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Cooperation in Viral Movement: The Geminivirus BL1 Movement Protein Interacts with BR1 and Redirects It from the Nucleus to the Cell Periphery

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For plant viruses to systemically infect a host requires the active participation of viral-encoded movement proteins. It has been suggested that BL1 and BR1, the two movement proteins encoded by the bipartite geminivirus squash leaf curl virus (SqLCV), act cooperatively to facilitate movement of the viral single-stranded DNA genome from its site of replication in the nucleus to the cell periphery and across the cell wall to adjacent uninfected cells. To better understand the mechanism of SqLCV movement, we investigated the ability of BL1 and BR1 to interact specifically with each other using transient expression assays in insect cells and *Nicotiana tabacum* cv Xanthi protoplasts. In this study, we showed that when individually expressed, BL1 is localized to the periphery and BR1 to nuclei in both cell systems. However, when coexpressed in either cell type, BL1 relocalized BR1 from the nucleus to the cell periphery. This interaction was found to be specific for BL1 and BR1, because BL1 did not relocalize the SqLCV nuclear-localized AL2 or coat protein. In addition, mutations in BL1 known to affect viral infectivity and pathogenicity were found to be defective in either their subcellular localization or their ability to relocalize BR1, and, thus, identified regions of BL1 required for correct subcellular targeting or interaction with BR1. These findings extend our model for SqLCV movement, demonstrating that BL1 and BR1 appear to interact directly with each other to facilitate movement cooperatively and that BL1 is responsible for providing directionality to movement of the viral genome.

INTRODUCTION

To successfully infect a host plant and cause disease, a plant virus must cross the barrier of the cell wall to move cell to cell and reach the phloem sieve elements. From the sieve elements, it systemically infects the host. Plant viruses accomplish this by encoding movement proteins (MPs), which are nonstructural proteins that are not essential for viral replication or encapsidation but are required for systemic infection of the host (Atabekov and Dorokhov, 1984; Hull, 1991). Our current understanding of MP function is based primarily on molecular studies of the single MP encoded by tobacco mosaic virus (TMV) and red clover necrotic mosaic virus. In vitro studies have shown each to be a sequence-nonspecific nucleic acid binding protein that appears to bind RNA in a cooperative manner (Citovsky et al., 1990, 1992; Fujiwara et al., 1993; Giesman-Cookmeyer and Lommel, 1993). In transgenic plants, the TMV 30-kD MP localizes to secondary plasmodesmata in primarily nonvascular cells (Ding et al., 1992) and increases the size exclusion limit (SEL) of plasmodesmata ~10-fold between mesophyll and bundle sheath cells. When microinjected into mesophyll cells, bacterially expressed fusions of the TMV or red clover necrotic mosaic virus MP increase measured SELs of plasmodesmata, and each MP rapidly moves from cell to cell and functions to move single-stranded RNA (Fujiwara et al., 1993; Waigmann et al., 1994). Based on these studies, it has been proposed that these MPs are molecular chaperones that bind the viral RNA genome and target it to plasmodesmata, where the MP functions to increase the SEL and thus facilitates movement of the viral genome to adjacent cells.

A second model has been proposed that is based primarily on electron microscopic studies of cauliflower mosaic virus (CaMV), cowpea mosaic virus, tomato ringspot virus, and tomato spotted wilt virus infections. In this model, the single viral-encoded MP is associated with tubular structures that contain viruslike particles and appear to extend from cell walls at or near plasmodesmata into adjacent uninfected cells. This has led to the suggestion that for these viruses, a virus particle or subviral nucleocapsid form may move in association with these tubular structures (van Lent et al., 1990; Perbal et al., 1993; Weiczorek and Sanfaçon, 1993; Kormelink et al., 1994). Few molecular studies exist to support this model, but transient expression assays in protoplasts do suggest that the single viral-encoded MP is sufficient to induce formation of the tubular structures (van Lent et al., 1991; Perbal et al., 1993).

The bipartite geminiviruses, such as squash leaf curl virus (SqLCV), are phloem limited and, having genomes of covalently

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closed circular single-stranded DNA (ssDNA), replicate in the nucleus. These viruses encode two MPs, BR1 and BL1, that act directly to promote viral movement (Brough et al., 1988; Etessami et al., 1988) and define viral host range and pathogenic properties (Ingham and Lazarowitz, 1993; Pascal et al., 1993; Ingham et al., 1995). Recent studies of SqLCV (Pascal et al., 1993, 1994; Ingham et al., 1995) and bean dwarf mosaic virus (BDMV; Noueiry et al., 1994) have provided the first insights into the mechanism by which BR1 and BL1 may act to facilitate viral short-distance (cell-to-cell) and long-distance (systemic) movement. We find that BR1 is a nuclear localized ssDNA binding protein and that BL1 localizes to plasma membrane and crude cell wall fractions from both infected and transgenic plants and to the periphery of recombinant baculovirus-infected Sf9 insect cells (Pascal et al., 1993, 1994). Based on these studies, we have proposed that BR1 and BL1 have distinct roles and act coordinately to facilitate viral movement. Our model predicts that BR1 is a nuclear shuttle protein that binds viral ssDNA and moves it to the cell periphery where, as the result of BL1 action, BR1–ssDNA complexes move locally to adjacent uninfected cells and also enter sieve elements. From the sieve elements, we suggest that BR1–ssDNA complexes may initiate infection at distal sites along the phloem. Whether SqLCV BL1 acts directly to alter the plasmodesmal SEL, as suggested by microinjection studies of BDMV BL1 in mesophyll cells (Noueiry et al., 1994), or facilitates movement by some other mechanism remains unclear.

Our model for SqLCV movement predicts that BL1 and BR1 interact directly to facilitate viral movement. To demonstrate this, we have established transient expression assays in Sf9 insect cells and *Nicotiana tabacum* cv Xanthi protoplasts and used these assays to investigate the localization of wild-type and mutant forms of BL1 and BR1 when each is expressed individually or together. These studies have been aided by the large collection of alanine scanning, deletion, and truncation mutants of *BL1* and *BR1* that we have generated by site-directed mutagenesis and have characterized as to their infectivity, pathogenicity, and host range properties (Ingham et al., 1995). Our results reported here demonstrate that BL1 and BR1 appear to interact directly, with BL1 redirecting BR1 from the nucleus to the cell periphery in both Sf9 cells and Xanthi protoplasts. These studies have also identified domains in BL1 required for its specific interaction with BR1 and its correct subcellular targeting to the cell periphery.

RESULTS

Transient Expression of BL1 and BR1 in Sf9 Cells and Tobacco Protoplasts

To examine the requirements for correct subcellular localization of BL1 and BR1, and their potential interactions with each other, we utilized transient expression assays to express each in Sf9 cells and tobacco (Xanthi) protoplasts. For expression in Sf9 cells, *BL1* or *BR1* was cloned as a transcriptional fusion to the *Autographica californica* nuclear polyhedrosis virus *gp64* promoter and upstream of the *gp64* 3′ untranslated termination region contained in the insect expression vector p166B-10 (Gary Blissard, personal communication), as diagrammed in Figure 1. This promoter is strongly expressed early during baculovirus infection. Using this vector, 20 to 30% of transfected Sf9 cells were found to maximally express BR1 or BL1 by 48 hr post-transfection, as assayed by immunofluorescent staining and confocal microscopy; sufficient protein was expressed to be detected on immunoblots (data not shown). As shown in Figure 2A, BR1 localized to the nuclei of transfected Sf9 cells, which is consistent with its nuclear localization in phloem parenchyma cells in infected pumpkin (Pascal et al., 1994). BL1 was localized to the periphery of transfected Sf9

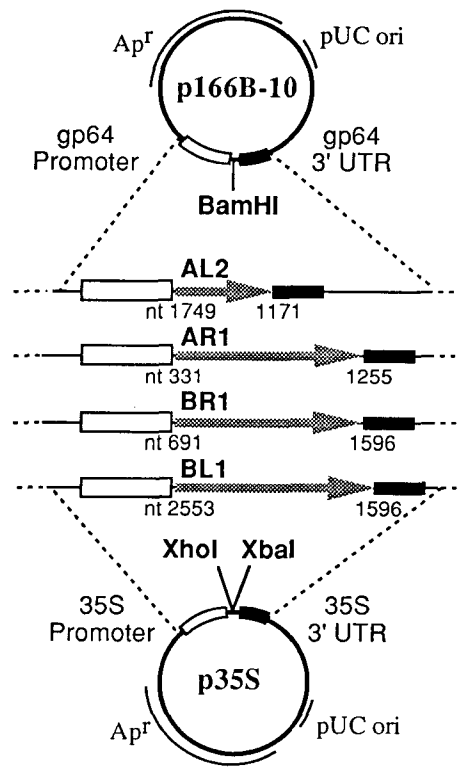

Figure 1. Expression Vectors Used for Transient Expression Assays in Sf9 Cells or Xanthi Protoplasts.

Shown are the orientation and nucleotide coordinates of the SqLCV *AL2*, *AR1* (CP), *BL1*, and *BR1* genes cloned as transcriptional fusions into each expression vector. (Top) p166B-10 showing locations of the *A. californica* nuclear polyhedrosis virus *gp64* promoter and 3′ untranslated region (3′ UTR) and the unique BamHI site used for cloning. (Bottom) p35S, derived by excision of the tobacco etch virus leader sequences and β-glucuronidase coding region from pRTL2-GUS: NIaΔBam (Restrepo et al., 1990), showing locations of the CaMV 35S promoter and termination sequences and the unique XhoI and XbaI sites used for cloning. Ap^r, ampicillin resistance; nt, nucleotide; ori, plasmid origin of replication.

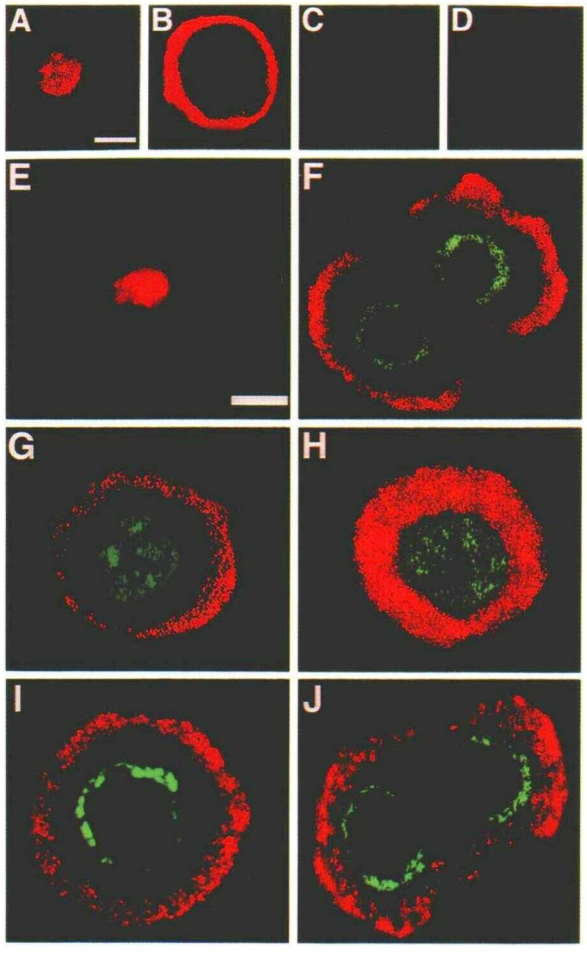

Figure 2. Localization of BR1, BL1, and BL1 Mutants in Sf9 Cells or Xanthi Protoplasts.

Cells transiently expressing BR1 or BL1 were incubated with the appropriate rabbit antisera, as indicated, followed by incubation with trimethylrhodamine-conjugated goat anti–rabbit secondary antibody. In **(F)** to **(J)**, cells were also counterstained with chromomycin A to show the location of the nuclei.

(A) Sf9 cells expressing BR1 and stained with anti-BR1 antisera.

(B) Sf9 cells expressing BL1 and stained with anti-BL1 antisera.

(C) Untransfected Sf9 cells stained with anti-BR1 antisera.

(D) Untransfected Sf9 cells stained with anti-BL1 antisera.

(E) Xanthi protoplasts expressing BR1 and stained with anti-BR1 antisera.

(F) Xanthi protoplasts expressing BL1 and stained with anti-BL1 antisera. Cells shown have recently divided.

(G) Sf9 cells expressing $BL1^{K140A/K142A}$ and stained with anti-BL1 antisera.

(H) Sf9 cells expressing $BL1^{W208A/K211A}$ and stained with anti-BL1 antisera.

(I) Xanthi protoplasts expressing $BL1^{K140A/K142A}$ and stained with anti-BL1 antisera.

(J) Xanthi protoplasts expressing $BL1^{W208A/K211A}$ and stained with anti-BL1 antisera. Cells shown have recently divided.

Bars in **(A)** and **(E)** = 10 μm.

cells (Figure 2B), which is consistent with its presence in plasma membrane–containing fractions from infected and transgenic plants (Pascal et al., 1993). No staining was observed when untransfected Sf9 cells were incubated with anti-BR1 or anti-BL1 antisera (Figures 2C and 2D) or when transfected cells were incubated with preimmune sera (data not shown; Pascal et al., 1994).

The same results were obtained for localization of BR1 and BL1 in tobacco protoplasts (Figures 2E and 2F). For these studies, *BL1* or *BR1* was expressed as a transcriptional fusion to the CaMV 35S promoter derived from the expression vector pRTL2-GUS:NIaΔBam (Restrepo et al., 1990) and utilized the 35S 3′ untranslated termination region (Figure 1). Approximately 20% of transfected Xanthi protoplasts were found to express BL1 or BR1 maximally by 24 hr post-transfection (data not shown). BR1 localized to the nuclei of these protoplasts (Figure 2E), as determined by phase contrast microscopy and colocalization with chromomycin A. No staining was observed with preimmune sera (data not shown). Interestingly, under the paraformaldehyde fixation conditions used, chromomycin A showed peripheral staining within the nuclei, as expected based on its direct binding to DNA (Figure 2F); however, BR1 was clearly localized within the nucleoplasm and was not associated with the chromatin (Figure 2E). Again, as observed in Sf9 cells, BL1 localized to the periphery of transfected Xanthi protoplasts (Figure 2F), with a somewhat more diffuse distribution at the cell periphery than was generally found in the insect cells.

Subcellular Localization of Mutated BL1 Proteins

Both missense and deletion mutants of *BL1* that decrease SqLCV infectivity and pathogenicity and affect viral host range have been identified (Ingham et al., 1995). For any mutated BL1 protein, these observed defects could be due to the misfolding of BL1, the inability of BL1 to interact with BR1 or other viral-encoded proteins, or incorrect subcellular targeting of BL1. Thus, to identify domains within BL1 required for its correct subcellular targeting, we used our transient expression assays in Sf9 cells and Xanthi protoplasts to investigate the subcellular localization of these mutated BL1 proteins.

As shown in Figures 2G and 2I and summarized in Table 1, BL1 alanine scanning mutants $BL1^{F35A}$, $BL1^{N67A}$, $BL1^{K79A}$, $BL1^{Y120A/Y121A}$, $BL1^{K140A/K142A}$, $BL1^{K147A/H148A}$, and $BL1^{E227A/E228A}$ and deletion mutants $BL1^{\Delta 11-23}$ and $BL1^{\Delta 194-293}$ correctly localized to the periphery of Sf9 cells and Xanthi protoplasts, their distribution being indistinguishable from wild-type BL1 in both timing and localization. Like wild-type BL1, these particular mutants first appeared at the cell periphery as early as 48 hr post-transfection in Sf9 cells and 24 hr post-transfection in Xanthi protoplasts, and they continued to accumulate at the cell periphery for up to 8 and 5 days, respectively. $BL1^{K79A}$ and $BL1^{E227A/E228A}$ are class I mutants that retain full wild-type levels of 100% infectivity in both pumpkin and squash and exhibit

Table 1. Phenotypes of BL1 Mutants: Subcellular Location and Ability To Relocalize BR1

BL1 Mutants[a]	Subcellular Localization: Sf9 Cells[b]	Subcellular Localization: Xanthi Cells[b]	BR1 Interaction[c]
Wild type	P	P	+
F35A	P	ND	+
N67A	P	ND	+
D78A/R80A	C	C	–
K79A	P	ND	+
K112A/D113A	ND	C	+
Y120A/Y121A	ND	P	+
K140A/K142A	P	P	–[d]
K147A/H148A	ND	P	–[d]
W208A/K211A	C	C	+
E227A/E228A	P	ND	+
N260A	C	C	–
Δ11–23	P	ND	+
Δ160–169	C	ND	–
Δ194–293	P	ND	+

[a] Shown are mutated amino acids (see text for details).
[b] P, cell periphery; C, cytoplasm; ND, not done.
[c] Indicated is the ability of BL1 to redirect BR1 from the nucleus, as assayed in Sf9 cells, Xanthi protoplasts, or both. (+), relocalized BR1; (–), BR1 remained in the nucleus.
[d] These BL1 mutants did not relocalize BR1 in Sf9 cells. In Xanthi protoplasts, these BL1 mutants transiently relocalized BR1 to the cell periphery at 24 hr, but from 48 to 120 hr, BR1 remained in the nucleus.

only short delays of 2 to 4 days in the initial appearance of disease symptoms (Ingham et al., 1995). *BL1*^{F35A}, *BL1*^{N67A}, *BL1*$^{K140A/K142A}$, *BL1*$^{K147A/H148A}$, and *BL1*$^{\Delta 194-293}$ are class II mutants that produce very attenuated disease symptoms and have reduced levels of infectivity in pumpkin of 56 to 70% of the wild-type levels, accompanied by long delays in the timing of disease symptom appearance (Ingham et al., 1995). All of these mutants also produce attenuated symptoms and have low levels of infectivity of 22 to 50% in squash but are no longer infectious for *N. benthamiana*. *BL1*$^{\Delta 11-23}$ and *BL1*$^{Y120A/Y121A}$ are class III mutants that are no longer infectious in pumpkin or *N. benthamiana* (Ingham et al., 1995).

In contrast to the correct targeting of these BL1 mutants, BL1$^{D78A/R80A}$, BL1$^{K112A/D113A}$, BL1$^{W208A/K211A}$, BL1^{N260A}, and BL1$^{\Delta 160-169}$ all mislocalized to the cytoplasm in both Sf9 cells and Xanthi protoplasts, as evidenced by uniform immunofluorescent staining throughout the cytoplasm (Figures 2H and 2J and Table 1). No accumulation at the cell periphery was observed for these particular BL1 mutated proteins, even following long incubation periods of up to 9 days post-transfection. The total intensities of immunofluorescent staining observed for these defective mutants were comparable to that seen for wild-type BL1 (compare Figures 2B and 2G), suggesting that these mutated BL1 proteins had turnover rates similar to that of wild-type BL1. *BL1*$^{D78A/R80A}$, *BL1*$^{K112A/D113A}$, *BL1*$^{W208A/K211A}$, *BL1*^{N260A}, and *BL1*$^{\Delta 160-169}$ have been tested for their infectivity and pathogenic properties (Ingham et al., 1995), and all are class II or III mutants that are highly defective in these traits. *BL1*$^{D78A/R80A}$, *BL1*$^{K112A/D113A}$, *BL1*$^{W208A/K211A}$, and *BL1*$^{\Delta 160-169}$ are all null mutants (class III) that have lost the ability to infect squash or *N. benthamiana*. *BL1*^{N260A} is a severely defective class II mutant that is no longer infectious for *N. benthamiana* and has low levels of infectivity in squash and pumpkin (11 and 40%, respectively; Ingham et al., 1995). This last mutant produces extremely mild, attenuated symptoms in cucurbit hosts, characterized by small chlorotic spots in the absence of downward leaf curl that appear with a very delayed time course 3 to 4 weeks later than symptoms in wild-type infections (Ingham et al., 1995). Thus, localization of mutated BL1 proteins to the cell periphery or cytoplasm in these transient expression studies was well correlated with the defects in infectivity and pathogenicity observed for these mutants in virus-infected plants.

BL1 Specifically Relocalizes BR1 from the Nucleus to the Cell Periphery

Our model for SqLCV MP function predicts that BR1 and BL1 directly interact to facilitate viral movement. To test this prediction, we coexpressed BR1 and BL1 in transiently transfected Sf9 cells and Xanthi protoplasts to examine whether the presence of either MP would alter the localization of the other MP. Coexpression of BR1 and BL1 did not alter the localization of BL1 in either Sf9 cells or Xanthi protoplasts. As shown in Figure 3C, BL1 remained localized to the periphery of Sf9 cells when coexpressed with BR1. The same was true in Xanthi protoplasts (data not shown). In contrast to these findings for BL1, when coexpressed with BL1 in either Sf9 cells or Xanthi protoplasts, BR1 was redirected from the nucleus to the cell periphery where BL1 was located (Figures 3A and 3B). This relocalization of BR1 to the cell periphery was quite stable, with BR1 found only at the periphery at all time points from 24 hr through 5 days post-transfection in Xanthi protoplasts and 24 hr through 9 days post-transfection in Sf9 cells.

To determine whether the ability of BL1 to redirect BR1 from the nucleus to the cell periphery was specific for BR1, we coexpressed BL1 with each of two other SqLCV proteins that are nuclear localized, namely, AL2 and coat protein (CP). AL2, a viral transcription factor (Sunter and Bisaro, 1992), and CP were each localized to nuclei of Sf9 cells or Xanthi protoplasts when expressed individually in our transient expression assays, as summarized in Table 2. When coexpressed with BL1, both AL2 and CP each remained in the nucleus (Figure 3D and Table 2), in stark contrast to the relocalization seen when BR1 was coexpressed with BL1. Hence, the ability of BL1 to relocalize SqLCV-encoded nuclear proteins is specific for BR1.

The fact that BR1 is redirected to the cell periphery where BL1 is found independent of cell type suggests that BR1 and BL1 directly interact with each other. To further investigate this interaction, as well as to potentially identify domains in BL1 required for this interaction, wild-type BR1 was coexpressed

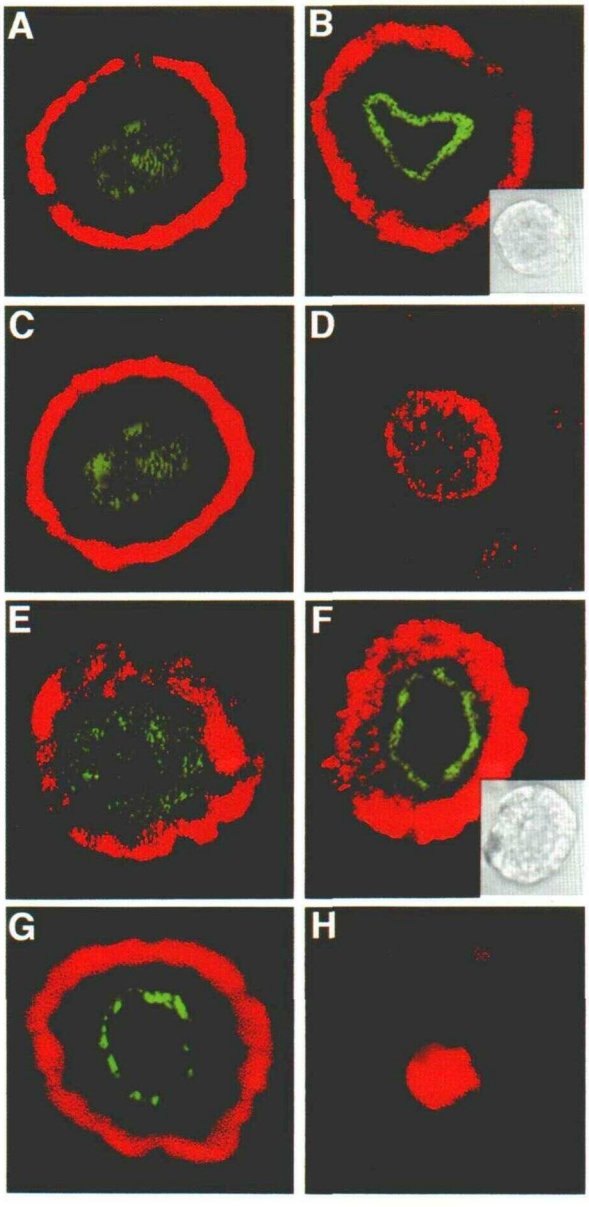

Figure 3. Ability of Wild-Type or Mutant BL1 To Relocalize BR1 or AL2 in Sf9 Cells or Xanthi Protoplasts.

Cells transiently coexpressing BL1 or BL1 mutants, as indicated, with either wild-type BR1 or AL2, were incubated with the appropriate rabbit antisera, followed by incubation with trimethylrhodamine-conjugated goat anti–rabbit secondary antibody. Fluorescein images show chromomycin A counterstaining to delimit the cell nuclei (not included in **[D]** and **[H]**).

(A) BR1 and BL1 coexpressed in Sf9 cells and stained with anti-BR1 antisera.

(B) BR1 and BL1 coexpressed in Xanthi protoplasts and stained with anti-BR1 antisera. Shown in inset is the phase image of the same cell.

(C) BR1 and BL1 coexpressed in Sf9 cells and stained with anti-BL1 antisera.

Table 2. Interaction of BL1 with SqLCV Nuclear Proteins BR1, AL2, and CP

Protein	– BL1[a]	+ BL1[a]
BR1	N[b]	P[b]
AL2	N	N
CP	N	N

[a] Shown is the subcellular location of the SqLCV protein when expressed in Sf9 cells or Xanthi protoplasts in the absence (– BL1) or presence (+ BL1) of wild-type BL1.
[b] N, nuclear; P, cell periphery.

with different BL1 mutants in our transient expression assays. When coexpressed with either $BL1^{K112A/D113A}$ or $BL1^{W208A/K211A}$, both of which localized to the cytoplasm of transfected cells (see Figures 2I and 2J), BR1 was relocalized to the cytoplasm where $BL1^{K112A/D113A}$ or $BL1^{W208A/K211A}$ was located and not to the cell periphery. This was true in both transfected Sf9 cells and Xanthi protoplasts (Figures 3E and 3F and Table 1). Thus, it again appears that BR1 and BL1 directly interact with each other. When coexpressed with the three other cytoplasmically localized BL1 mutants—$BL1^{D78A/R80A}$, $BL1^{\Delta 160-169}$, and $BL1^{N260A}$—BR1 was not relocalized but remained in the nucleus of Sf9 cells or Xanthi protoplasts (see Table 1).

Mutants $BL1^{\Delta 11-23}$, $BL1^{F35A}$, $BL1^{N67A}$, $BL1^{K79A}$, $BL1^{Y120A/Y121A}$, $BL1^{K140A/K142A}$, $BL1^{K147A/H148A}$, $BL1^{E227A/E228A}$, and $BL1^{\Delta 194-293}$ were all correctly localized to the periphery of transfected Sf9 cells or Xanthi protoplasts, their distribution being indistinguishable from wild-type BL1 (see Figure 2 and Table 1). Of these mutants, the N-terminal mutants $BL1^{\Delta 11-23}$, $BL1^{F35A}$, $BL1^{N67A}$, and $BL1^{K79A}$, the C-terminal mutant $BL1^{\Delta 194-293}$, and the centrally located mutant $BL1^{Y120A/Y121A}$ each redirected wild-type BR1 to the cell periphery. This redirection of BR1 was independent of cell type (Table 1). In contrast, mutants $BL1^{K140A/K142A}$ and $BL1^{K147A/H148A}$ were both defective in their ability to interact with BR1. Each of these mutants only transiently relocalized BR1 to the periphery of Xanthi protoplasts. When coexpressed with $BL1^{K140A/K142A}$ and $BL1^{K147A/H148A}$, BR1 was relocalized to the periphery of Xanthi protoplasts at 24 hr post-transfection; however, by 48 hr post-transfection, BR1 was relocated

(D) AL2 and BL1 coexpressed in Sf9 cells and stained with anti-AL2 antisera.

(E) BR1 and $BL1^{W208A/K211A}$ coexpressed in Sf9 cells and stained with anti-BR1 antisera.

(F) BR1 and $BL1^{W208A/K211A}$ coexpressed in Xanthi protoplasts and stained with anti-BR1 antisera. Shown in inset is the phase image of the same cell.

(G) and **(H)** BR1 and $BL1^{K140A/K142A}$ coexpressed in Xanthi protoplasts and stained with anti-BR1 antisera at 24 hr post-transfection in **(G)** or 48 hr post-transfection in **(H)**.

in the nucleus (Figures 3G and 3H and Table 1), where it remained throughout the 5 days of the assay. BL1$^{K140A/K142A}$ and BL1$^{K147A/H148A}$ were each found at the periphery of these cotransfected Xanthi protoplasts throughout the time course of the assay, and the amount of each mutated BL1 protein remained constant, as determined by the intensity of immunofluorescent staining (data not shown). This defect in the ability to interact with BR1 was even more extreme when assayed in Sf9 cells. When BL1$^{K140A/K142A}$ was coexpressed with BR1 in Sf9 cells, BR1 was never found at the cell periphery but remained in the nucleus at all times (Table 1; data not shown). These results suggest that the region surrounding the mutations in BL1$^{K140A/K142A}$ and BL1$^{K147A/H148A}$ is a domain required for BL1 to relocalize and thus specifically interact with BR1. These findings also revealed the first difference between Xanthi protoplasts and Sf9 cells in our assays for BL1–BR1 interactions. The subcellular localization of all of our BL1 mutants, as well as their ability to interact with BR1 and their infectivity defects when tested in plants, are summarized in Figure 4.

DISCUSSION

Plant virus movement is a dynamic process that requires that the viral genome be targeted to the cell periphery and directed locally to adjacent uninfected cells as well as to phloem sieve elements for systemic infection. Our previous studies of the in vivo subcellular localization and in vitro biochemical properties of SqLCV BR1 and BL1 have led us to propose that BR1 and BL1 act cooperatively to facilitate viral movement (Pascal et al., 1993, 1994). We have suggested that BR1 is a nuclear shuttle protein that binds newly replicated viral genomes and moves these into and out of the cell nucleus. According to this model, BL1 traps SqLCV BR1–ssDNA complexes in the cytoplasm and attracts these complexes to the cell periphery where BL1 acts to facilitate their movement to adjacent uninfected cells (Pascal et al., 1994; Ingham et al., 1995). In this study, we used transient expression assays in Sf9 cells and Xanthi protoplasts and our large collection of *BL1* mutants (Ingham et al., 1995) to investigate the dynamics of viral movement, in particular the interactions of BR1 and BL1. These cell culture model systems have allowed us to investigate the interactions of BL1 and BR1 in living cells, and the intracellular role of BL1 in viral movement and its subcellular targeting. As predicted by our model, we found that BR1 and BL1 do specifically and may directly interact with each other and that BL1 provides directionality to SqLCV movement.

When coexpressed with BR1, BL1 redirected BR1 from the nucleus to the cell periphery where BL1 itself was located. That BR1 and BL1 may interact directly is inferred from the finding that wild-type BR1 was relocalized to the cytoplasm when coexpressed with the cytoplasmically localized mutants BL1$^{K112A/D113A}$ and BL1$^{W208A/K211A}$. This interaction occurred in the absence of replicating viral DNA; however, the same results were obtained in Xanthi protoplasts transfected with intact SqLCV genomic DNA in which replicating viral DNA was present (data not shown). Although we cannot exclude a potential role for accessory cell proteins in this interaction, this interaction did occur independent of cell type, with BR1 relocalizing in the presence of wild-type BL1 or BL1 mutants in the same manner whether assayed in Sf9 insect cells or tobacco protoplasts. Given the central role of both BR1 and BL1 in determining viral host range (Ingham and Lazarowitz, 1993; Ingham et al., 1995), it seems unlikely that required accessory proteins would be expressed in both Sf9 cells, a non-host for SqLCV, and tobacco (*N. tabacum*), a permissive host for SqLCV movement (E. Pascal and S.G. Lazarowitz, unpublished data). Furthermore, given that this interaction can occur, whether BL1 is located at the cell periphery or throughout the cytoplasm, again makes it less likely that accessory proteins are involved. Thus, we suggest that BR1 and BL1 may interact directly. To date, we have been unable to demonstrate this interaction by coimmunoprecipitation of in vitro–synthesized proteins. It may be that, as our model suggests, the interaction of BR1 and BL1 is of a transitory nature and not sufficiently stable to be detected by immunoprecipitation. It is also possible that the epitopes recognized by our antibodies are masked

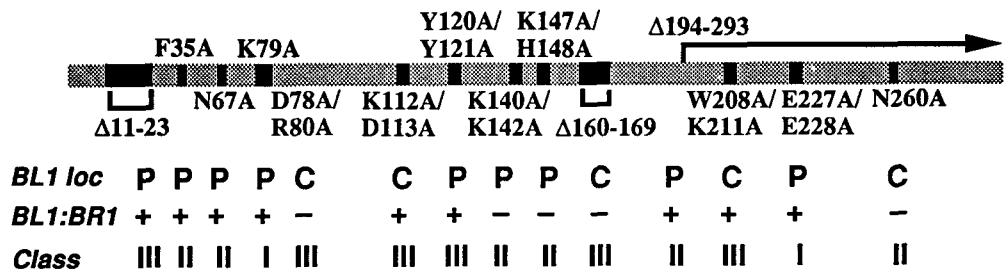

Figure 4. Summary of the Subcellular Location, BR1 Interactions, and Infectivity Phenotype of BL1 Mutants.

Diagrammed is the *BL1* coding sequence. Point mutations are indicated by black boxes, deletion mutants by brackets, and the C-terminal truncation Δ194–293 by an arrow. BL1 loc, subcellular location of BL1 as peripheral (P) or cytoplasmic (C); BL1:BR1, ability of the mutated BL1 protein to relocalize (+) or not relocalize (−) BR1; Class, infectivity defect of each mutant as characterized by Ingham et al. (1995).

in complexes formed between BR1 and BL1. Current in vitro binding assays using purified BR1 and BL1 overexpressed in Sf9 cells should directly address these issues.

Our findings demonstrate that the ability of BR1 and BL1 to interact with each other is an inherent property of, and is specific to, these two MPs. In addition to this interaction occurring independent of cell type, as discussed previously, BL1 did not relocalize other SqLCV-encoded nuclear-localized proteins, namely, CP and AL2. Both CP and AL2 remained in the nuclei of Sf9 or Xanthi cells when coexpressed with BL1; this is in striking contrast to the relocalization of BR1 that we observed. This makes sense in the context of viral multiplication, because both AL2 and CP function in the nuclei of infected cells: AL2, a transcription factor, activates viral gene expression from SqLCV double-stranded DNA templates located in the nucleus; and virions are found assembled only in the nucleus, the site of viral replication, with none having been reported in the cytoplasm of infected cells (Goodman, 1981). The different behavior of BR1 when coexpressed with BL1 further argues that as a nuclear-localizing protein, BR1 has properties quite distinct from those of either AL2 or CP.

What might these unique properties of BR1 be? For BL1 to perturb the nuclear localization of BR1 and redirect it to the cell periphery requires that both proteins at least transiently exist in the same subcellular compartment. The inability of BL1 to relocalize either AL2 or CP demonstrates that simple leakage of SqLCV proteins from the nucleus due to the documented cytopathic properties of BL1 (Pascal et al., 1993; Ingham et al., 1995) does not explain our findings. Rather, our results support our earlier suggestion that BR1 is a nuclear shuttle protein (Pascal et al., 1994). At equilibrium, BR1 is predominantly nuclear and does not have a large cytoplasmic pool (see Figures 2A and 2E). This is a common feature of other characterized nuclear shuttle proteins, such as nucleolin and B23/No38, in which the small cytoplasmic pools are not detected by conventional fractionation or immunological techniques (Borer et al., 1989; Laskey and Dingwall, 1993; Schmidt-Zachmann et al., 1993). We found the presence of BL1 to perturb the equilibrium of BR1, presumably by binding BR1 molecules as they transiently passed into the cytoplasm, thereby retaining them there.

Directionality is an important aspect of viral movement. Our results showed that BR1 does not provide directionality to SqLCV movement, but rather that BL1 acts to accomplish this through its interaction with BR1. Our coexpression studies in both Sf9 cells and Xanthi protoplasts demonstrated that BL1 acts to redirect BR1 from the nucleus to the cell periphery. Thus, as previously suggested (Pascal et al., 1994), it appears that one function of BL1 is to trap BR1–ssDNA complexes in the cytoplasm and redirect them to the cell plasma membrane for transport to adjacent uninfected cells. That BR1–ssDNA complexes move is supported by the properties of BR1 as an ssDNA binding protein (Pascal et al., 1994) and by the finding that CP positively interacts with the movement pathway, probably through its ability to increase the amount of viral ssDNA synthesis (Ingham et al., 1995). Localization of BL1 to the cell periphery is not required for it to interact with BR1 as cytoplasmically localized mutants BL1$^{K112A/D113A}$ and BL1$^{W208A/K211A}$ both redirected BR1 to the cytoplasm of Sf9 cells and Xanthi protoplasts. However, correct peripheral localization is required for proper BL1 function, as *BL1*$^{K112A/D113A}$ and *BL1*$^{W208A/K211A}$ and the cytoplasmically localized mutants *BL1*$^{D78A/R80A}$ and *BL1*$^{\Delta 160-169}$ are all class III null mutants having no infectivity in all hosts tested (Ingham et al., 1995).

That we can separate the ability of BL1 to interact with BR1 from the correct targeting of BL1 to the cell periphery has allowed us to identify domains in BL1 required for its specific interaction with BR1 or correct subcellular localization, as retention of either function suggested that the mutated protein under study was not globally misfolded (see Figure 4). That these domains are relevant in vivo can be concluded from the correlation between observed infectivity defects in the plant and the behavior of mutated BL1 proteins in our transient expression assays (Ingham et al., 1995). Thus, the mutations in BL1$^{K112A/D113A}$ and BL1$^{W208A/K211A}$ indicate that the regions surrounding residues 112 to 113 and 208 to 211 in BL1 are important for the correct subcellular targeting of BL1. Given the distribution of proline residues and hydrophobic regions in BL1, residues 112 to 113 may be exposed on the protein surface, and thus in the native protein they could be juxtaposed to the region of residues 208 to 211 to potentially form a domain required for correct targeting of BL1 to the cell periphery. Mutants BL1$^{K140A/K142A}$ and BL1$^{K147A/H148A}$ appear to define a region of BL1 delimited by mutants BL1$^{Y120A/Y21A}$ and BL1$^{\Delta 194-293}$, which is essential for BL1 to interact with BR1, as neither BL1$^{K140A/K142A}$ nor BL1$^{K147A/H148A}$ relocalized BR1, although each was correctly localized to the cell periphery. The entire region from residues 120 to 160 is neither highly charged nor hydrophobic; however, residues 140 to 148 are in a lysine-rich region of BL1 (^{136}KFKGKLKLSSAKH^{148}). These results suggest that the interaction of BL1 and BR1 is required for their correct functioning in viral movement in the plant. Consistent with this and of particular interest is our finding that both BL1$^{K140A/K142A}$ and BL1$^{K147A/H148A}$ only transiently relocalized BR1 to the cell periphery in Xanthi protoplasts (see Figure 3G). Thus, BL1$^{K140A/K142A}$ and BL1$^{K147A/H148A}$ are partially defective in their potential to interact with BR1. This correlates with the fact that *BL1*$^{K140A/K142A}$ and *BL1*$^{K147A/H148A}$ are class II mutants that have reduced infectivity and pathogenicity and a long delay in the appearance of disease symptoms in cucurbits (Ingham et al., 1995).

Certain class II or class III *BL1* mutants that are severely defective in infectivity or pathogenicity (*BL1*$^{\Delta 11-23}$, *BL1*^{F35A}, *BL1*^{N67A}, *BL1*$^{Y120A/Y121A}$, and *BL1*$^{\Delta 194-293}$) were not defective either in their targeting to the cell periphery or in their ability to relocalize BR1. This was expected because our transient expression systems only assayed for two properties of BL1, namely, its correct subcellular targeting and its ability to interact with BR1. Thus, *BL1*$^{\Delta 11-23}$, *BL1*^{F35A}, *BL1*^{N67A}, *BL1*$^{Y120A/Y121A}$, and *BL1*$^{\Delta 194-293}$ are presumed to be defective in other potential functions of BL1 not directly testable in our model systems.

One particularly interesting potential class of BL1 mutants consists of those that would have increased binding affinity for BR1. These would behave normally in our transient expression assays, but would be expected to be severely defective, according to our model, because BR1 movement complexes would be retained by BL1 in the infected cell and thus not released into adjacent uninfected cells.

Hence, all of our results taken together strongly support our proposed model for the function and cooperative interaction of BL1 and BR1 in facilitating movement of the SqLCV ssDNA genome. In vitro biochemical studies (Pascal et al., 1994), immunolocalization and cell fractionation studies in infected plants and cultured cells (Pascal et al., 1993, 1994), genetic epistasis studies (Ingham et al., 1995), and our demonstration here of the specific and cooperative interaction of BL1 and BR1 identify an intracellular pathway in which BR1–ssDNA complexes shuttle in and out of the nucleus and are trapped within the cytoplasm to be directed to the cell periphery by BL1. Several aspects of this model are at variance with, and difficult to reconcile with, the model proposed for BDMV based on microinjection of *Escherichia coli*–expressed BR1 and BL1 fusion proteins into *N. tabacum* mesophyll cells (Noueiry et al., 1994). This latter model posits that BL1 directly binds and moves viral double-stranded DNA and that BR1 is a nuclear-exiting factor that delivers the double-stranded DNA to BL1. These conclusions were based on cytoplasmic localization of BR1 and the inability to find BR1 in the nucleus. Clearly, the inability to find BR1 in the nucleus is a serious problem with these microinjection studies (Noueiry et al., 1994) because we find BR1 localized to nuclei in phloem cells and insect cells (Pascal et al., 1994) and, as shown above, mesophyll-derived cells (Xanthi protoplasts). At issue in interpreting the microinjection studies are the mode of preparation and the lack of proper post-translational modifications of the *E. coli*–expressed fusion proteins used, the large amounts of protein injected, and the lack of quantitation of the results (Noueiry et al., 1994). In addition, no direct binding of nucleic acids by BL1 or BR1 was demonstrated in the BDMV studies (Noueiry et al., 1994), and it remains to be shown whether the fluorescent dyes used remain bound to the nucleic acids following microinjection. Our studies reported here do not address directly the mechanism by which BL1 acts to facilitate movement of BR1-containing complexes across the cell membrane and wall. The microinjection studies of Noueiry et al. (1994) suggest that BDMV BL1 may act in a manner similar to the TMV 30-kD protein to affect plasmodesmal SELs. However, given the aforementioned problems and the lack of demonstrated relevance of their findings to the function of BL1 in virus-infected plants, it remains an open question whether BL1 functions through preexisting plasmodesmata or by some other mechanism to facilitate intercellular movement of the viral genome. Additional studies are needed to clarify the mechanism of action of BL1 in intercellular movement and to address the inconsistencies cited above.

The results reported here for SqLCV BL1 and BR1 demonstrate at least one mechanism involving specific protein–protein interaction by which directionality can be imposed on plant viral movement. These results have also begun to identify potential regions within BL1 required for its correct subcellular targeting to the cell periphery and its interaction with BR1. In addition to their intrinsic interest as facilitators of plant virus movement, further investigation of these MPs has broader implications for understanding intracellular trafficking in plants. Studies of BL1 should both reveal specific details about its ability to bind and direct BR1 and to help define those pathways by which peripheral membrane proteins are modified and properly targeted in plant cells. BR1 appears to be the only current example of a nuclear shuttle protein in plants and thus affords the opportunity to investigate the function of this interesting class of proteins in plant cells.

METHODS

Expression Vectors

Insect (*Spodoptera frugiperda*) cell expression vectors for transient transfection were constructed using p166B-10 (Gary Blissard, personal communication), a plasmid containing the promoter and terminator sequences from the *gp64* gene of the *Autographica californica* nuclear polyhedrosis virus, separated by a unique BamHI site for cloning (Figure 1) in the pBS (+) vector (Stratagene). Squash leaf curl virus (SqLCV-E, extended host range virus, genomic components A_E and B_E; Lazarowitz, 1991) *BR1* and *BL1* were excised from pGBR1 and pGBL1, respectively (Pascal et al., 1993, 1994), using the upstream HindIII and downstream XhoI sites in the polylinker flanking each coding region. Each was blunt ended using T4 DNA polymerase (Sambrook et al., 1989) and cloned into the blunt-ended BamHI site of p166B-10 using T4 polynucleotide ligase (Sambrook et al., 1989) to create pGP64-BR1E and pGP64-BL1E, respectively. *AL2* and *AR1* (coat protein gene) were excised from the SqLCV A_E component (Lazarowitz, 1991) by digestion with EcoRII and XhoI (nucleotides 1749 to 1171) or DdeI (nucleotides 331 to 1255), respectively. Each fragment was blunt ended with T4 DNA polymerase and cloned into the blunt-ended BamHI site of p166-10B, creating pGP64-AL2E and pGP64-AR1E, respectively.

The construction of *BL1* mutants and the characterization of their phenotype have been reported previously (Ingham et al., 1995). The coding region from each *BL1* mutant was cloned into p166B-10 as described above. For transient transfection assays in Sf9 cells, DNA (~200 μg) was prepared using the Wizard Midi Plasmid Preparation System (Promega) as recommended by the manufacturer, and the DNA was ethanol precipitated before it was used. DNA was stored at 4°C prior to transfection.

Plant expression vectors were derived from pRTL2-GUS:NIaΔBam (Restrepo et al., 1990). This vector was digested with XhoI and XbaI to remove the tobacco etch virus leader and the β-glucuronidase:NIa fusion, thus leaving the empty expression cassette with the cauliflower mosiac virus (CaMV) 35S promoter and terminator regions intact (see Figure 1). SqLCV *AL2*, *AR1*, *BR1*, *BL1*, and *BL1* mutants were then each cloned into this expression cassette essentially as described above, creating expression vectors p35S-AL2E, p35S-AR1E, p35S-BR1E, and p35S-BL1E and the corresponding mutant BL1-expressing plasmids (for example, p35S- $BL1E^{F35A}$). Specifically, the XhoI and XbaI sites of this expression cassette were blunt ended using T4 DNA polymerase, and the appropriate blunt-ended fragment for each

wild-type or mutant gene was inserted into these sites using T4 polynucleotide ligase (Sambrook et al., 1989). For the transient transfection assays in Xanthi protoplasts, plasmid DNA was purified by a single banding in CsCl gradients (Sambrook et al., 1989) and stored at 4°C prior to electroporation.

Site-Directed Mutagenesis of BL1

Alanine substitutions (alanine scanning; Cunningham and Wells, 1989) used to construct mutants $BL1^{K112A/D113A}$, $BL1^{Y120A/Y121A}$, and $BL1^{K147A/H148A}$ were introduced by site-directed mutagenesis using synthetic oligonucleotide primers and uracil-containing single-stranded DNA (ssDNA) templates, as previously described (Ingram et al., 1995).

Antisera

The generation of rabbit polyclonal antibodies raised against BR1 and BL1 expressed in *Escherichia coli* has been described previously (Pascal et al., 1993). For production of anti-AL2 and anti-AR1 antisera, pET-3b translational fusions (Studier et al., 1990) expressing AL2 or AR1 were constructed using the AvaII-XhoI fragment (nucleotides 1651 to 1169, amino acids 9 to 131) or the NcoI-XhoI fragment (nucleotides 410 to 1171, amino acids 4 to 251) of the SqLCV A_E component, respectively (Lazarowitz and Lazdins, 1991). Induction and expression of these AL2 and AR1 fusion proteins in *E. coli* were as described previously for BR1 and BL1 (Pascal et al., 1993). The pellet obtained from sonicated cells was washed with TEH (50 mM Tris-HCl, pH 8, 10 mM EDTA) containing 0.5% Triton X-100, followed by consecutive washes with TEH containing 1% Nonidet P-40 and TEH with 1 M urea. This final washed pellet was resuspended in sample buffer (60 mM Tris-HCl, pH 8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue), and the proteins were resolved on 10% acrylamide gels. Protein was visualized by briefly staining (~2 to 5 min) with 0.05% Coomassie Brilliant Blue R 250 in water, and the AR1 or AL2 band was excised and ground to a fine powder in liquid nitrogen. Rabbits were subcutaneously injected at eight sites along their flanks using ~0.5 mg of protein for initial injections and ~0.25 to 0.5 mg for subsequent boosts.

Transient Expression of SqLCV Proteins in Sf9 Cells

Sf9 cells were grown and maintained, and all transfections were performed at 26°C. Transfection of Sf9 cells by the $CaPO_4$ method was modified from Summers and Smith (1987). Briefly, ~10^6 cells were seeded into 60-mm tissue culture–treated Petri dishes and allowed to grow for 2 days in TMN-FH (Grace's salts plus 0.33% lactalbumin plus 0.33% Yeastolate [all from GIBCO BRL]) plus 10% fetal bovine serum (FBS; GIBCO BRL). Prior to transfection, the cells were incubated for 1 to 2 hr in 3 mL of Grace's salts containing 10% FBS and antibiotics (50 μg/mL gentamycin [Sigma], and 2.5 μg/mL amphotericin B [Sigma]). During this incubation period, 20 μg of the appropriate pGP64 expression plasmid was added to 1 mL of 2 × HEBS (274 mM NaCl, 12 mM dextrose, 10 mM KCl, 1.4 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM Hepes, pH 7.1), followed by the slow dropwise addition of 1 mL of 250 mM $CaCl_2$. A precipitate was allowed to form for 20 to 30 min at room temperature. The $CaPO_4$–DNA precipitate was then added dropwise to the Sf9 cells and incubated for an additional 4 hr. For coexpression studies, the two cotransfecting plasmids were each added at 20 μg to the transfection mixture described above.

Cells were washed once in fresh TMN-FH containing 10% FBS and antibiotics and incubated in this medium for 2 to 7 days. Cells were then removed from the dishes by gentle pipeting in TMN-FH and seeded into 10-mm chamber slides (Nunc, Naperville, IL). Cells were allowed to attach for 20 to 60 min. They were then gently rinsed with PBS and fixed by immersion in 95% ethanol at −20°C for 5 min. Cells were then stained with the appropriate antisera and trimethylrhodamine-conjugated goat anti–rabbit secondary antibody, as previously described (Lazarowitz, 1982). Samples were mounted in PBS containing 50% glycerol and visualized using a Bio-Rad MRC-1000 Krypton/Argon Dual Laser confocal system attached to an Optiphot microscope (Nikon, Melville, NY) at a final magnification of ×1500 for Sf9 cells and ×1000 for Xanthi protoplasts. In colocalization studies, nuclei were stained by incubation in 56 μM chromomycin A (Sigma) for 5 min (Leemann and Ruch, 1982), and the trimethylrhodamine and chromomycin A (fluorescein channel) images were superimposed.

Transient Expression of SqLCV Proteins in *Nicotiana tabacum* cv Xanthi Cells

Protoplasts of fast growing suspensions of Xanthi cells were prepared for electroporation essentially as described previously (Fromm et al., 1986). The protoplasts were resuspended at ~1.2 × 10^6 cells per mL. Twenty micrograms of the appropriate p35S expression vector and 100 μg of carrier salmon sperm DNA were added to 0.8 mL of protoplasts and electroporated at 360 V, 100 Ω, 25 μF in a Bio-Rad GenePulsar. Following incubation at 26°C for from 12 to 120 hr, ~1 × 10^5 cells were allowed to attach to chamber slides (Nunc) for 30 min in conditioned medium. Cells were fixed essentially as described by Liu et al. (1993). Briefly, cells were fixed with 4% paraformaldehyde in PME (50 mM Pipes, pH 6.9, 5 mM $MgSO_4$, 1 mM EGTA) for 1 hr, permeabilized with 0.5% Nonidet P-40 in PME for 30 min, and dehydrated in −20°C methanol for 10 min. Following rehydration in PME for 5 min, cells were prepared for indirect immunofluorescence staining and confocal microscopy as described above for Sf9 cells.

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