Bean dwarf mosaic virus* BV1 Protein Is a Determinant of the Hypersensitive Response and Avirulence in *Phaseolus vulgaris

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The capacities of the begomoviruses *Bean dwarf mosaic virus* (BDMV) and *Bean golden yellow mosaic virus* (BGYMV) to differentially infect certain common bean (*Phaseolus vulgaris*) cultivars were used to identify viral determinants of the hypersensitive response (HR) and avirulence (avr) in BDMV. A series of hybrid DNA-B components, containing BDMV and BGYMV sequences, was constructed and coinoculated with BDMV DNA-A (BDMV-A) or BDMV-A-green fluorescent protein into seedlings of cv. Topcrop (susceptible to BDMV and BGYMV) and the BDMV-resistant cvs. Othello and Black Turtle Soup T-39 (BTS). The BDMV avr determinant, in bean hypocotyl tissue, was mapped to the BDMV BV1 open reading frame and, most likely, to the BV1 protein. The BV1 also was identified as the determinant of the HR in Othello. However, the HR was not required for resistance in Othello nor was it associated with BDMV resistance in BTS. BDMV BV1, a nuclear shuttle protein that mediates viral DNA export from the nucleus, represents a new class of viral avr determinant. These results are discussed in terms of the relationship between the HR and resistance.

Plants have evolved an array of defense mechanisms to prevent or limit infection by viruses and other pathogens. In some situations, barriers appear to be formed prior to pathogen attack, whereas others require induction by the pathogen or the presence of pathogen-generated elicitors (Agrios 1997). One inducible mechanism that is associated with specific recognition and resistance to infection by a range of plant pathogens is the hypersensitive response (HR) (Dangl et al. 1996; Goodman and Novacky 1994; Morel and Dangl 1997). The HR is usually a highly pathogen-specific reaction involving rapid programmed cell death at the site of infection (Gilchrist 1998), as well as the activation of a cascade of signal transduction events leading to confinement of the pathogen to a localized area. The HR is considered to involve a gene-for-gene interaction (Flor 1971), in which a pathogen avirulence (avr) factor interacts with a host-encoded resistance (R) pro-

tein to activate a defense response (Gabriel and Rolfe 1990; Keen 1990; Lamb et al. 1989; Staskawicz et al. 1995). However, examples exist in which the HR and resistance have been uncoupled (Kim and Palukaitis 1997; Richael and Gilchrist 1999); therefore, the explicit role of the HR in the defense response remains to be elucidated.

With respect to RNA plant viruses, the best known examples involving the HR relate to infection by the tobamoviruses (Culver 1996; Goodman and Novacky 1994). Here, the viral determinant of HR or resistance (i.e., avr determinant) depends upon the specific host resistance gene and has been mapped to viral genes involved in each of the major steps of the tobamovirus life cycle. For *Tobacco mosaic virus* (TMV), the gene-for-gene interaction involves the coat protein (CP)-N' gene (Culver and Dawson 1989; Knorr and Dawson 1988; Saito et al. 1987), the replicase-N gene (Padgett and Beachy 1993), and the movement protein (MP)-*Tm-2* (Meshi et al. 1989) and *-Tm-2* genes (Weber et al. 1993). In the case of *Pepper mild mottle virus*, the CP interacts with the *L2* or *L3* R genes in *Capsicum* spp. (Berzal-Herranz et al. 1994).

Avirulence determinants have also been identified for other plant virus groups (Culver 1996). For example, in potato, the CP of *Potato virus X* (PVX) is an avr determinant for the *Nx* and *Rx* genes (Bendahmane et al. 1995; Kavanaugh et al. 1992), whereas the PVX 25-kDa MP is the determinant for the *Nb* gene (Malcuit et al. 1999). Additional examples include the p22 MP of *Tomato bushy stunt virus* in *Nicotiana edwardsonii* (Chu et al. 1999; Scholthof et al. 1995), the 2a polymerase gene of *Cucumber mosaic virus* (CMV) in cowpea (Kim and Palukaitis 1997), and the gene VI product of the double-stranded (ds) DNA virus, *Cauliflower mosaic virus* (Kiraly et al. 1999; Schoelz et al. 1986).

Members of the family *Geminiviridae* are small, single-stranded (ss) DNA plant viruses that replicate in the nucleus (Timmermans et al. 1994). The bipartite members of the genus *Begomovirus* have their genome divided between two approximately 2.6-kb DNA components, referred to as DNA-A and DNA-B. DNA-A encodes factors involved in viral DNA replication, gene expression, and encapsidation. The two genes encoded on DNA-B, *BV1* and *BC1*, are required for systemic infection (Brough et al. 1988; Etesami et al. 1988), and it has been further established that both the BV1 and BC1 proteins are required for cell-to-cell movement (Jeffrey et al. 1996; Sudarshana et al. 1998). The BV1 protein binds viral

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DNA and mediates its export out of the nucleus into the cytoplasm, whereas the BC1 protein also binds DNA and potentiates the cell-to-cell movement of viral DNA (Noueiry et al. 1994; Pascal et al. 1994; Rojas et al. 1998). BV1 is also a host range determinant (Ingham and Lazarowitz 1993; Ingham et al. 1995), whereas BC1 is a symptom determinant (Hou and Gilbertson, 1996; Hou et al. 2000; Ingham and Lazarowitz 1993; Ingham et al. 1995; Pascal et al. 1993; von Arnim and Stanley 1992a).

Bean dwarf mosaic virus (BDMV) is a bipartite begomovirus (Gilbertson et al. 1991a; Hidayat et al. 1993; Morales et al. 1990) that induces epinasty, dwarfing, and mosaic symptoms in common bean (*Phaseolus vulgaris* L.). However, BDMV does not infect a number of bean cultivars, including the Pinto bean cvs. Othello and UI 114 and cv. Black Turtle Soup T-39 (BTS) (Hidayat 1991; Morales et al. 1990; Wang et al. 1999). Recently, the BDMV DNA-A component (BDMV-A) was tagged with the green fluorescent protein (BDMV-GFP); this construct allowed the monitoring of viral cell-to-cell and long-distance movement (Sudarshana et al. 1998; Wang et al. 1999). Studies with BDMV-GFP established that BDMV resistance in Othello and BTS involved a block in long-distance transport and, in Othello, an HR was associated with resistance. The determinant of the HR in Othello was mapped to the BDMV DNA-B component (BDMV-B) (Wang et al. 1999).

To more precisely identify the BDMV avr determinant or determinants involved in the HR in Othello and the resistance responses in Othello and BTS, hybrid DNA-B components were engineered to have sequences of BDMV and *Bean golden yellow mosaic virus* (BGYMV), which can systemically infect these cultivars (Morales and Niessen 1988; Wang et al. 1999). Infection studies carried out with these constructs established that the BDMV BV1 is an elicitor of the HR in Othello. Furthermore, these experiments revealed that the BDMV BV1 is also an avr determinant in the resistance responses in Othello and BTS and that, in BTS, this occurs in the absence of an HR.

RESULTS

Infectivity of the HBDBG1 hybrid DNA-B component in Topcrop and Othello.

The determinant or determinants of the HR in Othello was previously mapped to BDMV-B on the basis of results obtained with infectious pseudorecombinants generated between BDMV and *Tomato mottle virus* (ToMoV) (Wang et al. 1999). However, ToMoV was considered inappropriate for further mapping of this determinant because it is poorly infectious in Topcrop (Hou et al. 1998) and does not systemically infect Othello (Wang et al. 1999). Therefore, BGYMV, which systemically infects Othello and other BDMV-resistant cultivars (Gilbertson et al. 1991b; Hidayat 1991; Morales and Niessen 1988; Wang et al. 1999), was selected for these studies.

To further map the BDMV avr determinant, a series of hybrid DNA-B components, having BDMV and BGYMV sequences, were constructed (Fig. 1). Viral DNA was introduced into epidermal cells of hypocotyls of bean seedlings (2 to 3 days old) via particle bombardment. BDMV systemically infected Topcrop and induced the HR-associated resistance in Othello, whereas BGYMV systemically infected Topcrop and

Othello. It is important to note that, by this method of inoculation, the infectivity of BGYMV in Othello was lower than that in Topcrop (Table 1). Infection studies were next conducted with the HBDBG1 hybrid, which contains the BDMV common region (CR) and untranslated sequences (UTRs) and the BGYMV BV1 and BC1 open reading frames (ORFs) (Fig. 1). When either wild-type BDMV-B or the HBDBG1 hybrid was coinoculated with BDMV-A-GFP into seedlings of Topcrop and Othello, extensive and comparable cell-to-cell movement was observed in cortical cells of hypocotyl tissues based on the detection of GFP fluorescence (compare Fig. 2A and E). However, in contrast to BDMV-B, the HBDBG1 hybrid did not elicit an HR in hypocotyl tissue of Othello when coinoculated with BDMV-A-GFP or BDMV-A (Table 1; Fig. 2D versus F). These results established that (i) BDMV-A-GFP mediated the replication of the HBDBG1 hybrid DNA-B; (ii) the BGYMV BV1 and BC1 proteins, expressed from HBDBG1, mediated the cell-to-cell movement of BDMV-A-GFP in a CP independent manner (the *GFP* gene is substituted in place of the *CP* gene in BDMV-A-GFP) (Sudarshana et al. 1998); and (iii) the BDMV HR determinant in Othello did not map to DNA-A or to the DNA-B CR or UTRs, but to the BV1 or BC1 ORFs.

The ability of HBDBG1 to mediate a systemic infection in Topcrop was next investigated. Control experiments conducted with BDMV-GFP resulted in high levels of infectivity and development of milder BDMV-like symptoms (Sudarshana et al. 1998) (Table 1). The HBDBG1 hybrid, coinoculated with BDMV-A or BDMV-A-GFP, was also highly infectious in Topcrop (approximately 90% infectivity), and both combinations induced mild BDMV-like symptoms (Table 1). Thus, in Topcrop, the BGYMV BV1 and BC1 proteins mediated CP-independent long-distance transport of BDMV-A and BDMV-A-GFP and a symptomatic systemic infection. However, in the BDMV background, the BGYMV BV1 and BC1 did not induce golden mosaic-like symptoms in Topcrop.

In Othello, wild-type BDMV-B, coinoculated with BDMV-A or BDMV-A-GFP, induced the HR in hypocotyl tissue (Fig. 2D) and failed to mediate long-distance transport of either component in inoculated seedlings. In contrast, BGYMV (Fig. 2B) or HBDBG1, coinoculated with BDMV-A or BDMV-A-GFP, did not induce the HR in hypocotyl tissue (Fig. 2F), and HBDBG1 mediated the long-distance transport of BDMV-A and BDMV-A-GFP and a symptomless systemic infection in inoculated seedlings (Table 1). These results established that the BDMV avr determinant in Othello also did not map to BDMV-A or the BDMV-B CR or UTRs, but to the BV1 or BC1 ORFs. However, the HBDBG1 hybrid was far less efficient in mediating systemic infections by BDMV-A or BDMV-A-GFP in Othello compared with Topcrop (Table 1), indicating that there are differences in the efficiency by which the BGYMV BV1 and BC1 proteins mediate long-distance movement of these components in these two cultivars.

To determine whether the BDMV HR or avr determinants are the BV1 or BC1 ORFs or proteins, three-component complementation experiments with BV1 and BC1 frameshift (*fs*) mutants were next performed. For these experiments, BDMV BV1_{fs} and BC1_{fs} mutants were used in combination with HBDBG1 BGYMV BC1_{fs} or BV1_{fs} mutants, respectively, to provide heterologous functional BV1/BC1 combinations. These DNA-B combinations were coinoculated into hypocotyl

Fig. 1. *Bean dwarf mosaic virus* (BDMV) and *Bean golden yellow mosaic virus* (BGYMV) DNA-B constructs used to identify the BDMV avirulence determinant (or determinants) and hypersensitive response in *Phaseolus vulgaris*. Solid and open boxes represent BDMV and BGYMV open reading frames (ORFs), respectively; arrows indicate the direction of transcription of the BV1 and BC1 ORFs; thick lines represent BDMV untranslated regions (UTRs) and thin lines represent BGYMV UTRs. The stop signals indicate introduced frameshift mutations. Restriction sites are indicated as follows: Ba = *Bam*HI, Bg = *Bgl*II, Bs = *Bsp*EI, C = *Cla*I, E = *Eco*RI, H = *Hind*III, N = *Nhe*I, S = *Sac*I, and X = *Xba*I; asterisks indicate introduced sites.

tissues with BDMVA-GFP. In general, the infectivity of these combinations was low, even in Topcrop (Table 2), which likely reflected the inefficient nature of these tripartite viruses. The observed high infectivity of the combination with the BDMV fs mutants (Table 2) was probably due to recombination as previously found by Noueiry (1995).

The tripartite virus having BDMVA-GFP, BGYMV BV1 (HBDBG-BC1_{fs}), and BDMV BC1 (BD-BV1_{fs}) moved efficiently cell to cell in hypocotyl tissues of Topcrop and Othello and, importantly, did not elicit an HR in Othello (Table 2). This virus induced a systemic infection and mild BDMV-like symptoms in 50% of inoculated Topcrop seedlings, whereas it induced a symptomless systemic infection in a small number (11%) of Othello seedlings (Table 2). These results suggested that the BDMV BC1 ORF/protein and the BV1 ORF/RNA transcript (provided by BD-BV1_{fs}) are not the BDMV HR/avr

determinants. In contrast, a tripartite virus having BDMVA-GFP, BDMV BV1 (BD-BC1_{fs}), and BGYMV BC1 (HBDBG-BV1_{fs}) was inefficient in cell-to-cell movement in Topcrop and Othello and did not elicit the HR in Othello hypocotyls (Table 2). This virus induced a symptomatic systemic infection in only 17% of inoculated Topcrop seedlings and did not systemically infect Othello seedlings. This suggested that, in these tripartite combinations, the BDMV BV1/BGYMV BC1 combination was less efficient than the BGYMV BV1/BDMV BC1 combination in facilitating BDMVA-GFP cell-to-cell and long-distance movement in both cultivars. The results of these experiments were consistent with the BDMV BV1 protein, rather than the ORF, being an avr determinant in Othello.

To further identify the BDMV HR/avr determinant in Othello, DNA-B hybrids having heterologous BV1-BC1 combinations provided from a single component were gener-

Table 1. Induction of the hypersensitive response (HR) and cell-to-cell or long-distance movement by *Bean golden yellow mosaic virus* (BGYMV), *Bean dwarf mosaic virus* (BDMV), and the HBDBG1 BDMV/BGYMV DNA-B hybrid in common bean (*Phaseolus vulgaris*) cvs. Othello and Topcrop

Virus ^a	Othello			Topcrop		
	HR ^b	Cell-to-cell ^c	Long-distance ^d	HR	Cell-to-cell	Long-distance
BGYMV-A + BGYMV-B	0/12 ^e	–	4/12 *	0/12	–	11/12 **
BDMV-A + BDMV-B	6/6	–	0/6	0/10	–	9/10 **
BDMVA-GFP + BDMV-B	43/54	43/54	0/12	0/31	25/31	25/31 *
BDMV-A + HBDBG1	0/10	–	9/30 +	0/10	–	9/10 *
BDMVA-GFP + HBDBG1	0/70	67/70	6/77 +	0/35	33/35	25/28 *
Gold	0/52	–	0/32	0/32	–	0/30

^a BDMVA-GFP = BDMV DNA-A (BDMV-A) tagged with the green fluorescent protein (GFP); HBDBG1 = DNA-B hybrid with the BDMV DNA-B (BDMV-B) common region and untranslated regions and the BGYMV BV1 and BC1 open reading frames; and Gold = gold particles used alone as a control.

^b HR was detected by examination of transverse hypocotyl sections, 4 to 5 days postinoculation (dpi), with fluorescence or light microscopy.

^c Cell-to-cell movement was assessed based on detection of GFP fluorescence, 4 to 5 dpi, in transverse hypocotyl sections with fluorescence microscopy; – indicates that cell-to-cell movement could not be observed when wild-type BDMV-A or BGYMV-A was inoculated.

^d Long-distance movement (systemic infection) was determined based on symptom development (indicated by asterisks) or polymerase chain reaction (PCR) analysis of total genomic DNA extracted from the third trifoliolate leaf, collected at 14 dpi. Relative degree of symptom development is indicated as follows: * = mild symptoms, ** = moderate symptoms, *** = severe symptoms, and + = no symptoms, but DNA-A and DNA-B were detected by PCR with degenerate primers (Rojas et al. 1993).

^e Number of plants in which either HR, GFP (cell-to-cell movement), or symptoms/viral DNA (long-distance movement) were detected/total number of seedlings inoculated. Numbers represent totals for three independent experiments.

Table 2. Induction of the hypersensitive response (HR) and cell-to-cell or long-distance movement by green fluorescent protein (GFP)-tagged *Bean dwarf mosaic virus* (BDMV), and tripartite combinations of BDMVA-GFP and BDMV or *Bean golden yellow mosaic virus* (BGYMV) DNA-B frameshift (fs) mutants in common bean (*Phaseolus vulgaris*) cvs. Othello and Topcrop

Component/open reading frame (ORF) combination ^a		Othello			Topcrop		
A	B	HR ^b	Cell-to-cell ^c	Long-distance ^d	HR	Cell-to-cell	Long-distance
BDMVA-GFP + BDMV-B		24/26 ^e	24/26	0/15	0/9	9/9	5/7 **
BDMVA-GFP + HBDBG1		0/18	17/18	2/13 +	0/15	14/15	5/11 *
BDMVA-GFP + HBDBGBV1 _{fs} + BD-BC1 _{fs}		0/35	2/35	0/35	0/6	2/6	1/6 *
BDMVA-GFP + HBDBGBC1 _{fs} + BD-BV1 _{fs}		0/34	30/34	2/19 +	0/9	6/9	4/8 *
BDMVA-GFP + HBDBGBV1 _{fs} + HBDBGBC1 _{fs}		0/41	6/41	0/24	0/12	8/12	1/15 *
BDMVA-GFP + BD-BV1 _{fs} + BD-BC1 _{fs}		3/12	3/12	0/6	0/12	10/12	9/12 *
Gold	...	0/30	–	0/30	0/9	0/9	0/9

^a BDMVA-GFP = BDMV DNA-A (BDMV-A) tagged with the GFP; BDB = BDMV DNA-B; HBDBG1 = DNA-B hybrid with the BDMV DNA-B (BDMV-B) common region and untranslated regions and the BGYMV BV1 and BC1 ORFs; HBDBGBV1_{fs} = HBDBG1 with an fs mutation in BGYMV BV1 ORF; HBDBGBC1_{fs} = HBDBG1 with an fs mutation in the BGYMV BC1 ORF; BD-BC1_{fs} = BDMV BC1 fs mutation; BD-BV1_{fs} = BDMV BV1 fs mutation (Noueiry et al., 1994); and Gold = gold particles used alone as a control.

^b HR was detected by examination of transverse hypocotyl sections, 4 to 5 days postinoculation (dpi), with fluorescence and light microscopy.

^c Cell-to-cell movement was assessed based on detection of GFP fluorescence, 4 to 5 dpi, in transverse hypocotyl sections with fluorescence microscopy; – indicates that cell-to-cell movement could not be observed.

^d Long-distance movement (systemic infection) was determined based on symptom development (indicated by asterisks) or by polymerase chain reaction (PCR) analysis of total genomic DNA extracted from the third trifoliolate leaf, collected at 14 dpi. Relative degree of symptom development is indicated as follows: * = mild symptoms, ** = moderate symptoms, *** = severe symptoms, + = no symptoms, but DNA-A and DNA-B were detected by PCR with degenerate primers (Rojas et al. 1993).

^e Number of plants in which either HR, GFP (cell-to-cell movement), or symptoms/viral DNA (long-distance movement) were detected/total number of seedlings inoculated. Numbers represent totals for three independent experiments.

ated. These constructs, HBDBG4 (BGYMV BV1/BDMV BC1) and HBDBG6 (BDMV BV1/BGYMV BC1), were first individually coinoculated with BDMV-A or BDMVA-GFP into seedlings of Topcrop to determine whether the BDMV or BGYMV BV1 and BC1 proteins were functionally interchangeable in a cultivar susceptible to both viruses. In contrast to the tripartite viruses, both hybrids induced systemic symptoms in all inoculated seedlings (Table 3). The symptoms induced by both hybrids were BDMV-like, but slightly milder than those induced by the HBDBG1 hybrid. These experiments established that, in Topcrop, the BDMV or BGYMV BV1 and BC1 proteins were functionally interchangeable and, in either combination, could mediate the cell-to-cell movement and long-distance transport of BDMV-A and BDMVA-GFP and a symptomatic systemic infection (Table 3).

Parallel experiments were conducted with Othello, in which the HBDBG4 (BGYMV BV1/BDMV BC1) or HBDBG6 (BDMV BV1/BGYMV BC1) were individually coinoculated into seedlings with BDMV-A or BDMVA-GFP. In these experiments, both hybrid DNA-B components mediated the efficient cell-to-cell movement of BDMVA-GFP in hypocotyl tissue (Table 3; Fig. 2I and G). The BGYMV BV1/BDMV BC1 (HBDBG4) combination did not elicit an HR (Table 3; Fig. 2J), whereas the BDMV BV1/BGYMV BC1 combination (HBDBG6) did (Table 3; Fig. 2H). These results indicated that the BDMV determinant of the HR in Othello is the BV1 ORF.

However, neither hybrid DNA-B component mediated the long-distance transport of BDMV-A (with the exception of a single symptomless plant for HBDBG4) (Table 3) or BDMVA-GFP (Table 3). Thus, the HR does not appear to be necessary for BDMV resistance in Othello, and neither the BGYMV BV1 or BC1, alone in the BDMV background, was sufficient to overcome the resistance of Othello under these inoculation conditions.

Mapping the BDMV *avr* determinant in BTS.

To further explore the relationship between the HR and BDMV resistance in common bean, these DNA-B hybrids were used to map the BDMV *avr* determinant or determinants of the non-HR resistance response in BTS. In these experiments, the HBDBG1, HBDBG4, and HBDBG6 hybrid DNA-B components were individually coinoculated with either BDMV-A or BDMVA-GFP into hypocotyl tissues of BTS and Topcrop. Control experiments confirmed that BTS was resistant to infection by BDMV, whereas BGYMV systemically infected BTS seedlings and induced vein yellowing in the primary leaves and golden mosaic in trifoliolate leaves (Table 4).

Consistent with previous results, all three hybrids induced a symptomatic systemic infection in Topcrop (Table 4). In BTS, BDMV-B and all three hybrids mediated the extensive cell-to-cell movement of BDMVA-GFP in hypocotyl tissue, and no HR was observed for any of these combinations (Table 4).

Fig. 2. Presence or absence of the hypersensitive response in common bean (cv. Othello) hypocotyls inoculated with various geminiviral constructs. Fluorescent images of transverse sections depicting green fluorescent protein (GFP) fluorescence in hypocotyl tissues inoculated with **A**, *Bean golden yellow mosaic virus* (BGYMV)-GFP; **C**, *Bean dwarf mosaic virus* (BDMV)-GFP; **E**, BDMVA-GFP+HBDBG1 (BGYMV BV1/BC1); **G**, BDMVA-GFP+HBDBG6 (BDMV BV1/BGYMV BC1); **I**, BDMVA-GFP+HBDBG4 (BDMV BC1/BGYMV BV1), or **K**, gold particles alone. **B**, **D**, **F**, **H**, **J**, and **L**, bright-field images of sections presented in **A**, **C**, **E**, **G**, **I** and **K**, respectively. All images were collected with a fluorescence microscope (Nikon Opiphot-2). Arrows without labels identify the cortex/phloem (vascular) boundary. C, E, HR, and Xy identify the location of the cortex, epidermis, hypersensitive response, and xylem, respectively. **B**, Scale bar = 250 μ m and is common to all images presented.

Hybrids HBDBG1 (BGYMV BV1/BC1) and HBDBG4 (BGYMV BV1/BDMV BC1), coinoculated with BDMV-A or BDMVA-GFP, mediated long-distance transport of both components in BTS and a symptomatic systemic infection (Table 4). Interestingly, the symptoms induced by HBDBG1 were BDMV-like, whereas those induced by HBDBG4 were BDMV-like, but included yellow mosaic in trifoliolate leaves. In contrast, hybrid HBDBG6 (BDMV BV1/BGYMV BC1), coinoculated with BDMV-A or BDMVA-GFP, did not mediate long-distance

transport of either component in BTS (based on the failure to observe disease symptoms or detect viral DNA in trifoliolate leaves of inoculated seedlings) (Table 4). These results mapped the BDMV *avr* determinant in BTS to the BV1 ORF.

DISCUSSION

In this study, we conducted experiments to map viral HR or *avr* determinants involved in a resistance response to a bipar-

Table 3. Induction of the hypersensitive response (HR) and cell-to-cell or long-distance movement by *Bean golden yellow mosaic virus* (BGYMV), *Bean dwarf mosaic virus* (BDMV), and BDMV/BGYMV DNA-B hybrids (HBDBG4 and HBDBG6) in common bean (*Phaseolus vulgaris*) cvs. Othello and Topcrop

Virus components ^a		ORFs ^b		Othello			Topcrop		
A	B	BV1	BC1	HR ^c	Cell ^d	LD ^e	HR	Cell	LD
BGYMV-A	BGYMV-B	BG	BG	0/16 ^f	–	7/21 **	0/10	–	12/12 ***
BDMV-A	BDMV-B	BD	BD	16/16	–	0/10	0/11	–	12/12 **
BDMVA-GFP	BDMV-B	BD	BD	16/16	16/16	0/10	0/12	12/12	8/8 **
BDMV-A	HBDBG4	BG	BD	0/16	–	1/30 +	0/12	–	8/8 *
BDMVA-GFP	HBDBG4	BG	BD	0/16	16/16	0/16	0/12	12/12	8/8 *
BDMV-A	HBDBG6	BD	BG	12/16	–	0/8	0/12	–	8/8 *
BDMVA-GFP	HBDBG6	BD	BG	15/16	16/16	0/8	0/12	12/12	8/8 *
Gold	...	...	...	0/16	–	0/8	0/10	–	0/12

^a BDMVA-GFP = BDMV DNA-A (BDMV-A) tagged with the green fluorescent protein (GFP); HBDBG4 = DNA-B hybrid with the BDMV common region (CR), untranslated regions (UTRs), BC1 open reading frame (ORF), and the BGYMV BV1 ORF; HBDBG6 = DNA-B hybrid with the BDMV CR, UTRs, and BV1 ORF, and the BGYMV BC1 ORF; and Gold = gold particles used alone as a control.

^b BG = BGYMV BV1 or BC1 ORF; BD = BDMV BV1 or BC1 ORF.

^c HR was detected by examination of transverse hypocotyl sections, 4 to 5 days postinoculation (dpi), with fluorescence and light microscopy.

^d Cell = cell-to-cell movement was assessed based on detection of GFP fluorescence, 4 to 5 dpi, in transverse hypocotyl sections with fluorescence microscopy; – indicates that cell-to-cell movement could not be observed when wild-type BDMV-A or BGYMV-A was inoculated.

^e LD = long-distance movement (systemic infection) was determined based on symptom development (indicated by asterisks) or by polymerase chain reaction (PCR) analysis of total genomic DNA extracted from the third trifoliolate leaf, collected at 14 dpi. Relative degree of symptom development is as follows: * = mild symptoms, ** = moderate symptoms, *** = severe symptoms, + = no symptoms, but DNA-A and DNA-B were detected by PCR with degenerate primers (Rojas et al. 1993).

^f 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 independent experiments.

Table 4. Induction of the hypersensitive response (HR) and cell-to-cell or long-distance movement by *Bean golden yellow mosaic virus* (BGYMV), *Bean dwarf mosaic virus* (BDMV), and BDMV/BGYMV DNA-B hybrids (HBDBG4 and HBDBG6) in common bean (*Phaseolus vulgaris*) cvs. Black Turtle Soup T39 and Topcrop

Virus components ^a		ORFs ^b		Black Turtle Soup T-39			Topcrop		
A	B	BV1	BC1	HR ^c	Cell ^d	LD ^e	HR	Cell	LD
BGYMV-A	BGYMV-B	BG	BG	0/12 ^f	–	9/12 **	0/10	–	12/12 ***
BDMV-A	BDMV-B	BD	BD	0/12	–	0/10	0/11	–	12/12 ***
BDMVA-GFP	BDMV-B	BD	BD	0/12	12/12	0/4	0/12	12/12	8/8 **
BDMV-A	HBDBG1	BG	BG	0/12	–	8/12 *+	0/12	–	8/8 **
BDMVA-GFP	HBDBG1	BG	BG	0/12	12/12	5/10 *	0/12	12/12	8/8 *
BDMV-A	HBDBG4	BG	BD	0/12	–	8/12 *+	0/12	–	8/8 **
BDMVA-GFP	HBDBG4	BG	BD	0/12	12/12	6/12 *	0/12	12/12	8/8 *
BDMV-A	HBDBG6	BD	BG	0/12	–	0/12	0/12	–	8/8 **
BDMVA-GFP	HBDBG6	BD	BG	0/12	12/12	0/12	0/12	12/12	8/8 *
Gold	...	...	...	0/12	–	0/10	0/10	–	0/12

^a BDMVA-GFP = BDMV DNA-A (BDMV-A) tagged with the green fluorescent protein (GFP); HBDBG4 = DNA-B hybrid with the BDMV common region (CR), untranslated regions (UTRs), BC1 open reading frame (ORF), and the BGYMV BV1 ORF; HBDBG6 = DNA-B hybrid with the BDMV CR, UTRs, and BV1 ORF, and the BGYMV BC1 ORF; and Gold = gold particles used alone as a control.

^b BG = BGYMV BV1 or BC1 ORF; BD = BDMV BV1 or BC1 ORF.

^c HR was detected by examination of transverse hypocotyl sections, 4 to 5 days postinoculation (dpi), with fluorescence and light microscopy.

^d Cell = cell-to-cell movement was assessed based on detection of GFP fluorescence, 4 to 5 dpi, in transverse hypocotyl sections with fluorescence microscopy; – indicates that cell-to-cell movement could not be observed when wild-type BDMV-A or BGYMV-A was inoculated.

^e LD = long-distance movement (systemic infection) was determined based on symptom development (indicated by asterisks) or by polymerase chain reaction (PCR) analysis of total genomic DNA extracted from the third trifoliolate leaf, collected at 14 dpi. Relative degree of symptom development is as follows: * = mild symptoms, ** = moderate symptoms, *** = severe symptoms, + = no symptoms, but DNA-A and DNA-B were detected by PCR with degenerate primers (Rojas et al. 1993).

^f 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 independent experiments.

tite begomovirus. Previously, we established that (i) BDMV resistance in Othello and BTS involved a block in long-distance movement and (ii) the BDMV HR determinant in Othello mapped to BDMV-B (Wang et al. 1999). To further map the BDMV HR or avr determinants, we used BGYMV, which systemically infects Othello and BTS and does not elicit the HR in Othello. Here, we hypothesized that a specific BDMV sequence or sequences or encoded protein or proteins would be the avr determinants, whereas the homologous factor or factors in BGYMV act as virulence factors. Using a series of hybrid DNA-B components, together with markers for cell-to-cell movement (GFP-tagged BDMV-A) and resistance (i.e., HR development in Othello or systemic infection in Othello and BTS), the BV1 ORF/protein was identified as a viral determinant of the HR (in Othello) and avirulence (in BTS).

Studies conducted with the HBDBG1 (BGYMV BV1/BC1 in the BDMV DNA-B background) and BDMVA-GFP in bean hypocotyl tissues established that the BDMV CR or UTR sequences allowed for replication of this hybrid DNA-B component mediated by the BDMV replication-associated protein (Rep) protein. The capacity of HBDBG1 to mediate a systemic infection in Topcrop, when coinoculated with either BDMV-A or BDMVA-GFP, further established that (i) the BGYMV MPs recognize and interact with BDMV-A to mediate its cell-to-cell and long-distance transport and (ii) that CP (i.e., virions) is not required for movement or development of disease symptoms. These findings are consistent with our previous results showing that BGYMV MPs can mediate cell-to-cell movement of BDMVA-GFP (Sudarshana et al. 1998) and the nonspecific DNA binding properties of the BV1 and BC1 (Rojas et al. 1998). These results are also consistent with previous reports showing complementation of function among MPs of Western Hemisphere begomoviruses (Frischmuth et al. 1993; Hofer et al. 1997; Hou and Gilbertson 1996; Hou et al. 1998; Petty et al. 1995; Schaffer et al. 1995; Sung and Coutts 1995). It is worth noting that this complementation may not extend to the more distantly related Old World begomoviruses (von Arnim and Stanley 1992b).

In the BDMV-resistant Othello and BTS, the HBDBG1 hybrid did not elicit the HR (Othello) and mediated long-distance transport and systemic infection by BDMVA and BDMVA-GFP in both cultivars. These results were consistent with the BDMV-B CR and UTR sequences not being determinants of HR or avr. However, compared with BGYMV, the incidence of systemic infection was low (particularly in Othello) and plants infected with HBDBG1 were symptomless (in Othello) or showed attenuated symptoms (in BTS), indicating a deficiency in the capacity of the hybrid virus to infect these BDMV-resistant cultivars. Studies with the tripartite combinations in Othello implicated the BDMV BV1 as the BDMV avr determinant, but these studies were not conclusive due to the poor level of infectivity of these tripartite viruses (even in Topcrop).

To resolve this problem, the HBDBG4 (BGYMV BV1/BDMV BC1) and HBDBG6 (BDMV BV1/BGYMV BC1) hybrids were constructed. Both constructs mediated symptomatic systemic infections by BDMV-A and BDMVA-GFP in Topcrop, which established that the BDMV/BGYMV MPs are functionally interchangeable in a host susceptible to both viruses. These results also suggested that the BDMV/BGYMV MPs may interact with each other as pro-

posed for the *Squash leaf curl virus* (SLCV) BV1 and BC1 (Sanderfoot and Lazarowitz 1995). However, in a similar study with BGYMV (Guatemala isolate) and *Tomato golden mosaic virus* (TGMV) in Topcrop, a TGMV BV1/BGYMV BC1 hybrid was infectious, whereas a BGYMV BV1/TGMV BC1 hybrid was not (Schaffer et al. 1995). Thus, the BV1 and BC1 proteins of Western Hemisphere begomoviruses are not necessarily functionally interchangeable. The ability to functionally interchange the BDMV or BGYMV BV1 or BC1 proteins likely reflects the fact that both viruses are adapted to common bean and, thus, that these proteins efficiently interact with appropriate host factors. It was also interesting that none of the HBDBG hybrids, in combination with BDMV-A or BDMVA-GFP, induced golden mosaic symptoms. This indicates that BGYMV factors in addition to BV1 and BC1 (i.e., DNA-B CR, UTRs, DNA-A sequences, and gene products in various combinations) are involved in the induction of this symptom phenotype. This is consistent with findings that the TGMV or BGYMV hybrids also did not induce golden mosaic symptoms in Topcrop (Schaffer et al. 1995), and the concept that symptom induction by bipartite geminiviruses is a complex phenomenon involving multiple viral genes (Hou et al. 1998).

As with the HBDBG1 hybrid, the HBDBG4 and HBDBG6 hybrids were less infectious or caused attenuated symptoms in Othello and BTS compared with Topcrop. In BTS, HBDBG4 was infectious and induced attenuated symptoms (compared with BGYMV), whereas HBDBG6 failed to mediate a systemic infection by BDMV-A or BDMVA-GFP; these results mapped the BDMV avr determinant to the BV1 ORF. The differences in the responses of these common bean cultivars to infection mediated by the HBDBG hybrids may be due to the higher levels of resistance to BGYMV infection in common bean genotypes of Mesoamerican origin, such as Othello and BTS, compared with genotypes of Andean origin, such as Topcrop (Garrido-Ramirez et al. *in press*; Gilbertson et al. 1991b; Morales and Niessen 1988). For example, accumulation of BGYMV (Guatemalan isolate) in equivalent leaves of sap-inoculated plants was considerably reduced in Mesoamerican cv. Pinto 114 compared with Topcrop (Gilbertson et al. 1991b). The mechanism of this resistance is not known, but could be due to interference with viral replication or movement. In terms of the HBDBG hybrids, less efficient replication of BDMV-A and BDMVA-GFP in Othello and BTS compared with Topcrop would have a negative impact on infectivity. Another possibility is that the capacity of the hybrid viruses to mediate long-distance transport of BDMV-A and BDMVA-GFP might be influenced by the growth habit of the cultivar. Thus, the HBDBG hybrids may not efficiently mediate long-distance transport of BDMV-A and BDMVA-GFP in the rapid-growing indeterminate Othello; whereas, in Topcrop, a determinant bush type, these hybrids may be more efficient in mediating long-distance transport and systemic infection. In terms of the HBDBG4 and HBDBG6 hybrids, inefficiencies in the interactions between the BDMV and BGYMV MPs also could negatively impact long-distance transport in Othello and BTS. Regardless of the mechanism, these results suggest that BGYMV has additional virulence factors, other than BV1 and BC1, that allow it to infect Othello and BTS, and that equivalent BDMV factors may interact less efficiently with host factors in these cultivars.

The finding that the hybrid having the BDMV BV1 (HBDBG6) elicited the HR, whereas that with the BGYMV BV1 (HBDBG4) did not, mapped the determinant of the HR to the BV1 ORF. Results with the tripartite combinations, in which viruses having the frameshifted BV1 ORF failed to elicit the HR, are consistent with the hypothesis that the BV1 protein, rather than the ORF or mRNA, is the HR elicitor. The fact that the HBDBG4 hybrid (BGYMV BV1/BDMV BC1), coinoculated with BDMVA or BDMVA-GFP, failed to mediate a systemic infection in Othello, despite not eliciting an HR in inoculated hypocotyl tissue, suggests that the HR is not necessary for BDMV resistance. Indeed, there are other examples where the HR has been uncoupled from resistance, such as the CMV-cowpea interaction, where an inhibition (resistance) response was shown to be independent of the HR (Kim and Palukaitis 1997). The fact that the HR does not appear to be necessary for BDMV resistance in Othello also raises the question of whether the genes involved in BDMV resistance in Othello and BTS are the same or different. The answer to this question will have to be determined by genetic studies on the host side.

Finally, insight into the manner by which the BDMV BV1 acts as an HR/avr determinant can be gained from an analysis of the role played by this protein in the viral life cycle. The BV1 protein is a nuclear shuttle, and facilitates the export of viral ss- and dsDNA from the nucleoplasm to the cytoplasm, where the BC1 protein mediates the cell-to-cell movement of the DNA through plasmodesmata (Noueiry et al. 1994; Rojas et al. 1998). The role of the BDMV BV1 protein as an HR/avr determinant may involve active recognition at the nuclear pore complex by an endogenous receptor. This may occur within the nucleoplasm or the cytoplasm. Here, it is interesting to note that certain bacterial avr factors are also targeted to the nucleus, where recognition and initiation of the HR and host defense response may occur (Gabriel 1997). An alternative explanation is that an inefficient interaction of the BDMV BV1 with host factors in Othello and BTS allows for activation of host defense responses and a restriction of long-distance movement. In contrast, the BGYMV BV1 protein is either not recognized in Othello and BTS, is recognized but suppresses the host defense response, or simply interacts more efficiently with host factors such that it allows for BGYMV to escape the host defense response. Irrespective of the mechanism and site of action, the BDMV BV1 represents an additional class of viral HR/avr determinants (Culver 1996). Further insight into the mechanism or mechanisms of BDMV resistance in Othello and BTS will require genetic analyses or the cloning and characterization of the host genes involved in the resistance.

MATERIALS AND METHODS

BDMV and BGYMV infectious clones and constructs.

Infectious 1.5-mer clones of BDMV-A (pBDA1.5), BDMV-B (pBDB1.5), BGYMV (Mexican strain, BGMV-MX; hereafter referred to as BGYMV) DNA-A (BGMV-A; pBGMXA1.5), BGYMV DNA-B (BGMV-B; pBGMXB1.5), and BDMVA-GFP (pBDAGFP) have been previously described (Garrido-Ramirez and Gilbertson 1996; Hou et al. 1998; Sudarshana et al. 1998). BGMVA-GFP (pBGAGFP) was constructed using the approach described in Sudarshana

et al. (1998). BDMV BV1 and BC1 fs mutants have also been described (Noueiry et al. 1994). An infectious 1.5-mer hybrid DNA-B having the BDMV CR and UTR sequences and BGYMV BV1 and BC1 ORFs was engineered. For this purpose, oligonucleotide primer pairs (OBDBv2096E and OBDBc563N, and OBDBv2237N and OBDBc496H; primer sequences available upon request) and Ultma (Perkin-Elmer, Foster City, CA, U.S.A.) were used in polymerase chain reaction (PCR) to amplify two fragments, each containing the BDMV CR and UTRs from pBDB1.5, and to introduce appropriate restriction sites into these fragments. PCR parameters used for all steps of the plasmid constructions were as follows: one cycle of 94°C, 4 min; hot start at 80°C, 2 min; 45°C, 1 min; and 72°C, 2 min; followed by nine cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 3 min; and a final extension cycle of 72°C, 10 min. The PCR-amplified BDMV-B fragments were cloned with the Zero blunt cloning system (Invitrogen, Carlsbad, CA, U.S.A.) to generate the recombinant plasmids pBD1 and pBD2, respectively. The BDMV DNA-B CR and UTRs were excised from pBD1 as an *NheI/XhoI* fragment, and subcloned into pBD2 to generate pBD3, which contained two copies of the CR and UTRs.

Oligonucleotide primers OBGBv505N and OBGBc2180N were used to amplify a 1,600-bp BGYMV DNA-B fragment from pBGMXB1.5. This fragment contained the BGYMV BV1 and BC1 ORFs with introduced *NheI* restriction sites at the beginning of each ORF. This PCR-amplified fragment was cloned using the Zero blunt system to generate pBG1, and the nucleotide sequence from each of the introduced *NheI* sites up to the *ClaI* site in BV1 and the *BglII* site in BC1 verified. The 1.1-kb *ClaI/BglII* fragment of pBG1 was then replaced with the equivalent fragment from pBGMXB1.5 (wild-type BGYMV DNA-B) to generate pBG1A. The *NheI* fragment, containing the BGYMV BV1 and BC1 ORFs, was excised from pBG1A and cloned into *NheI*-digested pBD3 to generate pHDBG1, after which the sequences around the *NheI* sites were verified. This 1.5-mer construct contained two BDMV CRs and UTRs and the BGYMV BV1 and BC1 ORFs (Fig. 1).

BV1 and BC1 fs mutants in pHDBG1.

Oligonucleotide primers OBDBGBV1mut and OBV1c1044 and PCR were used to amplify a 500-bp BGYMV BV1 fragment from pBGMXB2, and to introduce *NheI* and *HindIII* restriction sites into the 5' and 3' ends, respectively, and an fs mutation into the 5' end of BV1. The PCR-amplified BV1 fragment was cloned with the Zero blunt system to generate pBGBV1_{fs}. A 1,400-bp *XhoI/ClaI* fragment from pHDBG1 was subcloned into pKS+ (Stratagene, La Jolla, CA, U.S.A.) to generate pBGBV1. This plasmid was digested with *NheI* and *ClaI*, and the 200-bp *NheI/ClaI* BV1 fragment was exchanged with the corresponding fragment from pBGBV1_{fs} to produce pBGBV1_{fs}2. The 1,400-bp *XhoI/ClaI* fragment from pBGBV1_{fs}2 was excised and exchanged with the corresponding fragment in pHDBG1 to generate pHDBG1_{fs}. This 1.5-mer construct contained two BDMV-B CRs and UTRs, and the BGYMV BV1_{fs} and BC1 ORFs (Fig. 1).

To generate a BC1_{fs} mutant, oligonucleotide primers OBDBGBC1mut and OBC1v1426 and PCR were used to amplify an 800-bp BGYMV BC1 fragment from pBGMXB2 and to introduce *NheI* and *BamHI* restriction sites into the 5' and 3' ends, respectively, and an fs mutation into the 5' end of

BC1. The PCR-amplified BC1 fragment was cloned with the Zero blunt system to generate pBGBC1_{fs}. A 1,200-bp *Hind*III fragment from pHDBG1 was subcloned into pKS+ to generate pBGBC1. This plasmid was digested with *Sac*I and *Nhe*I (Fig. 1), and the 300-bp *Sac*I/*Nhe*I BC1 fragment was exchanged with the corresponding fragment from pBGBC1_{fs} to produce pBGBC1_{fs}2. The 1,200-bp *Hind*III fragment from pBGBC1_{fs}2 was excised and exchanged with the corresponding fragment in pHDBG1 to generate pHDBGBC1_{fs}. This 1.5-mer construct contained two BDMV-B CRs and UTRs, and the BGYMV BV1 and BC1_{fs} ORFs (Fig. 1).

Generation of hybrids containing BGYMV BV1/BDMV BC1 and BDMV BV1/BGYMV BC1.

The recombinant plasmid pHDBG1 was digested with *Eco*RI to release a 3.5-kb fragment that contained the BDMV CR and UTR sequences and the BGYMV BV1 and BC1 ORFs. This 3.5-kb fragment was subcloned into pALTER (Promega Corp., Madison, WI, U.S.A.) to generate pHDBG2, in order to facilitate site-directed mutagenesis. Primer OBDBc1309X was used with the Altered Sites II Mutagenesis System (Promega Corp.) to introduce an *Xba*I site into the 3' end of the BGYMV BV1 ORF in pHDBG2 to generate pHDBG3. Primers OBDBv1297X and OBDBc2248S and PCR were used to amplify a 900-bp fragment from pBDB1.5 that contained the 3' end of the BV1 ORF and the entire BC1 ORF; these primers also introduced an *Xba*I site into the 3' end of the BDMV BV1 ORF and a *Spe*I site into the 5' end of the BC1 ORF. This PCR-amplified fragment was cloned with the Zero blunt system to generate pBDBXS, and the sequence verified. The *Xba*I/*Spe*I BDMV BV1/BC1 fragment from pBDBXS was excised and cloned into *Xba*I/*Nhe*I-digested and dephosphorylated pHDBG3 to generate pHDBG4, which contained the BGYMV BV1 ORF and the remainder of the BDMV-B sequence (Fig. 1).

Primers OBDBGH2 and OBDBGH3 were used with the Altered Sites II Mutagenesis System to introduce *Bsp*EI and *Xba*I sites into the 5' and 3' ends of the BGYMV BV1 ORF, respectively, in pHDBG2 to generate pHDBG5. Primers OBDBv554B and OBDBc1309X and PCR were used to amplify a 700-bp fragment from pBDB1.5 that contained the BDMV BV1 ORF with *Bsp*EI and *Xba*I sites in the 5' and 3' ends, respectively. This PCR-amplified fragment was cloned with the Zero blunt system to generate pBDBBX, and the sequence of the cloned product verified. The *Bsp*EI/*Xba*I BDMV BV1 fragment from pBDBBX was excised and cloned into *Bsp*EI/*Xba*I-digested and dephosphorylated pHDBG5 to generate pHDBG6, which contained the BGYMV BC1 ORF and the remainder of the BDMV-B sequence (Fig. 1).

Inoculation of *Phaseolus vulgaris* seedlings by particle bombardment.

Seeds of *Phaseolus vulgaris* L. (common bean cvs. Topcrop, Othello, and BTS) were germinated at room temperature on moist filter paper in plastic petri dishes. Bean seedlings were inoculated by particle bombardment as previously described (Papomatas et al. 1994; Sudarshana et al. 1998). Seedlings were coinoculated with each of the DNA-B constructs and either BDMV-A or BDMV-A-GFP and grown as previously described (Sudarshana et al. 1998).

Detection of GFP expression and the HR.

Expression of GFP, at the whole plant level, was detected with a handheld long-wavelength UV lamp (UVP, Upland, CA, U.S.A.). To detect the HR, seedlings were carefully removed from the soil approximately 5 days postinoculation (dpi), and freehand transverse-sections of hypocotyl tissue were prepared and examined with light and fluorescent microscopy, as previously described (Sudarshana et al. 1998; Wang et al. 1999). To assess systemic infection, seedlings were grown for 14 to 21 days, and then observed for disease symptom development and the presence of GFP in newly emerged leaves. In some cases, symptomless plants were tested for systemic infection by PCR with degenerate primers.

DNA extraction and PCR detection of geminiviral DNA.

DNA extraction and PCR parameters used to amplify geminivirus DNA fragments have been previously described (Gilbertson et al. 1991c; Rojas et al. 1993). DNA extracts were prepared from the first trifoliate leaves (approximately 15 dpi). Viral DNA fragments were amplified using the degenerate PCR primer pairs PAC1v1978/PAV1c970, PGFPv67/PGFPc94, or PBC1v2040/PBV1c970 (Garrido-Ramirez and Gilbertson 1998; Rojas et al. 1993; Sudarshana et al. 1998). Aliquots of PCR reactions were examined by electrophoresis in 0.7% agarose gels in 0.5 Tris-borate-EDTA buffer.

DNA sequence analyses.

DNA sequencing was conducted by the University of California-Davis Sequencing Facility or Davis Sequencing (Davis) with a Perkin-Elmer/ABI 377 automated DNA sequencer using either dRhodamine terminator or Big Dye terminator chemistry (Perkin-Elmer/ABI, Foster City, CA, U.S.A.). DNA sequence analyses were performed using the GCG sequence analysis software package (version 9.1; Genetics Computer Group, Madison, WI, U.S.A.).

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