in insect cells, we found that transient expression of MP-GFP alone did not result in tubule formation, but only in the development of foci associated with plasmodesmata (Fig. 2.8A), while expression of wild type CaMV MP with MP-GFP

yielded a tubule with the base embedded in the cell wall and tip extended into the cytoplasm (Fig. 2.8B). These findings are in agreement with those from earlier work, in which electron micrographs showed the protrusion of CaMV tubules into the cytoplasm (Conti *et al*., 1972). Furthermore, the tubules formed from the CaMV MP are similar in structure and orientation to those produced by the GFLV MP (Amari *et al*., 2010). Since AtSRC2.2 is associated with CaMV MP, we sought to determine whether the association would extend to the tubules formed from CaMV MP within cells. Co-agroinfiltration of AtSRC2.2-RFP, MP-GFP and wild type MP revealed that of 45 tubules that contained AtSRC2.2, it was present at the base in 76.2% of the samples (Fig. 2.8C-E) and at the tip of 23.8% of the MP tubules (Fig. 2.8F-H). We concluded from these experiments that AtSRC2.2 is capable of a close association with tubules formed from the MP of CaMV.

P6 I-LBs are associated with plasmodesmata.

AtSRC2.2 physically interacted with the P6 protein and also associated with the plasmodesmal-localized proteins PDLP1 and CaMV MP. To determine whether P6 I-LBs are localized to plasmodesmata, we investigated the association P6-RFP I-LBs with the plasmodesmal markers PDLP1 and aniline blue. Aniline blue is a fluorescent dye that stains callose, a polysaccharide that accumulates around the openings of plasmodesmata (Northcote *et al*., 1989; Thomas *et al*., 2008). PDLP1-GFP and P6-RFP were co-

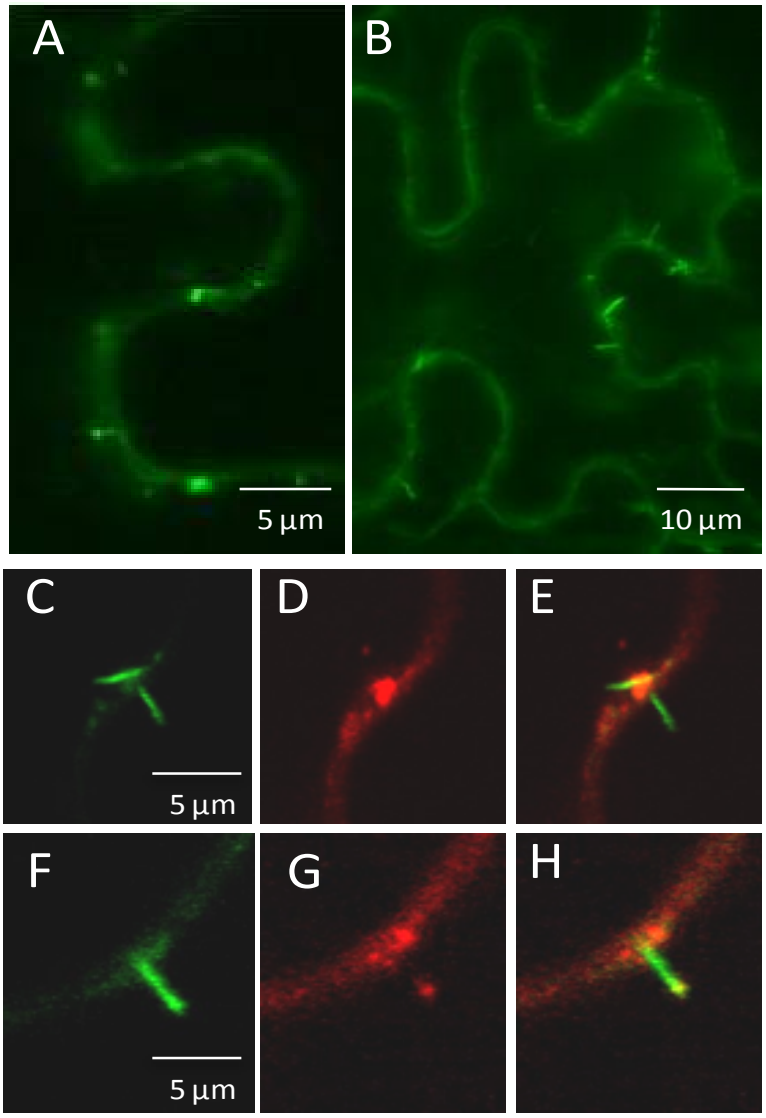

Figure 2.8 AtSRC2.2-RFP co-localizes with CaMV P1-GFP at the base of tubule structures in *N. benthamiana* leaf cells. (A) P1-GFP forms punctate spots that are associated with plasmodesmata. (B) Co-agroinfiltration of unmodified P1 with P1-GFP leads to the formation and labeling of tubule structures. (C-E) Co-agroinfiltration of unmodified P1, P1-GFP and AtSRC2.2-RFP illustrates that AtSRC2.2-RFP co-localizes with P1-GFP at the base of tubule structures. (C) P1-GFP. (D) AtSRC2.2-RFP. (E) Overlay of panels C and D. (F-H) Co-agroinfiltration of unmodified P1, P1-GFP and AtSRC2.2-RFP shows that AtSRC2.2-RFP may also be co-localized with P1-GFP at the tip of tubule structures. (F) P1-GFP. (G) AtSRC2.2-RFP. (H) Overlay of panels F and G.

agroinfiltrated into *N. benthamiana* leaves and 19 different fields were examined by confocal microscopy. Of 357 punctate spots associated with PDLP1-GFP, 163 (45.6%) were adjacent to the P6-RFP I-LBs (Fig 2.9A-C). We also examined the localization of PDLP1-GFP + P6-RFP in plasmolyzed cells, and under these conditions P6-RFP I-LBs were co-localized with PDLP1-GFP (data not shown). When P6-RFP-agroinfiltrated tissue was stained with aniline blue, we counted 185 foci for aniline blue and 247 P6-RFP I-LBs. Of the aniline blue foci, 87 (47%) were adjacent to P6-RFP I-LBs (Fig 2.9D-E). These results show that P6-I-LBs were associated with a significant proportion of markers for plasmodesmata. The lack of overlapping signal between the plasmodesmal markers and P6 IBs may be due to the inability of P6 I-LBs to enter plasmodesmata. However, once this physical barrier is eliminated by the effects of plasmolysis, then P6-RFP is capable of co-localization with PDLP1-GFP.

P6-GFP is co-immunoprecipitated with PDLP1-RFP.

The co-localization of P6-RFP with PDLP1-GFP suggested that the two proteins might physically interact; this potential interaction was explored in a co-immunoprecipitation assay. *N. benthamiana* plant tissues were agroinfiltrated with P6-RFP, PDLP1-GFP, or the combination of P6-RFP with PDLP1-GFP, and protein extracts from infiltrated tissues were either used immediately for western blots or incubated with GFP antibodies immobilized onto sepharose beads. For the western blots, both P6-RFP and PDLP1-GFP were readily detected when expressed individually or when co-

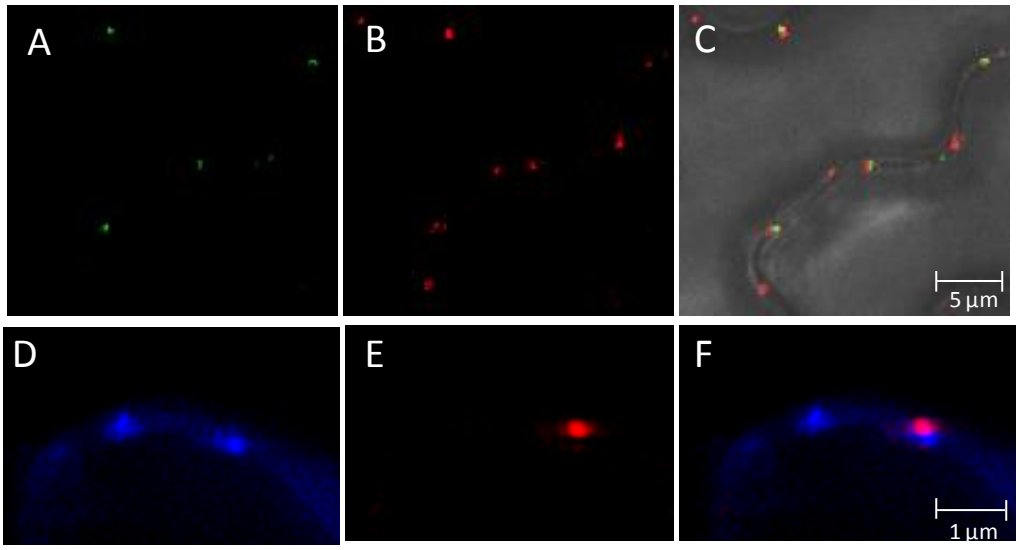

Figure 2.9 Association of P6-RFP with the plasmodesmal protein PDLP1-GFP and with aniline blue. Panels A – C illustrate the association of P6-RFP with PDLP1 in *N. benthamiana* leaf tissue after co-agroinfiltration. (A) PDLP1-GFP. (B) P6-RFP. (C) Overlay of panels A and B. Panels D – F illustrate the association of P6-RFP with aniline blue in *N. benthamiana* leaf tissue after agroinfiltration. (D) cell walls stained with aniline blue. (E) P6-RFP. (F) Overlay of panels E and F.

expressed (Fig. 2.10A and B). For the co-IP, a protein of about 90 kDa corresponding to P6-RFP was detected upon co-immunoprecipitation with PDLP1-GFP (Fig. 2.10D Lane 4), but was not detected when PDLP1-GFP was omitted from the co-immunoprecipitation assay (Fig. 2.10C Lane 2). With these results, we concluded that the P6-RFP is co-immunoprecipated with PDLP1-GFP.

CaMV local and systemic symptom development is unaffected in an AtSRC2.2 Arabidopsis T-DNA knockout line.

A previous paper had examined the influence of the PDLPs on GFLV and CaMV infections. PDLPs 1-8 are each incorporated into tubules formed from the GFLV MP, and T-DNA knockouts of individual PDLP proteins did not inhibit tubule formation (Amari *et al*., 2010). A triple T-DNA knockout of *pdlp1*, *pdlp2*, and *pdlp3* (named *pdlp 1/2/3*) exhibited a significant reduction in the number of cells developing tubules formed after addition of the GFLV MP, indicating that the PDLPs contribute to tubule formation and are functionally redundant (Amari *et al*., 2010). Similarly, the timing of infection of the *pdlp 1/2/3* mutant plants by GFLV and CaMV was delayed relative to wild type Col-0 plants, and in the case of CaMV fewer plants became infected. However, even with the triple knockout, infection by either virus was not abolished (Amari *et al*., 2010).

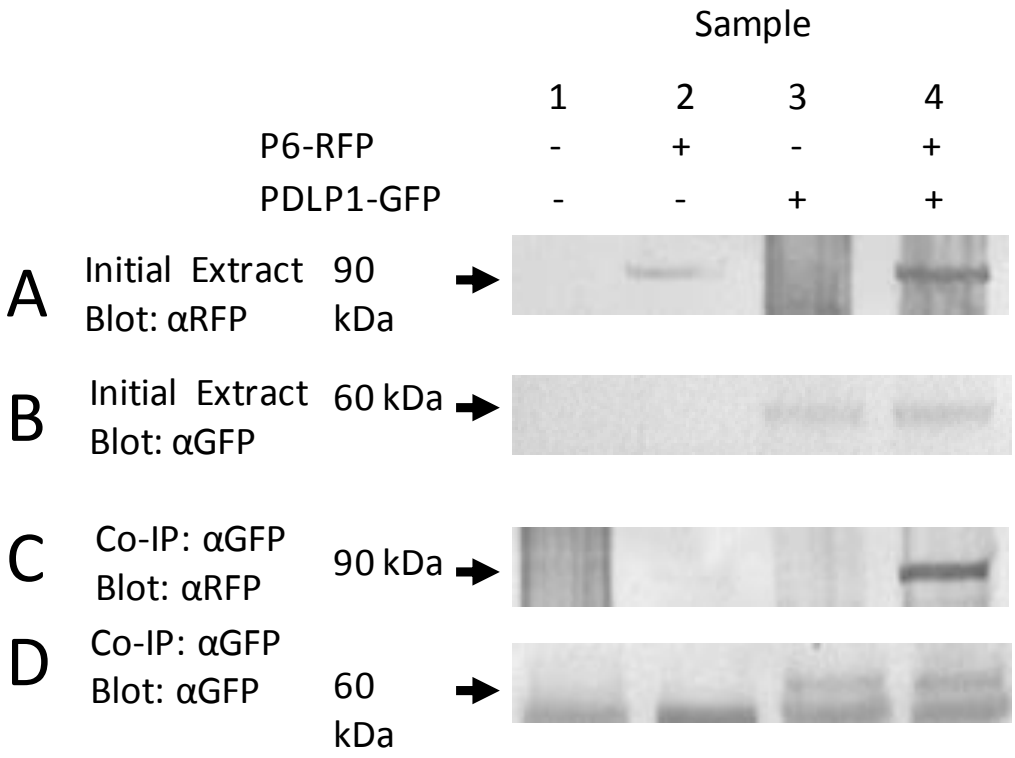

Figure 2.10 Co-immunoprecipitation of PDLP1-GFP with P6-RFP after co-agroinfiltration of *N. benthamiana* leaves. Lane 1, Mock-inoculated control leaf; Lane 2, Expression of P6-RFP; Lane 3, Expression of PDLP1-GFP; Lane 4, Co-expression of P6-RFP and PDLP1-GFP. (A) Western blot for total proteins input probed against RFP antibodies. (B) Western blot for total protein input probed against GFP antibodies. (C) Western blot for proteins immunoprecipitated using GFP antibodies and probed against RFP antibodies. (D) Western blot for proteins immunoprecipitated using GFP antibodies and probed against GFP antibodies.

To investigate the contribution of AtSRC2.2 to the CaMV infection process, we examined the capacity of CaMV to infect the *AtSRC2.2* T-DNA knockout line SALK 111179, a line in which the T-DNA is inserted into exon I (Figs. 2.2 and 2.11). The use of the T-DNA specific primer LBa1 and the gene-specific primers C2_TDNA-Fwd and C2_TDNA-Rev was important to characterize two T-DNA insertions in tandem. The forward insertion resides 85 nucleotides upstream of the start codon, while the reverse insertion is found 61 nucleotides downstream of the start codon (Fig. 2.11A). However, the initial characterization of the line SALK-111179 showed also the amplification of the wild type gene, therefore confirming the information provided by The Arabidopsis Information Resource (TAIR), which states that the initial seed stock is heterozygous for the T-DNA insertion at the AtSRC2.2 loci. Homozygous plants were allowed to self-pollinate to produce enough homozygous seed for analysis of RNA expression and CaMV inoculation experiments.

To investigate the potential for transcripts produced in the homozygous T-DNA knockout line, we isolated total RNA from the homozygous plants and treated the RNA preparation with DNaseI to degrade Arabidopsis DNA. Reverse transcription PCR (RT-PCR) performed with primers A and C, designed to amplify a region that spans the T-DNA insertions in the homozygous plants, yielded a PCR product of approximately 900 bp only in the wild type Col-0 (Fig. 2.11B), indicating that no transcript was initiated from the AtSRC2.2 promoter. However, RT-PCR analysis conducted with primers B and C, designed to amplify a region of the AtSRC2.2 gene located downstream of the T-DNA insertions, amplified a 570 bp band present in two different total RNA preparations of homozygous T-DNA mutant lines and Col-0 plants (Fig. 2.11C). Furthermore, it seems

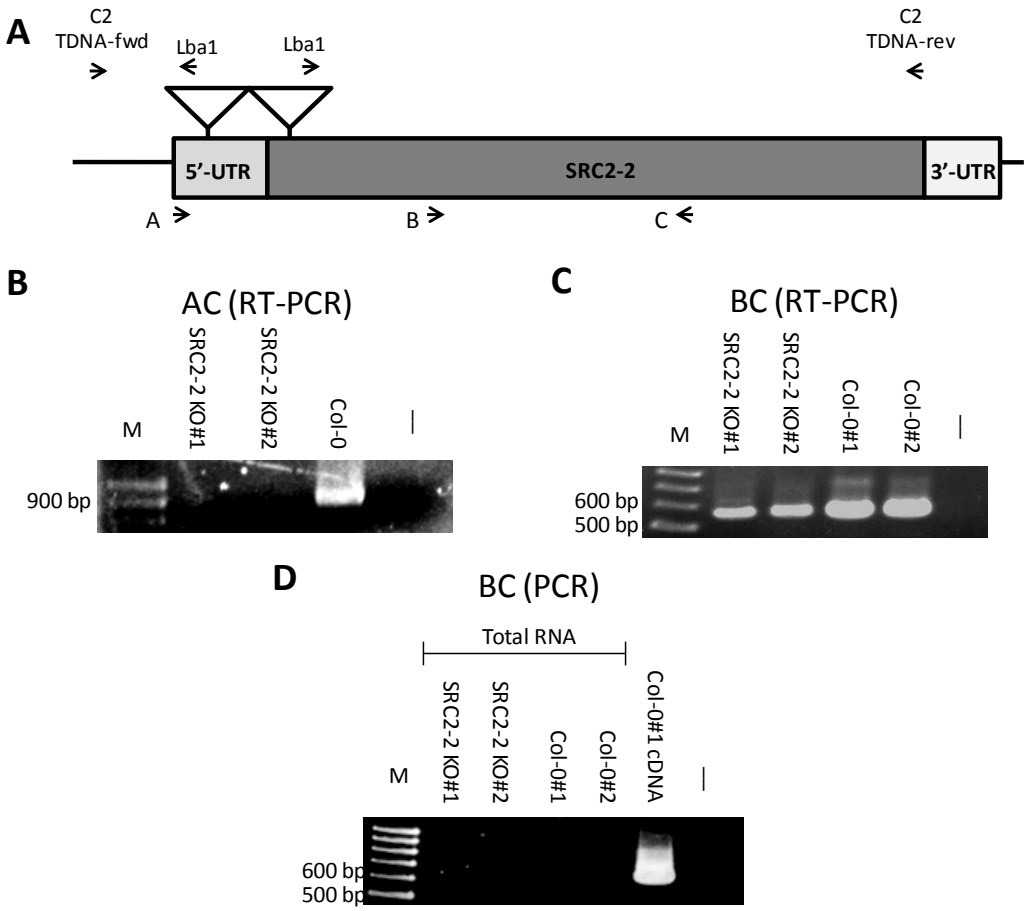

Figure 2.11. Characterization of the AtSRC2.2 T-DNA mutant line SALK_111179. A. Gene-specific primers C2_TDNA-Fwd and C2_TDNA-Rev and T-DNA specific-primer Lba1 were used to characterize the T-DNA insertions. B-D. Detection of AtSRC2.2 expression levels in homozygous AtSRC2.2 T-DNA mutant SALK_111179 plants. 1µg of cDNA samples was used to perform RT-PCR. B. AtSRC2.2 transcripts were not detected in AtSRC2.2 cDNA using the primers A and C, which insert would span the T-DNA insertions. Col-0 cDNA and water were used as positive and negative controls, respectively. C. SRC2.2 transcript was detected with primers B and C which amplify a fragment downstream of the T-DNA insertions. D. DNA contamination was not detected in total RNA samples used for RT-PCR experiments in A and B. PCR was performed using primers B and C and total RNA for both AtSRC2.2 mutant plants and the control Col-0. Col-0 cDNA and water were used as positive and negative controls, respectively.

that the transcript levels are reduced in the homozygous T-DNA insertional lines as those in Col-0. Since the amplification observed in Figure 2.11C could also be possible if there were DNA remnants in the RNA preparations, we sought to confirm that the DNaseI treatment had been successful and that the amplification in the RT-PCR experiments could only occur if we used cDNA as a template. Consequently, we set up a PCR reaction in which total RNA from Col-0 and the mutant lines was used as template. In this case we used the same primers and conditions as those in Figure 2.11C. Amplification of a 570 bp band occurred only in the positive control that had Col-0 cDNA instead of total RNA (Fig. 2.11D).

Similar results were reported by Ojangu and coworkers (2007) when they characterized an Arabidopsis line homozygous for a T-DNA insertion in the myosin XI-K gene. The authors detected XI-K-specific transcripts in wild type and mutant plants through RT-PCR. However, Northern blot analysis did not detect XI-K transcripts in the mutant plants but it did in the wild type. Hence, it is likely that the AtSRC2.2 transcripts detected in our RT-PCR analysis are not functional.

Upon inoculation with CaMV virions, the *AtSRC2.2* mutant plants developed local lesions and systemic symptoms at a slightly delayed rate to that observed in wild type Col-0 plants (Fig. 2.12), but this delay was not significant, because in a subsequent experiment, the results were reversed (data not shown). We concluded that the development of local lesions and systemic symptoms was not detectably delayed relative to CaMV infections of wild type Col-0 plants. An analysis of the Arabidopsis genome sequence indicated the existence of several potential homologs of AtSRC2.2 (Table 2.1),

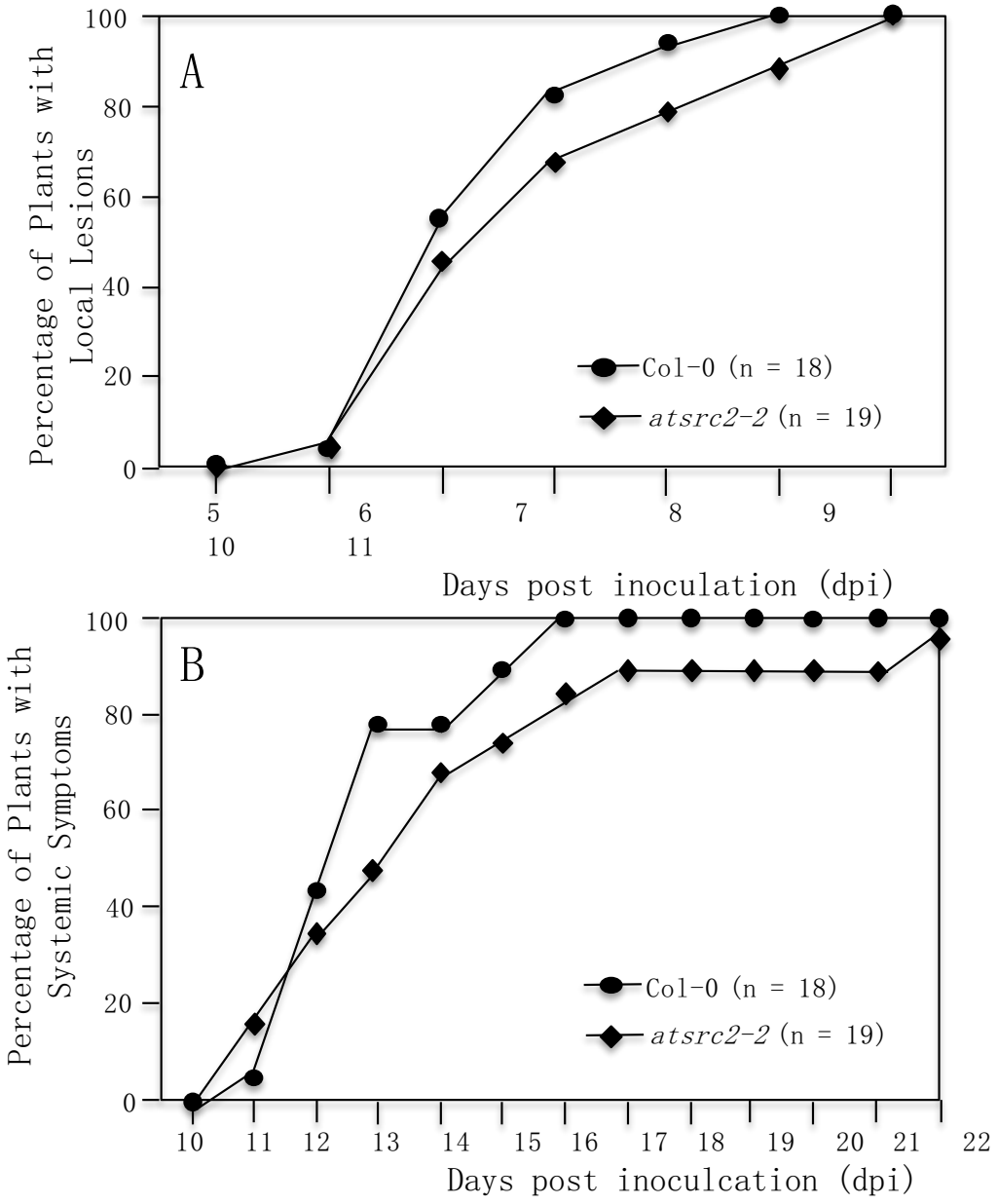

Figure 2.12 Development of local (A) and systemic symptoms (B) in *A. thaliana* wild type Col-0 (circles) and the *atsrc2.2* T-DNA insertion line SALK 111179 (diamonds).

which might obscure an effect of the *AtSRC2.2* T-DNA knockout on CaMV infections, similarly to the findings for PDLP.

DISCUSSION

It is generally accepted that CaMV virions accumulate in P6 IBs after their assembly. Most electron micrographs of P6 IBs show a single, very large, amorphous inclusion body in the cell, with individual virions embedded in the matrix (Conti *et al*., 1972; Fujisawa *et al*., 1967). Electron microscopy has also shown that CaMV moves from cell-to-cell as virions through tubules (Conti *et al*., 1972; Stavolone *et al*., 2005). What is not understood is how the virions move from the site of synthesis, the P6 IBs, to the plasmodesmata. For many viruses, models for intracellular movement of the virus have involved the viral MP (Epel, 2009; Harries *et al*., 2010; Liu and Nelson, 2013; Patarroyo *et al*., 2013; Peña and Heinlein 2013; Schoelz *et al*., 2011; Tilsner and Oparka 2012). However in the case of CaMV, Stavolone and coworkers (2005) emphasize that the CaMV MP and virion/VAP complex may travel independently to the plasmodesmata and may first encounter each other at the entrance to the plasmodesmata. Consequently, the evidence suggests that the CaMV MP does not contribute to the intracellular trafficking of CaMV virions to the plasmodesmata.

By contrast, previous experiments had indicated that the P6 protein might have a role in intracellular movement of the virus. Ectopic expression of a P6-GFP fusion protein in *N. benthamiana* had shown that P6-GFP I-LBs were capable of associating with and moving on microfilaments (Harries *et al*., 2009a). Furthermore, treatment of plant tissues with latrunculin B blocked the development of CaMV local lesions in *N.*

edwardsonii. Since latrunculin B disrupts the structure of microfilaments, Harries and coworkers (2009a) suggested that intracellular movement of P6 IBs on actin microfilaments might be essential for the CaMV infection process. A later study showed that the P6 protein interacts with the host protein CHUP1, a protein necessary for movement of chloroplasts on microfilaments in response to changes in light intensity (Angel *et al*., 2013). Angel and coworkers (2013) suggested that P6 IBs are able to associate with and move on microfilaments through their interaction with CHUP1. However, although both studies (Angel *et al*., 2013; Harries *et al*., 2009a) contributed to a mechanistic explanation for the movement of P6 IBs on microfilaments, what was missing was any indication of a destination for P6 I-LB's within the cell.

Electron micrographs of P6 IBs in CaMV-infected cells have not indicated an association with plasmodesmata (Conti *et al*. 1972; Cecchini *et al*. 1997; Fujisawa *et al*. 1967; Rubio-Huertos *et al*. 1968; Shalla *et al*. 1980; Stavolone *et al*. 2005; Stratford *et al*. 1988). However, the inability to find CaMV IBs adjacent to plasmodesmata in infected plants might be explained by the differences in when CaMV-infected plant tissues were examined versus tissues agroinfiltrated with P6-GFP. Electron micrographs have been published of mature CaMV infections in leaves that had already developed systemic symptoms, typically between 21 and 40 dpi. In systemically-infected leaves, cells contain one or a few, very large CaMV IBs (Shalla *et al*. 1980), and the infection front would have already moved through that tissue. By contrast, upon agroinfiltration of P6-GFP, plant cells contain numerous, small I-LBs that are capable of moving on microfilaments (Angel *et al*. 2013; Harries *et al*. 2009a; Laird *et al*. 2013). Larger P6-

GFP I-LBs are also present in the cell, but they tend to be stationary (Angel *et al*. 2013). We hypothesize that the P6 protein forms aggregates that transiently associate with plasmodesmata early in the infection in the cell, but as the infection matures the P6 protein forms larger aggregates that eventually lose the capacity for intracellular movement. Indeed, Rubio-Huertos and coworkers (1968) found that CaMV IBs observed in cytoplasm at 21 dpi consisted of four to five particles surrounded by a very dense, granular material, whereas larger aggregates of particles and electron dense material were seen at 28 – 35 dpi.

Here we show that P6 I-LBs interacted with AtSRC2.2 and PDLP1, two proteins associated with plasmodesmata. These proteins co-localized in punctate locations in the cell periphery, as expected for plasmodesma-associated proteins. Furthermore, the association of P6 I-LBs with plasmodesmata occurs independently of the presence of CaMV MP. Our evidence for these conclusions is based on yeast two-hybrid, co-immunoprecipitation and co-localization assays, the latter with proteins shown to serve as reliable markers for the plasmodesmata (PDLP1 and CaMV MP: Figs. 2.5 and 2.10). The pull-down assays between P6 and CaMV MP (Hapiak *et al*., 2008) further suggest that P6 I-LBs associate with plasmodesmata. This additionally was supported through the consistent association of P6 I-LBs with cell wall regions stained with aniline blue, a marker for plasmodesmata (Thomas *et al*., 2008). Furthermore, we were able to establish that AtSRC2.2 becomes incorporated into the base and tip of tubules formed from the CaMV MP. The location of AtSRC2.2 at the base of tubules is similar to the location of PDLP1 when tubules were formed with GFLV MP (Amari *et al*. 2010). Intriguingly,

confocal microscopy indicated that P6 IBs might not directly enter the plasmodesmata, as P6 was found adjacent to these plasmodesmal markers rather than co-localizing with them.

Members of the PDLP family had previously been shown to interact with the MPs of CaMV and GFLV. The PDLPs form a small family of eight proteins that are trafficked along the secretory pathway to plasmodesmata, where they are incorporated into the plasma membrane that lines the plasmodesmata (Amari *et al*., 2011; Thomas *et al*., 2008). Amari and coworkers (2010; 2011) suggested that the PDLP proteins might act as receptors for the MPs of GFLV or CaMV. Our observation that P6 protein interacts with PDLP1 and that P6 I-LBs can be located adjacent to plasmodesmata adds another level of complexity to models proposed for cell-to-cell movement of CaMV, as the PDLPs, or at a minimum PDLP1, may also serve as a receptor for the CaMV P6 protein.

A BLAST analysis of the full-length AtSRC2.2 protein sequence against the Arabidopsis proteome showed that the proteins most closely related to AtSRC2.2 are AtSRC2.3 (At4g15755), and AtSRC2.1 (At1g09070) (Table 2.1) (Kim *et al*., 2008; Zhang *et al*. 2013). Our work in this paper provides the first characterization of AtSRC2.2. No information has yet been reported on the function of AtSRC2.3. AtSRC2.1 has been shown to utilize the vesicular trafficking machinery to move rapidly from the ER to protein storage vacuoles where it is internalized into the vacuole (Oufattole *et al*., 2005). AtSRC2.1 binds to the sequence motif PIEPPPHH, present in the C-terminal end of a membrane protein that is itself trafficked from the ER to

vacuoles. This finding suggests that AtSRC2.1 utilizes a membrane anchor protein for its observed intracellular trafficking. The sequence motifs of AtSRC2.1 that direct it to the vacuole, or are necessary for binding to PIEPPPHHH, have not been identified. Consequently, it is not possible to predict whether AtSRC2.2 would have those same properties. However, our observation that at least a portion of AtSRC2.2 can be found in plasmodesmata, a location not reported for AtSRC2.1, suggests a divergence in function between AtSRC2.2 and AtSRC2.1.

One structural feature identified in AtSRC2.2 is its C2 domain, a lipid-binding domain that is present in a large number of eukaryotic proteins. C2 domains are typically coupled with other functions on the protein such as kinases, but also have been linked with cytoskeletal interactions and vesicular trafficking. A large number of protein families carrying distinct C2 domains have been identified, some of which are plant-specific (Zhang and Aravind, 2010). Plant proteins that contain C2-domains have been implicated in responses to biotic and abiotic stress (Kim *et al*., 2004; Zhang *et al*., 2012), suppression of programmed cell death (Yang *et al*., 2006), and cell-to-cell movement of plant viruses (Lewis and Lazarowitz, 2010). Since C2 domains have such a diversity of sequences (Zhang and Aravind, 2010), only a subset can be revealed through homology search of the Arabidopsis proteome that specifically targets the C2 domain. Of the subset of Arabidopsis proteins whose C2 domain is related to AtSRC2.2, the functions of most have not been characterized. However, one notable exception is Synaptogtagmin4 (AtSYT4, Table 2.2). AtSYT4 belongs to a small family of five proteins that contain two C2 domains, C_2A and C_2B, both of which are found towards the C-terminal end of the

protein; the C2 domain of AtSRC2.2 is homologous to C_2A of AtSYT4 (36% identity, Table 2.2). Although not much is known about AtSYT4, another synaptotagmin, AtSYTA, has been implicated in the movement of several viruses, including the *Begomoviruses Cabbage leaf curl virus* and *Squash leaf curl virus*, as well as the *Tobamovirus* TMV through an interaction with their respective MPs (Lewis and Lazarowitz, 2010). Since SYTA also regulates endocytosis and endosome recycling, Lewis and Lazarowitz (2010) have suggested that virus MPs might utilize SYTA and its recycling activity when trafficking to the PD. It is intriguing to note that AtSYTA has also been implicated in modulating freezing tolerance (Yamazaki *et al*., 2008), another feature that is common to the SRC2 proteins.

Although the function of the interaction between AtSRC2.2 with P6 still must be clarified, it is important to note that this interaction led to the discovery that P6 interacts with PDLP1 and that P6 I-LBs can be found adjacent to plasmodesmata. The localization of P6 I-LBs next to plasmodesmata suggests that P6 IBs may directly deliver CaMV virions to the CaMV MP within the plasmodesmata. This mechanism of transfer would also be in agreement with models advanced by Stavolone and coworkers (2005) for CaMV intracellular transport where the CaMV virion/VAP complex is postulated to travel independently of CaMV MP to the plasma membrane. We suggest that multiple proteins, such as the PDLP proteins and AtSRC2.2 might have a role in the transfer of CaMV virion/VAP complex from their site of assembly to the CaMV MP at the plasma membrane.

Table 2.2 Arabidopsis proteins that share homology with the C2 domain of AtSRC2.2 (aa 6 – 112)

Description	Coverage	Identity	E value[a]	Accession
AtSRC2.2 (At3g16510)	100%	100%	4e-74	NP188272.1
AtSRC2.3 (At4g15755)	99%	51%	1e-33	NP680701.1
C2 domain containing (At4g15740)	98%	52%	3e-27	NP193309.1
AtSRC2.1 (At1g09070)	92%	50%	1e-23	NP563835.1
C2 domain containing (At3g62780)	92%	45%	2e-17	NP191837.1
C2 domain containing (At2g13350)	97%	34%	5e-09	NP178968.1
C2 domain containing (At2g33320)	98%	33%	2e-08	NP180890.1
C2 domain containing (At3g05440)	91%	29%	2e-08	NP187195.1
C2 domain containing (At1g04540)	98%	31%	9e-08	NP171948.1
C2 domain containing (At3g04360)	92%	29%	1e-06	NP566225.4
Synaptotagmin-4 (At5g11100)	94%	36%	7e-05	NP196671.2
C2 domain containing (At4g01200)	57%	39%	2e-04	NP192029.1
C2 domain containing (At3g55470)	92%	28%	4e-04	NP001078296.1
C2 domain containing (At1g63220)	100%	29%	5e-04	NP176511.4
C2 domain containing (At1g50570.2)	92%	32%	73-04	NP200364.2

Only C2 domains that have a cutoff value greater than 1e-03 are listed.

Tilsner *et al*. (2013) recently suggested that replication of PVX might be coupled with trafficking of the virus through the plasmodesma. In this model, PVX replication complexes are able to move on the ER network and some become anchored at the PD. As viral RNA is replicated it is diverted through the PD and is also encapsidated. In this paper and in previous work (Angel *et al*., 2013; Harries *et al*., 2009a), we have shown that ectopically expressed P6 I-LBs are capable of movement on the cytoskeleton and also have the capacity for association with PD. Other research has emphasized that P6 inclusion bodies are considered the sites for reverse transcription and virion assembly. Collectively, these studies suggest a link between replication and movement of CaMV.

MATERIALS AND METHODS

Plants and Viruses.

All plants were propagated under greenhouse conditions at the University of Missouri (Columbia, MO). Virions of CaMV W260 strain were partially purified from infected turnip leaves (*Brassica rapa* subsp. rapa cv. Just Right), according to Schoelz *et al*. (1986), and mechanically inoculated onto leaves of Arabidopsis plants (Kloek *et al*., 2001).

Yeast Two-Hybrid Analysis.

Yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S (Paris, France). The coding sequence for the full length CaMV P6 protein was PCR-amplified from plasmid pW260 (Schoelz and Shepherd, 1988) and cloned into pB29 as an N-terminal fusion to LexA (N-P6-LexA-C). The sequence of the entire construct was verified and used as a bait to screen an *A. thaliana* cDNA library within a pP6 prey vector produced from 1-week old seedlings. pB29 and pP6 are derived from the original pBTM116 (Vojtek and Hollenberg, 1995)and pGADGH (Bartel *et al*., 1993) plasmids, respectively. Eighty one million clones (8-fold the complexity of the library) were screened using a mating approach with Y187 (mata) and L40ΔGal4 (mata) yeast strains

as previously described (Fromont-Racine *et al*., 1997). Eighty five His+ colonies were selected on a medium lacking tryptophan, leucine and histidine. The prey fragments of the positive clones were amplified by PCR, sequenced at their 5' and 3' junctions, and the resulting sequences were used to identify the corresponding interacting genes in the GenBank database (NCBI).

To identify the domains of P6 that interact with AtSRC2.2, a second Y2H assay was performed using only the 629 nt region of *A. thaliana* AtSRC2.2 identified in the Hybrigenics Y2H. This region corresponding to the positions 28-657 of the AtSRC2.2 coding sequence (Salanoubat *et al*, 2000. AT3G16510) was amplified from the full-length complementary DNA (cDNA) clone U63853 obtained from the Arabidopsis Biological Resource Center (ABRC. The Ohio State University, Columbus, OH) by PCR, using forward and reverse primers containing 5'-end extensions with the *Eco*RI and *Xho*I sites, respectively. The PCR product was cloned into pGEM-T easy vector (Promega, Madison WI) for nucleotide sequence confirmation, and subsequently cloned into the yeast plasmid pJG4-5 (Gyuris *et al*., 1993), a plasmid that contained the activation domain (Li and Leisner, 2002). The four P6 self-association domains were previously cloned into the yeast plasmid pEG202 and the Y2H analysis was performed as described (Li and Leisner, 2002). All PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA), and all sequencing reactions were performed at the DNA Core Facility of the University of Missouri (Columbia, MO).

Clones of *A. thaliana* AtSRC2.2, AtPDLP1, CaMV MP, and CaMV P6.

The full-length cDNA clone U63853 of the Col-0 AtSRC2.2 gene was used as a template to amplify by PCR a DNA fragment corresponding to the 1,083 nt of the *AtSRC2.2* coding sequence and subsequent cloning into a pGEM-T Easy vector (Promega, Madison WI). The nucleotide sequence of the *AtSRC2.2* insert was determined at the DNA Core Facility at the University of Missouri to confirm that no mutations had been introduced during PCR. The 1,083 nt AtSRC2.2 fragment was cloned into pDONR-201 and then cloned into the pSITE expression vectors (Chakrabarty *et al*., 2007, Martin *et al*., 2009), resulting in a C-terminal fusion of AtSRC2.2 to either RFP or GFP, using Gateway Technology ® (Invitrogen, Carlsbad CA), following the manufacturer's instructions. The full-length P6 protein constructs, with GFP or RFP fused to its C-terminus, have been described previously (Angel *et al*. 2013). Additionally, pW260 served as template to amplify a 983 bp fragment corresponding to the MP with and without the stop codon. Subsequently, the insert was cloned into pDONR-201 and into selected pSITE vectors, creating fusions at the either N- and C-termini of MP with Green Fluorescent Protein (GFP). The Plasmodesmata-Localized Protein 1 fused to GFP (PDLP1-GFP) (Amari *et al*., 2010; Thomas *et al*., 2008) was provided by Dr. Andrew Maule (John Innes Centre, UK) via Dr. Richard Nelson. pSITE vectors containing the AtSRC2.2, CaMV MP, and P6 and PDLP1 sequences were electroporated into *A. tumefaciens* strain AGL-1 (Lazo *et al*., 1991). Candidate colonies

were selected on appropriate antibiotics, and screened for the presence of pSITE plasmids by colony PCR.

The *A. thaliana* sequences related to AtSRC2.2 were initially identified using Blastp (Altschul *et al*., 1990). The phylogenetic analysis of AtSRC sequences was developed in ClustalW by the Maximum likelihood method (Jones *et al*. 1992) and the bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985).

Agroinfiltration Transient Expression Assays and Confocal Microscopy.

Agrobacterium cultures containing pSITE vectors were agroinfiltrated into leaves of 8-12 week old *N. benthamiana* plants as described (Angel *et al*., 2013). To extend and enhance the transient expression of target proteins, they were co-agroinfiltrated with an Agrobacterium culture that expressed the *Tomato bushy stunt virus* P19 protein. The *p19* gene had been cloned previously in the *A. tumefaciens* binary vector pKYLX7 (Angel *et al*., 2011). The final optical density at 600nm for individual constructs was 1.0.

Confocal laser scanning microscopy was performed at the University of Missouri Molecular Cytology Core (Columbia, MO), using a Zeiss LSM 510 META microscope, under multitrack mode set with the following parameters for excitation/emission filters wavelengths: 488nm/501-530 for GFP, and 543nm/565-615nm for RFP. *N. benthamiana* leaves were observed between 2 and 4 days postinfiltration (dpinf) for

transient expression. Confocal images were processed using LSM software (Carl Zeiss, Peabody MA).

Co-immunoprecipitation Assays.

Co-immunoprecipitations were conducted as described (Lee *et al*., 2003), with minor modifications, as described (Angel *et al*., 2013). Proteins from total extracts, pull down assays, and the Co-IPs were run in an 8% SDS-PAGE and transferred to a 0.45µm PVDF-Plus membrane (GE Osmonics Inc., Minnetonka, MN). Western blot analyses were performed by incubating the blocked membrane with rabbit-anti-RFP (Invitrogen, Eugene, OR) or goat-anti-GFP (Santacruz Biotechnology, Santa Cruz, CA) antibodies at 1:5,000 or 1:1,000 dilutions respectively, in 2.5% dry skim milk in TBS- Tween 0.2%. Following several washes, alkaline phosphatase conjugates of donkey, anti-goat IgG and anti-rabbit IgG (Promega, Madison, WI), were used for GFP and RFP blots respectively, at a 1:7,500 (vol./vol.) dilution. After several washes, the blots were exposed to 10 ml of developing solution (100 mM Tris-HCl pH 9.0, 150 mM NaCl, 1mM $MgCl_2$, 66 µl NBT, 33 µl BCIP (Promega, Madison, WI). The reaction was stopped with running water and air dried at room temperature. To determine the specificity of the interaction between P6 with AtSRC2.2, a deletion of the first 253 codons of P6 was performed by PCR, using a forward primer that replaced the last residue of domain D2 (aa 253) with a start codon to create P6ΔD2. Sequencing, cloning and fusion of P6ΔD2 with RFP, resulting in

expression of P6ΔD2-RFP, were performed as described previously. Co-immunoprecipitations were performed following the same conditions described above and an extra reaction including full-length P6-RFP was used as positive control.

Characterization of T-DNA insertions and gene expression level of an AtSRC2.2 mutant line.

Seeds of T-DNA insertional line SALK_111179 were provided by the Arabidopsis Biological Resource Center at The Ohio State University. Information about the T-DNA the insertional line was obtained from The Arabidopsis Information Resource (TAIR. http://arabidopsis.org). To break dormancy, seeds were planted in pots with soil mixture and stored at 4°C for 3 days. Plants were subsequently grown in a Conviron CMP3023 growth chamber at 20°C with 16h light/ 8h dark cycles.

Leaf tissue from SALK_111179 plants was used for DNA isolation using the DNeasy® Plant Mini Kit (Quiagen). Venlo, The Netherlands). T-DNA insertion zygosity was determined for each plant through PCR. Briefly, three different PCR reactions were performed to amplify the T-DNA insertions and the wild type gene. Thus, to characterize both T-DNA insertions, the T-DNA-specific primer LBa1 (5'-TGG TTC ACG TAG TGG GCC ATC G-3') served as either reverse primer if mixed with the Gene-specific primers C2_TDNA-Fwd (5'-TGATTAAAGACGTGTCAAAGATC-3') or as a forward primer if mixed with the reverse gene-specific primer C2_TDNA-Rev (5'-

AGGTTAAAAATCAAAACCAATGTC-3'). Wild type AtSRC2.2 gene was amplified by using only the gene-specific primers C2_TDNA-Fwd and C2_TDNA-Rev in the same reaction (Fig. 2.11). To map the T-DNA insertions, PCR products from all three reactions were cloned into p-GEM T-easy (Promega, Madison WI) and sequenced.

Heterozygous plants were self-pollinated and allowed to produce seed. Zygosity of self-pollinated plants was determined as indicated earlier. Plants shown to be homozygous for the T-DNA insertions were self-pollinated and seeds were grown as explained above. F2 plants were used for confirmation of T-DNA insertion homozygosity and for mechanical inoculation with partially purified CaMV virions according to Schoelz *et al*, (1986). Experiments were carried out under greenhouse conditions. The number of plants with chlorotic primary lesions and evidence of systemic virus symptoms was evaluated every day.

To determine the effect of T-DNA insertions on RNA expression levels, total RNA was isolated from the F2 plants using the RNAqueous®-4PCR DNA-free, RNA Isolation for RT-PCR kit (Ambion. Life Technologies. Austin TX.). cDNA synthesis and RT-PCR was performed using the ImProm-II Reverse Transcription System (Promega, Madison WI) according to the manufacturer suggested procedure. RT-PCR reactions were performed using the gene-specific forward primers A (5'-TTACATAAATATTATTT-3') and B (5'-AGCTTCTTCATGGTTCTTCGT-3') and the reverse gene-specific primer C (5'-GACGAAGGCGCGAACCCGTGA-3'). Col-0 cDNA and water were used as positive and negative controls, respectively. To confirm that total RNA preparations were DNA-free, a PCR reaction was set up, using total RNA instead of

cDNA as template. PCR was performed using the primers B and C. Col-0 cDNA and water were used as positive and negative controls, respectively.

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CHAPTER III

DEVELOPMENT OF CHUP1/MYOSIN XI-2/MYOSIN XI-K DOUBLE AND TRIPLE KNOCKOUT *Arabidopsis thaliana* PLANTS AND ELUCIDATION OF BIOLOGICAL IMPACT ON *Cauliflower mosaic virus* INFECTION.

INTRODUCTION

To complete their life cycle, plant viruses have to replicate, encapsidate and move the virus throughout the plant. Since plant viruses are obligate parasites whose genomic capacity is small, each of their viral proteins is likely to have multiple functions and associate with multiple host factors. For many years, the movement of plant viruses was divided into the categories of intercellular movement and long distance transport through the vascular system. However in recent years there has been a growing awareness that intracellular movement of viral genomes and proteins may also be an essential step. In this process, plant viruses would need to transport their genomes from the initial site of replication within the cell to the plasmodesmata for movement to adjacent cells. This process could involve the interaction of viral proteins with host proteins and also the

cytoskeleton and endomembrane transport system (Angel *et al*, 2013; Nelson and Citovsky, 2005; Schoelz *et al*, 2011; Tilsner and Oparka, 2012).

Early studies aimed at determining the subcellular structures involved in intracellular movement of plant viruses pointed to the cytoskeleton and the endomembrane transport system as important players in trafficking of viral proteins and viral genomes (Boevink and Oparka, 2005; Harries *et al*, 2009a,b; Heinlein *et al*, 1995; McLean *et al*, 1995). Initially the focus was on the role that plant virus movement proteins (MPs) might have in intracellular movement. For example, it is generally accepted that several viral MPs use the endoplasmic reticulum (ER) to be trafficked to plasmodesmata (Ju *et al*, 2005; Haupt *et al*, 2005; Heinlein *et al*, 1998). Once at the plasmodesmata, MPs play an important role in the movement of viral particles or genomic nucleic acids to neighboring cells. In fact, many host/virus models for intracellular movement have focused exclusively on the role of the MP (Ashby *et al*, 2006; Boyko *et al*, 2007; Brandner *et al*, 2008; Gillespie *et al*, 2002; Kawakami *et al*, 2004; Sambade *et al*, 2008).

Nonetheless, as other studies have examined viral replication, they have shown that plant viruses replicate in association with cell membranes or in the case of *Cauliflower mosaic virus* (CaMV), viral replication occurs within inclusion bodies (IBs). These studies emphasize that there must be a mechanism for transport of the viral genome from the replication site to the MP at the plasmodesmata (Harries *et al*, 2009a,b; Harries and Ding, 2011; Schoelz *et al*, 2011). This transport step might be mediated by viral MPs, but there is evidence that other types of viral proteins, including proteins associated with replication or gene expression may also have an important function in

intracellular transport of the newly replicated viral nucleic acid to plasmodesmata (Angel *et al*, 2013; Cotton *et al*, 2009; Harries *et al*, 2009a,b; Kawakami *et al*, 2004; Liu *et al*, 2005).

For example, Harries and coworkers (2009a) showed that the CaMV IB protein P6, associates with endoplasmic reticulum, microtubules and microfilaments. They further showed that P6-GFP IBs were capable of movement on microfilaments. To investigate the role of microfilaments in CaMV infection, *N. edwardsonii* half leaves were infiltrated with the actin polymerization inhibitor latrunculin B, followed by inoculation with CaMV. Harries *et al* (2009a) found that necrotic local lesions induced by CaMV developed on the untreated half leaf, but no lesions developed on the half leaf treated with latrunculin B. This experiment suggested that the movement of P6 IBs on actin microfilaments is essential for CaMV infection (Harries *et al*, 2009a).

To investigate *Arabidopsis* proteins that might contribute to trafficking of P6 IBs on microfilaments, Angel *et al* (2013) characterized the association of P6 with the Chloroplast Unusual Positioning 1 (CHUP1) protein, which is located to the outer membrane of chloroplasts. CHUP1 was originally shown to interact with CaMV P6 in a yeast two-hybrid screen. It was subsequently shown that P6 and CHUP1 proteins are co-localized within the cell in transient expression assays and that CHUP1 and P6 can be co-immunoprecipitated (Angel *et al*, 2013). CHUP1 is essential for chloroplast movement on microfilaments in response to light intensity (Oikawa *et al*, 2003; 2008). Consequently, Angel and coworkers (2013) hypothesized that P6 might hijack CHUP1 for movement of P6 IBs on microfilaments. In Chapter II, I showed that a portion of ectopically expressed P6 IBs are associated with plasmodesmata. Taken together, these

studies are consistent with our model that suggests that P6 IBs utilize microfilaments for delivery of CaMV virions to plasmodesmata, where the CaMV MP then transports them to adjacent cells.

However, attempts to knock out the function of CHUP1 have not been successful in blocking CaMV infections. Angel and coworkers (2013) utilized virus induced gene silencing to abolish the function of CHUP1. They were able to show that chloroplasts in CHUP1-silenced *Nicotiana edwardsonii* plants were not capable of movement in response to a high light intensity treatment. Upon inoculation with CaMV, the CHUP1-silenced plants responded with a delay in local lesion formation, but the local lesion response was not abolished, which would be expected if CHUP1 were essential for movement of P6 IBs on microfilaments. Angel *et al*. (2013) suggested that functional redundancies with other proteins capable of trafficking on microfilaments, such as the myosins, might explain the susceptibility of CHUP1-silenced *N. edwardsonii* to CaMV infection.

Myosins are another set of host proteins that traffic cargo on actin microfilaments. Although myosins are clustered into 24 different types, only three of them, VIII, XI, and XIII, are specific to plants (Foth *et al*, 2006; Reddy, 2001; Yakamoto, 2007). These proteins are involved in cell processes such as cytoplasmic streaming, organelle, vesicle, and nuclear transport, membrane trafficking, cytokinesis, signal transduction and intercellular communication through plasmodesma (Avisar *et al*, 2008b; Yokota and Shimmen, 2011). Recently, several studies have suggested the role of plant myosins in trafficking plant viruses inter- and intracellularly (Avisar *et al*, 2008a; Harries *et al*, 2009b; Yuan *et al*, 2011). In particular, myosins XI-K and XI-2 have been shown to be

responsible for trafficking of some plant viruses (Harries *et al*, 2009b), as well as plasmodesmal-located proteins such as PDLP1, through the secretory pathway (Amari *et al*, 2011).

A common approach to determine the function of a gene product *in situ* under a specific treatment is the use of gene knockouts or null mutations (Krysan *et al* 1999). Insertional mutagenesis has been the preferred alternative for disrupting gene function in model plants such as *Arabidopsis thaliana* (Alonso *et al*, 2003; Bechtold *et al*, 1993; Feldman, 1991; Galbiati *et al*, 2000; Koncz *et al*, 1989; Krysan *et al*, 1999). Transfer-DNA (T-DNA) insertion into genes of interest has been proven to be a successful mutagen for genome-wide mutagenesis (Krysan *et al*, 1999). The foreign DNA not only interrupts the expression of the gene it was inserted into but also acts as a marker for identification of the mutation. If there is a large population of T-DNA-mutated lines available, there is a good chance of finding a plant with a T-DNA inserted into any gene of interest (Krysan *et al*, 1999).

In this study we characterized the biological impact of CHUP1, Myosin XI-2 and Myosin XI-K, on CaMV infection. We initially tested Arabidopsis single knockout lines for each of these genes and observed that CaMV infected the single knockouts just as in the wild type Col-0. Functional redundancies might be the cause for the lack of a biological effect in single knockout lines infected with CaMV. Therefore, we developed different combinations of double and triple mutants and observed that in several experiments infection development in double and triple mutants occurred at a slower rate than Col-0. Furthermore, one out of two experiments showed a four-day delay in both local lesion and systemic infection development in the *chup1 xi-2 xi-k* triple mutant.

Timing of symptom development in the second experiment was accelerated by four days, therefore making it challenging for us to compare CaMV infection data between tests. Further tests will be necessary to determine if CaMV infections truly are slower in the double and triple knockout lines than in Col-0 or the single knockout lines.

RESULTS AND DISCUSION

Biological impact of *chup1* T-DNA knockout on CaMV infection of Arabidopsis.

In a previous study, the CHUP1 gene in *N. edwardsonii* was silenced through the use of a VIGS vector and CHUP1 silenced plants were inoculated with CaMV 21 days after the initiation of silencing (Angel *et al.,* 2013). *N. edwardsonii* responds to CaMV infection with a hypersensitive response (Schoelz *et al.,* 1986); consequently, it was only possible to record the local lesion response of the plants, since systemic movement of the virus was blocked by the plant defense response. Furthermore, this study showed that the local lesion response of *N. edwardsonii* occurred over an extended period of time. Local lesion development began at approximately 10 days post-inoculation (dpi) and new lesions continued to develop every day up to 18 – 20 dpi. At this point, the response of the plant to CaMV lesion development was largely completed, and the rate of new lesion development was dramatically slowed. Since individual lesions induced in *N. edwardsonii* by CaMV infection grew very slowly from one day to the next, it was not possible to assess changes in size of individual lesions, an approach that had been used for viruses such as TMV (Harries *et al.,* 2009b). Instead, Angel and coworkers (2013) counted the number of lesions every day and in this manner measured the rate of lesion development. They found that the rate of CaMV lesion development in the CHUP1-

silenced *N. edwardsonii* leaves was significantly slower than in lesion development in *N. edwardsonii* treated with a VIGS vector that lacked the CHUP1 insert. The results obtained with the CHUP1-silenced plants were consistent with a model in which intracellular movement of CaMV P6 IBs is mediated by CHUP1. However, it also indicated that functional redundancies might exist in the plant that would compensate for the lack of CHUP1 protein.

Arabidopsis thaliana responds to CaMV infection differently than *N. edwardsonii*, as *A. thaliana* is susceptible to CaMV. Consequently, with Arabidopsis it was important to develop a different type of evaluation system than that used for *N. edwardsonii*. For example, Arabidopsis leaves inoculated with CaMV respond with chlorotic primary lesions rather than necrotic lesions. Furthermore, differences in the size of Arabidopsis leaves relative to those of *N. edwardsonii* necessitated a different approach towards the analysis of CaMV infections. With *N. edwardsonii*, it is possible to count several hundred individual lesions on a single leaf, whereas it would be difficult to count more than 20 lesions on an Arabidopsis leaf. However, another approach towards assessing the rate of infection in inoculated leaves would be to note the first appearance of primary lesions in a plant, and this is the approach we took for the analysis of CaMV infections in Arabidopsis. A primary lesion induced by CaMV infection of Arabidopsis ecotype Col-0 is illustrated in Fig. 3.1A. Another significant difference between Arabidopsis and *N. edwardsonii* is that CaMV is able to move systemically in Arabidopsis, whereas it is localized to the inoculated leaf in *N. edwardsonii*. Thus, we could also track the first appearance of systemic symptoms in individual plants. Systemic symptoms induced by CaMV infection of Arabidopsis ecotype Col-0 is illustrated in

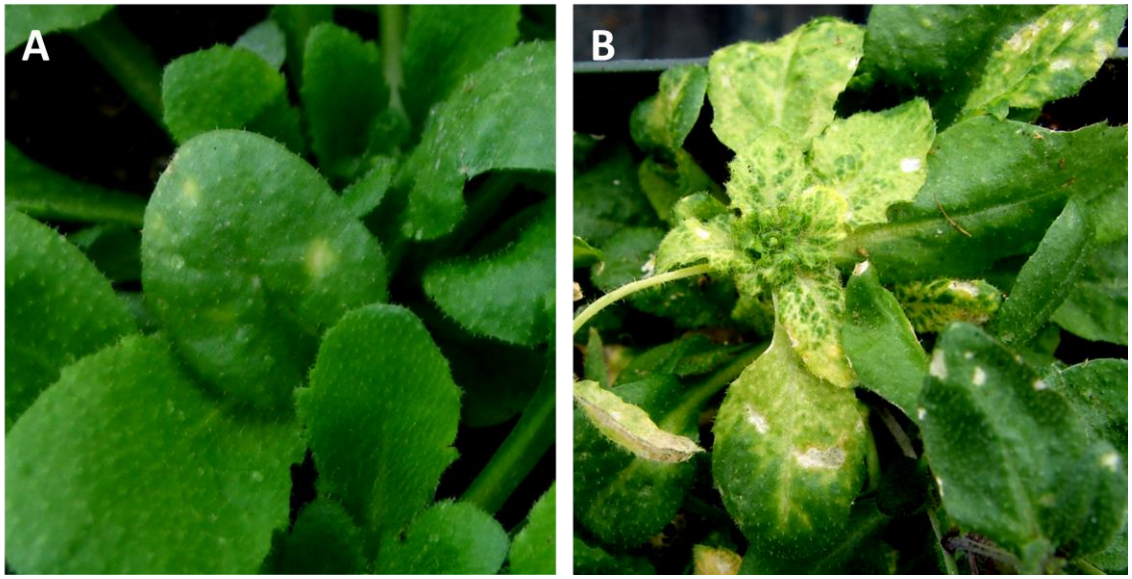

Fig 3.1 CaMV infection in *A. thaliana*. (A) Local lesions appear on inoculated leaves at approximately 7 dpi. (B) Systemic infection is observed in young tissue approximately at 15 dpi.

Fig. 3.1B. In this instance we hypothesized that any deficiencies in intracellular movement would be magnified, or at the very least reproduced, as the virus moved from cell-to-cell in the inoculated leaf and then to upper non-inoculated leaves. It is also significant to note that since we were assessing putative changes in movement of the virus, we would expect that the plant would become fully infected, as once a cell became infected the virus would replicate as in the wild type Col-0 plants.

To further examine the effect of CHUP1 on CaMV infections, Dr. Carlos Angel obtained the *chup1* T-DNA knockout line SALK_129128C and verified that it was homozygous for the placement of the T-DNA within the CHUP1 gene. Previous studies using SALK_129128C had shown that chloroplasts were unable to relocate within the cell in response to high light intensity (Oikawa *et al.*, 2003; 2008). Dr. Angel was able to reproduce this assay, as illustrated in Fig. 3.2. In this assay a narrow band of plant tissue for both wild type Col-0 and the *chup1* T-DNA knockout line was subjected to a high light intensity light source for one hour. After one hour of treatment, the band of Col-0 plant tissue exposed to high light intensity had become chlorotic, as the chloroplasts had moved within the cell to avoid potential photodamage. By contrast, the band of plant tissue of the *chup1* T-DNA knockout that was exposed to high light intensity was no different than the surrounding tissue. This simple assay was used to show that the *chup*1 T-DNA knockout line was homozygous for the insertion of the T-DNA in the *CHUP1* coding sequence. In our CaMV infection assay, the primary lesion response of the *chup1* T-DNA knockout line was delayed by approximately one day, relative to the wild type Col-0 (Fig. 3.3A). In an analysis of the systemic symptoms, the population of the chup1

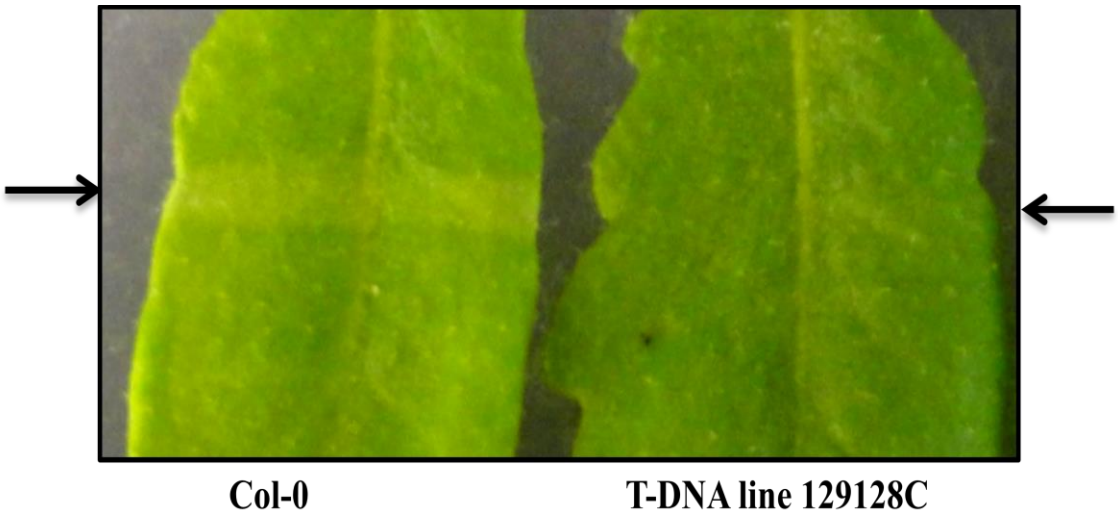

Fig 3.2 White band assay for chloroplast avoidance relocation in *A. thaliana* wild type Col-0 and *chup1* mutant SALK 129128C, after treatment with high intensity white light. Leaves were covered with a black cardboard sheet that contained an open slit and then irradiated for one hour with a strong white light at 700-800 µmol/m^2/sec.

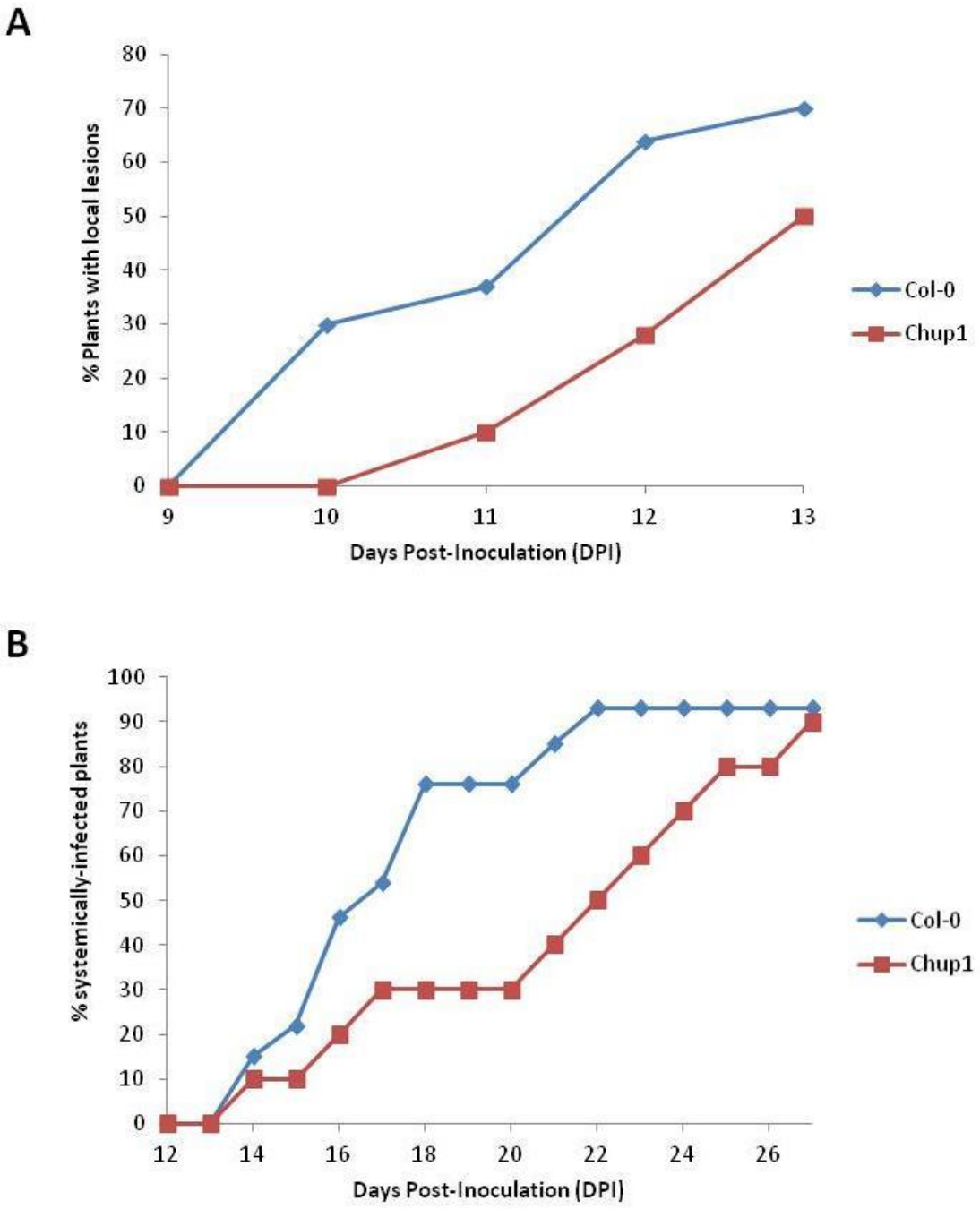

Fig 3.3 Development of local (A) and systemic symptoms (B) in *A. thaliana* wild type Col-0 and the *chup1* T-DNA insertion line SALK 129128C.

plants appeared to respond more slowly than Col-0, but there did not appear to be a delay in the initial appearance of systemic symptoms (Fig. 3.3B). However, repetitions of this test indicated that the response of the *chup1* T-DNA knockout line was not delayed relative to Col-0 (Fig. 3.4).

Although we were unable to show that *chup1* T-DNA knockout line exhibited a delayed response to CaMV infection and thus, it did not invalidate the results obtained with CaMV infection of *N. edwardsonii*. There are significant differences in the pathosystems, as *N. edwardsonii* responds to CaMV with HR, whereas Arabidopsis is susceptible. Furthermore, we were using a different assay to examine the rate of lesion development in *N. edwardsonii* than that used in Arabidopsis, and this might also account for the different results. Finally, even with *N. edwardsonii* we had hypothesized that functional redundancies in the host might account for the ability of local lesions to develop in *N. edwardsonii*, so these redundancies might mask any deficiency in CaMV movement in Arabidopsis.

Biological impact of myosin XI-K, XI-2, VIII-A, and VIII-B individual T-DNA knockouts on CaMV infection of Arabidopsis.

The myosins XI-2, XI-K and VIII have each been shown to contribute to the movement of plant viruses (Avisar *et al*, 2008a; Harries *et al*, 2009b; Yuan *et al*, 2011). For each myosin, its role in infection has been implicated either through T-DNA knockouts in

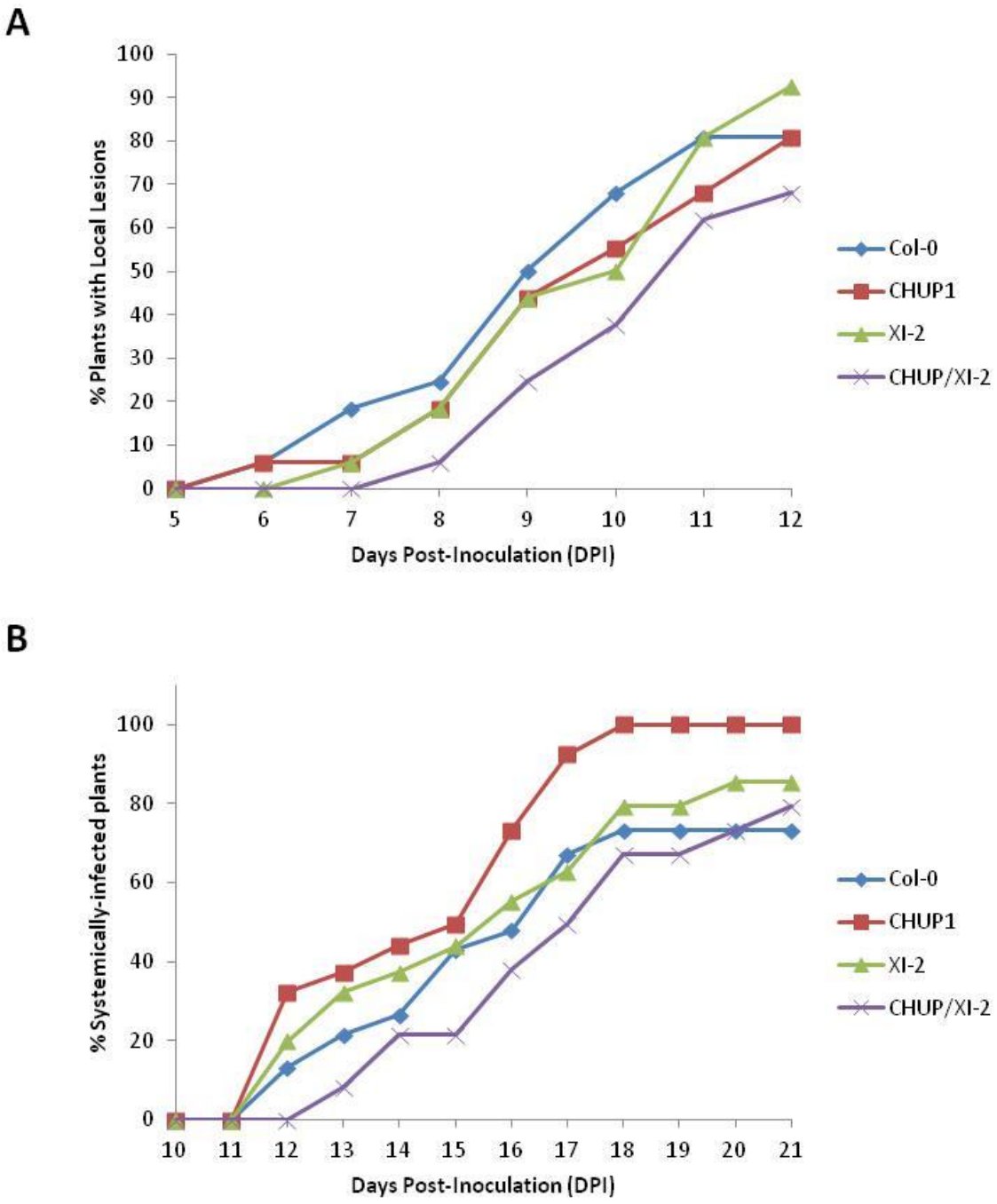

Fig 3.4 Performance of CaMV infection in *A. thaliana* T-DNA insertional single mutants for the genes CHUP1, myosin XI-2 and the double mutant CHUP1 XI-2. Charts show local lesion (A) and systemic infection development (B) in this test.

Arabidopsis or through virus –induced gene silencing (VIGS) in *N. benthamiana*. For example, Harries *et al.* (2009b) utilized a *Tobacco rattle virus* (TRV) VIGS system to silence specific myosins in *N. benthamiana* and followed with inoculation of Tobacco mosaic virus tagged with GFP (TMV-GFP). The influence of a myosin on TMV-GFP local movement was assessed through the measurement of the area of individual lesions at 3 dpi. Harries *et al.,* (2009b) found that the area of TMV-GFP lesions in *N. benthamiana* plants silenced for myosin XI-2 was approximately 33% the area of lesions in the *N. benthamiana* plants inoculated with the empty TRV vector. By contrast, silencing of myosins VIII-1, VIII-2 or XI F had no effect on the lesion area of TMV-GFP. This study implicated myosin XI-2 in movement of TMV. However, it also suggested that in the case of TMV, functional redundancies might be responsible for a reduced rate of growth of the TMV-GFP lesions in the myosin XI-2 plants.

To determine the effect of knocking out individual myosins on CaMV infection, we initially inoculated leaves of homozygous single T-DNA knockout lines for the genes myosin XI-2 and myosin XI-K with partially purified CaMV virions. We compared the development of the infection in the single knockout lines and the wild type control Col-0.

After four replicates we found that local lesions appeared on inoculated leaves of wild-type plants (Fig. 3.5A) at approximately 7 days post inoculation (dpi) and the percentage of plants with local lesions increased over time up to approximately 12 dpi, when nearly all of the plants exhibited local lesions (Fig. 3.5A). In the case of the XI-2 knockout line, we observed a one-day delay in the development of local lesions in three out of four tests, whereas with XI-K knockout line, we only observed a one-day delay in two out of four tests. The delay in local response was also reflected in a delayed systemic

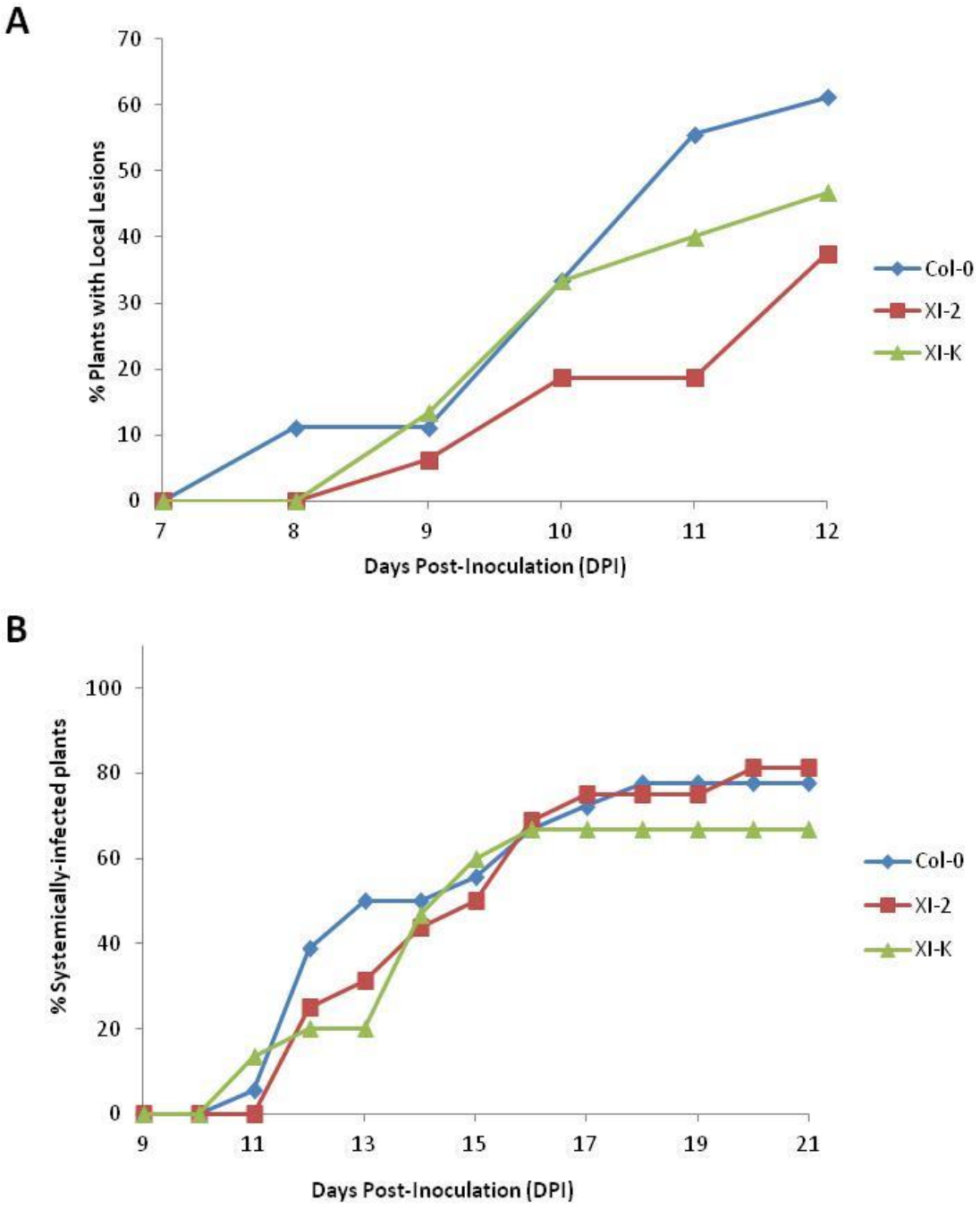

Fig 3.5 Performance of CaMV infection in *A. thaliana* T-DNA insertional single mutants for the genes myosin XI-K and myosin XI-2. Development of local lesions (A) and systemic infection (B) is shown for the tested mutants and the wild type Col-0.

response (Fig. 3.5B). In the case of Col-0, systemic symptoms typically began to appear 10 to 11 days after inoculation. With the XI-2 T-DNA knockout line, we observed a delay of one or more days in four out of four tests, whereas with XI-K we observed a delay in two out of four tests. This indicated that individual myosins XI-2 and XI-K might contribute to the movement of CaMV, but the effect was subtle, as once infections were observed in one plant, the number of plants increased at a similar rate as with the wild type Col-0 (Fig. 3.5B).

By contrast, CaMV infections in the myosin VIIIA and myosin VIIIB knockout lines were nearly indistinguishable from the infections in wild type Col-0. In the case of the myosin VIII-A and VIIIB knockout lines, a one-day delay in the development of local lesions was observed in only one out of four tests for each (Fig. 3.6A). A similar effect was observed with the development of systemic symptoms, as there was a one-day delay seen in only two of four tests for VIIIA, and only in one of four tests for VIIIB (Fig. 3.6B).

Biological impact of CHUP1, Myosin XI-K and Myosin XI-2 double and triple knockouts on CaMV infection

Although several studies have evaluated the impact of individual myosins on virus infections (Avisar *et al*, 2008a; Harries *et al*, 2009b; Yuan *et al*, 2011), no one has looked at crossing T-DNA knockout lines to evaluate the susceptibility of double and

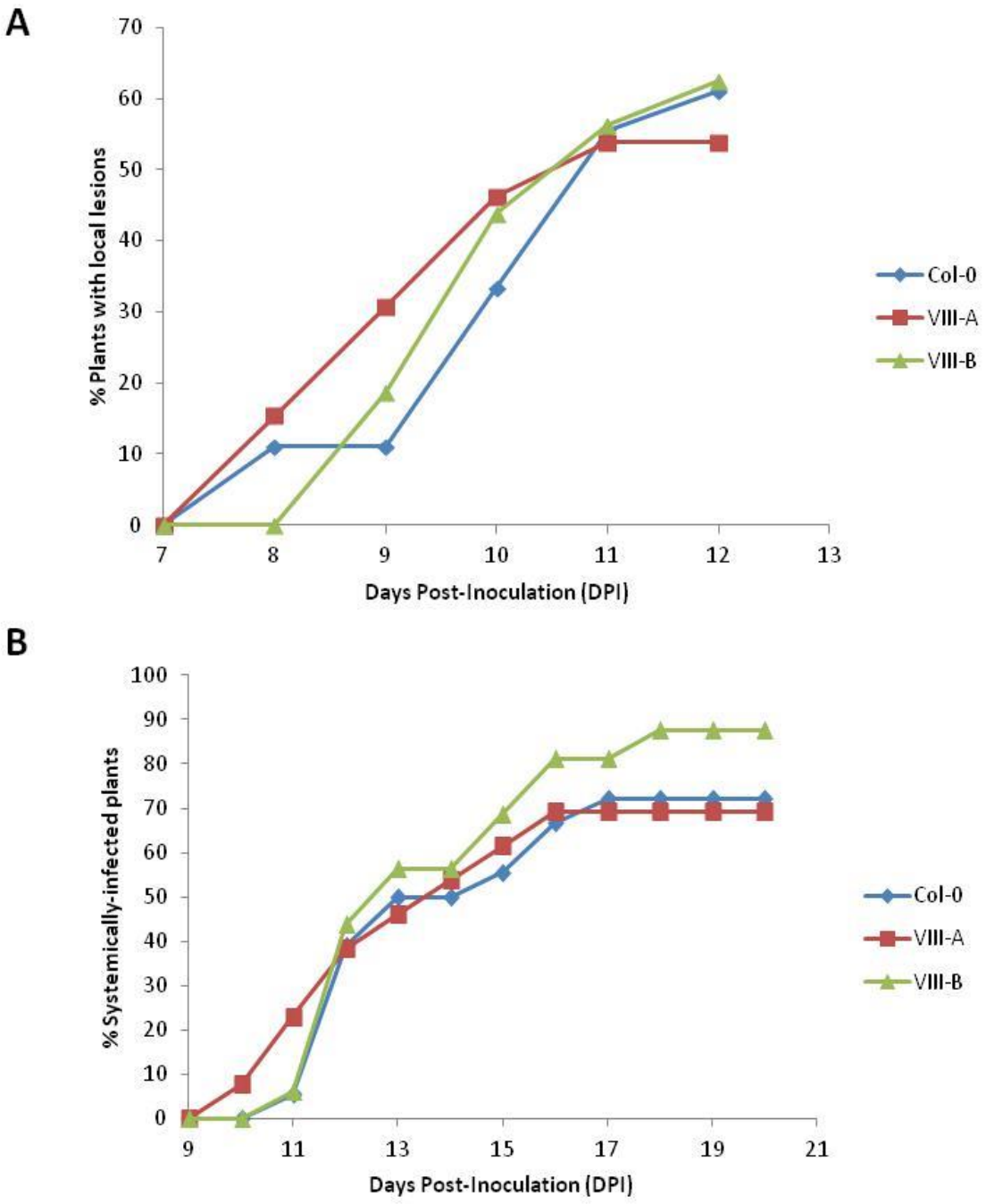

Fig 3.6 Performance of CaMV infection in *A. thaliana* T-DNA insertional single mutants for the genes myosin VIII-A and VIII-B. Development of local lesions (A) and systemic infection (B) is shown for the tested mutants and the wild type Col-0.

triple knockouts to virus infection. Since myosins have an important role in trafficking of organelles on microfilaments, one potential issue might concern the viability of double and triple knockouts of myosin genes. However, these combinations have already been shown to be viable. For example, in examining the movement of Golgi bodies within the cell, Peremyslov and coworkers (2013) made a series of double and triple knockout lines for different combinations of myosins. Some combinations did negatively affect growth of Arabidopsis, especially the triple knockout lines, but even with the triple knockout of myosins *myoB1*, *myoB2* and *myoB3* the plants were approximately 8% smaller than the wild type Col-0 plants (Peremyslov *et al*, 2013).

We hypothesized that functional redundancies between CHUP1 and specific myosins might explain the susceptibility of plants to virus infection. To test this hypothesis, we crossed the homozygous *chup*1 T-DNA line with T-DNA lines for myosin XI-2 and XI-K to create all combinations of double and triple knockouts. PCR was used to screen for homozygous mutants. In regard to the *chup1/xi-2* mutant, 48 F2 plants were screened to identify two that were homozygous. For the other two double knockout lines, *chupi/xi-k* and *xi-2/xi-k*, only 18 plants were necessary to be screened in each case in order to obtain one homozygous double mutant. Surprisingly, to obtain a homozygous triple mutant line we screened a total of 136 plants. All combinations were viable, as illustrated in Fig. 3.7.

The double and triple knockout lines were subsequently evaluated for their susceptibility to CaMV. Two experiments illustrated in Figures 3.4 and 3.8 evaluated the susceptibility of the double mutant *chup1/xi-2*. In these experiments, the slowest rate of development for local lesions (Figs. 3.3A and 3.8A) as well as systemic movement (Figs.

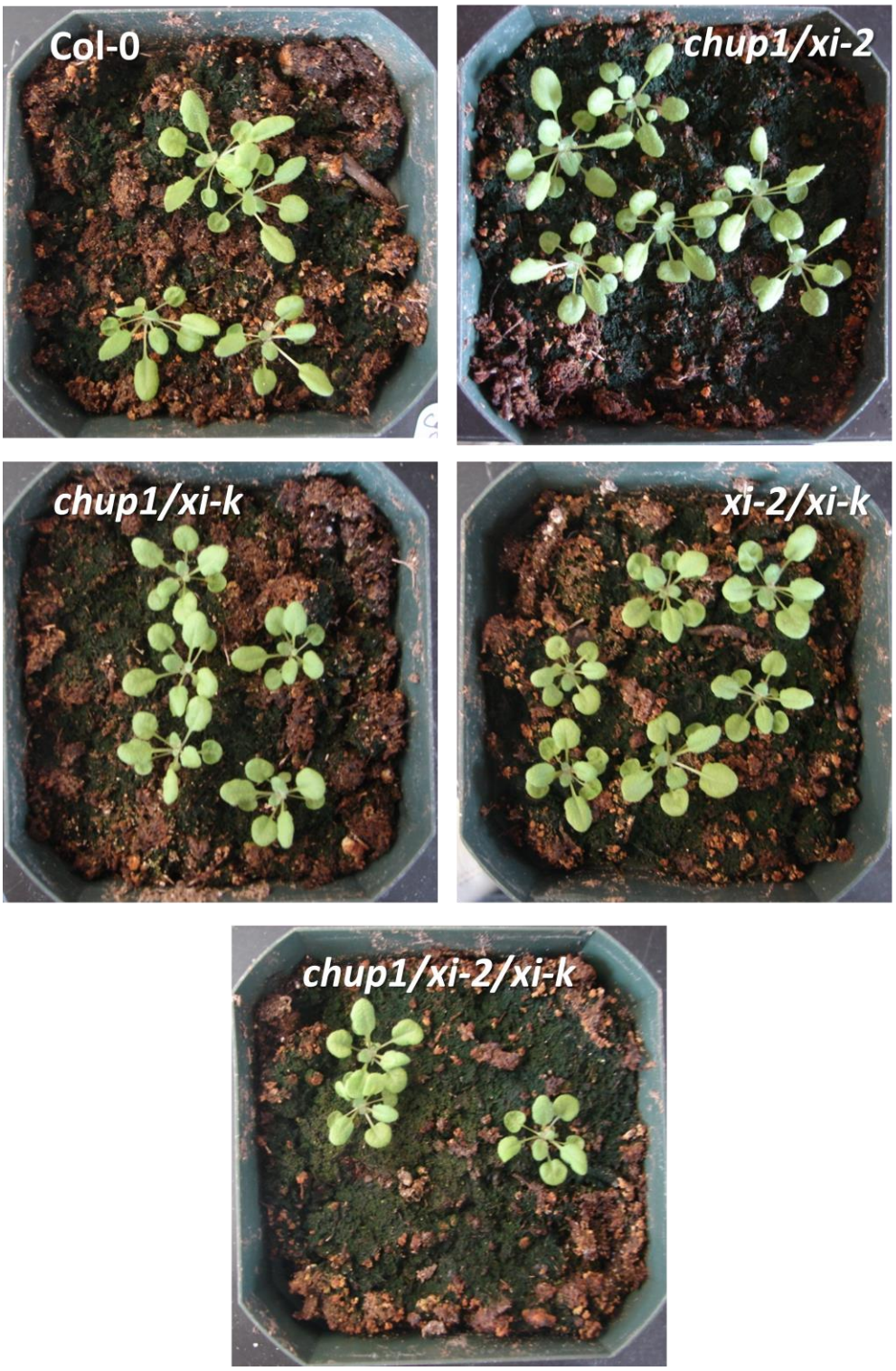

Fig. 3.7 Phenotypes observed for double and triple mutants grown at the same time as the wild type Col-0. No apparent phenotype was observed in any of the mutants.

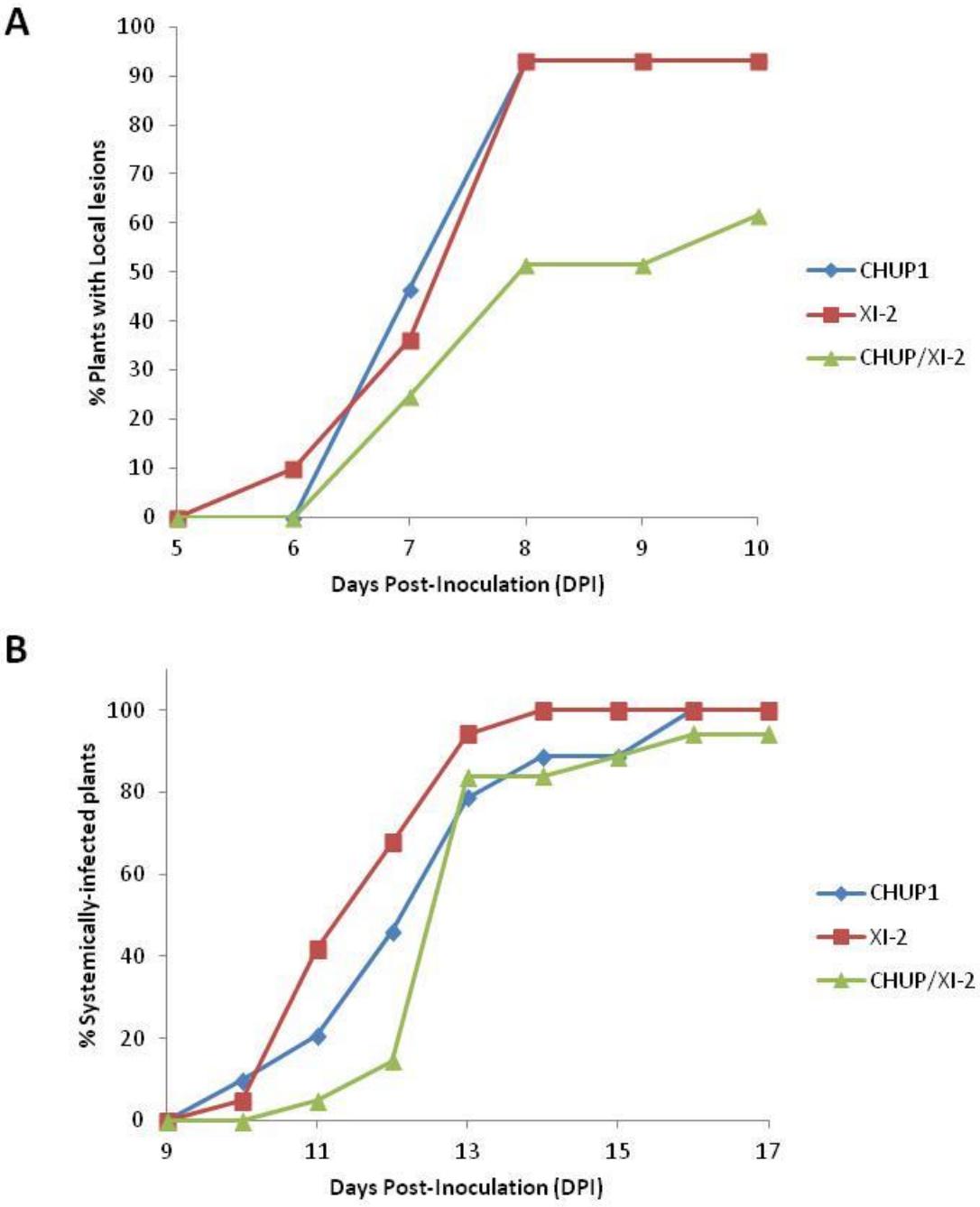

Fig 3.8 Performance of CaMV infection in *A. thaliana* T-DNA insertional single mutants for the genes CHUP1, myosin XI-2 and the double mutant CHUP1 XI-2. Charts show local lesion (A) and systemic infection development (B) in this test.

3.4B and 3.8B) was observed with the double mutant. Two additional tests were performed to evaluate the susceptibility of all combinations of double knockout lines (*chup1/xi-2*, *chup1/xi-k*, *xi-2/xi-k*) as well as the triple knockout line (*chup1/xi-2/xi-k*). In the first experiment, the local lesion response of all of the double knockout lines was delayed by one day, relative to wild type Col-0, whereas with the triple knockout line, the initiation of the local lesion response was delayed by two days (Fig. 3.9A). Interestingly, the systemic symptom response of the double and triple knockout lines was also delayed, but there were differences in the timing of symptom development between the double and triple knockout lines. Relative to Col-0, the line *chup1/xi-2* exhibited a one-day delay, the line *chup1/xi-k* exhibited a two-day delay, the line *xi-2/xi-k* exhibited a three-day delay, and the triple knockout line exhibited a four-day delay (Fig. 3.9B).

A second virus inoculation experiment was carried out with the double mutants *chup1/xi-2* and *xi-k/xi-2*, the triple mutant, and Col-0. The double mutant *chup1/xi-k* was not included due to a lack of seeds. Local lesion development in this experiment started with Col-0 at 6 dpi, and we observed a one-day delay in the initiation of local lesions for the *chup1/xi-2* double mutant. The triple mutant and *xi-2/xi-k* double mutant showed local lesions the same day as that in Col-0. However, the percentage of plants showing local lesions was approximately 30% less than Col-0 and the double and triple knockout plants developed symptoms at a slower rate than Col-0 (Fig. 3.10A). The systemic symptoms were first observed in 42% of Col-0 and 6% of the triple mutant plants at 10 dpi (Fig. 3.10B). The double mutants *chup1 xi-2* and *xi-k/xi-2* showed a one-day delay in developing systemic infection.

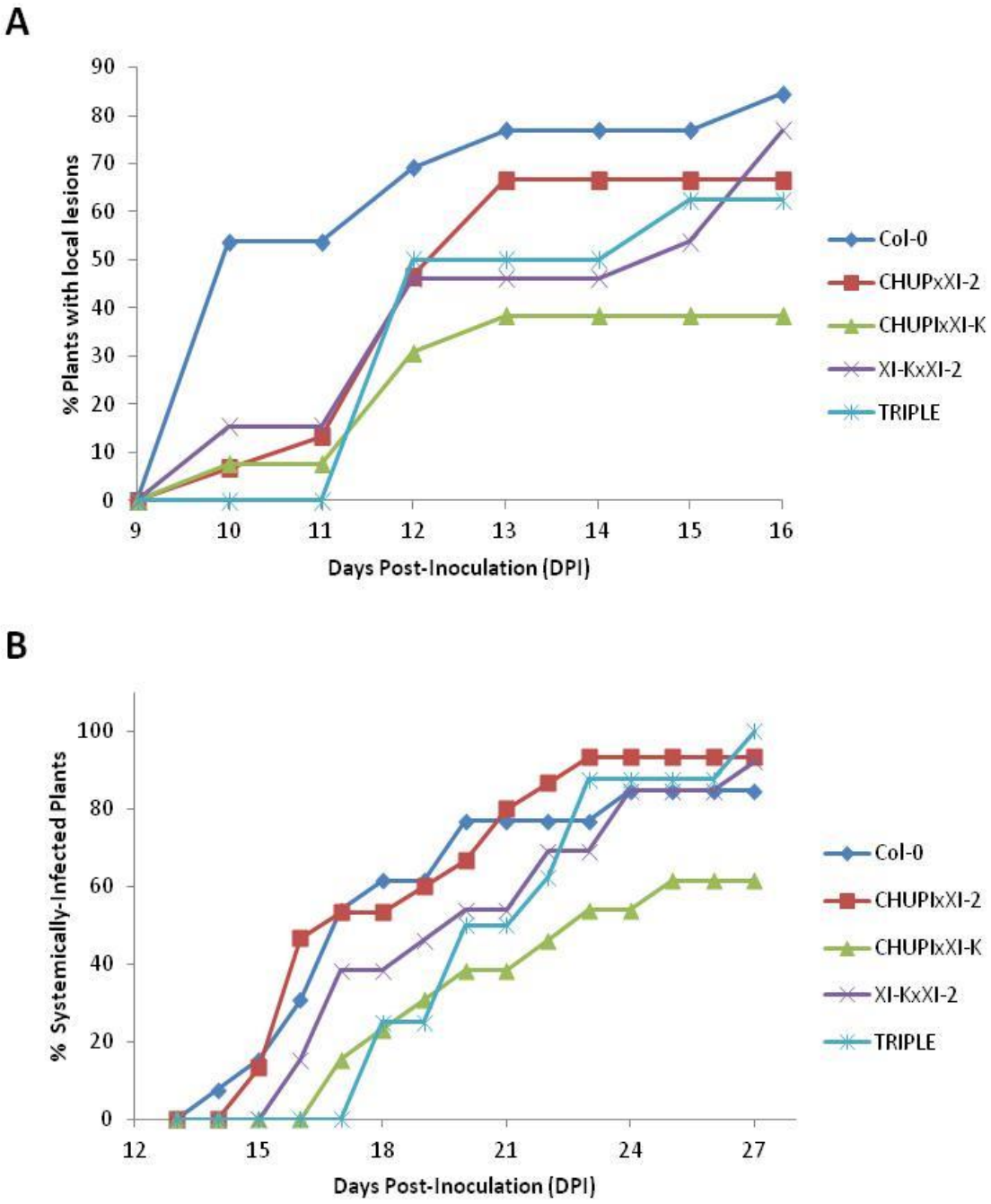

Fig 3.9 Performance of CaMV infection in *A. thaliana* T-DNA insertional double mutants *chup1/xi-k*, *chup1/xi-2*, *xi-k/xi-2* and the triple mutant *chup1/xi-2/xi-k*. Charts show local lesion (A) and systemic infection development (B) in this test.

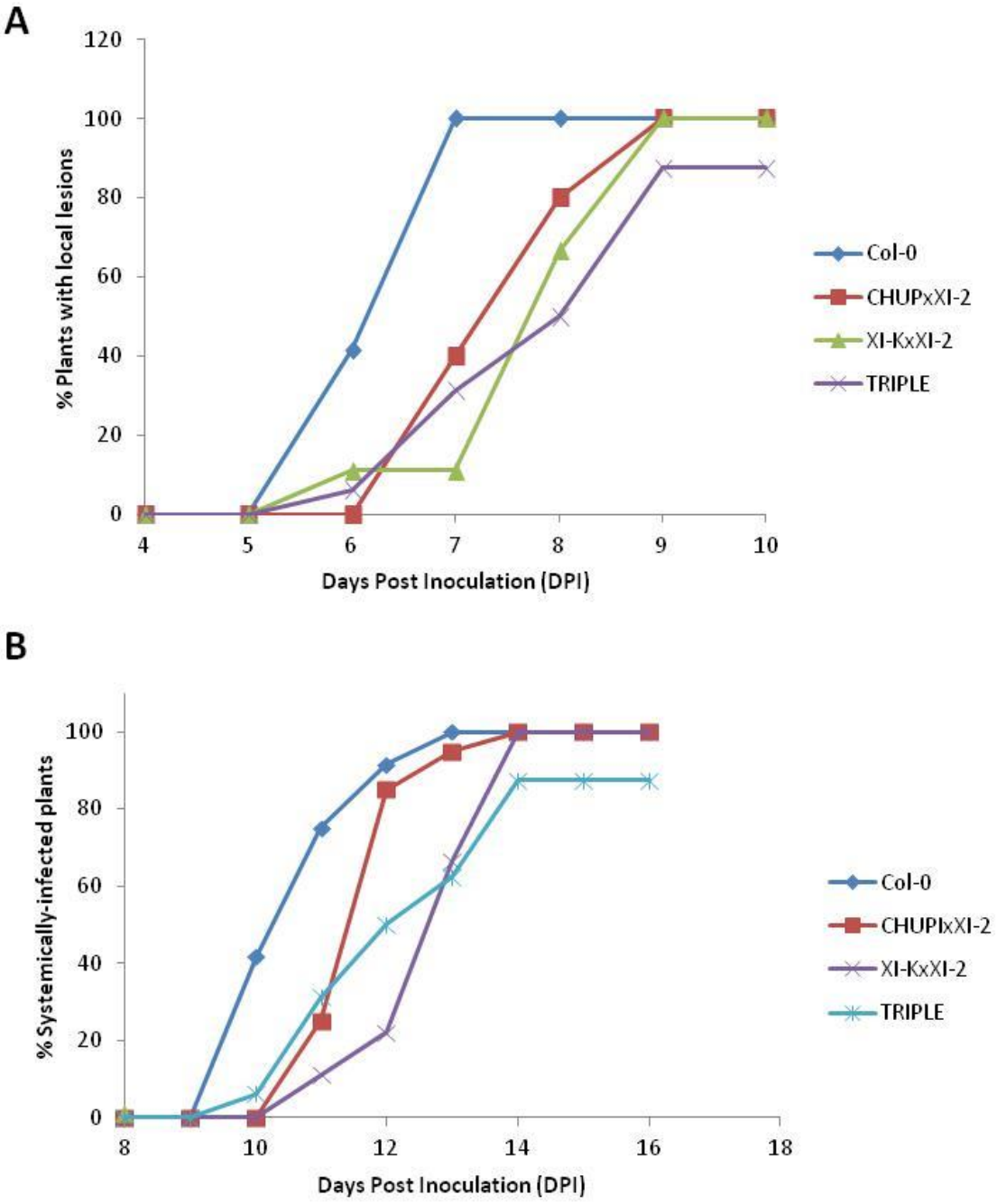

Fig 3.10 Performance of CaMV infection in *A. thaliana* T-DNA insertional double mutants *chup1/xi-2*, *xi-k/xi-2* and the triple mutant *chup1/xi-2/xi-k*. Charts show local lesion (A) and systemic infection development (B) in this test.

One difference between the first and second tests concerns the timing of symptoms, as local and systemic symptom development was accelerated in the second test by four days. The accelerated symptom development in test two might represent a more conducive environment for CaMV infections, and might have the effect of compressing any differences observed between Col-0 and the knockout lines. Although both tests indicated that CaMV symptom development was delayed in the knockout lines, they also illustrated the challenges of making comparisons between tests. Further tests will be necessary to determine if CaMV infections truly are slower in the double and triple knockout lines than Col-0 or the single knockout lines.

MATERIALS AND METHODS

Plant Material and Growth Conditions.

Seeds of *Arabidopsis thaliana* homozogous T-DNA insertional mutants for the gene CHUP1 (AT3G25690. Line SALK_129128C) were obtained from the Arabidopsis Biological Resource Center (ABRC, The Ohio State University. Columbus, OH, U.S.A.). *Arabidopsis thaliana* homozogous T-DNA insertional mutants for the genes Myosins XI-2 (AT5G43900, Line SALK_055785C) and XI-K (AT5G20490, Line SALK_067972C) were provided by Dr. Richard S. Nelson from The Samuel Roberts Noble Foundation (Ardmore, OK., U.S.A.). To break dormancy, seeds were planted in pots with soil mixture and stored at 4°C for 3 days. Plants were subsequently grown in a Conviron CMP3023 growth chamber at 20°C with 16h light/ 8h dark cycles.

Development of CHUP1, Myosins XI-2 and XI-K double and triple T-DNA Insertional mutants.

Crosses between homozygous T-DNA mutant lines were performed according to Faure *et al* (2002), to create the combinations *chup1/xi-k, chup1/xi-2, xi-k/xi-2*. The latter

was crossed with homozygous *chup1* plants to develop the triple knockout *chup1/xi-k/xi-2*. Leaf tissue from F1 plants was used for DNA isolation using the DNeasy® Plant Mini Kit (Quiagen. Venlo, The Netherlands) to verify that the plants were true hybrids and then they were allowed to self to identify the homozygous double and triple knockouts. Two different PCR reactions were performed to genotype each gene of interest. One reaction used two gene-specific primers, whereas the second reaction involved the T-DNA-specific primer LBa1 (5'-TGG TTC ACG TAG TGG GCC ATC G-3') and one of the gene-specific primers used in the first PCR reaction. The CHUP1-specific primers were CHUP1_2F (5'-TGG TGA ATC CGT AGC CAT AAC AAC-3') and CHUP1_3R (5'-AAG TCC CCTTGT GTC TCC ACA TCC-3'). The myosin XI-K-specific primers were XI-K_507F (5'-TGG GGA AAG TGG TGC TGG-3') and XI-K_935R (5'-TCC TCG GTG TCA TCC ACT CC-3'). The myosin XI-2-specific primers MYA-2_2691F (5'-TGA AGA GCT GAC CTG GAG ATT G-3') and MYA-2_3234R (5'-CTT TTC CAA GTT CGT TTG GTG GC-3'). Homozygous plants were self-pollinated and allowed to produce seed. Plants from the F2 progeny were used for CaMV infection assays. Characterization of the *chup1/xi-2* double mutant lines were characterized by Yu Zhang from the Virus Biotechnology lab at the University of Missouri (Columbia, MO, U.S.A).

Virus Purification and Inoculation.

To prepare the inoculum for the Arabidopsis infection assays, CaMV strain W260 was inoculated to turnips (*Brassica rapa* subsp. rapa cv. Just Right). The CaMV virions

were partially purified from systemically infected turnip leaves and concentrated according to Schoelz *et al* (1986), with one additional step. After ultracentrifugation and resuspension in H_2O, the virion preparation was subjected to a low speed centrifugation step (12,000 x g for 10 min) to remove any plant debris that had been carried through the ultracentrifugation step. Arabidopsis plants were 5 – 6 weeks old at inoculation, and immediately before inoculation they were lightly dusted with Celite. The CaMV virion preparation was inoculated to three leaves on each plant by applying 20 µl onto a flattened glass rod and then gently rubbing the leaves. *A. thaliana* wild type Col-0 inoculated with purified CaMV virions was used as the control for each experiment. Experiments were carried out under greenhouse conditions. The number of plants with chlorotic primary lesions and evidence of systemic virus symptoms was evaluated every day.

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APPENDIX A

THE *Tobacco necrosis virus* COAT PROTEIN ELICITS A HYPERSENSITIVE RESPONSE IN *Nicotiana* SPECIES WITHIN THE *Alatae* SECTION.

INTRODUCTION

One of the most common strategies used by plants for defense against pathogen attack involves specific receptors for individual pathogens. These receptors are called resistance (R) genes, and upon recognition of a plant pathogen, either directly or indirectly, they trigger a cascade of plant defenses (Chisholm *et al*, 2006; Jones and Dangl, 2006). The pathogen proteins recognized by the R proteins were originally called avirulence (Avr) proteins (Flor, 1971). However, several years ago it was recognized that these proteins serve to facilitate the pathogen infection, in some instances through suppression of plant defenses, so they are now called effectors and the plant defense response is considered effector-mediated immunity (ETI) (Jones and Dangl, 2006). The Resistance-gene-mediated defense triggered by either the direct or indirect association of a particular pathogenic, avirulence determinant (Avr) with, a matching resistant (*R*) gene

that confers resistance to the pathogen in a strain-specific manner is one of the most intensively studied forms of ETI is the hypersensitive response (HR), a form of programmed-cell death that limits pathogen spread to initial infected cells and restraints pathogen growth (Goodman and Novacky, 1994). In the case of viruses, HR development may occur as early as one day postinoculation (dpi) and as late as 10 dpi, depending on the virus-host combination (Angel and Schoelz, 2013).

Most plant *R* genes code for proteins classified within the nucleotide binding site-leucine rich repeat (NBS-LRR) family, and several *R* genes involved in resistance against plant viruses have been identified and cloned in the last decade (Collier and Moffett, 2009; Gururani *et al*, 2012). Furthermore, genome sequencing projects have allowed the identification of all genes that have the NBS-LRR motif in a plant genome. For example, the genome of *Arabidopsis thaliana* ecotype Col-0 contains 149 NBS-LRR-coding genes (Meyers *et al*, 2003). The identification of *R* genes through genome sequencing projects, however, has led to a mystery, because the pathogen Avr targets for most NBS-LRR genes present in a genome have not been determined.

One genus that has been valuable for the study of virus resistance is the genus *Nicotiana*, which includes 76 species clustered into 13 taxonomic sections (Clarkson *et al*, 2004; Knapp *et al*, 2004). The highest diversity within this genus occurs in South and North America with approximately 75% of the *Nicotiana* species distributed between 12 sections. The remaining 25% of the species, which are all members of the *Suaveolentes* section, is mostly found in Australia. Only one species has been found in Africa (Clarkson *et al*, 2004). Although *N. benthamiana* and *N. tabacum* have been frequently used in the study of plant virology, *N. glutinosa* has played an important role in the

characterization of resistance genes against plant viruses. *N. glutinosa* carries the *N* gene, a gene that encodes an R protein that triggers resistance to TMV and was one of the first *R* genes to be discovered and cloned (Holmes 1938, 1946; Whitham *et al*, 1994). Similarly, several *Nicotiana* species have also been successfully used to identify viral Avr determinants. *Cauliflower mosaic virus* protein P6 triggers HR in *N. edwardsonii* and *N. glutinosa*, and it was the first viral protein shown to be an Avr determinant (Schoelz *et al*, 1986). Two years later, the TMV coat protein was conclusively identified as an avr determinant in *N. sylvestris* (Knorr and Dawson, 1988). TMV infectious clones were used to determine that the 183-kDa replicase protein of TMV is recognized by the *N* gene (Padgett and Beachy, 1993), and later, agroinfiltration subsequently showed that the HR determinant could be localized to the replicase region that contains the helicase domain (i.e. the P50 protein) (Erickson *et al*, 1999). The P50 protein, a subset of the replicase, has frequently been used to probe the interaction of the TMV with the N protein (Erickson *et al*, 1999; Ueda *et al*, 2006), but it is significant to note that the P50 protein does not exist in TMV infections.

Previous studies have successfully characterized tombusvirus Avr determinants in *Nicotiana* species (Angel and Schoelz, 2013; Angel *et al*, 2011; Scholthof *et al*, 1995). One of the Avr determinants identified was the coat protein (CP) of *Tomato bushy stunt virus* (TBSV), which was shown to trigger HR in *Nicotiana* species of the section *Alatae* (Angel and Schoelz, 2013). To explore the possibility of sources of resistance to other viruses in the *Tombusviridae*, we I chose to characterize potential Avr determinants in *Tobacco necrosis virus* (TNV) TNV is a necrovirus with an uncapped, nonpolyadenylated positive-sense single-stranded RNA (+ssRNA) genome of about 3.8

kb that is encapsidated into small icosahedral viral particles that have a diameter of 28 nanometers (Russo *et al*, 1994; Shen and Miller, 2004). TNV has been transmitted experimentally to at least 88 species in 37 dicotyledonous and monocotyledonous families. It is unusual because it elicits HR in the majority of these species (Price, 1940). The only hosts shown to be systemically infected by TNV are *Anthriscus cerefolium* and *Trachymene caerulea* (Price, 1940).

In the present study, 20 *Nicotiana* species were tested for their response to *Tobacco necrosis virus*. We found that responded to TNV infection with HR; in fact, TNV was able to move systemically in only one of the species, *N. benthamiana*. To identify potential TNV proteins that trigger HR, we expressed the TNV CP through agroinfiltration and found that HR is elicited in species that belong to section *Alatae*. Finally, agroinfiltration of a combination of TNV-CP deletion and point mutants showed that the coat protein rather than viral RNA was responsible for HR elicitation.

RESULTS

Survey of *Nicotiana* species for resistance to TNV virion inoculations

To examine the reaction of *Nicotiana* species to TNV, TNV virions were inoculated to leaves of 20 *Nicotiana* species (Table A.1), species that had previously been used to characterize the avirulence proteins present in the TBSV genome (Angel and Schoelz, 2013). Nineteen of the twenty *Nicotiana* species responded with necrotic local lesions on inoculated leaves between 2 to 5 days post inoculation (dpi) (Table A.1). Necrotic lesions varied in size, number per leaf, and timing. For example, *N. quadrivalvus* responded with large necrotic lesions that coalesced to cover nearly the entire leaf (Fig. A.1a), whereas *N. forgetiana* accession TW50, included in the same test, responded with fewer lesions (Fig. A.1b). Although *N. benthamiana* also developed necrotic local lesions upon inoculation of TNV virions (Fig. A.1c), TNV was able to spread systemically in this plant, as upper non-inoculated leaves developed a systemic necrosis symptom (Fig. A.1d). *N. benthamiana* was the only species that TNV was able to move systemically. Consequently, the reactions of 18 of the *Nicotiana* species were classified as an HR type resistance (Table A.1). Only one *Nicotiana* species, *N. otophora*, responded to TNV virion inoculation with chlorotic local lesions (Fig. A.1e), which developed much more slowly than the necrotic responses of the other *Nicotiana* species. Interestingly, *N. otophora* also was resistant to TNV, as no symptoms developed on upper, non-inoculated

Table A.1. Response of 20 *Nicotiana* species to inoculation of TNV or TBSV virions and to agroinfiltration of selected viral proteins.

Nicotiana spp.	Section	TNV Virion Inoc.	Agroinfiltration of TNV CP
N. langsdorffii	*Alatae*	HR[a]	HR
N. longiflora	*Alatae*	HR	HR
N. bonariensis	*Alatae*	HR	HR
N. alata	*Alatae*	HR	HR
N. forgetiana	*Alatae*	HR	HR
N. plumbaginifolia	*Alatae*	HR	nr[b]
N. quadrivalvus	*Polydicliae*	HR	nr
N. clevelandii	*Polydicliae*	HR	nr
N. edwardsonii[c]	*Undulatae/Polydicliae*	HR	nr
N. glutinosa	*Undulatae*	HR	nr
N. arentsii	*Undulatae*	HR	nr
N. undulata	*Undulatae*	HR	nr
N. tabacum	*Nicotiana*	HR	nr
N. sylvestris	*Sylvestres*	HR	nr
N. otophora	*Tomentosae*	CLL[d]	nr
N. tomentosiformis	*Tomentosae*	HR	nr
N. repanda	*Repandae*	HR	nr
N. glauca	*Noctiflorae*	HR	nr
N. rustica	*Rusticae*	HR	nr
N. benthamiana	*Suaveolentes*	Susc.[e]	nr

[a]HR – necrotic local lesions, no development of systemic symptoms.
[b]nr – no visible reaction.
[c]*N. edwardsonii* is an artificial species hybrid between *N. glutinosa* and *N. clevelandii* (Christie 1969).
[d]CLL, chlorotic local lesions, no development of systemic symptoms.
[e]Susceptible – symptoms develop in upper, non-inoculated leaves.

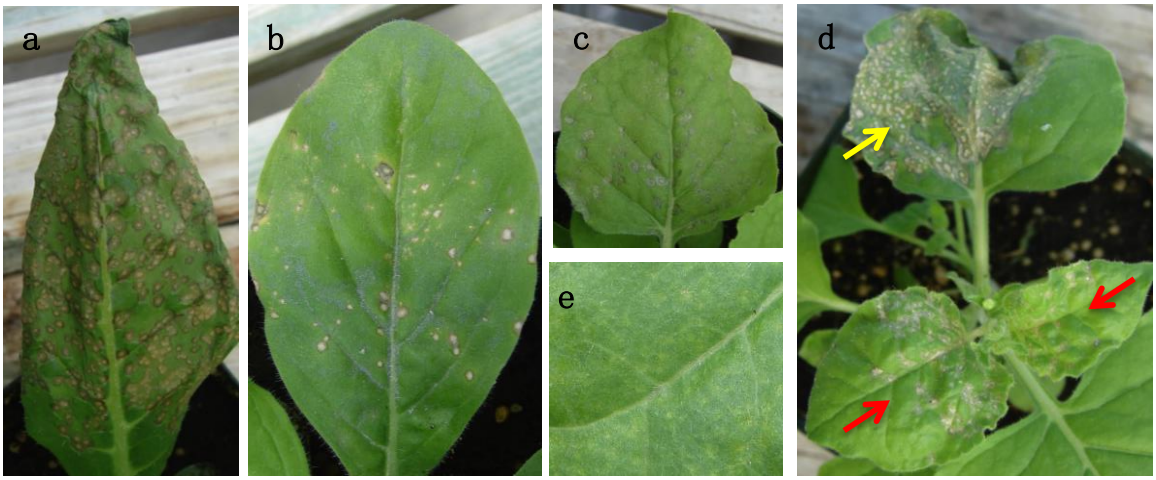

Figure A.1. Response of *Nicotiana* species to TNV virion inoulation. a) *N. quadrivalvus* at 3 dpi. B) *N. forgetiana* TW50 at 5 dpi. c) *N. benthamiana* at 3 dpi. d) *N. benthamiana* at 5 dpi. e) *N. otophora* at 9 dpi. The red arrows indicate *N. benthamiana* leaves exhibiting systemic symptoms, whereas the yellow arrow indicates lesions in an inoculated leaf.

leaves. Figures A.2-A.5 illustrate the diversity of responses of all 20 *Nicotiana* species to TNV virion inoculations.

The TNV coat protein triggers HR in *Nicotiana* species in section *Alatae*

To investigate whether the TNV coat protein was capable of triggering HR in any *Nicotiana* species, we cloned the TNV coat protein coding sequence into the *Agrobacterium tumefaciens* binary vector pKYLX7 to create pCP-29 (Fig. A.6). Upon agroinfiltration into leaves of each of the 20 *Nicotiana* species, pCP-29 elicited HR in several species in section *Alatae* (Table A.1), including the species *N. langsdorffii*, *N. longiflora*, *N. bonariensis*, *N. alata*, and *N. forgetiana*. Of the six species in section *Alatae*, only *N. plumbaginifolia* failed to respond to agroinfiltration of pCP-29 with an HR. Typical reactions are illustrated in Fig. A.7. HR was initiated in *N. langsdorffii* by pCP-29 agroinfitlration as early as 2 dai (days after infiltration), whereas no reaction was observed in *N. plumbaginifolia* at the same timepoint (Fig. A.7). The TNV coat protein did not elicit HR in any of the other *Nicotiana* species included in our study (Table 1). Interestingly, the same *Nicotiana* species that responded to agroinfiltration of the TNV coat protein with HR also responded with HR to agroinifltation of the TBSV coat protein in an earlier paper (Angel and Schoelz, 2013).

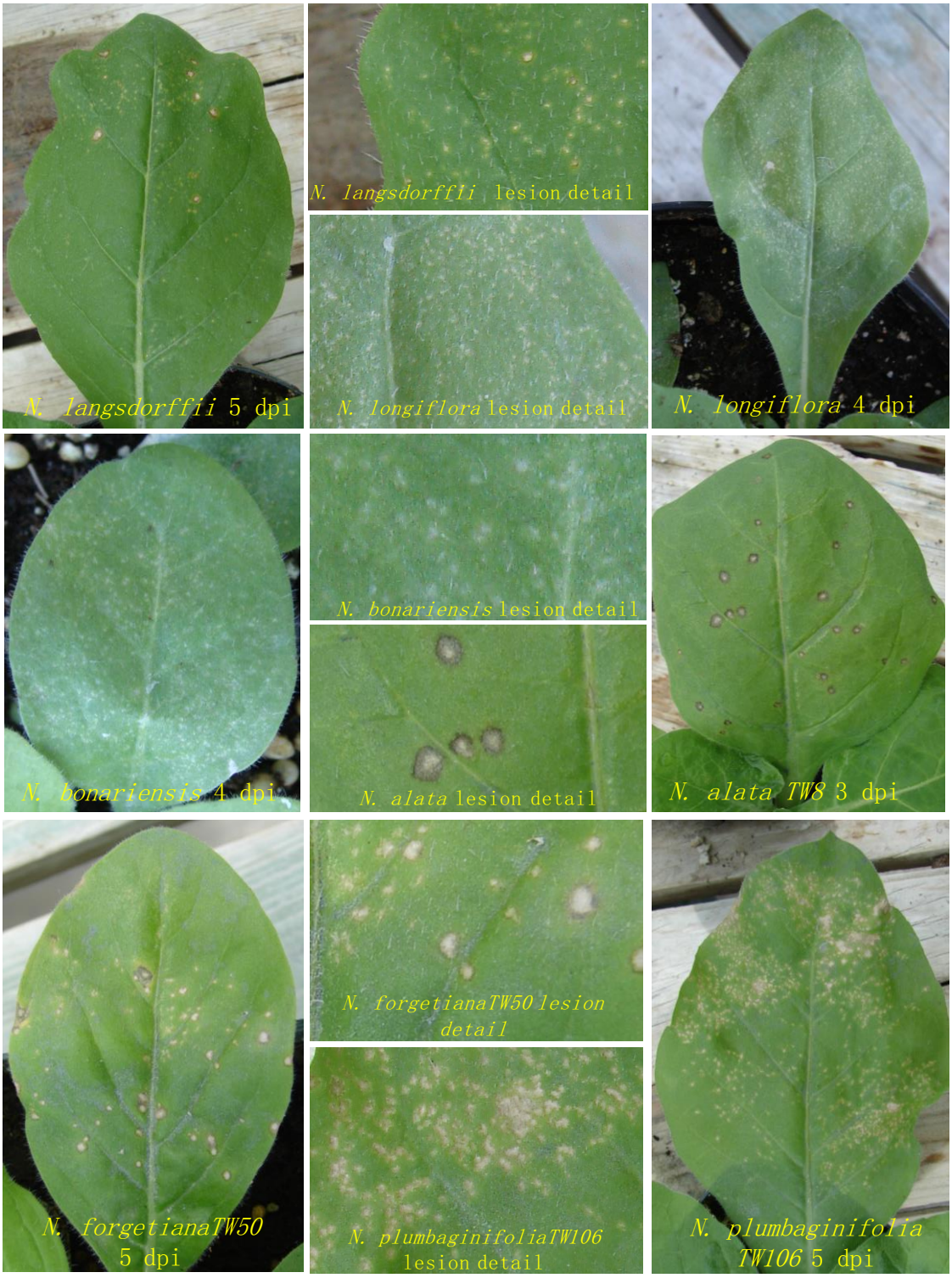

Figure A.2. Response of *Nicotiana* species to TNV virion inoculation. with HR. The photos in the central column are an expansion of a portion of the whole leaf photos in the side columns to illustrate local lesions in greater detail.

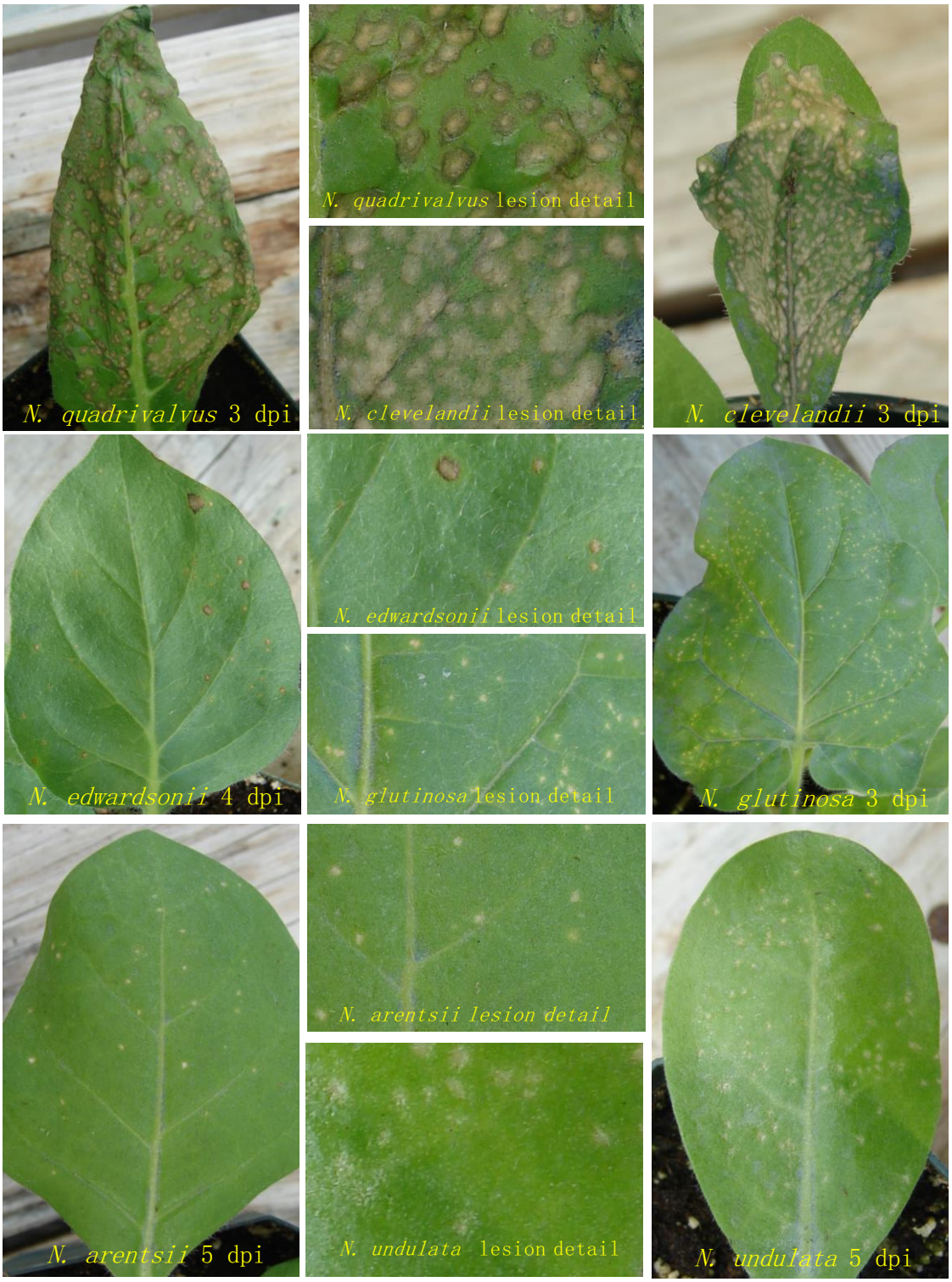

Figure A.3. Response of *Nicotiana* species to TNV virion inoculation. with HR. The photos in the central column are an expansion of a portion of the whole leaf photos in the side columns to illustrate local lesions in greater detail.

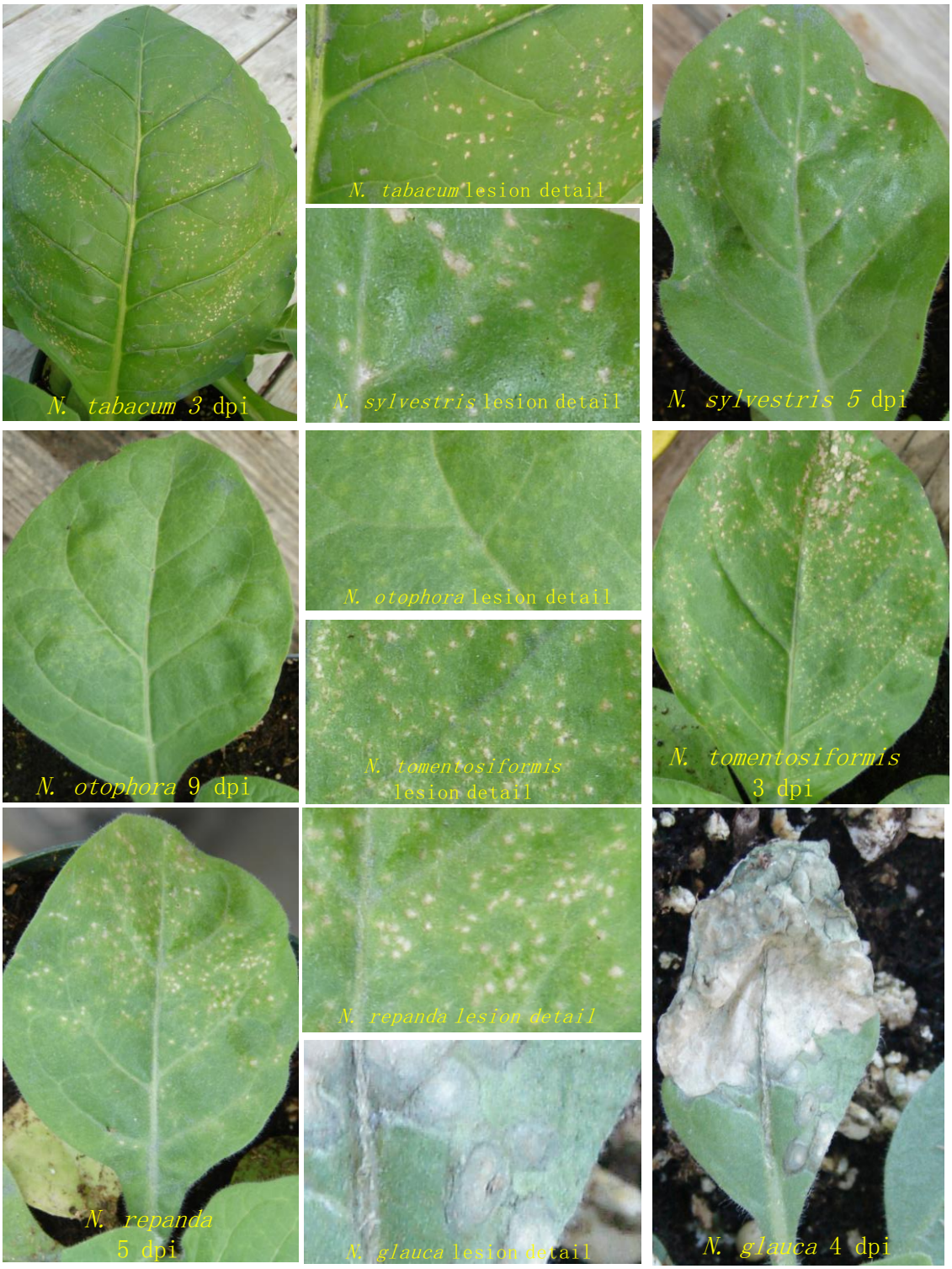

Figure A.4. Response of *Nicotiana* species to TNV virion inoculation. with HR. The photos in the central column are an expansion of a portion of the whole leaf photos in the side columns to illustrate local lesions in greater detail.

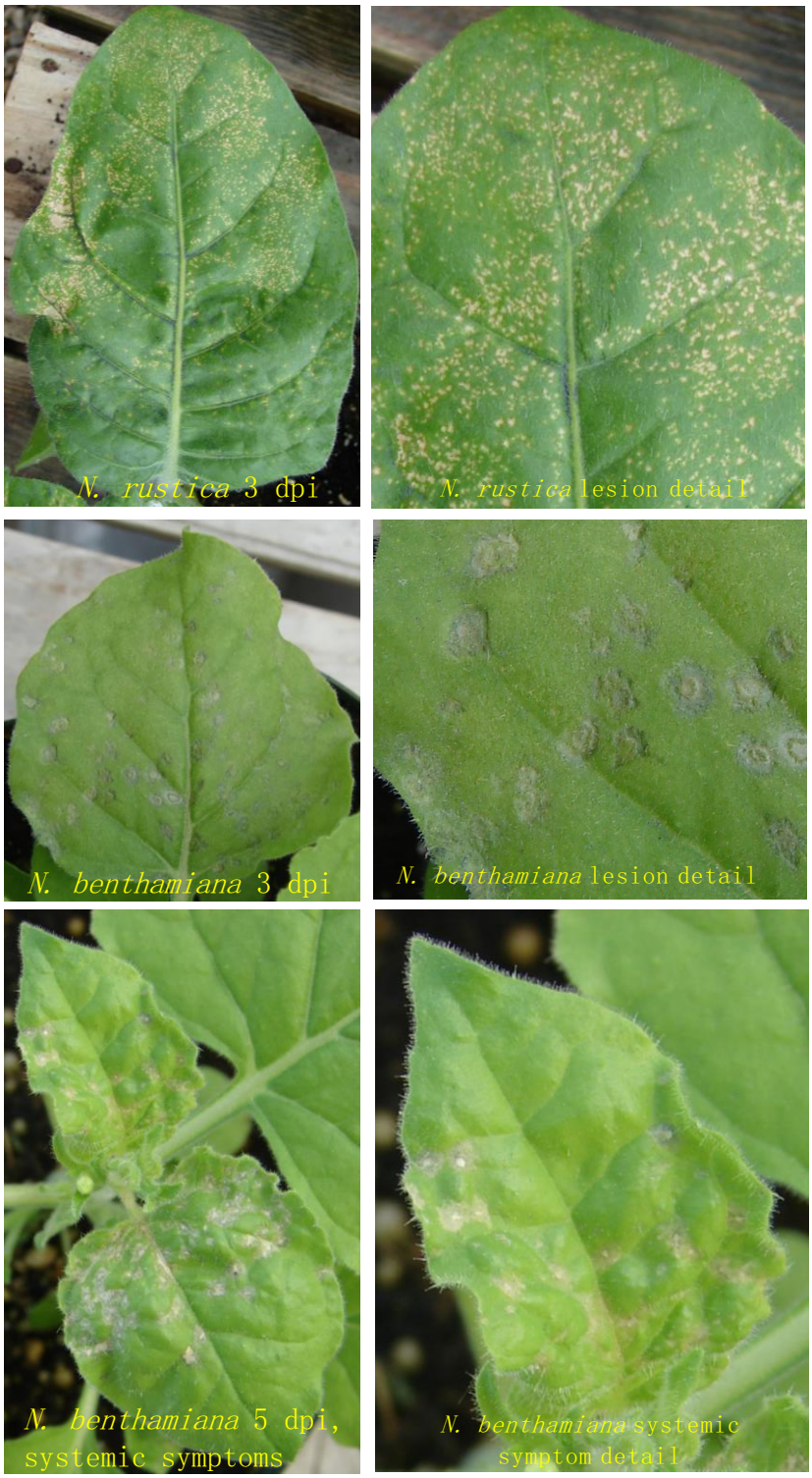

Figure A.5. Response of *Nicotiana* species to TNV virion inoculation. with HR. The photos in the right column are an expansion of a portion of the whole leaf photos on the left side columns to illustrate local lesions or systemic symptoms in greater detail.

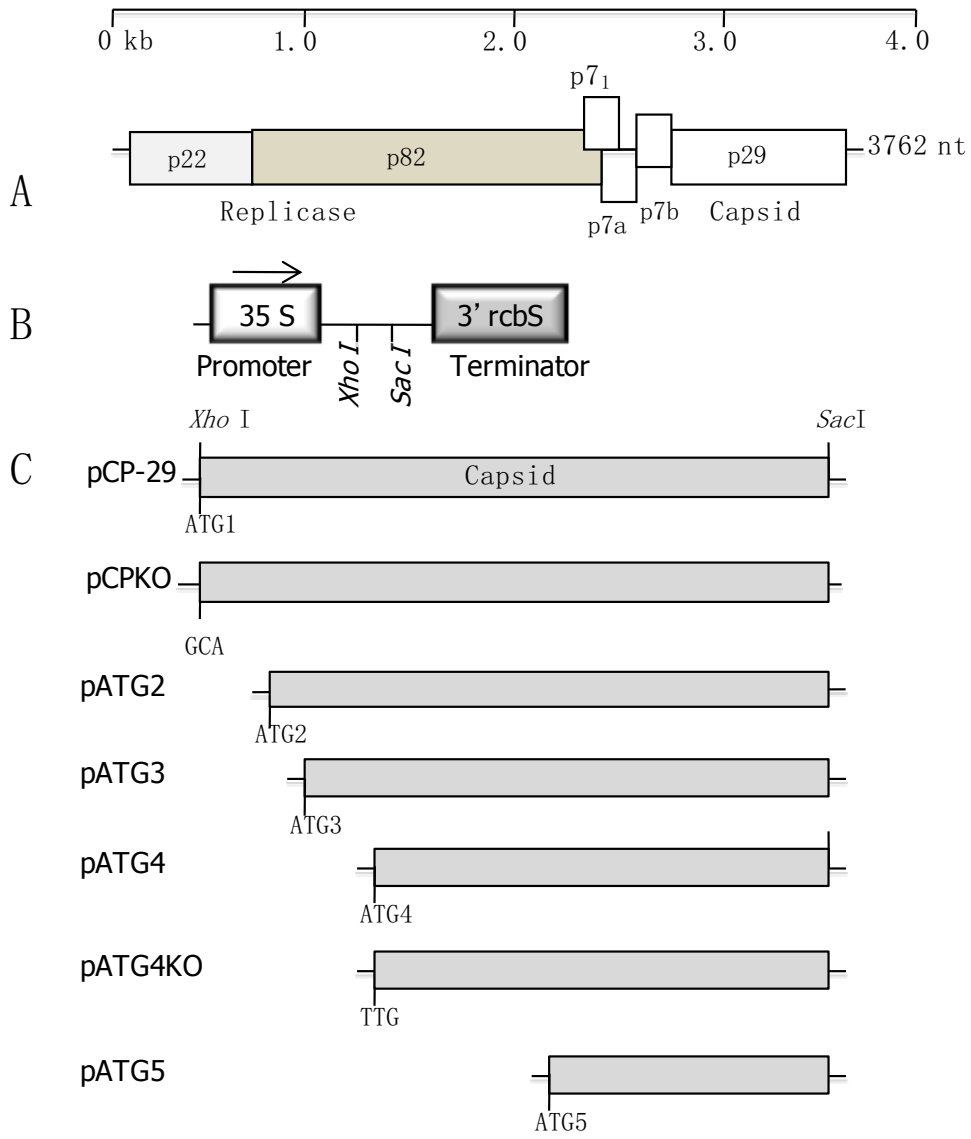

Figure A.6. Organization of the genome of TNV-D^H, and T-DNA constructs used for transient expression of the TNV CP in *A. tumefaciens*. A) TNV-D^H genome structure. B) Agrobacterium binary vector pKYLX7. The TNV CP constructs were inserted between the *Xho*I and *Sac*I sites for expression in plants. C) Truncated and mutated coat proteins tested for hypersensitive response in *Nicotiana* spp. The number associated with ATG indicated the position of that start codon in the coat protein sequence.

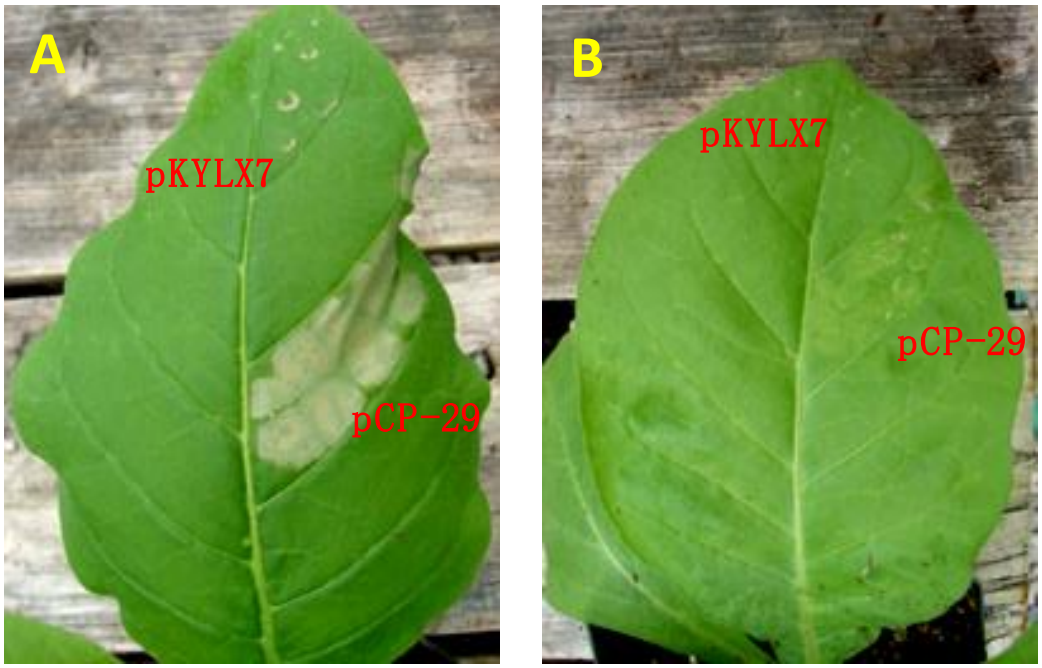

Figure A.7. Reaction to the expression of pCP-29 in plants of the section *Alatae*. A. Shows a typical HR reaction in *N. langsdorfii* at 2 dai. B. No reaction observed in *N. plumbaginifolia* at 2 dai. pKYLX7 indicates the panels infiltrated with the empty vector control.

To investigate whether the TNV coat protein or nucleic acid sequence was responsible for eliciting HR, the coat protein start codon was mutated from ATG to GCA, and this mutated sequence was inserted into pKYLX7 to create pCPKO. Agroinfiltration of pCPKO into *N. langsdorffii* resulted in the development of an HR that could not be distinguished in appearance or timing from the wild type TNV construct pCP-29 (Fig. A.8A). An inspection of the *p29* nucleotide sequence revealed additional start codons that could theoretically allow for initiation of translation and synthesis of a truncated coat protein sequence (Fig. A.9). To investigate whether initiation of translation at downstream start codons might result in a CP capable of initiating HR in *Nicotiana* species, we created a series of deletion mutants in which translation would be initiated at each of the start codons in the CP sequence (Fig. A.2C). Each of these constructs was cloned into pKYLX7 and agroinfiltrated into leaf panels of *N. langsdorffii* along with pCP-29 and the pKYLX7 empty vector. Constructs pATG2, pATG3 and pATG4 consistently elicited HR (Fig. A.8B), indicating that the first 77 codons of *p29* were not necessary for HR elicitation (Fig. A.9). By contrast, the capacity of construct pATG5 to elicit HR was abolished (Fig. A.8B).

To determine if the truncated ATG4 protein or its nucleotide sequence was responsible for HR, we mutated the start codon of construct pATG4 to TTG (construct pATG4KO), and found that this construct was unable to elicit HR upon agroinfiltration into *N. langsdorffii* (Fig. A.8C and D). To investigate whether any coat protein epitopes could be detected upon agroinfiltration of pATG4KO into *N. benthamiana* leaf tissues, we utilized an ELISA (Table A.2). Three days after agroinfiltration of leaf panels, plants

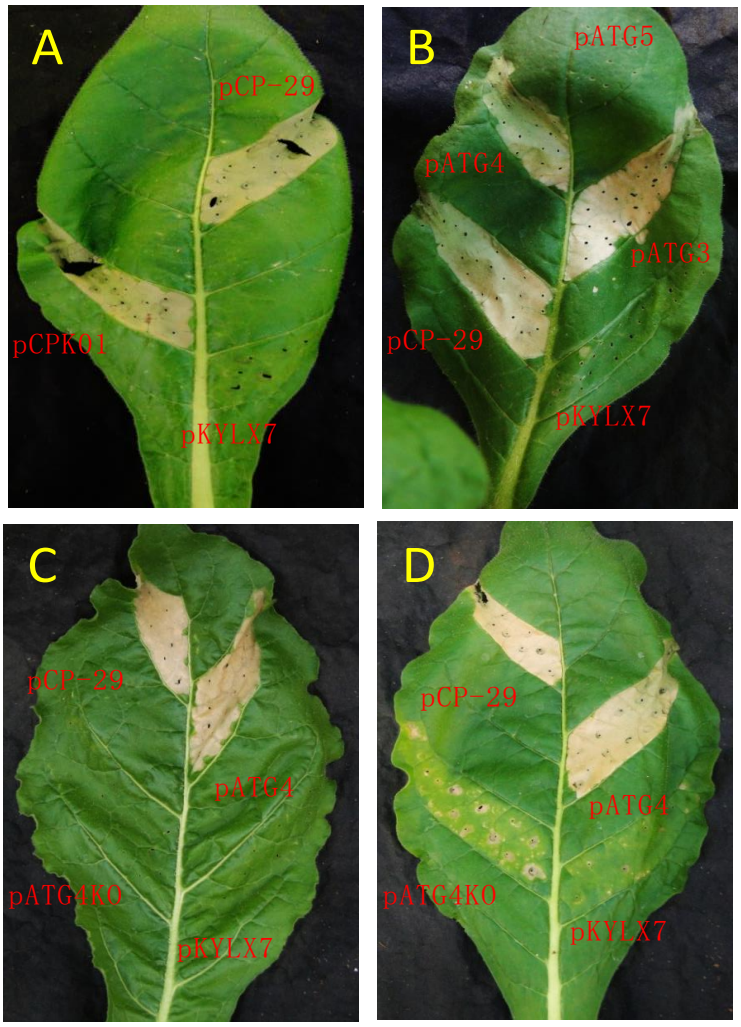

Figure A.8. TNV CP is an avirulence determinant, which activity is abolished upon mutation of the fourth start codon. (A) The susbstitution of the initial start codon (ATG GCA) in pCPKO1 (panel 2) does not affect its recognition in *N. langsdorfii*. pCP-29 (panel 1) and empty vector (panel 3) were used as controls. (B) Serial deletions in start codons 3 (panel 3) and 4 (panel 2) does not affect the elicitation of HR in *N. langsdorfii*. However, deletion performed from the ATG 5 (1) abolishes such response . pCP-29 (panel 4) and empty vector (panel 5) were used as controls. (C and D) A functional fourth start codon in TNV-CP is required for the elicitation of HR. Agroinfiltration of the wild type pCP-29 (panel 1), pCPATG4 (panel 2), pCPΔATG4 (panel 3) and empty vector (panel 4) was carried out in *N. longiflora* (D) and *N. langsdorfii* (C). Pictures were taken at 3 dpi.

Figure A.9. Nucleic acid and amino acid sequence of the TNV coat protein gene. Start codons are highlighted in yellow, with the single letter amino acid code listed underneath the nucleotide sequence. The start codond in bold, capital letters illustrate the boundaries of the truncated clones used for agroinfiltration. The nucleotide numbering of the coat protein sequence is illustrated on the right column.

Table A.2. Mutation of the start codon in pATG4 abolishes protein expression and consequently, HR elicitation. ELISA experiments were not able to detect protein expression in pATG4KO. Three replicates were performed. Mean, Standard Deviation and Stardard Error were calculated for earch treatment.

Treatment	**Absorbance at 405 nm**
Empty Vector	0.018 ± 0.031
pCP-29	0.693 ± 0.200
pATG4	1.222± 0.279*
pATG4KO	0.004± 0.008
TNV-infected tissue	2.465 ± 0.424

*Comparison of protein expression between pCP-29 and pATG4 thourgh T-test, showed a P-value < 0.05, indicating statistical significant difference in protein expression.

samples were tested for the expression of TNV coat protein constructs. TNV epitopes were readily detected in the plant samples agroinfiltrated with the full length coat protein and with pATG4, whereas no TNV coat protein epitopes were detected in leaf samples agroinfiltrated with pATG4KO. This study showed that the coat protein of TNV, rather than the viral RNA, was responsible for eliciting HR in *N. langsdorffii* and *N. longiflora*. Furthermore, the first 77 amino acids of the P29 protein did not contribute to HR-elicitation. These results were similar to those found in an analysis of the TBSV coat protein elicitor, in which the first 79 codons could be deleted and the truncated TBSV coat protein triggered HR in *N. langsdorffii* (Angel *et al*., 2013).

DISCUSSION

In this study we screened a total of 20 *Nicotiana* species belonging to 10 of 13 taxonomic sections for resistance to the necrovirus TNV strain D^H, (Table A.1) (Clarkson *et al*, 2004; Knapp *et al*, 2004). Of 20 species, 19 were resistant to TNV virion inoculation and most of the resistant plants (18 of 20 species) responded with HR-type resistance. This is remarkable because it indicates that most *Nicotiana* species have a receptor that recognizes some aspect of TNV. Of the two exceptions, *N. otophora* responded with chlorotic local lesions at 4 dpi and no symptom development in upper, non-inoculated leaves was observed, indicating that this plant reacted to TNV with non-necrotic resistance. Only one *Nicotiana* species, *N. benthamiana*, a species that originated in Australia, showed clear signs of systemic viral infection (Table A.1; Fig. 2). Interestingly enough, almost identical results have been previously reported in *Nicotiana* species infected with tombusviruses *Cymbidium ringspot virus* (CymRSV) , *Tomato bushy stunt virus* (TBSV) and *Cucumber necrosis virus* (CNV) (Angel and Schoelz, 2013; Angel *et al*, 2011). These viruses belong to the same viral family as TNV. The only hosts that distinguished the tombusviruses from TNV were *N. clevelandii* and *N. quadrivalvus*, as both of these plants were susceptible to the tombusviruses (Angel *et al*., 2011).

Resistance against plant viruses and other pathogens was explained several decades ago in the "gene-for-gene" model, which proposes that pathogens have avirulence (*Avr*) genes whose protein products can be detected by the plant surveillance

system composed of resistance (*R*) gene products (Flor, 1971). This recognition event initiates a cascade of plant defense reactions that limit the spread of a viral pathogen to a small number of infected cells in the inoculated leaf (Martin *et al*, 2003; Baker *et al*, 1997; Hammond-Kosack and Jones, 1996). When a cell death pathway is initiated in these locally infected cells, this is manifested in the formation of a necrotic local lesion.

The first viral Avr determinant was initially identified through gene swaps between infectious virus clones of CaMV (Daubert *et al*. 1984). However, since the development of transient expression assays, the identification of Avr gene products has become more simplified, as individual pathogen genes can be evaluated as Avr determinants. In one approach, a putative viral *Avr* gene can be delivered and expressed into plant cells though T-DNA delivery by *Agrobacterium tumefaciens* (i.e., agroinfiltration). *Nicotiana* species and in particular *N. benthamiana*, have been widely used for agroinfiltration because of they are highly receptive to this procedure (Angel *et al*. 2011; Goodin *et al*. 2008; Erickson *et al*. 1999).

A previous study has shown that although almost all *Nicotiana* species respond to tombusviruses with HR, the *Nicotiana* species do not target a single protein of the tombusviruses as the Avr determinant. In fact, three different tombusvirus proteins elicit HR in specific *Nicotiana* species. The tombusvirus P19 protein triggers HR in *N. sylvestris* and the close relative *N. tabacum*, as well as *N. bonariensis* and *N. plumbaginifolia*. The P22 protein triggers HR in *N. glutinosa* and the close relative *N. edwardsonii*, as well as *N. forgetiana*. Finally, the TBSV CP triggers HR in six of seven *Nicotiana* species in section *Alatae*; the species that respond to the TBSV CP with HR

include *N. alata*, *N. langsdorffii*, *N. mutabilis*, *N. forgetiana*, *N. bonariensis*, and *N. longiflora* (Angel *et al*., 2011; Angel *et al* 2013).

Since TNV belongs to the same family (*Tombusviridae*) as TBSV, CymRSV and CNV, we evaluated the capacity of the TNV-D^H CP to elicit a resistance response in *Nicotiana* species. The genomic sequence of this TNV strain has previously been characterized by Molnar and co-workers (1997). Interestingly, the TNV genome does not contain counterparts to P19 or P22, but its CP is phylogenetically related to the CPs of other tombusviruses. Upon agroinfiltration of the TNV CP, only species classified within the section *Alatae* responded with HR. Similar to the results obtained with the TBSV coat protein, most of the *Nicotiana* species in this section recognized CP-29, with the exception of *N. plumbaginifolia*. Interestingly enough, *N. plumbaginifolia* had previously been shown to detect and respond to the expression of TBSV silencing suppressor P19 instead of the TBSV CP (P41) (Angel and Schoelz, 2013). Since *N. plumbaginifolia* responds to inoculation of TNV-D^H virions with HR, some protein other than the CP must be eliciting this response, perhaps the TNV P7a or P7b proteins. However, we cannot verify the role for those proteins until the respective tests are performed.

Previously, a study performed with CymRSV implied that the RNA sequence for the CP, rather than the CP itself, was responsible for triggering HR in the solanaceous host *Datura stramonium* (Szittya and Burgyán, 2001). In this study, a frameshift after the third start codon was created, which theoretically, would alter the coat protein sequence after the 15th amino acid. This mutant clone was able to trigger HR in *D. stramonium*, even though most of the CP presumably would not be synthesized. The authors concluded that the RNA encoding the CymRSV coat protein was responsible for the

elicitation of HR. However, they did not show whether protein synthesis from their frameshift mutant was blocked in plants. In a study with TBSV, a relative of CymRSV, Angel and coworkers (2013) showed the opposite result; that the TBSV CP elicits HR in members of section Alatae. Angel *et al*. (2013) demonstrated that mutation of the start codon of the TBSV CP was not sufficient to abolish CP synthesis, likely because ribosomes could initiate translation of the CP sequence by utilizing downstream start codons. Furthermore, Desvoyes and Scholthof (2002) showed that an infectious TBSV clone that is unable to produce a coat protein due to a small in-frame deletion was prone to recombination *in planta* that ended up restoring the coat protein reading frame.

In the case of TNV CP, we observed that the CP coding sequence contained six start codons throughout the sequence, with the last start codon found only 17 codons from the end of the protein (Fig. A.9). As with the TBSV CP, we found that mutation of the first start codon had no effect on CP expression or elicitation of HR in *N. langsdorffii*. Furthermore, we found that we could eliminate the first 77 codons of the TNV CP without affecting HR elicitation, whereas with the TBSV CP, it was possible to eliminate the first 80 codons. Consequently, our work supports the hypothesis that the CP sequence is the Avr determinant, rather than the corresponding RNA sequence (Angel and Schoelz, 2013). Furthermore, the deletion analyses of both the TNV and TBSV CPs shows that the N-terminus is not involved in HR elicitation.

Nicotiana spp. has been the subject of several phylogenetic studies (Kelly *et al*. 2010; Lim *et al*. 2006; Clarkson *et al*. 2004; Knapp *et al*. 2004). This analysis might give us some insight about R-genes that detect Avr genes from close related plant viruses. The fact that TNV CP triggers HR in several members of the *Alatae* section indicates that

these species might possess a common R gene, whose product recognizes the CP, either through a direct or indirect interaction (i.e the guard hypothesis)(Dangl and McDowell 2006; Van der Biezen and Jones 1998). Another alternative is that the TNVP CP protein is targeted by various R proteins in *Alatae* species. However, whatever the scenario is, it is interesting that only species within the *Alatae* section contain the gene(s) that recognize the TNV CP. Furthermore, we still have the question about why *N. plumbaginifolia* did not trigger HR upon infiltration of TNV CP, or the CP of TBSV (Angel and Schoelz, 2011). One possible explanation for this might be the existence of phylogenetic conflicts for the discrimination of this specific species. Similar to *N. plumbaginifolia*, there were other 14 *Nicotiana* species that were resistant to the inoculation of TNV virions but did not elicit HR upon agroinfiltration of pCP-29. This suggests that other genes in the TNV genome act as avirulence determinants and are responsible for such a resistance response. Another alternative is that protein expression levels obtained through agroinfiltration are not satisfactory to trigger HR in *N. plumbaginifolia* and other hosts. Further research will be necessary to characterize other TNV genes that trigger a resistance response in the rest of the *Nicotiana* spp.

In addition to the CPs of TNV and TBSV, the CPs of several other viruses have also been found to serve as Avr determinants in plants. For example, the CP of TMV triggers HR in *N. sylvestris* in response to the N' gene; it was one of the first Avr determinants to be identified (Knorr and Dawson, 1988; Saito *et al*, 1987). The N' gene was recently cloned and shown to encode an NBS-LRR protein (Whitham *et al*, 1994). Interestingly, the CP of *Pepper mild mottle virus*, another tobamovirus, triggers HR in *Capsicum* plants that contain a series of R genes, the *L* genes (Berzal-Herranz *et al*, 1995;

de la Cruz *et al*, 1997; Gilardi *et al*, 1998). The CP of *Turnip crinkle virus* is responsible for triggering a resistance response in Arabidopsis ecotype Dijon (Oh *et al*, 1995; Wobbe and Zhao, 1998). The R gene that targets *Turnip crinkle virus* was one of the first R genes to be cloned, and it also encodes an NBS-LRR protein (Dempsey *et al*, 1997). Arabidopsis also contains another R gene that targets the CP of *Cucumber mosaic virus* (Takahashi *et al*, 2001). Finally, the CP of Potato virus X triggers extreme resistance that in potato plants that contain the *Nx* gene (Santa Cruz and Baulcombe, 1993). One advantage of studying viral CPs as Avr determinants is that in many cases the three dimensional structure of the protein has been determined. As we learn more about the structural features present in the CP responsible for triggering HR, this will provide valuable new information about how Avr proteins in their native form are ultimately recognized by the R proteins in plants.

MATERIALS AND METHODS

TNV virions and clones

The full length genome of TNV strain D^H was cloned into the plasmid pUC18 by Molnár and coworkers (1997). Both the plasmid and TNV virions were provided by Dr. Lóránt Király.

TNV Virion Inoculation in *Nicotiana* species

Seeds of different *Nicotiana* species were obtained from the U.S. Tobacco Germplasm Collection at North Carolina State University (Lewis and Nicholson, 2007), as described in Angel and Schoelz (2013). To break dormancy, seeds were treated for 30 min with commercial bleach at 50% strength (2.6% vol/vol NaOCl) (Burke, 1957). TNV- D^H virions were initially inoculated to *N. benthamiana* and infected tissue was frozen for further inoculations. For inoculation of test plants, plant tissues infected with TNV- D^H were ground with a mortar and pestle at a dilution of approximately 1:20 (wt/vol) with inoculation buffer (0.05M phosphate buffer pH 7.0) and gently rubbed onto *Nicotiana* leaves dusted with 600-mesh carborundum.

Construction of TNV-CP expression plasmids

To amplify the coat protein gene of TNV (pCP-29) by PCR from the TNV genomic clone, I used the oligonucleotides TNV-CP *XhoI* Fwd (5'-**CTCGAG**ATGCCTAAACGAGGAAGAGT-3') and TNV-CP *SacI* Rev (5'-**GAGCTC**CTAAATGTTAATGGTGGGAT - 3'). The forward primer included an *Xho*I site, whereas the reverse primer contained a *Sac*I site for evnetual cloning into the Agrobacterium binary vector pKYLX7 (Schardl *et al*., 1997). The TNV coat primer sequences matched the published genomic sequence of TNV-D^H (Molnár *et al*, 1997, National Center of Biotechnology Information [NCBI] accession number NC_003487.1). The PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles at 95°C for 1 min, 55°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 5 min. The PCR product was purified by agarose gel elution using the QIAquick gel extraction kit (Quiagen Inc., Valencia, CA, U.S.A.) and was cloned into pGEM-T-Easy (Promega Corp., Madison, WI, U.S.A.). White *Escherichia coli* colonies were selected on Luria Bertani (LB) media containing 40 ul Xgal (20mg/ml), 10 ul IPTG (20%) and kanamycin (50 µg/ml). Colonies were initially screened by colony PCR for the presence of the TNV-CP insert. Candidate clones were sequenced in both orientations by the DNA Core Facility at the University of Missouri (Columbia, MO, U.S.A.). Once the fidelity of the sequence was confirmed, the insert was transferred into a previously *Xho*I-*Sac*I digested pKYLX7 binary plasmid and was moved into *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al*, 1991) by electroporation with a

PG200 Progenetor II (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Transformants were selected on LB medium supplemented with kanamycin (50 µg/ml) and tetracycline (12.5 µg/ml).

The deletion of point mutants of pCP-29 were also created by PCR by utilizing the same reverse primer coupled with a different forward primer. The forward primer for pCPKO was CPKO *Xho*I fwd (5'-**CTCGAG**GCACCTAAACGAGGAAGAG-3'), the forward primer for pATG2 was ATG2 *Xho*I fwd (5'-**CTCGAG**ATGGAACGCGCACTTGTCAAC-3'), the forward primer for pATG3 was ATG3 *Xho*I fwd (5'- **CTCGAG**ATGTCTTTCAGACCACTCACT-3'), the forward primer for pATG4 was ATG *Xho*I fwd (5'-**CTCGAG**ATGTCCACCTTCGTGGTCAAT-3'), the forward primer for pATG5 was pATG5 *Xho*I fwd (5'-**CTCGAG**ATGGCAATTGTGTACGATGCA-3'), and the forward primer of pATG4KO was ATG4KO *Xho*I fwd (5'-**CTCGAG**TTGTCCACCTTCGTGGTCAAT-3'). In each case, the PCR product was first cloned into pGem-T-Easy, its nucleotide sequence confirmed, and then moved into the binary vector pKYLX7. All of the primers used throughout the study were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.).

Agroinfiltration Assays

A. tumefaciens strain AGL1 was grown in 3 ml LB broth supplemented with kanamycin (50 µg/ml) for 24 h at 28°C in an incubator shaker at 220 rpm. From each

initial culture, 500 µl was added to flasks with 40 ml LB broth containing kanamycin (50µg/ml), and the cultures gorwn for an additional 24 h. Bacteria were then sedimented by centrifugation at 14000 g for 10 min and resuspended in 20 ml of infiltration solution (3.9 g/l MES, 20g/l Sucrose, 10 g/l Glucose, pH 5.4) supplemented with 20 µl 0.2M acetosyringone. Cells were incubated overnight at 28°C and 220 rpm and cultures subsequently diluted to an OD_{600} 1.0 immediately before infiltration into *Nicotiana* leaves as described in Angel and Schoelz (2013).

ELISA tests for TNV-CP detection.

To detect the TNV-CP and other deletion mutants, epitopes were expressed through agroinfiltration into *N. benthamiana* plants. Infiltrated tissue was collected at 3 dpi and ground with mortar and pistil at a 1:3 tissue/grinding buffer (1X phosphate buffered saline, 2% polyvinylpyrrolidone MW 40,000 g/mol, 0.2% bovine serum albumin and 0.05% Tween 20) ratio. Double antibody sandwich ELISA (DAS-ELISA) was carried out using TNV- serotype D coating and alkaline phosphatase-conjugated secondary antibodies purchased from AC diagnostics (Fayetteville, AR, U.S.A.). Coating antibody was diluted 1:200 using coating buffer (0.015M Na_2CO_3, 0.03M $NaHCO_3$, pH 9.6), whereas the conjugated antibody was diluted at the same ratio with grinding buffer. Colorimetric reactions with the substrate p-nitrophenyl phosphate were quantified at 405 nm using a Multiskan MCC-340 microplate reader (Thermo Fischer Scientific,

Cincinnati, OH, U.S.A.). All experiments were repeated three times. Absorbance reads at 405 nm were analyzed using the standard error and T tests.

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SUMMARY

For a viral infection to be successful, plant viruses must find a way to traffic their genomic material from the sites of replication within the cell to the plasmodesmata for movement to neighboring and distant cells. It is generally accepted that plant viruses must interact with a collection of host factors for intracellular trafficking to plasmodesmata.

It has been already established that CaMV virions accumulate in inclusion bodies (IBs) formed mainly by the viral protein P6. Furthermore, ectopic expression of only P6 has been shown to yield IBs that look similar to those IBs observed in CaMV infected tissue. Consequently, this approach is considered a viable option to study the subcellular localization and trafficking of P6 IBs. Previous studies have shown that P6 IBs are associated with the endoplasmic reticulum, actin microfilaments and microtubules. Furthermore, P6 IBs have been shown to move along microfilaments, and inhibition of actin polymerization caused an inhibition of lesion formation. Since CaMV virions accumulate in P6 IBs, we hypothesized that P6 IBs have a role on delivering CaMV virions to plasmodesmata.

We recently discovered that the P6 protein interacted with a C2 calcium-dependent membrane targeting protein (designated AtSRC2.2) in a yeast two-hybrid screen and confirmed this interaction through co-immunoprecipitation and co-localization assays in the CaMV host, *Nicotiana benthamiana*. An AtSRC2.2 protein fused to RFP was localized to the plasma membrane and specifically associated with plasmodesmata. The AtSRC2.2-RFP fusion also co-localized with two proteins previously shown to

associate with plasmodesmata: the host protein PDLP1 and the CaMV movement protein (MP). Since P6 IBs were found to co-localize with AtSCR2.2 and had previously been shown to interact with CaMV MP, we investigated whether a portion of the P6 IBs might also be associated with plasmodesmata. We examined the co-localization of P6-GFP IBs with PDLP1, the CaMV MP, and with aniline blue, a chemical stain for callose, and found that P6-GFP IBs were associated with each of these markers. Furthermore, a P6-RFP protein was co-immunoprecipitated with PDLP1-GFP.

In addition to the interaction of P6 with AtSRC2.2, the initial yeast two-hybrid screening also indicated an interaction of P6 with the chloroplast unusual positioning 1 (CHUP1. Angel *et al*, 2013). This protein was previously shown to locate to the outer membrane of the chloroplasts and assist in chloroplast movement in response to light intensity. Recently, CHUP1 was shown to interact with P6 and was implicated in trafficking of P6-IBs on actin microfilaments. Furthermore, ectopic expression of a truncated CHUP1, which is unable to traffic chloroplasts, inhibited the movement of P6 IBs. However, previous attempts to knock out the function of CHUP1 through virus-induced gene silencing in *Nicotiana benthamiana* have not been successful in blocking CaMV infections (Angel *et al*, 2013). This suggests the possibility of functional redundancies with other proteins for trafficking on microfilaments such as myosins, which have already been implicated in the transport of plant viruses inter and intracellularly. Particularly, myosins XI-K and XI-2 have been shown to be responsible for trafficking of some plant viruses (Harries *et al*, 2009).

One widely used approach to determine the function of a gene product *in situ* under a specific treatment is the use of gene knockouts or null mutations. Specifically, T-

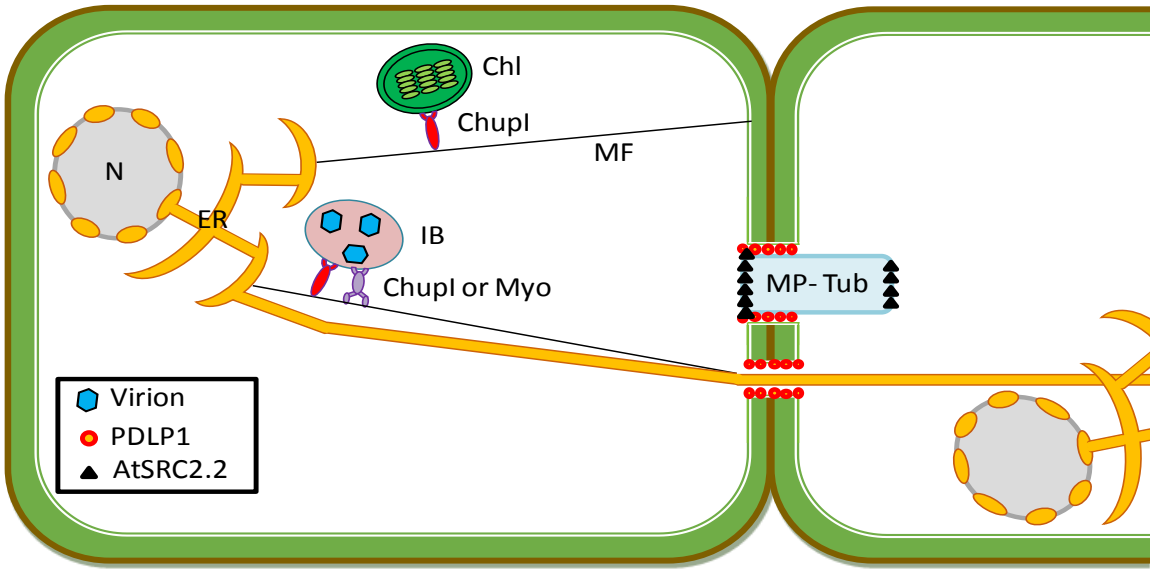
DNA insertional mutagenesis has been the preferred alternative for disrupting gene function in the model plant *Arabidopsis thaliana*. Thus, we characterized the biological impact of CHUP1, myosin XI-2, and myosin XI-K, on CaMV infection. We initially tested Arabidopsis single knockout lines for each of these genes and observed that CaMV infected the single knockouts just as in the wild type Col-0. Functional redundancies might be the cause for the lack of a biological effect in single knockout lines infected with CaMV. Therefore, we developed different combinations of double and triple mutants and observed that in several experiments infection development in double and triple mutants occurred at a slower rate than Col-0. Furthermore, one out of two experiments involving the *chup1 xi-2 xi-k* triple mutant, there was a four-day delay in both local lesion and systemic infection relative to Col-0. Timing of symptom development in the second experiment was accelerated by four days for Col-0 as well as for the T-DNA knockout lines, therefore making it challenging for us to compare CaMV infection data between tests. Although further tests will be necessary to determine if CaMV infections truly are slower in the double and triple knockout lines than Col-0 or the single knockout lines, we found a trend in the delay of local and systemic symptom development in some of the double mutants and the triple mutants.

Our evidence that a portion of P6-GFP IBs associate with AtSRC2-2, PDLP1, and CaMV MP at plasmodesmata supports a model (Fig. S1) in which P6 IBs would traffic along microfilaments through the use of microfilament-associated motor proteins such as CHUP1, myosin XI-2 or myosin XI-K (Fig. S1a). At plasmodesmata, PDLP1 and probably other host proteins will interact with the P1 protein of CaMV and yield the formation of a tubule-like structure that modifies the structure of the plasmodesma.

AtSRC2.2 will associate with the tubules at each end of the tubule and along with PDLP1 may assist in docking the P6 IBs to the tubule (Fig. S1b). Here, the P6 IB will function to transfer CaMV virions directly to the tubule and these virions will pass to the adjacent cell to continue with the infection process.

Although key elements of the model could explain the movement of P6 IBs on microfilaments to plasmodesmata, further research will be necessary to clarify the roles of AtSRC2.2, the PDLP proteins, CHUP1 and myosins in the infection of CaMV, as well as other viruses.

A



B

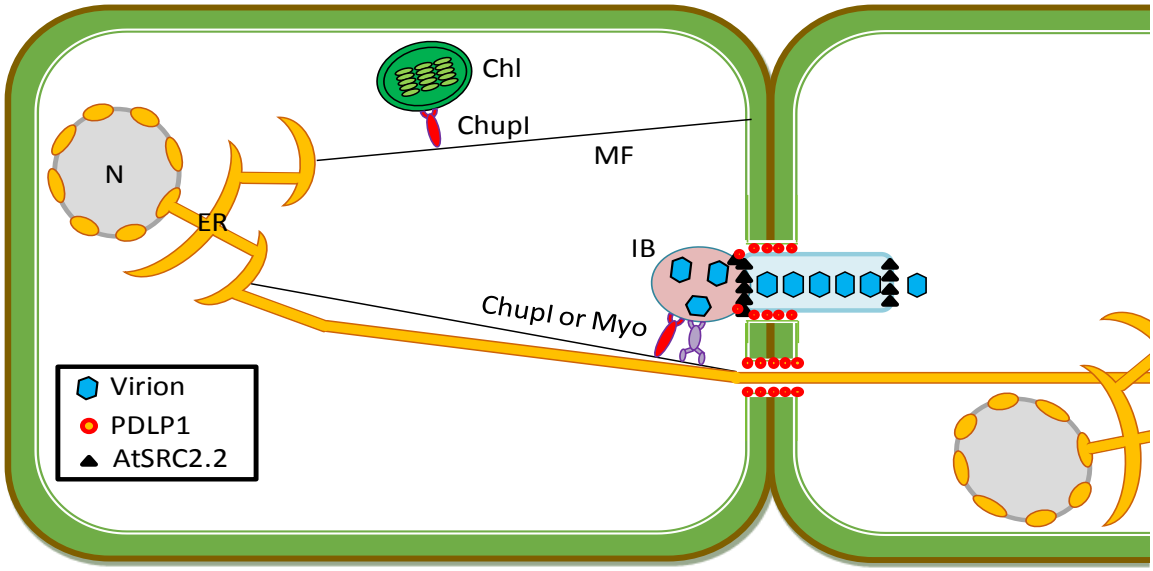


Figure S.1. Model for the intracellular movement of CaMV P6 IBs. N: Nucleus. Chl: Chloroplast. ER: Endoplasmic Reticulum. MF: Microfilaments. MP-tub: Tubule formed by the CaMV P1 movement protein. Myo: Myosin.

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VITA

Andres Rodriguez was born on January 5, 1984 in Tunja (Boyaca, Colombia-South America). He is son of Fernando Rodriguez Lopez, a systems engineer and Leonor Gonzalez de Rodriguez, an Architect. He is the third out of four kids.

Andres was always very interested in science and biology. After finishing high school, he decided to pursue a B.Sc. in Microbiology at the Pontificia Universidad Javeriana in Bogota, Colombia; one of the best universities in that country. At the end of his B.Sc. studies, he was accepted as an undergraduate intern at the National Coffee Research Center (CENICAFE) and performed a research project that involved the isolation and morphological characterization of the fungal micro flora associated with coffee crops. This project gave rise to a B.Sc thesis research project in which Andres performed the molecular characterization of a population of the fungus *Colletotrichum* associated with coffee plants. Furthermore, he used DNA samples of the African pathogenic species *C. kahawae* in order to develop polymorphic markers that can be used to differentiate between the Colombian and African species.

In 2009, Andres was accepted to the PhD program in Plant, Insect and Microbial Sciences at the University of Missouri-Columbia, under the supervision of Dr. James E. Schoelz. This document indicates the research projects in which Andres was involved during the course of his formation as a PhD in Plant, Insect and Microbial Sciences.