Association of the P6 protein of Cauliflower mosaic virus with plasmodesmata and plasmodesmal proteins

1Andres Rodriguez, 1Carlos A. Angel, 3Lindy Lutz, 2Scott M. Leisner, 2Richard S. Nelson, and 1James E. Schoelz

1Division of Plant Sciences, University of Missouri, Columbia, MO 65211; 2The Division of Plant Biology, The Samuel Roberts Noble Foundation, Ardmore OK 73401; 3Department of Biological Sciences, University of Toledo, Toledo OH 43606

Corresponding author: J. E. Schoelz, Telephone: (573) 882-1185; Fax: (573) 882-0588; E-mail: schoelzj@missouri.edu

Running head: Association of CaMV P6 protein with plasmodesmata
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 inclusion-like bodies (I-LBs) 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 have determined that the P6 protein interacts with a C2 calcium-dependent membrane targeting protein (designated AtSRC2.2) in a yeast two-hybrid screen and have 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 I-LBs co-localized with AtSCR2-2 and the P6 protein had previously been shown to interact with MP, we investigated whether P6 I-LBs might also be associated with plasmodesmata. We examined the co-localization of P6-RFP I-LBs with PDLP1-GFP and aniline blue (a stain for callose normally observed at plasmodesmata), and found that P6-RFP I-LBs were associated with each of these markers. Furthermore, P6-RFP co-immunoprecipitated with PDLP1-GFP. Our evidence that a portion of P6-GFP I-LBs associate with AtSRC2.2 and PDLP1 at plasmodesmata supports a model in which P6 IBs function to transfer CaMV virions directly to MP at the plasmodesmata.
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 lesion, 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 Füttener, 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. 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 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 (Stavolone 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 is incorporated into vesicles and is trafficked on the endomembrane system to reach the plasmodesma (Carluccio et al., 2014). These authors suggest that the CaMV MP is recycled in a vesicular transport pathway between plasmodesmata and early endosome compartments. The CaMV MP interacts with µA-Adaptin (Carluccio et al. 2014) and MPI7 (Huang et al., 2001), two proteins shown to have a role in vesicular trafficking. Once the MP arrives at plasmodesmata it interacts with the PDLP proteins, which comprise a family of eight proteins 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 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 of 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 (Supplemental Fig. S1) 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 (Suppl. Table S1; Suppl. Fig. S1B) (Kim et al., 2008; 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. 1, Suppl. Fig. S1A).

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. 1; Li and Leisner, 2002). Yeast cells co-transformed with the full length P6 fused to the LexA DNA-binding domain and 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. 2A). 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. 2B). 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 is partially co-localized 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 protein (RFP) coding sequence and cloned into an *A. tumefaciens* expression pSITE vector (Fig. 1, 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. 1, Angel et al., 2013; Harries et al., 2009a). Each construct was individually agroinfiltrated into *N. benthamiana* leaves to determine expression patterns. The protein AtSRC2.2-RFP was distributed throughout the cell, but also formed small punctate spots (Fig. 3A), whereas P6-GFP formed well-defined P6-GFP I-LBs of varying sizes (Fig. 3B) as previously described (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 frequently co-localized with P6-GFP in both small and large aggregates (Fig. 3C-E). We found that of 725 P6-GFP I-LBs examined, 32.7% also contained AtSRC2.2 (Fig. 3C). Significantly, AtSRC2.2 was co-localized with even the largest P6-GFP IL-Bs (Fig. 3E, white arrows). Since we did not observe such large aggregates of AtSRC2.2-RFP when it was agroinfiltrated by itself, the existence of the large aggregates suggested that a portion of AtSRC2.2-RFP was reclocalized into the P6-GFP 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. 4A, lanes 2, 4, and 6). Furthermore, antibodies to GFP and RFP did not cross react with each other at levels that would influence the co-IP results (Fig. 4A). 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. 4A, lane 6), but was not detected when P6-GFP was omitted from the co-immunoprecipitation assay (Fig. 4A, lane 2). With these results, we concluded that the co-immunoprecipitation of AtSRC2.2-RFP is dependent on its association with P6-GFP.

To further examine the interaction of the D2 domain of P6 with AtSRC2.2 during co-IP analysis, we divided the P6 protein into two sections. In the protein P6ΔD1D2-GFP, 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. 1). Agroinfiltration of P6ΔD1D2-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 (Fig. 4A, lanes 3 and 5) and confocal microscopy showed that P6ΔD1D2-GFP was unable to form I-LBs (Fig. 4B). In contrast to the full-length P6-GFP protein, a co-IP showed that the P6ΔD1D2-GFP protein was unable to associate with the AtSRC2.2-RFP fusion protein (Fig. 4A, lane 5). In the protein P6D1D2-GFP, the D1 and D2 domains of P6 were fused to GFP and P6 domains D3 and D4 were deleted (Fig. 1). The truncated protein P6D1D2-GFP interacted in a co-IP with AtSRC2.2-RFP (Fig. 5A), confirming that the D2 domain of P6 was responsible for the interaction of AtSRC2.2. Furthermore, AtSRC2.2-RFP was relocalized into the inclusion bodies formed from P6D1D2-GFP (Fig. 5B-D). 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).

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 interior of the plasmolyzed cell, whereas AtSRC2.2-RFP remained primarily at the periphery (Fig. 6A-C). In addition, we co-agroinfiltrated AtSRC2.2-RFP with the plasmodesmal marker protein aquaporin AtPIP2A-GFP (Nelson et al., 2007) and cells were subsequently plasmolyzed. In contrast to free GFP, AtSRC2.2-RFP was co-localized with AtPIP2A-GFP (Fig. 6D-F). 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. 3A) 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 also serve as plasmodesmal markers and influence CaMV infection (Amari et al., 2010; Thomas et al., 2008). The MP-GFP protein formed punctate spots (Fig. 6G), 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. 6H), but also formed punctate spots that co-localized with the MP-GFP protein (Fig. 6I). 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. 6J-L) 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. 6L, white arrows), but both PDLP1 and AtSRC2.2 were retained in the area of the cell wall where they also co-localized (Fig. 6L, 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 and PDLP1 are associated with the base and tip of 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. 7A), 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. 7B). 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. 7C-E) and at the tip of 23.8% of the MP tubules (Fig. 7F-H). We concluded from these experiments that AtSRC2.2 is capable of a close association with tubules formed from the MP of CaMV.
Amari and coworkers (2010) had shown that the PDLP1 protein was incorporated into tubules formed by the GFLV MP. To determine if PDLP1 was found in the tubules formed from the CaMV MP, we created a CaMV MP-RFP construct and subsequently co-agroinfiltrated PDLP1-GFP, CaMV MP-RFP and unmodified CaMV MP into *N. benthamiana* leaves. The PDLP1-GFP protein appeared to be incorporated into a higher percentage of tubules than AtSRC2.2-GFP, as PDLP1-GFP was observed in 55.9% of the tubules (38 out of 68 total) whereas AtSRC2.2 was observed in 39.8% (45 out of 113) of tubules. As with atSRC2.2, we found that PDLP1-GFP was found at the base and tip of the tubules formed from CaMV MP (Fig. 7I-K). Of the 38 tubules that contained PDLP1-RFP, the protein was found at the base in 60.5% of the tubules and at the tip in 39.5% of the tubules. The images in Fig. 7 are the first that illustrate the incorporation of host proteins into tubules formed from the CaMV MP.

**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-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 8A-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 (Fig. 8D-F). 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 8G-I). 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 the cell was plasmolyzed, the portion of PDLP1 that remained with the plasma membrane in the plasmolyzed cell may have
become accessible to P6-RFP and co-localization of PDLP1-GFP with P6-RFP therefore possible.

**P6-GFP is co-immunoprecipitated with PDLP1-RFP.** The association 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-expressed (Fig. 9, panels a 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. 9, panel c Lane 4), but was not detected when PDLP1-GFP was omitted from the co-immunoprecipitation assay (Fig. 9, panel c Lane 2). With these results, we concluded that the P6-RFP is co-immunoprecipitated 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 *pdlp*1, *pdlp*2, and *pdlp*3 (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).

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 (Suppl. Fig. S1). 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 (Suppl. Fig. S2), 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* (Suppl. Fig. S1), 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. Furthermore, the fixation of infected tissue for electron microscopy might also disrupt a transient association. 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. AtSRC2.2 and PDLP1 proteins co-localized with each other in
punctate locations in the cell periphery, as expected for plasmodesma-associated proteins. The CaMV P6 protein was co-immunoprecipitated with each host protein and a portion of the P6-GFP I-LBs were co-localized with AtSCR2.2-RFP. 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. 6-9). 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) (Suppl. Table S1) (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 synaptotagmin4 (AtSYT4, Suppl. Table S2). AtSYT4 belongs to a small family of five proteins that contain two C2 domains, C2A and C2B, both of which are found towards the C-terminal end of the protein; the C2 domain of AtSRC2.2 is homologous to C2A of AtSYT4 (36% identity, Suppl. Table S2). 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 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.

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 EcoRI and XhoI 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, AtPDL1, 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 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) based on the JTT matrix and the bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) (Suppl. Fig. S1). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 7 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 165 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).
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). For visualization with aniline blue, plant tissues were excited with a 405 nm laser and the emitted light was captured at 460-500 nm, as described in Thomas et al (2008).

Co-immunoprecipitation Assays.

Co-immunoprecipitations were conducted as described (Lee et al., 2003), with minor modifications, as described by Angel et al. (2013), including detergents Triton X-100 (0.1%) and NP40 (0.5%) in the extraction buffer. 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 MgCl₂, 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 P6ΔD1D2. Sequencing, cloning and fusion of P6ΔD1D2 with RFP, resulting in expression of P6ΔD1D2-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.

ACKNOWLEDGMENTS

The authors thank Dr. David Braun for helpful advice, Dr. Aleksandr Jurkevic (University of Missouri) for his assistance with confocal microscopy, and Sandra Valdes for technical assistance. This project was supported by the Agriculture and Food Research Initiative Competitive Grants Program No. 2010-65108-20525 from the USDA National Institute of Food and Agriculture and The Samuel Roberts Noble Foundation, Inc.

LITERATURE CITED


forms tubular structures which extend from the surface of infected protoplasts. Virology **195**: 281-285.


**Schoelz JE, Shepherd RJ, Daubert SD** (1986) Gene VI of CaMV encodes a host range determinant. Molecular and Cellular Biology **6**: 2632-2637.


FIGURE LEGENDS

Figure 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. The NLSa sequence corresponds to the nuclear localization signal of influenza virus. The NLS sequence corresponds to the nuclear localization signal of human ribosomal protein L22 (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.

Figure 2 AtSRC2.2 preferentially interacts with D2 of P6 in a yeast two-hybrid analysis. (A) Interaction of AtSRC2.2 with the full-length CaMV P6. (B) Interaction of 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, the striped box represents the transcriptional activator domain and black boxes show the DNA-binding domain.
Figure 3  AtSRC2.2-RFP is partially co-localized with P6-GFP upon co-agroinfiltration into *N. benthamiana* leaves. (A) Expression of AtSRC2.2-RFP alone. (B) Expression of P6-GFP alone. (C-E) Co-agroinfiltration of AtSRC2.2-RFP with P6-GFP. (C) Expression of AtSRC2.2-RFP. (D) Expression of P6-GFP. (D) Overlay of photos C and D. Picture was taken at 3 dpi. White arrows indicate AtSRC2.2-RFP aggregates of a size that only exist when co-agroinfiltrated with P6-GFP.

Figure 4  Co-Immunoprecipitation studies with AtSRC2.2-RFP with P6-GFP and subcellular localization of P6\(^{\Delta D1D2}\)-GFP. (A) 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\(^{\Delta D1D2}\)-GFP; Lane 4, Expression of P6-GFP; Lane 5, Co-expression of P6\(^{\Delta D1D2}\)-GFP with AtSRC2.2-RFP; Lane 6, Co-expression of P6-GFP with AtSRC2.2-RFP. Panel a. Western blot for total proteins probed with RFP antibodies. Panel b. Western blot for total protein probed with GFP antibodies. Panel c. Co-immunoprecipitation of proteins using GFP antibodies and probed in a Western blot with RFP antibodies. (B) Localization of P6\(^{\Delta D1D2}\)-GFP expressed in *N. benthamiana* leaves at 3 dpi.

Figure 5  Co-immunoprecipitation and co-localization of P6D1D2-GFP with AtSCR2.2-RFP. Co-immunoprecipitation of P6D1D2-GFP with AtSRC2.2-RFP after co-agroinfiltration of *N. benthamiana* leaves. Lane 1, Mock-inoculated control leaf; Lane 2, Expression of AtSRC2.2-RFP; Lane 3, Expression of P6D1D2-GFP; Lane 4, Co-expression of AtSRC2.2-RFP and P6D1D2-GFP. Panel a. Western blot for total proteins probed with RFP antibodies. Panel b. Western blot for total proteins probed with GFP antibodies. Panel c. Co-immunoprecipitation of proteins using GFP antibodies and probed in a Western blot with RFP antibodies. Panel d. Co-immunoprecipitation of proteins using GFP antibodies and probed in a Western blot with GFP antibodies. Photos B – D illustrate re-localization of AtSCR2.2 with P6D1D2-GFP. (B) AtSRC2.2-RFP. (C) P6D1D2-GFP. (D) Overlay of B and C.
**Figure 6** Association of AtSRC2.2 with the membrane marker protein AtPIP2A and with plasmodesmal marker proteins PDLP1 and CaMV MP. Photos A-C show that AtSRC2-2RFP does not colocalize with free GFP in a plasmolyzed cell (A) AtSCR2.2-RFP. (B) Free GFP. (C) Overlay of photos A and B. Photos D-F illustrate colocalization of AtSRC2.2 with the plasma membrane marker protein aquaporin AtPIP2A-GFP in a plasmolyzed cell. (D) SRC2.2-RFP. (E) AtPIP2A-GFP. (F) Overlay of photos D and E. Photos G-I illustrate colocalization of CaMV MP-GFP with AtSRC2.2-RFP in *N. benthamiana* leaf tissue. (G) MP-GFP. (H) AtSRC2.2-RFP. (I) overlay of G and H. Photos J-L illustrate that CaMV AtSRC2.2-RFP is co-localized with PDLP1-GFP in both the membrane and cell wall of plasmolyzed *N. benthamiana* cells. (J) PDLP1-GFP. (K) AtSRC2.2-RFP. (L) overlay of J and K. *N. benthamiana* cells were plasmolyzed by infiltration of 30% glycerol. The white arrows illustrate co-localization of AtSRC2.2-RFP and PDLP1-GFP in the plasmolyzed membrane, whereas the yellow arrows illustrate co-localization in the cell wall. The higher magnifications of the co-localized signals in the insets are highlighted by the purple arrows in photos D and G.

**Figure 7** AtSRC2.2 and PDLP1 co-localize with CaMV P1 at the base and tip 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 photos C and D. (F-H) Co-agroinfiltration of unmodified P1, P1-GFP and AtSRC2.2-RFP shows that AtSRC2.2-RFP also co-localized with P1-GFP at the tip of tubule structures. (F) P1-GFP. (G) AtSRC2.2-RFP. (H) Overlay of photos F and G. (I-K) Co-agroinfiltration of unmodified P1, P1-RFP and PDLP1-GFP shows that PDLP1-GFP may also be co-localized with P1-RFP at the tip and base of tubule structures. (I) P1-RFP. (J) PDLP1-GFP. (K) Overlay of photos I and J.

**Figure 8** Association of P6-RFP with the plasmodesmal protein PDLP1-GFP and with aniline blue. Photos 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 A and B. Photos D - F illustrate co-localization of P6-RFP with PDLP1-GFP plasmolyzed cells of N. benthamiana. (D) PDLP1-GFP. (E) P6-RFP. (F) Overlay of D and E. Photos G – I illustrate the association of P6-RFP with aniline blue in N. benthamiana leaf tissue after agroinfiltration. (G) cell walls stained with aniline blue. (H) P6-RFP. (I) Overlay of G and H.

**Figure 9** 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. **Panel a.** Western blot for total proteins probed with RFP antibodies. **Panel b.** Western blot for total proteins probed with GFP antibodies. **Panel c.** Co-immunoprecipitation of proteins using GFP antibodies and probed in a Western blot with RFP antibodies. **Panel d.** Co-immunoprecipitation of proteins using GFP antibodies and probed in a Western blot with GFP antibodies.
Figure 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. The NLSa sequence corresponds to the nuclear localization signal of influenza virus. The NLS sequence corresponds to the nuclear localization signal of human ribosomal protein L22 (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.
Figure 2 AtSRC2.2 preferentially interacts with D2 of P6 in a yeast two-hybrid analysis. (A) Interaction of AtSRC2.2 with the full-length CaMV P6. (B) Interaction of 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, the striped box represents the transcriptional activator domain and black boxes show the DNA-binding domain.
Figure 3  AtSRC2.2-RFP is partially co-localized with P6-GFP upon co-agroinfiltration into *N. benthamiana* leaves. (A) Expression of AtSRC2.2-RFP alone. (B) Expression of P6-GFP alone. (C-E) Co-agroinfiltration of AtSRC2.2-RFP with P6-GFP. (C) Expression of AtSRC2.2-RFP. (D) Expression of P6-GFP. (D) Overlay of photos C and D. Picture was taken at 3 dpi. White arrows indicate AtSRC2.2-RFP aggregates of a size that only exist when co-agroinfiltrated with P6-GFP.
Figure 4 Co-Immunoprecipitation studies with AtSRC2.2-RFP with P6-GFP and subcellular localization of P6ΔD1D2-GFP. (A) 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 P6ΔD1D2-GFP; Lane 4, Expression of P6-GFP; Lane 5, Co-expression of P6ΔD1D2-GFP with AtSRC2.2-RFP; Lane 6, Co-expression of P6-GFP with AtSRC2.2-RFP. **Panel a.** Western blot for total proteins probed with RFP antibodies. **Panel b.** Western blot for total protein probed with GFP antibodies. **Panel c.** Co- immunoprecipitation of proteins using GFP antibodies and probed in a Western blot with RFP antibodies. (B) Localization of P6ΔD1D2-GFP expressed in *N. benthamiana* leaves at 3 dpi.
**Figure 5** Co-immunoprecipitation and co-localization of P6D1D2-GFP with AtSCR2.2-RFP. Co-immunoprecipitation of P6D1D2-GFP with AtSCR2.2-RFP after co-agroinfiltration of *N. benthamiana* leaves. Lane 1, Mock-inoculated control leaf; Lane 2, Expression of AtSRC2.2-RFP; Lane 3, Expression of P6D1D2-GFP; Lane 4, Co-expression of AtSRC2.2-RFP and P6D1D2-GFP. **Panel a.** Western blot for total proteins probed with RFP antibodies. **Panel b.** Western blot for total proteins probed with GFP antibodies. **Panel c.** Co-immunoprecipitation of proteins using GFP antibodies and probed in a Western blot with RFP antibodies. **Panel d.** Co-immunoprecipitation of proteins using GFP antibodies and probed in a Western blot with GFP antibodies. Photos B – D illustrate re-localization of AtSCR2.2 with P6D1D2-GFP. (B) AtSRC2.2-RFP. (C) P6D1D2-GFP. (D) Overlay of B and C.
Figure 6 Association of AtSRC2.2 with the membrane marker protein AtPIP2A and with plasmodesmal marker proteins PDLP1 and CaMV MP. Photos A-C show that AtSRC2-2RFP does not colocalize with free GFP in a plasmolyzed cell (A) AtSCR2.2-RFP. (B) Free GFP. (C) Overlay of photos A and B. Photos D-F illustrate colocalization of AtSCR2.2 with the plasma membrane marker protein aquaporin AtPIP2A-GFP in a plasmolyzed cell. (D) SRC2.2-RFP. (E) AtPIP2A-GFP. (F) Overlay of photos D and E. Photos G-I illustrate colocalization of CaMV MP-GFP with AtSRC2.2-RFP in *N. benthamiana* leaf tissue. (G) MP-GFP. (H) AtSRC2.2-RFP. (I) overlay of G and H. Photos J-L illustrate that CaMV AtSRC2.2-RFP is co-localized with PDLP1-GFP in both the membrane and cell wall of plasmolyzed *N. benthamiana* cells. (J) PDLP1-GFP. (K) AtSRC2.2-RFP. (L) overlay of J and K. *N. benthamiana* cells were plasmolyzed by infiltration of 30% glycerol. The white arrows illustrate co-localization of AtSCR2.2-RFP and PDLP1-GFP in the plasmolyzed membrane, whereas the yellow arrows illustrate co-localization in the cell wall. The higher magnifications of the co-localized signals in the insets are highlighted by the purple arrows in photos D and G.
Figure 7 AtSRC2.2 and PDLP1 co-localize with CaMV P1 at the base and tip 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 photos C and D. (F-H) Co-agroinfiltration of unmodified P1, P1-GFP and AtSRC2.2-RFP shows that AtSRC2.2-RFP also co-localized with P1-GFP at the tip of tubule structures. (F) P1-GFP. (G) AtSRC2.2-RFP. (H) Overlay of photos F and G. (I-K) Co-agroinfiltration of unmodified P1, P1-RFP and PDLP1-GFP shows that PDLP1-GFP may also be co-localized with P1-RFP at the tip and base of tubule structures. (I) P1-RFP. (J) PDLP1-GFP. (K) Overlay of photos I and J.
**Figure 8** Association of P6-RFP with the plasmodesmal protein PDLP1-GFP and with aniline blue. Photos 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 A and B. Photos D - F illustrate cos-localization of P6-RFP with PDLP1-GFP plasmolyzed cells of *N. benthamiana*. (D) PDLP1-GFP. (E) P6-RFP. (F) Overlay of D and E. Photos G – I illustrate the association of P6-RFP with aniline blue in *N. benthamiana* leaf tissue after agroinfiltration. (G) cell walls stained with aniline blue. (H) P6-RFP. (I) Overlay of G and H.
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. **Panel a.** Western blot for total proteins probed with RFP antibodies. **Panel b.** Western blot for total proteins probed with GFP antibodies. **Panel c.** Co-immunoprecipitation of proteins using GFP antibodies and probed in a Western blot with RFP antibodies. **Panel d.** Co-immunoprecipitation of proteins using GFP antibodies and probed in a Western blot with GFP antibodies.