The N-terminus of the *Begomovirus* Nuclear Shuttle Protein (BV1) Determines Virulence or Avirulence in *Phaseolus vulgaris*

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The *BV1* gene of the bipartite *Begomovirus* genome encodes a nuclear shuttle protein (NSP) that is also an avirulence determinant in common bean. The function of the NSP of two common bean-infecting bipartite begomoviruses, *Bean dwarf mosaic virus* (BDMV) and *Bean golden yellow mosaic virus* (BGYMV), was investigated using a series of hybrid DNA-B components expressing chimeric BDMV and BGYMV NSP, and genotypes of the two major common bean gene pools: Andean (cv. Topcrop) and Middle American (cvs. Alpine and UI 114). BDMV DNA-A coinoculated with HBDBG4 (BDMV DNA-B expressing the BGYMV NSP) and HBDBG9 (BDMV DNA-B expressing a chimeric NSP with the N-terminal 1 to 42 amino acids from BGYMV) overcame the BDMV resistance of UI 114. This established that the BDMV NSP is an avirulence determinant in UI 114, and mapped the domain involved in this response to the N-terminus, which is a variable surface-exposed region. BDMV DNA-A coinoculated with HBDBG10, expressing a chimeric NSP with amino acids 43 to 92 from BGYMV, was not infectious, revealing an essential virus-specific domain. In the BGYMV background, the BDMV NSP was a virulence factor in the Andean cv. Topcrop, whereas it was an avirulence factor in the Middle American cultivars, particularly in the absence of the BGYMV NSP. The capsid protein (CP) also played a gene pool-specific role in viral infectivity; it was dispensable for infectivity in the Andean cv. Topcrop, but was required for infectivity of BDMV, BGYMV, and certain hybrid viruses in the Middle American cultivars. Redundancy of the CP and NSP, which are nuclear proteins involved directly or indirectly in viral movement, provides a masking effect that may allow the virus to avoid host defense responses.

Additional keywords: disease resistance, geminivirus.

The family *Geminiviridae* includes plant-infecting viruses that possess a small, circular, single-stranded DNA genome encapsidated in twinned icosahedral particles (Hanley-Bowdoin et al. 1999; Rojas et al. 2005; Timmermans et al. 1994). Mem-

bers of the genus *Begomovirus* are transmitted by whiteflies and have either a monopartite (approximately 3.0 kb) or bipartite (approximately 5.0 kb) genome. The bipartite *Begomovirus* genome is composed of two approximately 2.6-kb DNA components, referred to as DNA-A and DNA-B. The DNA-A and DNA-B components of a *Begomovirus* sp. share an approximately 200-nucleotide sequence referred to as the common region (CR), which contains the viral origin of replication and regulatory sequences (Hanley-Bowdoin et al. 1999, 2004; Stanley 1985). The DNA-A component has four or five genes (*AV1*, *AC1*, *AC2*, *AC3*, or [*AC4*]), encoding proteins involved in viral replication, transcription, encapsidation, and suppression of host defenses; whereas DNA-B has two genes, *BV1* and *BC1*, which encode a nuclear shuttle factor (NSP) and a cell-to-cell movement protein (MP), respectively. The NSP and MP are required for viral cell-to-cell and long-distance movement, and play important roles in symptom development and host range. In the current model for bipartite *Begomovirus* cell-to-cell movement, BV1 coordinates the movement of viral DNA from the nucleus to the cytoplasm through the nuclear pore complex (NPC), and BC1 mediates cell-to-cell movement across the cell wall via plasmodesmata (PD) (Gafni and Epel 2002; Lazarowitz and Beachy 1999; Noueiry et al. 1994; Rojas et al. 2005; Sanderfoot and Lazarowitz 1995). What is not well understood is the form or forms in which the virus moves cell-to-cell and long distance, and the nature of NSP and MP interactions with each other and host factors in facilitating movement.

Bean dwarf mosaic virus (BDMV) from Colombia and *Bean golden yellow mosaic virus* from southern Mexico (BGYMV-MX) are bipartite begomoviruses that infect common bean (*Phaseolus vulgaris* L.). Symptoms induced by BDMV include stunting and dwarfing of infected plants and epinasty and mottling of leaves (Morales et al. 1990), whereas those induced by BGYMV include stunted and distorted plant growth and a striking green-yellow mosaic in leaves (Garrido-Ramirez et al. 2000a). In addition to the different symptoms induced by these viruses, they also show differential pathogenicity in common bean cultivars (Morales and Niessen 1988; Morales et al. 1990; Seo et al. 2004).

Cultivated varieties of common bean can be divided into two major gene pools: Andean (large-seeded cultivars such as snap and dark red kidney beans) and Middle American (small- to medium-seeded cultivars such as black, pinto, and Great Northern beans). These two gene pools reflect the two major domestication origins of this New World crop (Gepts and Bliss

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1985; Singh et al. 1991). The majority of *Begomovirus* disease resistance in common bean has been identified in Middle American genotypes (Morales 2001; Morales and Niessen 1988; Morales and Singh 1991; Morales et al. 1990; Seo et al. 2004; Singh et al. 2000). Thus, Andean cultivars (e.g., cv. Topcrop) are susceptible to both BDMV and BGYMV; whereas Middle American cultivars (e.g., cvs. Othello, Black Turtle Soup [BTS], and Pinto UI 114) are resistant to BDMV and susceptible or moderately resistant to BGYMV (Garrido-Ramirez et al. 2000b; Hidayat et al. 1993; Morales and Niessen 1988; Morales et al. 1990; Seo et al. 2004; Wang et al. 1999).

A BDMV green fluorescent protein (GFP) reporter, with the *GFP* gene inserted in place of the capsid protein (CP) gene, has been used to investigate viral cell-to-cell and long-distance movement in common bean. This reporter revealed that CP is not required for viral infection in cv. Topcrop; and that BDMV resistance in cv. Othello occurs via a block in long-distance movement, which often (but not always) is associated with a hypersensitive response (HR) in the vasculature (Sudarshana et al. 1998; Wang et al. 1999). In contrast, BDMV resistance in cv. BTS also involves a block in long-distance movement, but is not associated with an HR (Garrido-Ramirez et al. 2000b; Seo et al. 2004; Wang et al. 1999). In another study with the BDMV-GFP reporter, BDMV/*Tomato mottle virus* (ToMoV) pseudorecombinants and a series of BDMV/BGYMV hybrid viruses were used to map a viral determinant of avirulence and the HR in cv. Othello to the BDMV *BV1* gene (Garrido-Ramirez et al. 2000b; Wang et al. 1999).

In the present study, we used a new series of hybrid DNA-B components expressing chimeric BDMV/BGYMV NSP and common bean cultivars representing both gene pools to further map the avirulence determinant to the N-terminus of the BDMV NSP. In the BGYMV background, the BDMV *BV1* acted as a virulence factor in the Andean cv. Topcrop, whereas it was an avirulence factor in the Middle American cvs. Alpine and UI 114. Infectivity studies also revealed that the CP is a determinant of pathogenicity in a common bean gene pool-specific manner. Thus, pathogenicity of BGYMV and BDMV in common bean is a function of the avirulence and virulence activity of NSP, other viral factors (e.g., CP and viral genetic background), and the host genetic background (e.g., gene pool).

RESULTS

In this study, the *Begomovirus* sp.–common bean interaction was further investigated using a series of hybrid DNA-B components (Fig. 1A and B). The infectivity of these hybrid components was assessed in cultivars representing Andean (Topcrop) and Middle American (UI 114 [Pinto bean type] and Alpine [Great Northern type]) common bean gene pools. In preliminary particle bombardment inoculation experiments, BDMV was highly infectious (approximately 94%) in Topcrop and induced typical dwarfing and mosaic symptoms (Fig. 2A). In contrast, UI 114 was resistant (symptomless phenotype and no viral DNA detected in newly emerged leaves) (Fig. 2C), and this resistance response was associated with a vascular HR (Table 1). In Alpine, an intermediate disease phenotype was observed in which 34% (25/73) of inoculated plants developed mild mosaic symptoms (Fig. 2B), despite development of the vascular HR in most plants (Table 1). Similar infectivity results were observed when these cultivars were inoculated with BDMV by sap inoculation (data not shown).

Equivalent experiments performed with the BDMV-GFP reporter revealed a cultivar-dependent role for the CP in viral infection. As previously reported, the BDMV-GFP reporter was highly infectious in Topcrop (Garrido-Ramirez et al. 2000b; Sudarshana et al. 1998; Wang et al. 1999) and induced mild

BDMV-like symptoms and no HR in bombarded hypocotyl tissues (Table 1; Fig. 3H and H'). Middle American cv. UI 114 was resistant to BDMV-GFP; however, in contrast to wild-type BDMV, cv. Alpine was resistant to BDMV-GFP (Table 1). The resistance in both cultivars to BDMV-GFP was associated with the vascular HR. These results are consistent with those of Seo and associates (2004), showing that i) BDMV requires the CP for infection of certain Middle American cultivars, ii) CP is not required for induction of the vascular HR, and iii) the vascular HR is not strictly correlated with resistance.

The BDMV and BGYMV NSP are determinants of avirulence and virulence, respectively, in cv. UI 114.

Infection studies next were performed in these common bean cultivars with the HBDBG4 and HBDBG6 constructs described by Garrido-Ramirez and associates (2000b). HBDBG4 and HBDBG6 are BDMV DNA-B hybrids with the BGYMV *BV1* and *BC1* open reading frames (ORF), respectively (Fig. 1A and B). Common bean seedlings were individually coinoculated with these constructs and BDMV-A or BDMVA-GFP, and the results are summarized in Table 1. Both hybrid DNA-B components, together with BDMV-A or BDMVA-GFP, were highly infectious in cv. Topcrop and induced BDMV-like symptoms and no vascular HR. In cv. Alpine, both hybrid DNA-B components, together with BDMV-A, were moderately infectious and induced mild mosaic symptoms (Table 1). However, when coinoculated with BDMVA-GFP, only HBDBG4 (BDMV *BC1*+BGYMV *BV1*) was infectious in Alpine, although at lower rates (11%) and with milder symptoms compared with BDMV-A and HBDBG4. In cv. UI 114, BDMV-A and HBDBG4 was infectious, inducing BDMV-like symptoms in approximately 17% of inoculated plants. All other combinations were not infectious in this cultivar. Here, it is important to note that none of the plants coinoculated with BDMVA-GFP and HBDBG4 developed the vascular HR, whereas Alpine and UI 114 plants coinoculated with BDMVA-GFP and HBDBG6 did (albeit at lower frequencies than wild-type BDMV) (Table 1; Fig. 3A, A', B, B', I, and J).

These results are consistent with the BDMV NSP acting as an avirulence factor in UI 114 because BDMV-A and HBDBG4 (with the BGYMV *BV1*) overcame the BDMV resistance. The fact that BDMVA-GFP and HBDBG4 was not infectious in UI 114 indicates that the BDMV CP also is required to break the resistance. Consistent with this notion, in Alpine, the BDMV NSP was an avirulence factor only in the absence of CP (Table 1; compare results for BDMV-A and HBDBG6 versus BDMVA-GFP and HBDBG6).

N-terminus of *BV1* determines virulence or avirulence in UI 114.

The BDMV and BGYMV NSP proteins are each composed of 256 amino acids, and are 74% identical. Most of the differences reside in the N-terminus (Fig. 1B and 2M). To map the avirulence domains in the BDMV NSP, a series of hybrid BDMV DNA-B components were generated that would express chimeric NSP (composed of BDMV and BGYMV sequences). These components and the BGYMV portion in the chimeric NSP are as follows: HBDBG9 with the N-terminal 1 to 42 amino acids, HBDBG10 with amino acids 43 to 92, HBDBG11 with amino acids 110 to 167, and HBDBG12 with amino acids 185 to 250 (Fig. 1A and B). These four hybrid DNA-B components were coinoculated with BDMV-A or BDMVA-GFP into seedlings of Topcrop, Alpine, and UI 114. The results of these experiments are presented in Table 2.

Hybrid HBDBG9 coinoculated with BDMV-A or BDMVA-GFP was highly infectious in Topcrop and induced BDMV-like symptoms (Fig. 2D) with no vascular HR (Table 2; Fig. 3K).

In Alpine, HBDBG9 coinoculated with BDMV-A was moderately infectious (approximately 63%) and induced mild BDMV-like symptoms (Table 2; Fig. 2E), whereas it was not infectious in Alpine with BDMVA-GFP. The vascular HR was observed in Alpine seedlings coinoculated with HBDBG9 and BDMV-A or BDMVA-GFP (Table 2; Fig. 3D and D'). In the BDMV-resistant UI 114, HBDBG9 coinoculated with BDMV-A was infectious and induced severe BDMV-like symptoms, but at relatively low rates (approximately 14%) (Table 2; Fig. 2F). In contrast, HBDBG9 was not infectious with BDMVA-GFP. The vascular HR developed in hypocotyls of UI 114

seedlings coinoculated with HBDBG9 and BDMV-A or BDMVA-GFP (Table 2; Fig. 3C, C', and L). These results indicate that i) this chimeric NSP was functional, ii) the avirulence domain is located in the N-terminus of the BDMV NSP, and iii) the CP was necessary for HBDBG9 to overcome the resistance of UI 114. The infectivity of these hybrid viruses was not correlated with HR development, although it is interesting to note the HR development was more frequent for hybrid viruses with BDMVA-GFP compared with BDMV-A (Table 2).

Hybrid HBDBG10 coinoculated with BDMV-A or BDMVA-GFP was not infectious, even in Topcrop. This was based upon

Fig. 1. Bean dwarf mosaic virus (BDMV) and Bean golden yellow mosaic virus (BGYMV) DNA-A and DNA-B constructs used to study BV1 function in *Phaseolus vulgaris*. **A**, Linear maps showing cloned multimeric DNA components used in this study. Red and blue boxes represent BDMV and BGYMV open reading frames (ORFs), respectively; the yellow box represents the BDMV common region (CR); and the green box represents the green fluorescent protein (GFP) gene in place of the BDMV AV1 ORF. Restriction sites used for generating the BDMV/BGYMV hybrid DNA-B components are shown below the appropriate construct. **B**, Circular maps of the constructs shown in A. Each BDMV DNA-B component was coinoculated individually (by particle bombardment) into common bean seedlings with either BDMV DNA-A (BDMV-A) or BDMVA-GFP. **C**, Schematic illustration of the method of plant inoculation and analysis of inoculated plants. Expression of GFP was detected at 24 to 48 h postinoculation (hpi). Hypersensitive response (HR) was detected 4 to 11 days postinoculation (dpi) using freehand transverse sections of hypocotyl tissue and light and fluorescent microscopy. Systemic infection and symptom development were detected at 14 to 21 dpi. Infections in selected plants were confirmed by polymerase chain reaction or sequencing.

M

HBDBG9

BDMV BV1 (1) MYGLRNKRGSSFSQRRFYRSRSSLNRLSANKRHDGKRRAMNP (42)
 BGYMV BV1 (1) MYTSKYKRGSFNYQRRGYRSRSGFRRTSIVKRHDGKRRQHOS (42)
 Consensus (1) MY K KRGS QRR YSR S R S KRHDGKRR N (42)

Fig. 2. Disease symptoms in bean plants inoculated with *Bean dwarf mosaic virus* (BDMV) or viruses with hybrid DNA-B components expressing chimeric BDMV/*Bean golden yellow mosaic virus* (BGYMV) nuclear shuttle proteins (NSPs). Seedlings were inoculated via particle bombardment and plants were photographed at 14 to 21 days postinoculation. **A**, Cv. Topcrop (Andean gene pool) infected with BDMV; **B**, cv. Alpine (Middle American gene pool) infected with BDMV; **C**, resistant reaction of cv. UI 114 (Middle American gene pool) inoculated with BDMV; **D**, Topcrop infected with HBDBG9 and BDMV-A; **E**, Alpine infected with HBDBG9 and BDMV-A; **F**, UI 114 infected with HBDBG9 and BDMV-A; **G**, Topcrop infected with HBDBG11 and BDMV-A; **H**, Alpine infected with HBDBG11 and BDMV-A; **I**, resistant reaction of UI 114 inoculated with HBDBG11 and BDMV-A; **J**, Topcrop infected with HBDBG12 and BDMV-A; **K**, Alpine infected with HBDBG12 and BDMV-A; **L**, resistant reaction of UI 114 inoculated with HBDBG12 and BDMV-A; and **M**, alignment of N-terminal 42 amino acids of the BDMV and BGYMV NSPs. Amino acids shown with a light background are identical, those with a dark background are similar, and those not highlighted are different.

the failure to observe symptoms or to detect viral DNA in newly emerging leaves of inoculated plants (Table 2). Consistent with these results, GFP fluorescence was observed only in single cells of hypocotyls of all three cultivars coinoculated with BDMV-A-GFP and HBDBG10; nor was the vascular HR observed in these inoculated hypocotyls (Table 2; Fig. 3E, E', and M). Together, these results indicate that this chimeric NSP was nonfunctional.

Hybrid HBDBG11 coinoculated with BDMV-A or BDMV-A-GFP was highly infectious in Topcrop and induced BDMV-like symptoms (Fig. 2G). In Alpine, HBDBG11 coinoculated with BDMV-A induced BDMV-like symptoms in approximately 19% of inoculated plants (Table 2; Fig. 2H), whereas HBDBG11 coinoculated with BDMV-A-GFP was not infectious. In UI 114, HBDBG11 was not infectious with BDMV-A or BDMV-A-GFP (Fig. 2I). Nearly identical infectivity results were obtained with HBDBG12 (Table 2; Fig. 2J through L). The vascular HR was detected in hypocotyls of Alpine and UI 114 seedlings coinoculated with HBDBG11 or HBDBG12 and BDMV-A or BDMV-A-GFP (Fig. 3F, F', G, G', and O); however, HR was more frequent and intense with BDMV-A-GFP compared with BDMV-A (Table 2).

Hybrid HBDBG12 coinoculated with BDMV-A or BDMV-A-GFP consistently showed delayed cell-to-cell movement compared with viruses having HBDBG9 and HBDBG11. For example, in all three cultivars, HBDBG12 coinoculated with BDMV-A-GFP moved approximately two to three cells by 48 h postbombardment (based on observation of GFP fluorescence), whereas HBDBG9 or HBDBG11 coinoculated with BDMV-A-GFP had moved approximately four to five cells in the same time period. Similarly, HR development in hypocotyls of Alpine and UI 114 coinoculated with HBDBG12 and BDMV-A-GFP, was delayed (7 to 8 days postbombardment [dpb]) compared with that in hypocotyls coinoculated with HBDBG9 or HBDBG11 and BDMV-A-GFP (4 to 5 dpb). Finally, systemic infection and symptom development in Topcrop seedlings coinoculated with HBDBG12 and BDMV-A or BDMV-A-GFP were delayed (8 to 9 dpb) compared with seedlings coinoculated with HBDBG9 or HBDBG11 and BDMV-A or BDMV-A-GFP (4 to 5 dpb). This delay was particularly evident in primary leaves, where no symptoms developed in plants infected with viruses having HBDBG12, whereas symptoms of vein clearing, mosaic, and crumpling consistently developed in seedlings infected with viruses having HBDBG9 or HBDBG11.

BDMV BV1 function in the BGYMV background.

To further investigate the role of the BDMV NSP in pathogenicity and host resistance, it was exchanged with the BGYMV CP gene in BGYMV DNA-A to generate BGA-BDBV1. To allow for assessment of BDMV NSP function in BGA-BDBV1 in the absence of the BGYMV NSP, a frameshift (fs) mutation was introduced into the BGYMV BV1 gene in BGYMV DNA-B, generating BGYMVB_{fs} (Fig. 4A and B). Additional constructs used in these experiments included multimeric clones of BGYMV-A and BGYMV-B (together referred to as BGYMV) and BGYMV-A-GFP and BGYMV-B (together referred to as BGYMV-GFP) (Garrido-Ramirez et al. 2000a and b).

In Topcrop, BGYMV and BGYMV-GFP had high rates of infectivity, 91 and 78%, respectively (Table 3). BGYMV induced typical golden mosaic and leaf distortion symptoms (Fig. 5A), whereas BGYMV-GFP induced attenuated symptoms, including yellow blotches and crumpling (Fig. 5B). In Alpine and UI 114, BGYMV was moderately infectious, 41 and 39%, respectively (Table 3); and induced golden mosaic symptoms (Fig. 5G and J, respectively). In contrast, BGYMV-GFP had considerably lower rates of infectivity in both of these cultivars, approximately 10 and 11%, respectively (Table 3), and symptoms were attenuated (Fig. 5H and K). These results are in agreement with the capacity of BGYMV to infect Andean and Middle American cultivars (Morales and Niessen 1988), but also demonstrate that the CP is a BGYMV pathogenicity factor, especially in Middle American genotypes.

BGA-BDBV1 coinoculated with BGYMV-B was highly infectious in Topcrop (100%). Furthermore, this virus induced striking chlorosis symptoms in trifoliate leaves, which were more severe than those induced by wild-type BGYMV (Table 3; compare Fig. 5C and D with A). However, in Alpine and UI 114, BGA-BDBV1 coinoculated with BGYMV-B was less infectious than BGYMV (17 versus 41 and 14 versus 39%, respectively) (Table 3), although plants infected with BGA-BDBV1 and BGYMV-B developed golden mosaic symptoms (Fig. 5I and L). Thus, the infectivity rates of BGA-BDBV1 and BGYMV-B in Alpine and UI 114 were more similar to those of BGYMV-GFP (Table 3; Fig. 5H and K). Thus, in Topcrop, the BDMV NSP acted as a pathogenicity factor in the BGYMV background, increasing symptom severity. However, in the Middle American cultivars, it was not clear whether the impaired infectivity of BGA-BDBV1 and BGYMV-B was due

Table 1. Induction of the hypersensitive response (HR), cell-to-cell and long-distance movement by *Bean dwarf mosaic virus* (BDMV), a BDMV green fluorescent protein (GFP) reporter, and BDMV/*Bean golden yellow mosaic virus* DNA-B hybrids in common bean (*Phaseolus vulgaris*)

Virus components ^a			Topcrop			Alpine			Pinto UI 114		
A	B	BV1/BC1	HR ^b	Cell ^c	LD ^d	HR	Cell	LD	HR	Cell	LD
BDMV-A	BDMV-B	BD/BD	0/33	—	29/31***	28/30	—	25/73** + CP	23/31	—	0/68 + CP
BDMV-A-GFP	BDMV-B	BD/BD	0/17	15/17	18/20**	24/26	26/26	0/17 – CP	20/24	24/24	0/12 – CP
BDMV-A	HBDBG4	BG/BD	0/17	—	11/12***	0/12	—	13/18** + CP	0/16	—	3/18*** + CP
BDMV-A-GFP	HBDBG4	BG/BD	0/17	16/17	11/13**	0/12	12/12	2/18* – CP	0/15	15/15	0/18 – CP
BDMV-A	HBDBG6	BD/BG	0/14	—	12/13***	9/15	—	12/18** + CP	7/15	—	0/18 + CP
BDMV-A-GFP	HBDBG6	BD/BG	0/16	16/16	11/17**	13/18	18/18	0/18 – CP	6/18	18/18	0/18 – CP
Gold	...	...	0/12	—	0/12	0/12	—	0/12	0/12	—	0/12

^a BDMV-A-GFP = BDMV DNA-A (BDMV-A) tagged with GFP; HBDBG4 = DNA-B hybrid with the BDMV common region (CR), untranslated regions (UTR), BC1 open reading frame (ORF), and the BGYMV BV1 ORF; HBDBG6 = DNA-B hybrid with the BDMV CR, UTR, and BV1 ORF, and the BGYMV BC1 ORF; and gold = gold particles alone.

^b HR was detected by examination of transverse hypocotyl sections, 4 to 11 days postinoculation (dpi), with fluorescence and light microscopy. Number of plants in which either HR, GFP (cell-to-cell movement), symptoms, or viral DNA (long-distance movement) were detected/total number of seedlings inoculated. Numbers represent totals for three or more independent experiments.

^c Cell = cell-to-cell movement, assessed based on detection of GFP fluorescence, 4 to 11 dpi, in transverse hypocotyl sections with fluorescence microscopy; – indicates that cell-to-cell movement could not be assessed when wild-type BDMV-A was inoculated.

^d LD = long-distance movement (systemic infection), determined based on symptom development (indicated by asterisks) or by polymerase chain reaction analysis of total genomic DNA extracted from the third trifoliate leaf, collected 14 to 21 dpi. CP = capsid protein. Relative degree of symptom development is as follows: * = mild symptoms, ** = moderate symptoms, and *** = severe symptoms.

to BDMV NSP acting as an avirulence factor or the absence of the BGYMV CP.

To test the hypothesis that the BGYMV NSP, expressed from the wild-type BGYMV DNA-B, may have masked the avirulence function of the BDMV NSP expressed from BGA-BDBV1, infectivity experiments were next performed with BGYMVB_{fs}. In Topcrop, BGA-BDBV1 coinoculated with BGYMVB_{fs} was moderately infectious (57%) and induced yellow vein and leaf mottling symptoms, which were less severe than those induced by wild-type BGYMV (Fig. 5E and F). In contrast, BGA-BDBV1 coinoculated with BGYMVB_{fs} was not infectious in Alpine or UI 114, consistent with the BDMV NSP conferring avirulence to BGYMV in these cultivars (Table 3). Interestingly, none of the viruses with BGA-BDBV1 elicited the vascular HR in Alpine or UI 114 (Table 3).

DISCUSSION

We have continued our studies of the *Begomovirus* sp.–common bean interaction using hybrid viruses, GFP reporters, and common bean cultivars with differential *Begomovirus* susceptibility. Functional domains of the BDMV NSP were identified using hybrid BDMV DNA-B components expressing chimeric BDMV/BGYMV NSP. With the exception of HBDBG10, most of the chimeric NSP proteins were functional and mediated systemic infection of the hybrid viruses in the susceptible

Topcrop (Table 2). This is consistent with previous results showing that the MPs of these two New World bean-infecting begomoviruses are functionally interchangeable (Garrido-Ramirez et al. 2000b).

Three common bean cultivars, representing the two major gene pools and showing differential susceptibility to BDMV and BGYMV, were used to reveal the specificity of NSP function. The capacity of the BDMV-A and HBDBG4 (BDMV BC1 and BGYMV BV1) to overcome the BDMV resistance in UI 114 extended previous results that the BDMV NSP acts as an avirulence determinant in Middle American genotypes. The finding that only HBDBG9, but not HBDBG10, HBDBG11, and HBDBG12, overcame the resistance mapped the avirulence determinant to the N-terminus of the BDMV NSP. This is perhaps not surprising because this region is i) the most divergent region of the protein (Fig. 1B, and 2M), ii) high in basic amino acid residues that are likely to be involved in interactions with proteins or nucleic acids, and iii) predicted to be on the surface of the folded protein (data not shown). Thus, this region of the BDMV NSP may be recognized by a plant host factor, mediating the activation of a host defense response that blocks long-distance movement. Furthermore, because the NSP mediates nucleocytoplasmic transport of viral DNA, this putative host factor may reside in or around the nucleus or NPC. The BGYMV NSP may not trigger this defense response, possibly because it is not recognized by this putative host factor. Alternatively, the

Fig. 3. Development of the vascular hypersensitive response (HR) in bean hypocotyls bombarded with the cloned DNA components of *Bean dwarf mosaic virus* (BDMV), BDMV expressing the green fluorescent protein (BDMV-GFP), or viruses with hybrid DNA-B components expressing chimeric BDMV/*Bean golden yellow mosaic virus* (BGYMV) nuclear shuttle proteins (NSPs). **A**, Cv. Alpine (Middle American gene pool) coinoculated with HBDBG4 (BDMV BC1+BGYMV BV1) and BDMVA-GFP; **B**, cv. UI 114 coinoculated with HBDBG6 (BDMV BV1+BGYMV BC1) and BDMVA-GFP; **C**, UI 114 coinoculated with HBDBG9 and BDMVA-GFP; **D**, Alpine coinoculated with HBDBG9 and BDMVA-GFP; **E**, cv. Topcrop (Andean gene pool) coinoculated with HBDBG10 and BDMVA-GFP; **F**, Alpine coinoculated with HBDBG11 and BDMVA-GFP; **G**, UI 114 coinoculated with HBDBG12 and BDMVA-GFP; and **H**, Topcrop coinoculated with BDMVA-GFP and BDMV-B. **A'–H'**, Bright-field images of sections presented in A–H, respectively. **I**, UI 114 coinoculated with HBDBG4 and BDMV-A; **J**, Alpine coinoculated with HBDBG6 and BDMV-A; **K**, Topcrop coinoculated with HBDBG9 and BDMV-A; **L**, UI 114 coinoculated with HBDBG9 and BDMV-A; **M**, Alpine coinoculated with HBDBG10 and BDMV-A; and **N**, Alpine plant bombarded with gold particles alone showing no HR in hypocotyl tissues, compared with **O**, HR development in hypocotyl tissues of an Alpine plant coinoculated with HBDBG11 and BDMVA-GFP. Images are transverse hypocotyl sections collected with a fluorescence microscope (Nikon Opiphot-2). White arrows identify the cortex/phloem (vascular) boundary. C, EP, HR, and Xy identify the location of the cortex, epidermis, HR (shown with dark arrow), and xylem, respectively. Scale bar in A' = 250 µm and is common to images A through H, and A' through H'. Scale bar in I = 250 µm and is common to images I through M.

Table 2. Induction of the hypersensitive response (HR), cell-to-cell and long-distance movement by *Bean dwarf mosaic virus* (BDMV)/*Bean golden yellow mosaic virus* (BGYMV) BV1 hybrids in common bean (*Phaseolus vulgaris*)

Virus components ^a		Topcrop			Alpine			Pinto UI 114		
A	B	HR ^b	Cell ^c	LD ^d	HR	Cell	LD	HR	Cell	LD
BDMV-A	HBDBG9	0/12	–	31/32***	14/27 + CP	–	35/56**	8/27 + CP	–	7/52***
BDMVA-GFP	HBDBG9	0/20	19/20	16/21**	25/25 – CP	25/25	0/20	12/13 – CP	12/13	0/13
BDMV-A	HBDBG10	0/14	–	0/14	0/12	–	0/14	0/12	–	0/12
BDMVA-GFP	HBDBG10	0/16	0/16	0/12	0/16	0/16	0/12	0/16	0/16	0/12
BDMV-A	HBDBG11	0/12	–	23/25***	8/33 + CP	–	5/27**	2/12 + CP	–	0/16
BDMVA-GFP	HBDBG11	0/18	17/18	12/18**	39/41 – CP	40/41	0/25	26/29 – CP	25/29	0/16
BDMV-A	HBDBG12	0/12	–	21/23***	7/26 + CP	–	5/28**	2/12 + CP	–	0/18
BDMVA-GFP	HBDBG12	0/16	15/16	12/18**	30/32 – CP	30/32	0/22	27/29 – CP	28/29	0/18
Gold	...	0/33	–	0/42	0/31	–	0/44	0/24	–	0/38

^a BDMVA-GFP = BDMV DNA-A (BDMV-A) tagged with the green fluorescent protein (GFP); HBDBG9 = DNA-B with the hybrid BV1 open reading frame (ORF) encoding the N-terminal 1 to 42 amino acids from BGYMV NSP in the BDMV DNA-B background; HBDBG10 = DNA-B with the hybrid BV1 ORF encoding amino acids 43 to 92 from BGYMV NSP in the BDMV DNA-B background; HBDBG11 = DNA-B hybrid with the hybrid BV1 ORF encoding amino acids 110 to 167 from BGYMV NSP in the BDMV DNA-B background; HBDBG12 = DNA-B hybrid with the hybrid BV1 ORF encoding amino acids 185 to 250 from BGYMV NSP in the BDMV DNA-B background; and Gold = gold particles used alone as a control.

^b HR was detected by examination of transverse hypocotyl sections, 4 to 11 days postinoculation (dpi), with fluorescence and light microscopy. Number of plants in which either HR, GFP (cell-to-cell movement), symptoms, or viral DNA (long-distance movement) were detected/total number of seedlings inoculated. Numbers represent totals for three or more independent experiments.

^c Cell = cell-to-cell movement, assessed based on detection of GFP fluorescence, 4 to 11 dpi, in transverse hypocotyl sections with fluorescence microscopy; – indicates that cell-to-cell movement could not be assessed when wild-type BDMV-A was inoculated.

^d LD = long-distance movement (systemic infection), determined based on symptom development (indicated by asterisks) or by polymerase chain reaction analysis of total genomic DNA extracted from the third trifoliate leaf, collected 14 to 21 dpi. CP = capsid protein. Relative degree of symptom development is as follows: * = mild symptoms, ** = moderate symptoms, and *** = severe symptoms.

BGYMV NSP may be more efficient in mediating cell-to-cell movement, thereby allowing the virus to “outrun” or avoid the NSP-triggered defense response. We favor the latter scenario, because even wild-type BGYMV was not highly infectious in UI 114, which is consistent with *Begomovirus* resistance in this cultivar. This also may explain the low frequency with which HBDBG4 and HBDBG9 (with wild-type BDMV DNA-A) were able to overcome the BDMV resistance of UI 114.

The NSP expressed by HBDBG10, which has BGYMV amino acids 43 to 92, did not mediate viral movement or development of the vascular HR. This hybrid, coinoculated with BDMV-A, also was not infectious in *Nicotiana benthamiana*, a solanaceous host of BDMV; nor was the hybrid BV1 gene able to mediate an infection when expressed from the BGYMV background (data not shown). Thus, this chimeric NSP is non-functional, although it is not clear which aspect of NSP function is impaired. In mediating nuclear export of viral DNA, NSP must shuttle into and out of the nucleus (Noueiry et al. 1994; Pascal et al. 1994), bind DNA (Rojas et al. 1998) and interact with viral (e.g., MP) or host factors (e.g., acetyl transferase) (McGarry et al. 2003). The function of the HBDBG10

NSP could be impaired simply due to a structural alteration (e.g., improper protein folding), or in a virus-specific interaction domain. Nuclear import of NSP is mediated by N-terminal nuclear localization signals (NLS), and mutations within these signals negatively impact nuclear targeting and viral infectivity (Kass et al. 2006; Noueiry et al. 1994; Sanderfoot et al. 1996). Two potential NLS motifs are found in the BDMV NSP (bipartite NLS-A [residues 27 to 39, R(L/T)S(A/D)(N/V)KRHDGKRR] and NLS-B [residues 81 to 96, (S/Q)(K/L)GKMEPNR(S/C)R SYIKL]). However, although part of the NLS-B motif is in the portion of the protein that was exchanged, these residues are conserved in the BDMV and BGYMV NSP, suggesting that nuclear import would still occur. The predicted chimeric NSP also has no change in the complement of positively charged amino acid residues or overall charge, indicating that it likely retains the capacity to bind DNA. An interesting possibility is the identification of a phosphorylation motif in this region of the BDMV NSP that is lacking in the BGYMV NSP. This motif may be essential for a BDMV-specific function involved in viral infectivity. Support for this concept comes from reports that NSP is phosphorylated in plants (Pascal et al. 1994). Fur-

Fig. 4. Linear maps showing cloned multimeric DNA components of *Bean golden yellow mosaic virus* (BGYMV), BGYMV expressing the green fluorescent protein (BGYMV-GFP), and a hybrid virus with the *Bean dwarf mosaic virus* (BDMV) BV1 gene in the BGYMV background. **A**, Linear maps. Red and blue boxes represent BDMV and BGYMV open reading frames, respectively; purple boxes represent the BGYMV common region (CR); and the green box represents the GFP gene in place of the BGYMV capsid protein (CP) gene in BGYMV-GFP. Restriction sites used for generating these constructs are shown below the maps. **B**, Circular maps of the DNA components of BGYMV (BGYMV DNA-A and DNA-B), BGYMV-GFP (BGYMV DNA-A with the GFP gene [BGA-GFP] and BGYMV DNA-B), BGYMV DNA-A with the BDMV BV1 gene exchanged for the CP gene and wild-type BGYMV DNA-B, and BGYMV DNA-A with BDMV BV1 and BGYMV DNA-B with a BV1 frameshift mutation.

thermore, NSP also interacts with three *Arabidopsis* leucine-rich-repeat receptor-like kinases (LRR-RLK) and a praline-rich extension-like receptor protein kinase (PERK), and these interactions play a role in virus infection and pathogenicity (Florentino et al. 2006; Fontes et al. 2004).

The infectivity of the HBDBG11 hybrid in Topcrop was similar to that of wild-type BDMV, indicating that exchange of

this highly conserved NSP domain (Fig. 1B) did not adversely affect protein function. In contrast, hybrid HBDBG12, expressing a chimeric NSP with the C-terminal part of the BGYMV NSP, had delayed cell-to-cell movement, HR induction, and systemic infection. This impairment in NSP function may be due to subtle alterations in NSP sequence or structure that effect protein–protein or protein–nucleic acid interactions. One such

Table 3. Induction of the hypersensitive response (HR), cell-to-cell and long-distance movement by *Bean golden yellow mosaic virus* (BGYMV), BGYMV expressing green fluorescent protein (GFP), and *Bean dwarf mosaic virus* (BDMV) BV1 in common bean (*Phaseolus vulgaris*)

Virus components ^a		Topcrop			Alpine			Pinto UI 114		
A	B	HR ^b	Cell ^c	LD ^d	HR	Cell	LD	HR	Cell	LD
BGYMV-A	BGYMV-B	0/33	–	40/44***	0/22	–	11/27**	0/12	–	7/18***
BGYMAV-GFP	BGYMV-B	0/26	26/26	38/49**	0/18	18/18	3/31*	0/12	12/12	2/19*
BGA-BDBV1	BGYMV-B	0/33	–	32/32****	0/26	–	4/23**	0/15	–	4/28**
BGA-BDBV1	BGYMVB _{fs}	0/28	–	21/37**	0/33	–	0/29	0/23	–	0/19
Gold	...	0/15	–	0/15	0/15	–	0/15	0/15	–	0/15

^a BGYMAV-GFP = BGYMV DNA-A (BGYMV-A) with GFP in place of the capsid protein (CP); BGA-BDBV1 = BGYMV-A with BDMV BV1 in place of the CP open reading frame (ORF); BGYMVB_{fs} = BGYMV DNA-B (BGYMV-B) with a frameshift mutation in the BV1 ORF; and Gold = gold particles alone.

^b HR was detected by examination of transverse hypocotyl sections, 4 to 11 days postinoculation (dpi), with fluorescence and light microscopy. Number of plants in which either HR, GFP (cell-to-cell movement), symptoms, or viral DNA (long-distance movement) were detected/total number of seedlings inoculated. Numbers represent totals for three or more independent experiments.

^c Cell = cell-to-cell movement, assessed based on detection of GFP fluorescence, 4 to 11 dpi, in transverse hypocotyl sections with fluorescence microscopy; – indicates that cell-to-cell movement could not be observed when wild-type BDMV-A was inoculated.

^d LD = long-distance movement (systemic infection), determined based on symptom development (indicated by asterisks) or by polymerase chain reaction analysis of total genomic DNA extracted from the third trifoliate leaf, collected at 14 to 21 dpi. Relative degree of symptom development is as follows: * = mild symptoms, ** = moderate symptoms, *** = severe symptoms, and **** = very severe symptoms.

Fig. 5. Disease phenotypes in bean plants inoculated with *Bean golden yellow mosaic virus* (BGYMV), BGYMV expressing the green fluorescent protein (BGYMV-GFP), or hybrid viruses with the *Bean dwarf mosaic virus* (BDMV) BV1 gene in the BGYMV background. Seedlings were inoculated via particle bombardment, and plants photographed at 14 to 21 days postinoculation. **A**, Cv. Topcrop (Andean gene pool) infected with BGYMV; **B**, Topcrop infected with BGYMV-GFP; **C** and **D**, Topcrop infected with BGYMV DNA-A (BGA) with the BDMV BV1 gene (BDBV1) exchanged for the capsid protein gene (BGA-BDBV1); and **E** and **F**, Topcrop infected with BGA-BDBV1+BGYMV DNA-B (BGB) with a BV1 frameshift mutation (BGYMVB_{fs}). **G**, Cv. Alpine (Middle American gene pool) infected with BGYMV; **H**, Alpine infected with BGYMV-GFP; **I**, Alpine infected with BGA-BDBV1+BGB; **J**, cv. UI 114 infected with BGYMV; **K**, UI 114 infected with BGYMV-GFP; and **L**, UI 114 infected with BGA-BDBV1+BGB.

interaction is the postulated NSP–MP interaction, and mutational analyses of the *Squash leaf curl virus* (SLCV) BV1 has implicated this domain in nuclear targeting and BC1-dependent redirection from nucleus to cytoplasm (Sanderfoot et al. 1996).

The BDMV NSP function also was investigated in the BGYMV background, by inserting the BDMV BV1 gene in place of the CP gene in BGYMV DNA-A. The development of striking chlorotic symptoms induced by BGA-BDBV1 and BGYMV-B (expressing BDMV and BGYMV NSP) in Topcrop, together with the milder symptom phenotype induced by BGA-BDBV1 and BGYMVB_{fs} (expressing only BDMV NSP), established that the BDMV NSP is a pathogenicity determinant in common bean. It is possible that this more extensive chlorosis is a direct effect of increased levels of NSP on an endogenous host pathway (e.g., transport across the NPC) or a more general toxicity due to overexpression of NSP. Regardless, this finding is in agreement with a recent report indicating that the *Tomato leaf curl New Delhi virus* NSP is a pathogenicity determinant in tobacco and tomato (Hussain et al. 2005).

The next question was whether the BDMV NSP in the BGYMV background would act as a dominant avirulence factor in the Middle American cultivars. Although BGA-BDBV1 and BGYMV DNA-B was highly pathogenic in the Andean cv. Topcrop, it was poorly infectious in the Middle American cultivars. This suggested that expression of the BDMV NSP was deleterious, but not sufficient to confer complete avirulence. The finding that BGA-BDBV1 and BGYMVB_{fs} was not infectious in the Middle American cultivars is consistent with the BDMV NSP acting as a dominant avirulence factor in the BGYMV background, and also suggests that the BGYMV NSP may mask the BDMV NSP avirulence function. However, it is important to point out that the reduced infectivity of these viruses in the Middle American cultivars also reflects the absence of the CP, which plays an important role in the *Begomovirus* infection process in these cultivars.

It is well established that the CP of some bipartite begomoviruses is not required for systemic infection (Gardiner et al. 1988; Ingham et al. 1995; Padidam et al. 1995; Pooma et al. 1996; Sudarshana et al. 1998). However, it has become increasingly clear that CP plays a key role in many *Begomovirus* sp.–host interactions. In the case of BDMV, CP-independent long-distance movement is host and cultivar dependent (Seo et al. 2004; Wang et al. 1999). In the present study, BDMV, BGYMV, and the infectious hybrid viruses did not require CP for infectivity in the Andean cv. Topcrop, although infectivity was reduced and symptoms were attenuated (e.g., BGYMV-GFP). On the other hand, in the Middle American cultivars, CP was necessary for infectivity of BDMV and the hybrid viruses and for higher levels of infection by BGYMV (Table 2). Thus, these results confirm and extend the findings of Seo and associates (2004), showing that the *Begomovirus* CP is a gene pool–dependent pathogenicity factor in common bean.

It is not clear how the CP is enhancing *Begomovirus* infectivity in common bean. It may allow BDMV and BGYMV to avoid host defense responses in Middle American cultivars, perhaps via formation of a resistant nucleoprotein complex for long-distance movement (e.g., virions or virion-like structures); or by evasion or suppression of a host defense mechanism, such as gene silencing (Bisaro 2006; Rojas et al. 2005). In the present study, CP masked the avirulence of the NSP of BDMV and the hybrid viruses, as well as the development of the vascular HR. These results also are consistent with those of Ingham and associates (1995), who found that the SLCV CP masks defective phenotypes of certain BV1 mutants in pumpkin. Thus, CP may interact with the host factor (receptor) that triggers resistance upon recognition of NSP (e.g., LRR-RLK) (Fontes et al. 2004). Alternatively, CP could participate in the BV1-mediated

transport of the viral genome across the nuclear membrane, perhaps by affecting the accumulation or subcellular localization of viral ssDNA (Azzam et al. 1994; Ingham et al. 1995; McGarry et al. 2003; Qin et al. 1998).

Finally, the finding that viruses expressing the BDMV NSP consistently induced the vascular HR in the Middle American cultivars supports results of previous studies showing that the BDMV NSP is an HR determinant (Garrido-Ramirez et al. 2000b; Seo et al. 2004). The finding that the NSP of HBDBG9 broke the resistance of UI 114 and induced the vascular HR indicates that the domains involved in avirulence and HR are not the same. Moreover, BDMV and the hybrid viruses were infectious in Alpine despite HR development; whereas, in other cases, UI 114 plants were resistant in the absence of a visible HR. These results are in agreement with previous studies showing that BDMV resistance and HR are not tightly linked (Seo et al. 2004; Wang et al. 1999). Thus, it is clear that the HR is not the underlying mechanism of resistance but, rather, an indicator of an ongoing defense response. Interestingly, the BDMV NSP, expressed in place of the CP in the BGYMV background, did not induce the vascular HR, even though genetic evidence strongly supported functional expression. This may reflect a different pattern of expression, via the BGYMV CP promoter, or a role for other BDMV factors in HR development.

MATERIALS AND METHODS

Viral infectious clones and constructs.

Infectious 1.5-mer clones of BDMV-A (pBDA1.5), BDMV-B (pBDB1.5), BGYMV-MX (Mexican strain; hereafter referred to as BGYMV) DNA-A (BGYMV-A; pBGMXA1.5), BGYMV DNA-B (BGYMV-B; pBGMXB1.5), BDMV-A-GFP (pBDAGFP), BGYMV-A-GFP (pBGAGFP), and the two BDMV DNA-B hybrids, HBDBG4 and HBDBG6, have been described previously (Garrido-Ramirez et al. 2000a and b; Hou et al. 1998; Sudarshana et al. 1998).

Generation of BDMV/BGYMV BV1 hybrids.

BDMV/BGYMV BV1 hybrids were made by exchanging sequences of the BDMV BV1 with homologous BGYMV sequences. To accomplish these exchanges, restriction enzyme sites were introduced into the BDMV and BGYMV sequences via silent mutations. For HBDBG9, the sequence encoding the N-terminal 42 amino acids of BGYMV NSP was polymerase chain reaction (PCR) amplified from pBGMXB1.5 with primers (BGV9 and BGC9) that introduced a *SpeI* site at the 5' end and a *SacI* site at the 3' end of the fragment. Another two pairs of primers (BDV1/BDC9 and BDC1/BDV9) were used to PCR amplify the remainder of the BDMV DNA-B component from pBDB1.5 (approximately 0.6 and 3.2 kb, respectively). PCR conditions were as follows: 10 cycles with Pfu polymerase of: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by 72°C for 10 min. These three PCR-amplified fragments each were cloned with the Zero blunt system (Invitrogen, Carlsbad, CA, U.S.A.) and their sequences verified. These fragments were excised, gel-purified, and ligated together to create HBDBG9. A similar strategy was used to generate HBDBG10, 11, and 12. For HBDBG10, *NruI/HpaI* and *BglII* sites were introduced into the 5' and 3' ends, respectively, of the fragment encoding amino acids 43 to 92 of BGYMV NSP. For HBDBG11, *MluI* and *HindIII* sites were introduced into the 5' and 3' ends, respectively, of the fragment encoding amino acids 110 to 167 of BGYMV NSP. For HBDBG12, *MluI* and *HindIII* sites were introduced into the 5' and 3' ends, respectively, of the fragment encoding amino acids 185 to 250 of BGYMV NSP.

BGA-BDBV1 construct and introduction of the BV1 fs mutation into BGYMV BV1.

BGA-BDBV1 was generated by replacing the BGYMV CP ORF with the BDMV BV1 ORF. First, a BGYMV *EcoRI/SalI* fragment, which had the CR and the entire CP ORF, was subcloned from pBGMXA1.5 into pALTER (Promega Corp., Madison, WI, U.S.A.). The Altered Sites II Mutagenesis System (Promega Corp.) was used to introduce *BspEI* and *XbaI* sites, respectively, into the 5' and 3' ends of the BGYMV CP ORF. This fragment was subcloned as an *EcoRI/SalI* fragment into pKS to generate pBGAV1. Two primers and PCR were used to amplify a fragment from pBDB1.5 that contained the entire BV1 ORF; these primers introduced *BspEI* and *XbaI* sites into the 5' and 3' end, respectively, of the BV1 ORF. This PCR-amplified fragment was cloned with the Zero blunt system and the sequence verified. The *BspEI/XbaI* BDMV BV1 fragment then was excised and cloned into *BspEI/XbaI*-digested pBGAV1 to generate pBGBDBV1, which contained the BDMV BV1 ORF and the BGYMV DNA-A CR sequence. Finally, the BGYMV DNA-A monomer was excised as an *EcoRI* fragment from pBGMXA1.5 and ligated into pBDBV1 digested with *EcoRI* to generate BGA-BDBV1 (Fig. 4A).

A frameshift mutation was introduced into the BGYMV BV1 gene at the *StyI* site. Here, pBGMXB1.5 was digested with *StyI*, releasing the BGYMV DNA-B monomer. The ends of the fragment with the vector and the BGYMV 0.5-mer (approximately 4.3 kb) were filled in with Klenow fragment and ligated to generate pBGMXB0.5st. This plasmid was digested with *XhoI* and *SacI* to release a 1,671-bp fragment, which included the BGYMV BV1, with the frameshift mutation, CR, and the 5' end of the *BCI* gene (at the *SacI* site). pBGMXB1.5 was digested with *SacI* and *StuI* to release a 1,669-bp fragment, which had the 3' end of the *BCI* ORF (at the *SacI* site) and a complete CR. Finally, pBluescriptKS (pKS) was digested with *XhoI* and *EcoRV*, and the BGYMV *XhoI/SacI* (1,671 bp) and *SacI/StuI* (1,669 bp) fragments were ligated to generate the BGYMV-B_{fs} construct (Fig. 4A).

Plant material and inoculation.

DNA for particle bombardment inoculation was prepared with a mega plasmid kit (Qiagen, Santa Clarita, CA, U.S.A.) or a quantum midi-prep kit (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's recommendations. Common bean seeds (cvs. Topcrop, Alpine, and Pinto UI 114) were germinated at room temperature on moist filter paper in plastic petri dishes. Bean seedlings (2 to 3 days old) were inoculated by particle bombardment as previously described (Paplomatas et al. 1994; Sudarshana et al. 1998). Inoculated seedlings were planted in soil and grown in a controlled-environment chamber (Convion PGR15; 290 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 16-h photoperiod, 28°C day and 24°C night temperatures, and relative humidity 50%).

Analysis of plants.

Expression of GFP at the whole-plant level was detected with a handheld long-wavelength UV lamp (UVP, Upland, CA, U.S.A.). At the tissue or cellular level, GFP was detected with a Nikon Optiphot-2 microscope (Tokyo). To detect the HR, seedlings were carefully removed from the soil approximately 5 dpb, and freehand transverse sections of hypocotyls were prepared and examined with light and fluorescence microscopy, as previously described (Garrido-Ramirez et al. 2000b; Sudarshana et al. 1998; Wang et al. 1999). For some BDMV/BGYMV hybrids, HR detection was carried out over a period of 4 to 11 days postinoculation (dpi).

To assess systemic infection, seedlings were grown for 14 to 21 days, and then observed for symptom development and, if appropriate, for the presence of GFP in newly emerged leaves.

In some cases, plants were tested for systemic infection by PCR and the nature of the virus confirmed by sequencing. For PCR analysis, DNA extracts were prepared from the youngest trifoliolate leaves (approximately 15 dpi), and viral DNA fragments were amplified using geminivirus degenerate PCR primer pairs (PAC1v1978/PAV1c496 and PBC1v2040/PBV1c970) (Rojas et al. 1993), BDMV BV1-specific primers (501V [5'-TCAGCTGTCAACGACGTGAA-3']/1476C [5'-CAGAGAGCGGAATCCAACAT-3']; BD64V [5'-AGTTTTTTTAAATCGCTTGTCCGC-3']/BD662C [5'-GCTGATGTTGTCATAAA-3']), and BGYMV BV1-specific primers (BG64V [5'-CAAGGTTTTTCGACGAACGTCAAT-3']/BG662C [5'-AGCAATATTAGCATACG-3']). PCR parameters were as follows: 25 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, followed by 72°C for 10 min. Aliquots of PCR reactions were examined by electrophoresis in 0.7% agarose gels in 0.5× Tris-borate-EDTA buffer. PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen) and sequenced by the University of California–Davis Sequencing Facility. Sequence analyses were performed using the Basic Local Alignment Search Tool (BLAST) program.

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