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Transgenic Tobacco Plants Expressing the Geminivirus BL1 Protein Exhibit Symptoms of Viral Disease

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Bipartite geminiviruses, such as squash leaf curl virus (SqLCV), encode two movement proteins (MPs), BR1 and BL1, that are essential for viral movement in and subsequent infection of the host plant. To elucidate the biochemical functions of these MPs and define their respective contributions to viral infection, we have generated transgenic *Nicotiana benthamiana* plants expressing SqLCV BR1 and BL1. Transgenic plants expressing BR1 or a truncated BL1 were phenotypically indistinguishable from wild-type *N. benthamiana.* In contrast, transgenic plants expressing full-length BL1, alone or in combination with BR1, were strikingly abnormal both in their growth properties and phenotypic appearance, with leaves that were mosaic and curled under, thus mimicking typical SqLCV disease symptoms in this host. BL1 was localized to the cell wall and plasma membrane fractions, whereas BR1 was predominantly in the microsomal membrane fraction. These findings demonstrate that expression of BL1 in transgenic plants is sufficient to produce viral disease symptoms, and they further suggest that BL1 and BR1 carry out distinct and independent functions in viral movement.

INTRODUCTION

How plant viruses move from the original site of infection to the surrounding cells and systemically invade the plant to cause disease is an important question in plant cell biology as well as in plant virology. Examination of this question has begun to provide insight into the interactions of plant viruses with their hosts and how their movement may alter or modify cellular connections in the host plant.

Plant viruses encode movement proteins (MPs), nonstructural proteins essential for infection that do not affect viral replication or encapsidation (Atabekov and Dorokhov, 1984; Hull, 1991). The best-characterized protein of this type is the 30-kD MP of tobacco mosaic virus (TMV) (Meshi et al., 1987). The 30-kD MP is a sequence-nonspecific nucleic acid binding protein (Citovsky et al., 1990), which has been shown to alter the size exclusion limit of plasmodesmata of nonvascular cells (Deom et al., 1990; Ding et al., 1992). In vitro and in vivo studies have led to a model for the cell-to-cell spread of TMV in which the MP binds to the viral single-stranded RNA (ssRNA) genome, flattening it into an elongated structure and targeting it to the plasmodesmata, where it also acts to increase the size exclusion limit and thereby facilitate passage of the ribonucleoprotein particle through the cell wall (Citovsky et al., 1992). This model has become the paradigm for the investigation of MP function in other plant viruses that have an RNA genome and/or RNA replicative intermediates.

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Geminiviruses are strikingly different in several respects from other plant viruses, including TMV. First, the viral genome consists of covalently closed circular ssDNA (Goodman, 1981). This poses two unique problems for viral movement: (1) a nuclear, rather than a cytoplasmic, compartmentalization of the viral genome necessitates transport of the viral genome and/or virions into and out of the nucleus, and (2) a DNA rather than an RNA binding protein may be required. A second distinctive feature of geminiviruses is their tissue specificity. Geminiviruses are generally phloem limited, a property that is shared with the luteoviruses but not with TMV and other plant viruses being intensively studied (Goodman, 1981; Matthews, 1991). This raises questions concerning the potential function of viral MPs in different cell types and their role in viral tissue and host specificity. Finally, bipartite geminiviruses encode two ~30-kD MPs, named BR1 and BL1 (Lazarowitz, 1992), with no striking homologies to other viral MPs. This suggests that BR1 and BL1 may have functions and/or protein–protein interactions distinct from those of other viral MPs.

Because of these differences, we have begun to characterize the BR1 and BL1 proteins from squash leaf curl virus (SqLCV), a member of the bipartite subgroup of geminiviruses. Two closely related SqLCVs have been cloned and characterized, SqLCV-E (extended host range) and SqLCV-R (restricted host range) (Lazarowitz, 1991; Lazarowitz and Lazdins, 1991). SqLCV-E is highly infectious in a broad range of hosts, which include pumpkin, squash, green bean, and *Nicotiana benthamiana* (hereafter given as tobacco). SqLCV-R is also highly infectious in tobacco but is less infectious in the other hosts.

As typical bipartite geminiviruses, SqLCV-E and SqLCV-R each have two ~2.7-kb genomic DNA components, both of which are essential for systemic infection. The A component encodes the viral coat protein (*AR1* gene) in addition to an essential viral replication protein (*AL1* gene) and regulatory proteins (*AL2* and *AL3* genes). The B component encodes the two viral MPs, BR1 and BL1 (Lazarowitz, 1992). A study of host range determinants in SqLCV has shown that similar to TMV (Meshi et al., 1989), the geminivirus MPs are important determinants of viral host range properties (Lazarowitz, 1991). Analysis of the bipartite tomato golden mosaic virus (TGMV) has also suggested that viral MPs play a role in determining the appearance of disease symptoms (von Arnim and Stanley, 1992).

To investigate the functions of the geminivirus MPs, we constructed transgenic tobacco plants expressing SqLCV-E BR1 and BL1 either singly or together. Tobacco was chosen as a fully permissive host for viral infection (Lazarowitz and Lazdins, 1991). As reported here, our characterization of independent lines for these three different transgenic genotypes demonstrates that plants expressing BR1 are phenotypically indistinguishable from wild-type tobacco, whereas plants expressing BL1 mimic geminivirus disease symptoms in this host. Subcellular fractionation studies show that BR1 localizes primarily to the cell microsomal membrane fraction. BL1 partitions with the plasma membrane and a crude cell wall fraction, whereas BR1 is not present to any great extent in these fractions under the conditions used. These results suggest that BR1 and BL1 may perform independent functions in viral movement.

RESULTS

Genetic and Phenotypic Characterization of Transgenic Lines

To construct transgenic tobacco lines expressing SqLCV-E *BR1* or *BL1*, either singly (hereafter *BR1* plants or *BL1* plants) or together (hereafter *BL1*::*BR1* plants), we utilized an Agrobacterium plant transformation vector into which we could clone one or two separate expression cassettes, as shown in Figure 1 (see also Methods). For plants expressing only *BL1* or only *BR1*, transcriptional fusions of *BR1* or *BL1* under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985) were made by directly cloning each gene with its own translational initiation and termination signals into pMON530 (Rogers et al., 1987) (Figures 1A and 1B), thereby generating pSQBR1E and pSQBL1E (Figure 1C). The insertion of two separate expression cassettes was accomplished by constructing a transcriptional fusion of the *BR1* gene to place it behind the enhanced CaMV 35S promoter in pMON921 (Kay et al., 1987), followed by insertion of this expression cassette into the unique NotI site in pSQBL1E (Figure 1C; see Methods) to generate pSQBL1::BR1E. Each of these three expression vectors was then transformed into explants of tobacco and regenerants were selected based on resistance to kanamycin (kan^r) (Rogers et al., 1986b).

As summarized in Table 1, 13 to 20 separate lines were characterized for each transformed genotype. The genotype of each transformed line was verified by polymerase chain reaction (PCR) amplification of genomic DNA from primary transformants (T_1) and F_1 progeny, using primers specific for pMON530 or pMON921 expression cassette sequences flanking the cloning site, followed by DNA gel blotting and hybridization with gene-specific probes. As shown in Figure 2, an amplified PCR product of the expected size (~1 kb) was detected in each line, and hybridization with *BR1*- or *BL1*-specific probes confirmed the identity of the inserted transgene(s).

Further genetic characterization of these transgenic lines included segregation analysis of the kan^r marker contained on the pMON530 vector and analysis of genomic DNA on DNA gel blots. In most of the lines, kan^r segregated in the F_1 seedlings as a dominant Mendelian trait with a ratio of 3:1 or higher of kanamycin-resistant (kan^r) to kanamycin-sensitive (kan^s) plants, the higher ratio being indicative of insertions at multiple loci (data not shown). In a few lines, the segregation ratio of kan^r:kan^s plants was less than 3:1. This was found to correlate with a reduced germination rate, which we attribute to a detrimental genomic location of the pMON530 insert. Genomic DNA gel blots were used to confirm the independence of the different transgenic lines and to determine the structure at the integrated site(s) (data not shown). Thus, the phenotypes of the different lines, as described below, are due to the particular SqLCV-E gene inserted into the plant genome and not the particular site of insertion.

As shown in Figure 3 and summarized in Table 1, striking phenotypic differences were evident among the different transgenic lines in both the primary T_1 transformants and F_1 progeny. Lines containing only *BR1* were phenotypically normal in every aspect. These plants grew and matured at a normal rate, were fully fertile, and were indistinguishable in appearance from wild-type tobacco (compare Figure 3C to 3B). Two *BR1* lines were slightly mosaic (Table 1). In marked contrast to the *BR1* lines, both *BL1* and *BL1*::*BR1* lines exhibited phenotypes that resembled SqLCV disease symptoms in tobacco (Table 1; Figures 3A, 3D, and 3E). This symptomatic phenotype included a characteristic mosaic pattern and curling under of the leaves (epinasty). In several cases, the plants were also stunted and bushy, with many lateral branches originating from the base of the plant. The plants displaying symptoms also exhibited other striking growth defects. Compared to wild-type tobacco, *BL1* and *BL1*::*BR1* plants were slow to shoot and slow to root (several weeks to months longer than *BR1* or wild-type plants), and many did not establish well-developed roots. Correlating with this slow pattern of growth and development was a decrease in fertility. Although most lines did set normal numbers of flowers, a majority of the seed pods were empty. In extreme cases associated with the most severe phenotypes,

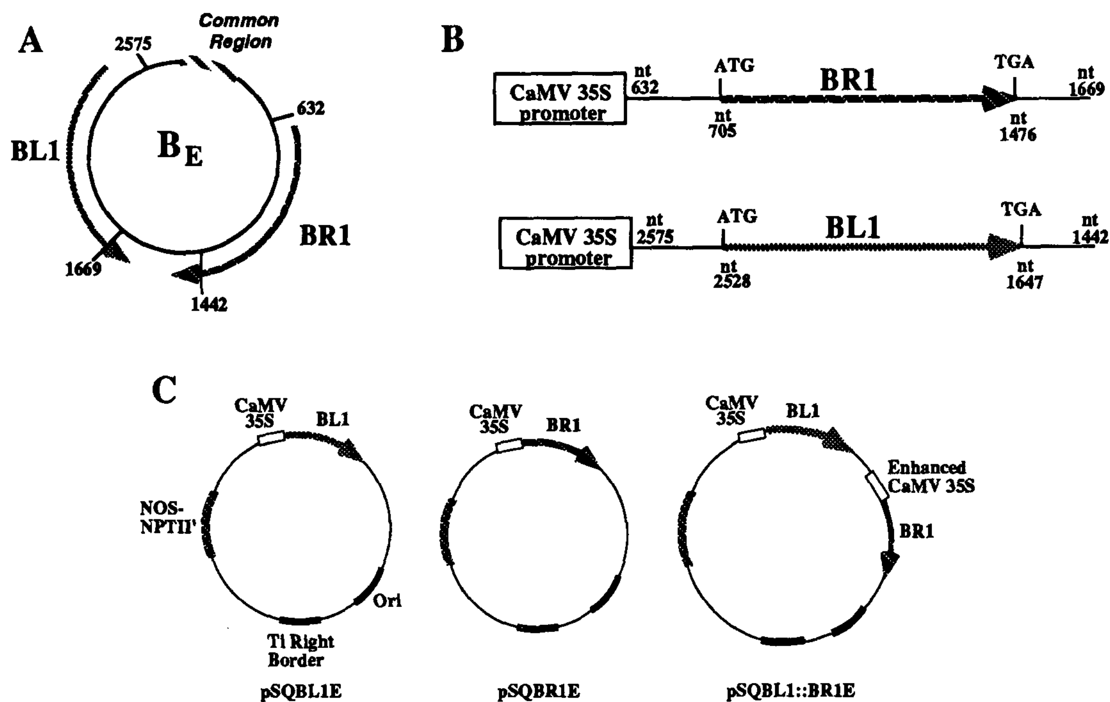

Figure 1. Construction of MP Expression Vectors.

(A) The B component of SqLCV-E. The open reading frames of *BR1* and *BL1* are denoted by arrows. The hatched box represents the common region necessary for replication of the B component in viral infection. Relevant cloning sites are marked (see Methods).

(B) Transcriptional fusions of *BR1* and *BL1* to the CaMV 35S promoter. The positions of translational start and termination codons are noted. The nucleotide numbers at either end show the regions of SqLCV-E B component cloned into the expression vectors. An alternate 5' end at nucleotide 632 was also used for BR1 fusions (see Methods for details).

(C) pMON expression vectors containing the *BR1* and *BL1* transcriptional fusions. pSQBL1E contains the CaMV 35S-*BL1* fusion. pSQBR1E contains the CaMV 35S-*BR1* fusion. pSQBL1::BR1E is derived from pSQBL1E and in addition contains a transcriptional fusion of *BR1* to the enhanced CaMV 35S promoter. NOS, nopaline synthase gene; NPTII, neomycin phosphotransferase II gene; nt, nucleotide.

the T_1 plants were sterile. However, many of the *BL1* and *BL1::BR1* lines were at least ~20 to 25% fertile.

As found in other studies of transgenic plants (Rogers et al., 1986b), varying degrees of expressivity were observed for the visible symptomatic phenotype of the *BL1* and *BL1::BR1* lines (Table 1). Some lines were mosaic with little leaf curl, whereas other lines were epinastic with only slight or no mosaic pattern. Several lines were both epinastic and mosaic, with the severity of the phenotype and low fertility correlating as already noted. In the representative lines tested, the slow

Table 1. Phenotypes of Transgenic Tobacco Plants Expressinng SqLCV-E MPs

Genotype	No. of Lines Generated	Leaf Phenotype[a]			
		Curly[b]	Mosaic[c]	Curly + Mosaic	Normal[d]
BR1	13	0	(2)[e]	0	11
BL1	17	8	2	2	5
BL1::BR1	20	5	4	10	1
Δ*BL1*	16	0	0	0	16

[a] Number of individual T_1 transgenic lines displaying the indicated phenotype.
[b] Uniform green color and curling under.
[c] Chlorotic splotches over the leaf surface.
[d] Indistinguishable in appearance from untransformed wild-type tobacco.
[e] Mild general chlorosis not typical of viral-induced mosaic pattern.

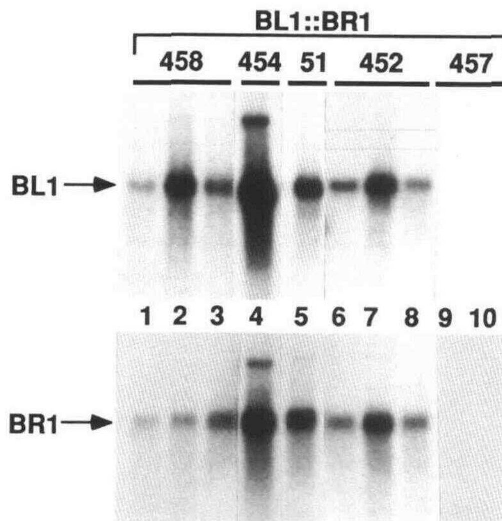

Figure 2. PCR Analysis of Genomic DNA from Transgenic Tobacco Lines.

DNA gel blots of PCR-amplified products from genomic DNA of representative F_1 plants. Genomic DNA samples from independent *BL1::BR1* lines were hybridized with a *BL1*-specific probe (top panel) or a *BR1*-specific probe (bottom panel). The *BL1* and *BR1* products, ~1.2 and 1.0 kb, respectively, are marked with arrows. The numbers above the top panel correspond to the *BL1::BR1* line numbers. Numbers 1 to 10 between the panels indicate the corresponding lanes containing the same DNA sample. The larger band in lane 4 is of unknown origin and does not occur reproducibly.

growth and symptomatic phenotype of these transgenic plants cosegregated with the *kan*^{r} marker as a dominant Mendelian trait (data not shown). Based on DNA gel blots of genomic DNA, the number of inserts did not appear to correlate with the severity of the phenotype (data not shown). In the comparison of the T_1 transgenic lines (Table 1), it appeared that a greater number of *BL1::BR1* plants displayed a mosaic leaf phenotype as compared to the *BL1* lines. Although this may suggest that the presence of *BR1* enhances the *BL1* phenotype, further analyses will be necessary to confirm this observation.

To further demonstrate the role of *BL1* in the production of this SqLCV diseaselike phenotype, we constructed Δ*BL1* transgenic tobacco expressing only the N-terminal 193 amino acids of the BL1 protein (see Methods). Of the 16 *kan*^{r} PCR positive lines, none developed the epinastic or mosaic traits (Table 1) characteristic of the transgenic lines expressing the full-length *BL1*. All of the Δ*BL1* lines grew at rates comparable to wild-type (untransformed) tobacco and were indistinguishable from wild-type tobacco in all aspects (Figure 3F). These results demonstrated that the expression of the full-length BL1 protein is sufficient to produce the SqLCV diseaselike phenotype in transgenic plants.

One line, *BL1::BR1-457*, derived from the *BL1::BR1* transformation was *kan*^{r} but phenotypically normal. PCR analysis showed that neither *BR1* nor *BL1* was present in this line (Figure 2, lanes 9 and 10). Genomic DNA gel blot analysis demonstrated that *BL1::BR1-457* carries a partially deleted pMON530 insert (data not shown). This further supports our conclusion that expression of *BL1* is responsible for the production of SqLCV disease symptoms in these transgenic plants.

Expression of *BL1* and *BR1* Genes in Transgenic Plants

To examine the expression of the *BL1* and *BR1* genes in the transgenic plants, total RNA was isolated from T_1 and F_1 plants and hybridized with *BR1*- and *BL1*- specific probes. As shown in Figure 4, the *BR1* probe hybridized with a 1.1-kb transcript in *BR1* and *BL1::BR1* plants. This transcript was not present in control samples of untransformed (data not shown) or *BL1* plants (Figure 4). Importantly, the phenotypically normal *BR1* lines were found to express appreciable amounts of *BR1* RNA. In some cases, these levels were significantly higher than those found in symptomatic *BL1::BR1* plants (Figure 4). For example, the *BR1-31* line had the highest accumulation of *BR1* RNA, far greater than any *BL1::BR1* plant, yet it showed no aberrant phenotype and was indistinguishable from wild-type tobacco in growth rate and fertility. The lower level of *BR1* RNA in the *BL1::BR1* plants may be due to a positional effect on the downstream CaMV 35S promoter that controls *BR1* expression. Whatever the explanation, the expression of *BR1* does not correlate with any visible phenotype.

In contrast to the pattern of *BR1* expression, the accumulation of *BL1* RNA does appear to correlate with symptom appearance in the transgenic plants. The *BL1* probe specifically hybridized to a 1.3-kb RNA that was present in all symptomatic *BL1* and *BL1::BR1* plants tested (Figure 4, bottom panels). In the *BL1* plants, the severity of the symptomatic and aberrant growth phenotypes paralleled the RNA levels. T_1 plants such as *BL1-8* and *BL1-12*, which had the highest levels of *BL1* RNA, exhibited the most dramatic leaf curl (Figure 4). This pattern was also observed in young F_1 plants from a number of *BL1* lines. *BL1-30* and *BL1-31* exhibited the most severe leaf curl of the lines examined, and, accordingly, they had the highest accumulation of *BL1* RNA (Figure 4). Thus, the presence and overall levels of *BL1* RNA correlated with the appearance of SqLCV-like phenotypes in the transgenic plants. The phenotypically normal Δ*BL1* plants expressed appreciable levels of RNA, comparable to the *BL1* transcript levels found in the symptomatic *BL1* plants (Figure 4; data not shown). This again demonstrates that the expression of full-length *BL1* is necessary for the SqLCV diseaselike phenotype in the transgenic plants.

Expression and Subcellular Localization of BL1 and BR1 Proteins

Subcellular fractionation of plant tissue expressing the TMV 30-kD MP has shown that this protein, although present in the

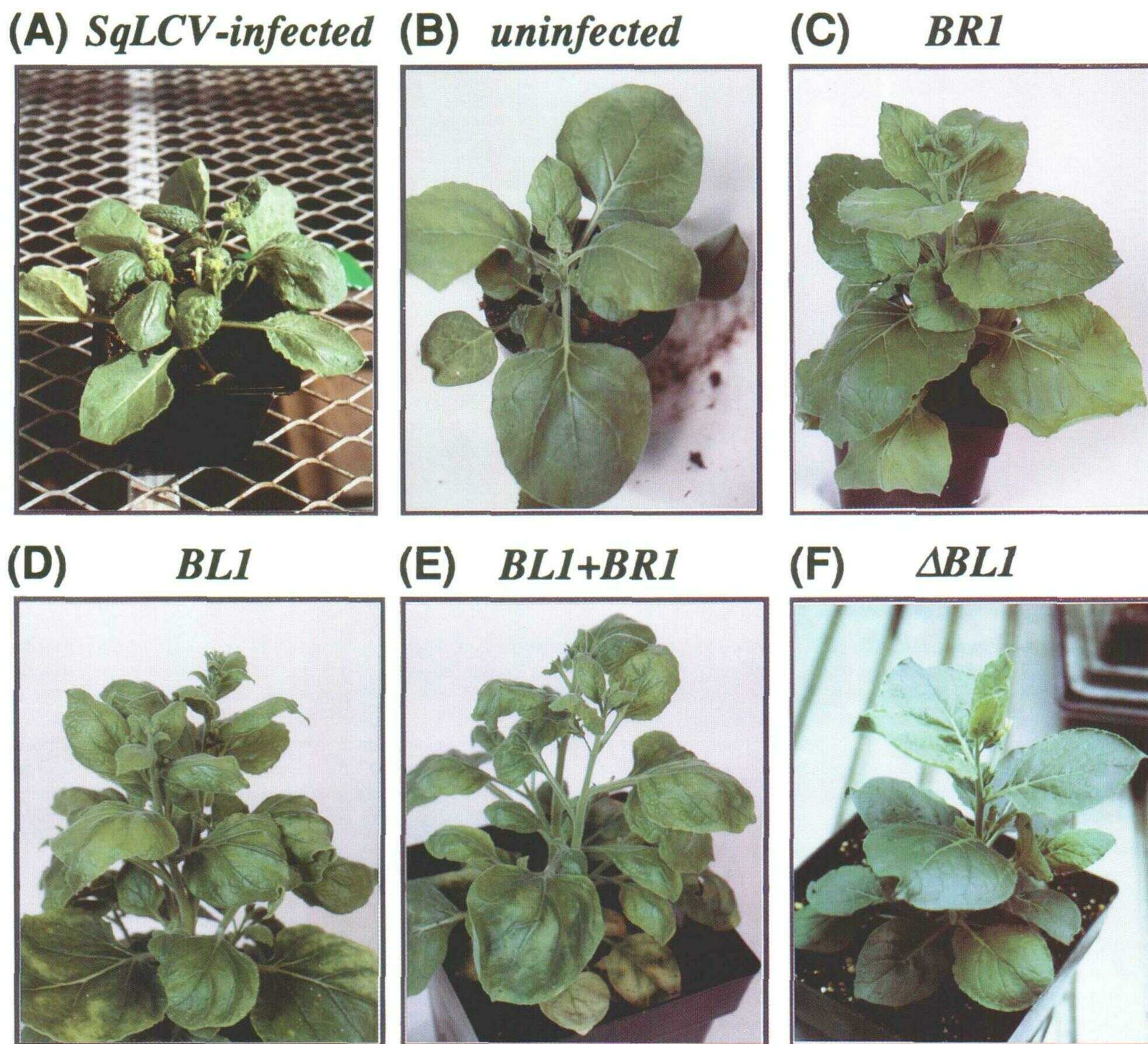

Figure 3. Phenotypic Comparison of Transgenic and Wild-Type Tobacco Plants.

(A) Wild-type tobacco infected with SqLCV-E showing typical disease symptoms of epinasty and mosaic.

(B) Wild-type uninfected tobacco showing typical flat, well-expanded large leaves.

(C) Transgenic *BR1* plant, *BR1-1* (F_1 plant).

(D) Transgenic *BL1* plant, *BL1-31* (F_1 plant).

(E) Transgenic *BL1::BR1* plant, *BL1::BR1-458* (F_1 plant).

(F) Transgenic Δ*BL1* plant (T_1 plant).

membrane and soluble fractions, also accumulates in the cell wall fraction (Deom et al., 1990; Berna et al., 1991). We employed a similar scheme to examine the localization and accumulation of the SqLCV-E BR1 and BL1 proteins in our transgenic lines. Differential centrifugation of plant extracts and treatment with denaturing agents were used to generate three protein-containing fractions: soluble protein (S30), crude cellular membrane (P30), and cell wall–associated proteins (P1). The presence of BR1 and BL1 in each fraction was detected by protein immunoblotting using polyclonal antisera generated against BR1 or BL1 expressed in bacteria (see Methods). Although only one representative transgenic line

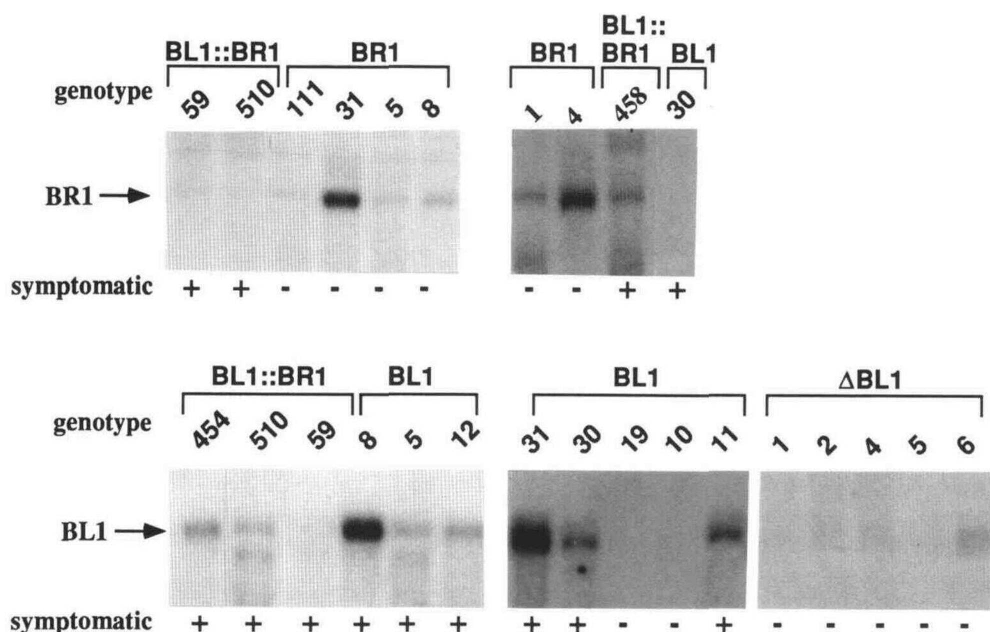

Figure 4. RNA Expression of *BL1* and *BR1* in Transgenic Lines.

Total RNA from transgenic plants was fractionated on formaldehyde-containing agarose gels, transferred to nylon membrane, and hybridized with probes specific for *BR1* (top panels) or *BL1* (bottom panels). Bracketed titles indicate the genotype (e.g., BL1::BR1 denotes transgenic *BL1::BR1* plants, BR1 denotes transgenic *BR1* plants, BL1 denotes transgenic *BL1* plants) and numbers indicate the particular transgenic line (e.g., *BL1::BR1-59*, *BR1-111*, *BL1-8*, and so forth). Although ΔBL1 contains only the N-terminal region of BL1, the transcriptional fusion is approximately the same size as full-length BL1 (see Methods for details). The phenotypes of the lines are noted below each lane (+ denotes leaf curl and/or mosaic pattern; – denotes plants exhibiting no differences in appearance from wild-type untransformed tobacco). Samples taken from T_1 plants are: *BR1-8*, *BL1-5*, *BL1-8*, *BL1-12* and all of *ΔBL1* (1, 2, and 4 to 6). All other samples are from F_1 plants, with the exception of *BL1::BR1-458*, which is from an F_2 plant.

of each type (*BL1*, *BR1*, *BL1::BR1*) is presented here, the same results were obtained for other lines.

As shown in Figure 5, BL1 protein was detected as a series of bands migrating between ~30 and 35 kD in extracts from both *BL1* and *BL1::BR1* transgenic plants. These bands were not present in control extracts (Figure 5A, lane 1), and they were not detected by the preimmune sera (data not shown). The predicted size of the BL1 protein is 33 kD; however, *BL1* expressed in baculovirus-infected Sf9 insect cells produces a similar pattern of bands to that of the extracts from transgenic *BL1* and *BL1::BR1* plants (Figure 5B, lane 1 compared to lanes 2 and 3; also Figure 5A, lanes 2 to 4). The presence of multiple, closely migrating bands near this size could be due to post-translational modification of BL1. Some of these bands may also be due to partial degradation of BL1. Whatever the explanation, it appears that the same alterations occur in the Sf9 insect cells as in the transgenic plants. The series of BL1 bands was found in the membrane (P30) fraction and also in the cell wall (P1) fraction in extracts from both *BL1* and *BL1::BR1* plants (Figure 5A, lanes 2 and 3; Figure 5B, lanes 2 and 3). A lower amount of BL1 was also detected in the soluble S30 fraction. Extracts of *ΔBL1* plants were tested on protein immunoblots with the anti-BL1 antibodies; however, no consistent bands corresponding to the ΔBL1 protein were detected (data not shown). This may be due to the instability of the truncated protein or the lack of reactive epitopes for the anti-BL1 antibodies in the ΔBL1 protein.

Antibodies raised against the BR1 protein recognized a discrete ~30-kD band, the size predicted for this protein. This band was present in extracts of *BR1* (Figure 5A, lanes 6 to 8) and *BL1::BR1* (Figure 5B, lane 6) transgenic plants but not in control extracts (Figure 5A, lane 5). BR1 in the transgenic extracts was also found to precisely comigrate with BR1 expressed in baculovirus-infected insect cells (Figure 5B, lanes 8 and 9). The anti-BR1 antibodies cross-reacted with a few lower and higher molecular weight bands of unknown identity in the transgenic extracts; however, these bands (but not the 30-kD band) were also recognized by the preimmune sera (data not shown) and were often present in control extracts (Figure 5A, lane 5). In tissue from *BR1* plants, BR1 protein was found primarily in the cell membrane P30 fraction, with a lower amount also detected in the soluble S30 and cell wall P1 fractions (Figure 5A, lanes 6 to 8). In *BL1::BR1* plants, BR1 protein was detected only in the cell membrane P30 fraction and not in

the other two protein fractions (Figure 5B, lanes 5 to 7). The levels of BR1 were generally much lower in *BL1::BR1* plants than in the lines that expressed BR1 alone (compare Figure 5B, lane 6 and Figure 5A, lane 7; data not shown). This is consistent with the RNA analysis, which indicated that *BR1* was expressed at a lower level in *BL1::BR1* plants compared to many of the *BR1* plants. The absence of BR1 in the other fractions may thus reflect the lower levels of BR1 protein in these particular plant extracts.

Subcellular Localization of BR1 and BL1 in Infected Plants

We compared the subcellular localization of BL1 and BR1 in infected plants to that found in extracts of transgenic tobacco. Tobacco and pumpkin plants were infected with SqLCV-E and subjected to the same fractionation schemes described above. Similar to the localization in *BL1* transgenic tobacco, BL1 protein was present primarily in the membrane fraction (P30) of infected pumpkin and tobacco tissues, as shown in Figure 6A. Lower amounts of BL1 were detected in the cell wall (P1) fraction, with very little detected in the S30 soluble fraction. In direct comparisons, BL1 was also found to be expressed in the SqLCV-infected tissues at comparable levels to BL1 in the transgenic *BL1* and *BL1::BR1* plants (data not shown).

The localization of BR1 in the SqLCV-infected tissues was also similar to that observed in the *BR1* transgenic tobacco lines. BR1 was found predominantly in the P30 membrane fraction in infected tobacco tissue (Figure 6B), similar to the fractionation of BR1 in *BR1* and *BL1::BR1* plants. In infected pumpkin tissue, BR1 fractionated more heterogeneously, accumulating in all three fractions (Figure 6B). In general, SqLCV-infected pumpkin had higher levels of BR1 than SqLCV-infected tobacco and appeared to accumulate more BR1 in the soluble and cell wall fractions. However, the BR1 levels in infected pumpkin were similar to those found in *BR1* transgenic plants (data not shown). The higher levels of BR1 protein or the presence of other viral proteins in the infected plants may contribute to its more heterogeneous distribution. Recently, von Arnim et al. (1993) reported the localization of the African cassava mosaic virus MPs in infected plants. Due to differences in fractionation procedures, their results cannot be directly compared to those reported here.

BR1 and BL1 Localize to Different Membrane Fractions

The localization of both BR1 and BL1 to a crude membrane fraction in both transgenic and infected tissues could suggest that BR1 and BL1 function together and perhaps even influence each other's subcellular distribution. To further localize the two MPs, we employed an aqueous two-phase partitioning method (Larsson, 1985; Bush, 1989) to separate plasma membrane from other cellular membranes. SqLCV-infected pumpkin tissue was fractionated and assayed by protein immunoblots for the presence of BL1 and BR1 in each separation phase. As shown in Figure 7, BR1 was detected only with the other cellular membranes (Figure 7, OM) and was not present in the plasma membrane fraction (Figure 7, PM). In contrast, BL1

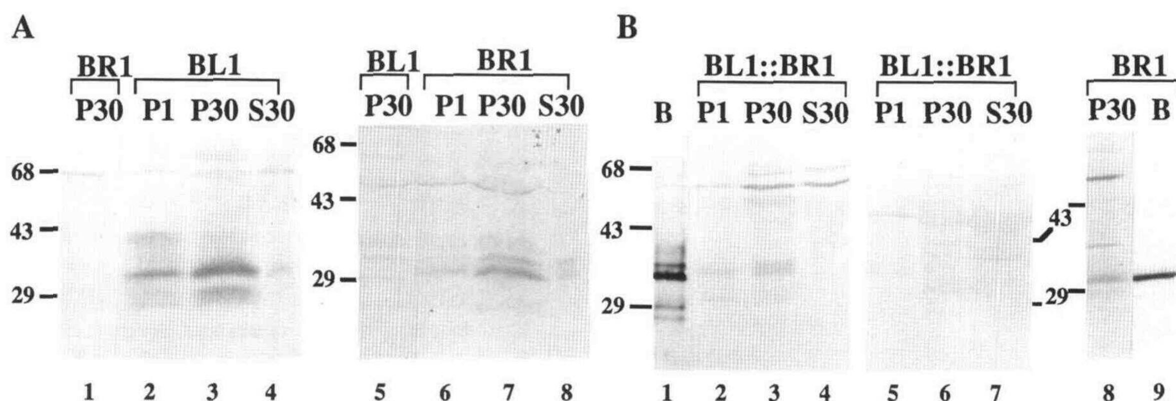

Figure 5. Protein Immunoblots of Subcellular Fractions from Transgenic Lines.

Tissue extracts from transgenic lines were fractionated into S30 soluble protein, P30 cellular membranes, and P1 cell wall fractions. Twenty microliters of each fraction (~50 μg of S30, 20 μg of P30, and 10 μg of P1) were separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose. Numbers to the side of each panel indicate protein molecular weight markers given in kilodaltons.

(A) Immunoblot of fractionated extracts from F_1 plants of *BL1-31* (lanes 2 to 5) and *BR1-4* (lanes 1 and 6 to 8) probed with anti-BL1 antisera (lanes 1 to 4) or anti-BR1 antisera (lanes 5 to 8).

(B) Immunoblot of fractionated extracts probed with anti-BL1 antisera (lanes 1 to 4) or anti-BR1 antisera (lanes 5 to 9). Lane 1 contains whole-cell extract of BL1 expressed in baculovirus-infected Sf9 cells. Lanes 2 to 7 contain fractionated extracts from an F_2 plant of *BL1::BR1-452*. Lane 8 contains the P30 fraction from a *BR1-4* fractionated extract. Lane 9 contains whole-cell extract of BR1 expressed in baculovirus-infected Sf9 cells.

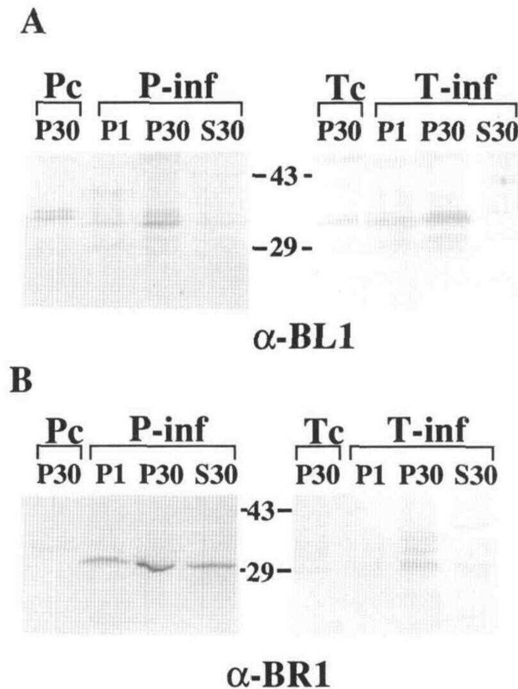

Figure 6. Protein Immunoblots of Fractionated Extracts from SqLCV-Infected Plants.

Fractionation and immunoblotting were performed as detailed in the Figure 5 legend.

(A) Immunoblots probed with anti-BL1 antisera. Pc is uninfected pumpkin. P-inf is pumpkin infected with SqLCV (see Methods for details). Tc is uninfected tobacco. T-inf is SqLCV-infected tobacco. Numbers between the panels indicate protein molecular weight markers given in kilodaltons.

(B) Immunoblots probed with anti-BR1 antisera. Abbreviations are the same as given in **(A)**.

fractionated to the plasma membrane fraction (Figure 7, PM). This distinct distribution of BR1 and BL1 suggests that their localization is independent of one another.

To confirm the independent localization of BL1, we assayed membrane localization in *BL1* transgenic tobacco. Similar to the results with infected tissue, BL1 localized primarily to the plasma membrane fraction, and only trace amounts of BL1 remained with the other cellular membranes (Figure 7). The identity of the fractions was confirmed by ATPase and chlorophyll assays. The plasma membrane fractions were enriched 5- to 10-fold in the specific activity of the sodium vanadate–sensitive ATPase, as compared with the total membrane fraction (data not shown). In addition, the levels of chlorophyll were low to undetectable in the plasma membrane fraction and 20- to 40-fold higher in the other cellular membrane fraction (data not shown). These results indicate that BL1 localization is independent of BR1 and that BL1 localizes predominantly to the plasma membrane in both infected and transgenic tissues.

DISCUSSION

Through genetic criteria, *BL1* and *BR1* in the bipartite geminiviruses have been assigned the roles of MPs. Mutations that disrupt either coding region prevent systemic infection without affecting viral replication and encapsidation (Brough et al., 1988; Etessami et al., 1988). Thus, these two proteins are likely to participate in the cell-to-cell and systemic transport of the virus in the plant. As an approach to elucidating the

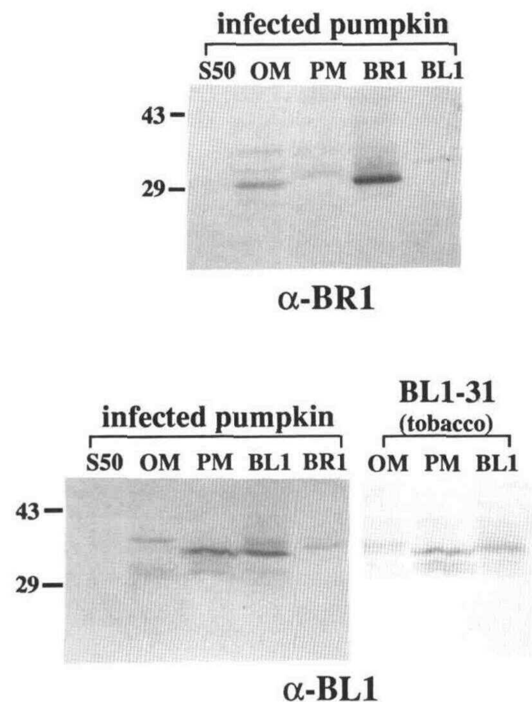

Figure 7. Aqueous Two-Phase Partitioning of Membrane Fractions from Transgenic and Infected Plants.

Aqueous two-phase partitioning was performed on membrane fractions from either SqLCV-infected pumpkin or transgenic tobacco line *BL1-31*. Immunoblotting was performed as detailed in the Figure 5 legend. The top panel was probed with anti-BR1 antisera, and the bottom panels were probed with antisera against BL1. S50 is the soluble fraction from the total cell extract. OM (other membrane) indicates that fraction of cellular membranes that are partitioned away from the plasma membrane. PM is the plasma membrane–containing fraction. Equal amounts of protein were loaded in the S50, OM, and PM lanes. Lanes marked BL1 and BR1 are total membrane fractions; however, they do not represent equivalent amounts of total protein and are merely included as reference markers for the specific BL1 and BR1 bands. Numbers to the side of each panel indicate protein molecular weight markers given in kilodaltons.

biochemical functions of the bipartite geminivirus MPs, as well as defining their respective contributions to viral infection, we generated transgenic tobacco plants expressing the SqLCV *BR1*- and *BL1*-encoded proteins. Our comparative analysis of plants expressing each SqLCV MP singly or together demonstrated that the expression of BL1 is sufficient to produce diseaselike symptoms, whereas the expression of BR1 has no apparent effects on the appearance or growth of tobacco.

Previous molecular genetic studies have shown that both viral movement and replication contribute to viral host range and pathogenicity in the bipartite geminiviruses (Lazarowitz, 1991; Lazarowitz et al., 1992). Analyses of geminivirus MPs in the context of viral infection have further suggested that *BL1* influences the symptom phenotype. The construction of chimeric B components from two different strains of TGMV demonstrated that the genomic segment encompassing the *BL1* coding region and the untranslated upstream sequences from within the start of the common region to the start of *BL1* is responsible for the differences in symptoms of these two TGMV strains (von Arnim and Stanley, 1992). Although these findings suggested that BL1 is a determinant of symptom appearance, contributions of the common region sequences to replication could not be excluded. Intriguingly, in previous studies of TGMV, integration of the B component in transgenic petunia or tobacco plants (Rogers et al., 1986a; Elmer et al., 1988) was not sufficient to produce a symptomatic phenotype. However, in these studies, *BR1* and *BL1* expression was dependent on the endogenous viral promoters and thus was likely to be very weak without the benefit of amplification normally provided by replication and transcriptional transactivation (Sunter and Bisaro, 1992) in the presence of the A component. In addition, petunia is not a host for systemic TGMV infection, and this could also explain the lack of symptom production.

Our ability to express each of the SqLCV MPs under the control of the constitutive CaMV 35S promoter, independent of viral infection and in a host normally permissive for SqLCV infection, has clearly shown that *BL1* both influences symptom production and, in fact, is sufficient for many of the phenotypic characteristics of SqLCV infection. Truncation of *BL1* in the Δ*BL1* transgenic plants does not produce a diseaselike phenotype and neither does the expression of the second SqLCV-E MP *BR1*. These results suggest that expression of full-length *BL1* leads to the epinasty and mosaic phenotypes of the plants. The levels of BL1 protein are comparable in extracts of both transgenic and SqLCV-infected plants, and given the levels detected, the phenotype of the *BL1* plants does not appear to be the result of a gross overexpression of BL1. In addition, the similar patterns of BL1 subcellular localization in extracts of transgenic and infected tissue suggest that BL1 interacts with the host plant in a similar manner in both cases to produce the leaf curl and mosaic phenotypes. It has been assumed that disease symptoms during geminivirus infection are the result of virus-induced necrosis in the phloem companion cells inhibiting general transport throughout the phloem (Goodman, 1981). Our results demonstrate that the expression of BL1, and not necrosis due to viral replication, is responsible for the disease symptoms. This suggests that BL1 may interfere with cell-to-cell movement in the vascular system. Future ultrastructural and metabolic studies of these transgenic plants will address this issue.

To our knowledge, SqLCV *BL1* is the one clear example of a viral MP producing the appropriate symptoms when expressed in transgenic plants generated from a permissive host. There have been previous reports of other plant virus proteins expressed in transgenic plants, most notably gene VI of CaMV, which is a translational transactivator of viral genes (Baughman et al., 1988; Goldberg et al., 1989; Takahashi et al., 1989; Zijlstra and Hohn, 1992), and the coat protein and MP of TMV (Abel et al., 1986; Deom et al., 1987). In the experiments with the TMV proteins, neither the coat protein nor the 30-kD MP produced a visible phenotype in transgenic tobacco (Abel et al., 1986; Deom et al., 1987). However, it has been reported that mutant TMV coat protein expressed in transgenic plants elicits a hypersensitive response (Culver and Dawson, 1991). The expression of CaMV gene VI in transgenic tobacco generated from nonhost species for this virus produces chlorotic and mosaic leaves and stunted growth (Baughman et al., 1988; Goldberg et al., 1989; Takahashi et al., 1989). These traits are similar to CaMV infection symptoms; however, these symptoms are not produced upon transformation of susceptible host species. In fact, gene VI expressed in hosts permissive for CaMV infection produced no abnormal phenotypes (Goldberg et al., 1989). Hence, each of these CaMV and TMV genes, although they may be in some capacity associated with symptom development, is not sufficient to cause the disease phenotype in susceptible host plants.

What are the functions of the bipartite geminivirus MPs? The requirement for two MPs in these viruses suggests that there may be partitioning of movement functions between *BL1* and *BR1*. One model for partitioning is that one MP participates in direct cell-to-cell movement in vascular cells, whereas the other is involved in long-distance (systemic) movement through the sieve elements. Our subcellular fractionation studies presented here and previous genetic studies are consistent with this model. Although both MPs are found in the microsomal membrane fraction, BL1 localizes predominantly to the plasma membrane, whereas BR1 fractionates with the other cellular membranes. In addition, BL1 localizes to the cell wall fraction. These distinct subcellular localizations of BL1 and BR1 suggest that they function in different cellular compartments, perhaps in different steps in viral movement. The different phenotypes of the *BL1* and *BR1* transgenic plants further demonstrate that BR1 and BL1 have distinct interactions in the plants. Previous studies of SqLCV have suggested that *BR1* may be dispensable for cell-to-cell movement. A mutant SqLCV B component (BR*) is able to amplify the SqLCV A component in agroinoculated tobacco leaf discs (Lazarowitz, 1991). This is presumably accomplished by direct cell-to-cell movement within the leaf disc, increasing the number of cells in which the SqLCV A component replicates. However, BR*,

which contains a missense mutation in *BR1*, is not able to infect tobacco systemically (Lazarowitz, 1991; Ingham and Lazarowitz, 1993). Based on these studies and the subcellular localization studies presented here, we suggest that BR1 participates in long-distance (systemic) movement and not in cell-to-cell movement of the virus, and that BL1 may be the MP responsible for cell-to-cell spread. These predications are testable and currently being investigated.

Several studies have suggested that long-distance and cell-to-cell movement may constitute two separable functions (reviewed in Hull, 1991). One such example is that of TMV. The TMV 30-kD protein is thought to participate in the cell-to-cell spread of the virus (Wolf et al., 1989; Citovsky et al., 1990; Deom et al., 1990). It has been shown that the TMV MP increases the size exclusion limits of plasmodesmata between mesophyll and bundle sheath cells but not between bundle sheath and phloem parenchyma cells (Ding et al., 1992). Because TMV must reach the phloem for systemic movement, Ding et al. (1992) have suggested that the TMV coat protein may participate in the movement of the virus from the bundle sheath cells into the vascular tissue by cooperating with the 30-kD MP. Studies of TMV coat protein mutants have also implicated the coat protein as having a role in systemic movement (Dawson et al., 1988; Saito et al., 1990). Thus, long-distance movement of TMV may require other viral proteins in addition to the MP. In contrast to the requirements of most plant viruses, the coat protein is not essential for systemic infection by the bipartite geminiviruses. Studies of TGMV and African cassava mosaic virus found that mutants lacking the coat protein gene could systemically infect *N. benthamiana* and cause disease, although the symptoms were delayed and attenuated (Gardiner et al., 1988; Klinkenberg et al., 1989). Thus, as suggested in the model discussed above, BR1 may function in long-distance viral systemic movement, partially able to carry out the process in the absence of coat protein and facilitating this when encapsidated virions are present. It is also possible that the BR1 MP is the sole determinant for long-distance movement and that differences observed in the presence or absence of the coat protein simply reflect the protection of the viral ssDNA genome from degradation when associated with coat protein.

We had anticipated that, as shown with TMV (Deom et al., 1987; Holt and Beachy, 1991), plants expressing the SqLCV MPs would complement viruses mutated in either of these genes. Unexpectedly, preliminary studies failed to detect complementation for any of our lines tested to date (E. Pascal and S. G. Lazarowitz, unpublished results). This does not seem to be related to the functional integrity of *BL1* and *BR1* in the transgenic plants, as we have obtained similar negative results with transgenic plants expressing other SqLCV proteins such as the AL2 transactivator or the coat protein (A. Sanderfoot and S. G. Lazarowitz, unpublished results). Thus, it is possible that the timing of expression for the viral proteins is critical and that the constitutive expression in the transgenic plants cannot correctly integrate with the viral program. It is also possible that given the potential for broad expression of the transgenes under the control of the CaMV promoter (Odell et al., 1985), compared to the phloem limitation of the virus (Goodman, 1981; Matthews, 1991), sufficient levels of viral proteins may not be expressed in the correct cell types for complementation. More precise localization of BR1 and BL1 in the transgenic lines and the use of tissue-specific promoters will address these points and have important implications for effective strategies to engineer plants resistant to infections by geminiviruses and perhaps other phloem-limited viruses.

Although many questions remain to be resolved, the expression of *BR1* and *BL1* MPs in transgenic plants independent of viral replication and infection has provided new insights into the roles of these MPs. We have clearly demonstrated that *BL1* is responsible for the production of phenotypic changes characteristic of viral disease symptoms and the specific association of the BL1 protein with the cell membrane and cell wall fractions. In addition, the distinct localization of BL1 and BR1 to separate membrane fractions along with the normal appearance of BR1-expressing transgenic tobacco indicate that the BL1 and BR1 MPs may have distinct and separable functions. Our transgenic plants will serve as useful tools to now address the multitude of possibilities for MP function. The ability to perturb the host in a manner similar to that of viral infection by simply expressing the *BL1* MP provides a new approach to address viral–host interactions and to further define the effects of viral infection on normal cellular processes. In particular, these transgenic plants open the way to identifying host proteins involved in the regulation of transport in the plant.

METHODS

Construction of Movement Protein Expression Vectors

BL1 was cloned as an AvaI-NdeI fragment between nucleotides 2575 and 1442 of the extended host range squash leaf curl virus (SqLCV-E) B component (Lazarowitz and Lazdins, 1991). After blunting with the Klenow fragment of *Escherichia coli* DNA polymerase I and ligation to EcoRI linkers (Bethesda Research Laboratories), this fragment was cloned into the unique EcoRI site of pMON530 (Rogers et al., 1987) to create pSQBL1E. For the creation of Δ*BL1*, the EcoRI fragment from pSQBL1E was cloned into the EcoRI site of pGEM7Z– (Stratagene) to create pGBL1. This plasmid was then cut with XbaI, blunt ended with the Klenow fragment, and religated to create a stop codon after amino acid 193 in BL1. This BL1 truncation was then cloned back into the SmaI site of pMON530 as a PvuII fragment to create pSQΔBL1. *BR1* was cloned in two ways, as an EcoRI-AvaI fragment (nucleotides 632 to 1669 of SqLCV-E B component) and as an MboII-AvaI fragment (nucleotides 579 to 1669). This was based on sequence data that identified two in-frame methionines 13 amino acids apart. Subsequent S1 mapping has demonstrated that the second methionine is the true start codon (S.G. Lazarowitz, unpublished data). Both fragments were blunt ended with the Klenow fragment and then ligated to BglII linkers (Bethesda Research Laboratories) and cloned into the unique BglII site in pMON530 to create pSQBR1E. In a similar manner, *BR1* was cloned into the unique BglII site of pMON921, fusing it with the enhanced cauliflower mosaic virus (CaMV) 35S promoter (Kay et al., 1987). This expression cassette was transferred as a NotI fragment into the unique NotI site in pSQBL1E to create pSQBL1::BR1E.

Expression cassettes constructed using either *BR1* fragment gave identical results in transformed *Nicotiana benthamiana* (hereafter referred to as tobacco).

Plant Transformation

The expression vectors were transferred to *Agrobacterium tumefaciens* carrying the disarmed plasmid TiT37SE by the triparental mating procedure (Rogers et al., 1986b). These Agrobacterium strains were used to transform leaf explants of tobacco by the methods of Rogers and coworkers (1986) with the following modifications: (1) explants from young sterile seedlings were wounded with blunt forceps and the nurse layer was omitted, and (2) calli and shoots were selected on kanamycin-containing media continuously throughout the regeneration procedure, with transfer to fresh plates every 3 to 4 weeks. *BL1* and Δ*BL1* plants were derived by transformation with Agrobacterium carrying pSQBL1E and pSQΔBL1, respectively. *BR1* lines were generated from tobacco transformed with pSQBR1E. Lines *BR1-9*, *BR1-14*, *BR1-18*, *BR1-34*, and *BR1-33* contained *BR1* starting at nucleotide 579. All other *BR1* lines contained *BR1* starting at nucleotide 632. *BL1*::*BR1* lines were generated from tobacco transformed with pSQBL1::BR1E. Lines *BL1*::*BR1-10*, *BL1*::*BR1-20*, and *BL1*::*BR1-51* through *BL1*::*BR1-59* contained *BR1* starting at nucleotide 579. All other *BL1*::*BR1* lines contained *BR1* starting at nucleotide 632.

Polymerase Chain Reaction Analysis

Leaves (~1 to 2 cm) were collected from primary transformants and F_1 seedlings. DNA was extracted with cetyltrimethylammonium bromide and 2 μL (of 50 μL total) was used as a substrate for polymerase chain reaction (PCR) analysis with specific primers (McGarvey and Kaper, 1991). The primers used are as follows: pMON530 cassette, 5′-CTGAAATCACCAGTCTCTCTC-3′ (upstream) and 5′-TGCCAAATGTTTGAACGATC-3′ (downstream); pMON921 NotI cassette, 5′-TGGAGAGGACACGCTGA-3′ (upstream) and 5′-GTCGAAACCGATGATACG-3′ (downstream). DNA from each sample was fractionated on 1.4% agarose gels, transferred to nylon membranes (Hoeffer), and hybridized with *BR1*- or *BL1*-specific probes (Southern, 1975; Reed and Mann, 1985). The DNA probes were labeled with α-^{32}P-dCTP by the random hexamer priming method (Feinberg and Vogelstein, 1984).

RNA Analysis

Approximately 100 mg of leaf tissue was ground in liquid nitrogen in an Eppendorf tube with a Kontes pestle. RNA extraction was performed using the method of Verwoerd et al. (1989). Concentrations were approximated by spectrophotometer readings at λ = 260 nm. Twenty micrograms of RNA was fractionated on 1.5% agarose gels containing formaldehyde and transferred to nylon membranes (Ausubel et al., 1989). Sizes were standardized to RNA markers (GIBCO BRL). Transfer and equal loading of samples were confirmed by methylene blue staining of the nylon following blotting. RNA blots were preincubated for 1 hr at 42°C in 5 × SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) containing 1% SDS, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidine, and 0.02% BSA), 100 mg/mL of denatured salmon sperm DNA, and 50% formamide. The blots were then hybridized at 42°C in the same buffer with the addition of dextran sulfate to a final concentration of 10% and the appropriate ^{32}P-labeled DNA probe (Feinberg and Vogelstein, 1984).

Subcellular Fractionation

Two grams of leaf tissue was ground to a fine powder under liquid nitrogen. Protein extraction and fractionation followed the procedure of Deom et al. (1990), with the following modifications: (1) extensive grinding in liquid nitrogen was substituted for the first step of the mechanical grinding, and (2) the tissues were not further ground after the addition of Triton X-100. Membrane and cell wall pellets were resuspended in 700 μL and 3 mL, respectively, of sample buffer (60 mM Tris-HCl, pH 8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue). The volume of the soluble S30 fraction was 10 mL. Twenty microliters of each fraction was loaded for gel analysis of each sample, except where otherwise noted. Protein amounts were approximated by staining of SDS–polyacrylamide gels with Coomassie brilliant blue R 250. Infected *Curcurbita maxima* (var Big Max; hereafter referred to as pumpkin) tissue was harvested at 15 days postinfection. Infected tobacco tissue was harvested at 22 days postinfection. Pumpkin and tobacco seedlings were infected with cloned genomic components of SqLCV-E by agroinoculation, as described previously (Lazarowitz and Lazdins, 1991).

Total cell extracts of baculovirus-expressed BL1 and BR1 in infected Sf9 cells were used as references for the size of each viral movement protein (MP). For this purpose *BL1* and *BR1* were cloned as transcriptional fusions (nucleotides 2575 to 1442 and nucleotides 679 to 1669, respectively) into the vector pVL1393 (Summers and Smith, 1987). All manipulations followed the procedures of Summers and Smith (1987).

Two-Phase Separation of Membrane Fractions

For partitioning membrane fractions, membranes were prepared according to the method of Bush (1989) from fresh, unfrozen SqLCV-infected pumpkin (40 g of tissue at 7 days postinfection) and transgenic BL1 tobacco (20 g of fully expanded leaf tissue from *BL1-31*). Plasma membranes were separated using the aqueous two-phase partition method (Larsson, 1985) under the conditions described by Bush (1989). ATPase and chlorophyll assays followed the methods of Bush (1989). Protein was quantitated using the Bio-Rad protein assay. Equal amounts of protein from each fraction were used for SDS-PAGE analysis.

Protein Gels and Immunoblots

Proteins were separated by SDS-PAGE on 12% discontinuous buffer gels (Laemmli, 1970) and transferred to nitrocellulose in 20 mM sodium phosphate, pH 6.8. BL1 and BR1 were detected by rabbit polyclonal antisera raised against bacterial-expressed BL1 or BR1, respectively (see below), followed by incubation with alkaline phosphatase–conjugated anti–rabbit antibodies (Promega). Following each antibody incubation, washes were performed with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate *p*-toludine salt were used for color development (Harlow and Lane, 1988).

Antibodies directed against BL1 and BR1 were raised against translational fusions of BL1 (amino acids 11 to 279) and BR1 (amino acids 12 to 233) to the N-terminal 12 amino acids of T7 gene 10 in the pET-3

vectors (pET-3a and pET-3b, respectively) (Studier et al., 1990) expressed in *E. coli* BL21 (DE3) pLysS. Growth and induction followed the methods of Studier et al. (1990). Cell pellets were washed and sonicated in HEMG (25 mM Hepes-KOH, pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol). Following centrifugation at 10,000*g*, the pellet was washed successively in HEMG with 0.05% Triton X-100, HEMG with 2 M urea, and HEMG with 0.1% SDS. The final pellet was resuspended in sample buffer and loaded onto 10 to 15% acrylamide gradient gels for SDS-PAGE. Protein was visualized by Coomassie brilliant blue R 250 staining in water. The BL1 or BR1 protein band was excised and ground to a fine powder in liquid nitrogen. Rabbits were injected with 0.5 to 1.0 mg of protein for the initial injection and subsequent boosts. The antibody pellet from a 50% ammonium sulfate precipitation was used at 1:2000 dilution for all protein immunoblots.

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