Analysis of Cell-to-Cell and Long-Distance Movement of a Bean Dwarf Mosaic Geminivirus–Green Fluorescent Protein Reporter in Host and Nonhost Species: Identification of Sites of Resistance

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A bean dwarf mosaic geminivirus–green fluorescent protein (BDMV-GFP) reporter system was employed to analyze the viral infection process in host and nonhost species. Five classes of BDMV/host interaction were identified: (i) adapted hosts (susceptible *Phaseolus vulgaris* cultivars) permissive for systemic infection; (ii) adapted hosts (resistant *P. vulgaris* cv. Othello) displaying the development of a hypersensitive response (HR) associated with resistance to systemic infection; (iii) adapted (resistant *P. vulgaris* cv. Black Turtle Soup T-39) and nonadapted (*Vigna unguiculata*) hosts in which cell-to-cell, but not long-distance, movement was permitted; (iv) nonadapted hosts (*Glycine max*) in which systemic infection was coat protein-dependent; and (v) nonhosts (*Cucurbita maxima*, *Gossypium barbadense*, and *Zea mays*) in which the virus was confined to inoculated cells. Confocal laser scanning microscopy, fluorescence microscopy, and histochemical analyses were used to identify the cellular distribution of BDMV-GFP and the host response to viral infection. With this approach, the HR in *P. vulgaris* cv. Othello was visualized within cells of the epidermis, cortex, and phloem of inoculated hypocotyls. Infection studies performed with four begomoviruses and infectious BDMV/tomato mottle geminivirus pseudorecombinants revealed that the HR determinant(s) mapped to the BDMV DNA-B component.

Additional keywords: BGMV, TLCrV, ToMoV.

Plant virus systemic infection is dependent upon the compatible interaction(s) between viral and host factors involved in (i) virus replication, (ii) cell-to-cell movement, and (iii) virus entry into and egress from the phloem long-distance translocation system (Dawson and Hilf 1992; Carrington et al. 1996; Gilbertson and Lucas 1996; Nelson and van Bel 1997). Dawson and Hilf (1992) recognized seven kinds of virus-host

interactions, ranging from complete plant susceptibility to true immunity. In situations where a virus has the capacity to infect plants, this interaction can be subdivided into adapted and nonadapted hosts. Here, adapted hosts have been defined as those in which a virus replicates efficiently and induces symptoms (generally, the natural host of the virus), whereas nonadapted hosts are those for which the virus has low infectivity and induces mild or no symptoms (Hou et al. 1998; Petty et al. 1995). Incompatibility between virus and host factors associated with any (or all) of the steps of the viral infection could impede this process and result in either limited or complete resistance to systemic infection. Relatively little information is currently available concerning the underlying mechanisms (viral and host factors) involved in host resistance to viral infection.

The Geminiviridae family comprises single-stranded DNA viruses that have distinctive, twinned icosahedral virions and are vectored by either leafhoppers, treehoppers, or whiteflies. These viruses are responsible for numerous diseases of economically important crops, such as beans, cassava, cotton, maize, pepper, and tomato. Whitefly-transmitted geminiviruses cause major losses in tropical and subtropical regions (Bock 1982; Brown and Bird 1992; Polston and Anderson 1997), and disease control currently depends upon extensive use of insecticides (Polston and Anderson 1997). A solution to this problem lies in the identification and utilization of the host factors (resistance) that interfere with the geminivirus systemic infection process. The magnitude of this challenge is reflected in the tremendous genetic diversity of this virus group. In contrast to the situation for viruses displaying wide host ranges (e.g., cucumber mosaic cucumovirus [CMV]; Palukaitis et al. 1992), there are numerous whitefly-transmitted geminivirus species that have narrow and often overlapping host ranges (Briddon and Markham 1995). Additionally, the pivotal role of the whitefly vector in driving geminiviral evolution (i.e., via pseudorecombination and recombination; Hou and Gilbertson 1996) further complicates the development of durable geminivirus-resistant plants.

Bean dwarf mosaic geminivirus (BDMV) represents a model system with which to dissect the molecular basis for a range of geminivirus-host interactions. BDMV infects common bean (*Phaseolus vulgaris* L.), in which it causes stunted

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plant growth as well as mosaic and mottling on systemically infected leaves (Morales et al. 1990). Differences in the ability of BDMV to systemically infect bean cultivars have been established (Hidayat 1991; Morales et al. 1990). Furthermore, BDMV is able to infect some species, such as soybean (*Glycine max* L.), without inducing symptoms (Gilbertson et al. 1991), whereas other plant species appear to act as non-hosts (Morales et al. 1990). On the virus side, infectious BDMV clones (Hidayat et al. 1993) have been engineered such that the coat protein (CP) gene (*CP*) has been replaced with a gene encoding a modified form of the green fluorescent protein (GFP) gene (*mGFP4*). Wild-type and GFP-tagged virus (BDMV-GFP) have been used to investigate the viral determinants for replication, cell-to-cell movement, and systemic movement (Noueiry et al. 1994; Sudarshana et al. 1998).

In the present study, we utilized the BDMV-GFP system to explore the basis of the BDMV-plant interaction for both host and nonhost species. Based on these studies, five distinct classes of BDMV-host interaction were characterized, including a resistance phenotype that was correlated with a hypersensitive reaction (HR). The viral determinant for induction of this HR was mapped to the BDMV DNA-B component.

RESULTS

Phaseolus vulgaris cv. Othello is resistant to BDMV.

Previous studies, in which whitefly and sap transmissions were employed, indicated that a number of *P. vulgaris* Pinto cultivars are resistant to BDMV infection (Hidayat 1991; Morales et al. 1990). To further investigate this disease resistance phenotype, bean seedlings of cvs. Topcrop and Othello, a Pinto bean cultivar, were coinoculated with 1.5-mers of BDMV-mGFP4 and BDMV-B (hereafter referred to as BDMV-GFP) or 1.5-mers of BDMV-A and BDMV-B (wild-type BDMV), by particle bombardment (Paplomatas et al. 1994; Sudarshana et al. 1998). As shown in Figure 1A and B, at 4 days post inoculation (dpi), GFP fluorescence (hereafter referred to as GFP) was detected in the inoculated hypocotyls of cvs. Topcrop and Othello, respectively, indicating that the virus replicated and moved cell to cell in epidermal cells of both cultivars. Analysis of cv. Topcrop primary leaves (4 dpi) revealed that BDMV-GFP had entered and replicated in cells associated with the higher-ordered veins; in Figure 1C, the presence of GFP is clearly visible in the second- through fourth-ordered veins. In contrast, GFP was not detected in equivalent primary leaves of cv. Othello (Fig. 1D). By 8 dpi, GFP was detectable in all vein orders within the primary leaves of cv. Topcrop (Fig. 1E), but not in equivalent leaves of cv. Othello (Fig. 1F). These results reflect observations made of over 100 inoculated seedlings of each bean cultivar, and establish that systemic infection of BDMV-GFP was blocked, or severely retarded, in cv. Othello.

Disease phenotypes in these cultivars, inoculated with either BDMV-GFP or wild-type BDMV, were consistent with the results obtained from BDMV-GFP-based experiments. Inoculated cv. Topcrop plants developed symptoms of stunted growth, with distorted and epinastic leaves, whereas equivalent cv. Othello plants were symptomless. Together, these results establish that *P. vulgaris* cv. Othello is resistant to infection by BDMV.

Site(s) of host resistance identified with BDMV-GFP.

To identify the explicit site at which BDMV infection was impaired in cv. Othello, two experimental approaches were employed: monitoring of GFP to follow the cell-to-cell and long-distance movement of BDMV; and detection of viral DNA by PCR (polymerase chain reaction), to confirm the tissue-specific distribution as revealed by GFP analysis. As shown in Figure 2, by 24 h post inoculation (hpi), BDMV-GFP had replicated in and moved cell to cell to a similar extent within the epidermis of cvs. Othello (Fig. 2A) and Topcrop (Fig. 2C) hypocotyls. Transverse sections taken 48 hpi revealed that BDMV-GFP had advanced through the cortex and into the phloem of both cvs. Othello (Fig. 2B) and Topcrop (Fig. 2D). Analysis of this early infection phase indicated that BDMV-GFP replication and cell-to-cell movement in infected hypocotyl tissues of these two common bean cultivars were similar.

In the ensuing period (3 to 4 dpi), during which time BDMV-GFP moved into the phloem and was detected in primary leaves of cv. Topcrop (Fig. 1C), a clear difference could be discerned between infected hypocotyl tissues of cvs. Othello (Fig. 2E) and Topcrop (Fig. 2G). Infected cortical cells of cv. Othello displayed early signs of collapse and degradation, whereas those of cv. Topcrop remained intact (Fig. 2E vs G). At this same time point, the phloem tissue of infected cv. Othello hypocotyls displayed significant differences in terms of the relative level of GFP and general cellular integrity, compared with the situation observed in cv. Topcrop hypocotyls. By 8 dpi, these differences between the two cultivars had become striking in their extent. The infected hypocotyl tissues of cv. Othello showed an advanced stage of collapse and cellular degradation, which was accompanied by an almost complete absence of GFP in these cortical and phloem tissues. In contrast, infected cv. Topcrop hypocotyl tissues retained structural integrity and a high level of GFP (Fig. 2F vs H). Furthermore, as illustrated in Figure 2F, the yellow autofluorescent signal that was detectable in infected cv. Othello hypocotyl tissue at 4 dpi (Fig. 2E) had intensified. In cv. Topcrop, this autofluorescence was not present at 4 dpi and, by 8 dpi, was present only to a limited extent (Fig. 2H).

Examination of sequential transverse sections, taken above (acropetal direction) the initial infection site (8 dpi), indicated the presence of limited GFP (in phloem cells) within a strong background of yellow autofluorescence in both the phloem and xylem in cv. Othello (Fig. 2I; note also the weak autofluorescence in cortical cell walls). Similar sections taken from cv. Topcrop revealed the presence of strong GFP in phloem cells and the absence of yellow autofluorescence (Fig. 2K). In infected cv. Othello plants (8 dpi), GFP was restricted to a region extending from the inoculation site to a maximum distance of 4.5 cm in the acropetal direction. Interestingly, in a small number of infected cv. Othello seedlings, GFP was detected within some root tips (data not shown). In contrast, in infected cv. Topcrop plants, GFP was detected continuously from the inoculated tissue, through the cotyledonary node, to the shoot apex, as previously reported (Sudarshana et al. 1998). These experiments established that BDMV-GFP was competent to move long distance within cv. Topcrop, whereas in cv. Othello this process was severely impaired. This conclusion gained support from analysis of sections taken 0.5 cm below the shoot apex, where GFP was found to be absent from

cv. Othello tissues (Fig. 2J), but was readily detectable in cv. Topcrop phloem tissue (Fig. 2L).

BDMV-induced HR in *P. vulgaris* cv. Othello.

A comparison between BDMV-GFP infection in cvs. Topcrop and Othello revealed (i) the site at which BDMV systemic infection was blocked in cv. Othello, and (ii) the nature of the tissue response to infection. To further characterize this cellular response in cv. Othello, a series of histochemical analyses was next performed. Figure 3A and B illustrate the extent to which BDMV-GFP-infected hypocotyl tissue (4 dpi) had undergone cellular collapse, reflecting the onset of necro-

sis. In contrast, no such necrotic response was observed in the equivalent tissue of cv. Topcrop (Fig. 3G and H). The response within the phloem tissue, in cv. Othello, was particularly noteworthy, in that the GFP signal was barely detectable (compared with the cortical tissue), and cell collapse and necrosis were prominent in the region from the cortex-phloem boundary on into the cambial layer (Fig. 3A vs G, B vs H).

Analysis of BDMV-GFP-infected (4 dpi) and control hypocotyl tissues, stained for phenolic compounds, provided further evidence in support of the hypothesis that an HR was induced in cv. Othello, but not in cv. Topcrop. As illustrated in Figure 3C, a strong phenolic reaction was detected within the

Fig. 1. Analysis of bean dwarf mosaic geminivirus–green fluorescent protein (BDMV-GFP) infection in *Phaseolus vulgaris* cvs. Topcrop (susceptible) and Othello (resistant). **A** and **B**, Fluorescent images of epidermal cells of the hypocotyls of cvs. **(A)** Topcrop and **(B)** Othello bombarded with gold particles coated with BDMVA-mGFP4 and BDMV-B. **C–F**, Fluorescent images taken in the plane of the vascular tissue within primary leaves of cv. Topcrop **(C, 4 days post inoculation [dpi]; E, 8 dpi)** and cv. Othello **(D, 4 dpi; F, 8 dpi)**. Vein orders indicated by arrows. Scale bar in **A** = 200 μ m and is common to all images presented.

phloem of infected cv. Othello, whereas equivalent uninfected tissues were devoid of any positive staining reaction (Fig. 3D). Phenolic staining in equivalent cv. Topcrop hypocotyl tissue was either not detected, or was present at low levels, within the

phloem of infected or control plants (Fig. 3I vs J). Similar results were obtained from tissues stained for suberin, in that positive staining was detected only at the cortex-phloem boundary of BDMV-GFP-infected cv. Othello (data not shown).

Fig. 2. Identification of the site at which the infection process of bean dwarf mosaic geminivirus–green fluorescent protein (BDMV-GFP) is blocked in the resistant *Phaseolus vulgaris* cv. Othello. **A** and **C**, Fluorescent images of epidermal cells of hypocotyls of *P. vulgaris* cvs. **(A)** Othello and **(C)** Topcrop seedlings bombarded with gold particles coated with BDMVA-mGFP4 and BDMV-B (24 h post inoculation [hpi]). **B** and **D**, Transverse sections depicting GFP fluorescence in BDMV-GFP-infected hypocotyl tissues of cvs. **(B)** Othello and **(D)** Topcrop at 48 hpi. **E–H**, Experimental details as in **B** and **D**, except that tissues were analyzed at either 4 days post inoculation (dpi) **(E, cv. Othello; G, cv. Topcrop)** or 8 dpi **(F, cv. Othello; H, cv. Topcrop)**. **I** and **K**, Transverse sections taken 1.5 cm above inoculation site imaged for cvs. **(F)** Othello and **(H)** Topcrop. **J** and **L**, Transverse sections of cvs. **(J)** Othello and **(L)** Topcrop taken 0.5 cm beneath the shoot apex of BDMV-GFP-infected seedlings. Abbreviations: B, boundary between cortex and vascular tissues; C, cortex; E, epidermis; Ph, phloem tissues; Xy, xylem tissues. Scale bar = 100 μ m and is common to all images presented.

Lignin analysis performed on infected (4 dpi) and control hypocotyl tissues indicated the induction of cell wall lignification within the phloem cells of cv. Topcrop was greater than that observed in cv. Othello (Fig. 3E, F, K, and L, respectively). BDMV-GFP infection of cvs. Othello and Topcrop appeared to have little, if any, effect on the extent of xylem lignification (Fig. 3E, F, K, and L, respectively).

An advanced stage of HR was observed in infected cv. Othello hypocotyl tissues at 8 dpi (Fig. 3M and N) whereas, in cv. Topcrop, the infection process had little detectable effect on hypocotyl tissue structure (cortex and phloem; Fig. 3S and T). The pattern of phenolic staining, within BDMV-GFP-infected cv. Othello hypocotyl tissue (8 dpi), had expanded compared with that observed in the 4 dpi tissue, and now encompassed the entire region of the phloem (Fig. 3O). At this stage of infection, cv. Othello cortical cells had undergone a complete collapse (Fig. 3O vs P), consistent with the minimal levels of GFP detected in this tissue (Fig. 3M). The level of phenolic staining in cv. Topcrop (8 dpi) had increased, but to a much lesser extent than that detected in cv. Othello (Fig. 3U vs O). Consistent with the pattern of GFP detection in these infected tissues (Fig. 3S), the cells of the cortex appeared to remain viable in cv. Topcrop (Fig. 3U and V). At this time point (8 dpi), a similar situation was detected in these cultivars with respect to suberin deposition (data not shown).

The level of lignin deposition, within the phloem cells of infected cv. Othello hypocotyls (8 dpi), had increased significantly over that detected in phloem tissue at 4 dpi (Fig. 3Q vs E). However, these deposits appeared in localized regions associated with the presence of phenolic compounds. Stronger lignification of the phloem cell walls was detected in cv. Topcrop (8 dpi; Fig. 3W and X). The perturbed condition of the phloem in cv. Othello both at 4 dpi and, in particular, at 8 dpi, where phloem cells had either collapsed or degraded into void areas (Fig. 3Q vs R), may explain the localized deposition of lignin in these tissues.

Detection of GFP is consistent with presence of viral DNA.

Tissue-specific distribution of BDMV DNA was determined in order to confirm that the absence of GFP, within the primary leaf and shoot apex of cv. Othello seedlings inoculated with BDMV-GFP, reflected a blockage to systemic infection. As shown in Table 1, plants inoculated with BDMV-GFP displayed a good correlation between the presence of viral DNA and detection of GFP. As expected, by 8 dpi, both BDMV DNA and GFP were detected in all cv. Topcrop tissues examined. Consistent with the data presented in Figures 1 and 2, viral DNA and GFP were only detected in the inoculated hypocotyl tissue of cv. Othello. Equivalent experiments conducted with wild-type BDMV established a viral DNA distribution similar to that detected for BDMV-GFP (Table 1). Because BDMV-GFP lacks the *CP*, these findings demonstrate that the BDMV *CP* is not a determinant of systemic infection in cv. Othello.

Analysis of BDMV-GFP distribution in other plants.

To investigate whether the host-BDMV interaction underlying resistance to systemic infection documented in cv. Othello occurs in other plants, experiments were conducted on additional commercial *P. vulgaris* cultivars. As illustrated in Table 2, BDMV-GFP was able to systemically infect and in-

duce typical symptoms in cv. White Kidney Bean. Alternatively, as with cv. Othello, BDMV-GFP failed to systemically infect (or induce symptoms) in cv. Black Turtle Soup T-39. Interestingly, and in contrast to cv. Othello, the block on systemic infection in cv. Black Turtle Soup T-39 occurred in the absence of a detectable HR (data not shown).

Infection studies performed on two soybean cultivars, Haro 15 and Williams, considered to be nonadapted hosts for BDMV (Gilbertson et al. 1991), demonstrated that BDMV-GFP had the capacity to replicate and move cell to cell in the inoculated hypocotyls (Tables 2 and 3). The pattern of viral spread was similar to that observed in common bean, in that BDMV-GFP moved from the epidermis through the cortex and into the phloem. However, in soybean, the extent and rate of viral movement initially were reduced in comparison with common bean (Table 3). Although BDMV-GFP accessed the phloem tissues of inoculated soybean hypocotyls, it was unable to establish a systemic infection. In contrast to the situation where the presence of the *CP* did not rescue the ability of BDMV to establish a systemic infection in cvs. Othello or Black Turtle Soup T-39, wild-type BDMV was able to systemically infect both soybean cultivars (Table 2). Consistent with soybean representing a nonadapted host of BDMV, infected cv. Haro 15 plants developed mild symptoms (restricted to chlorotic flecks), whereas infected cv. Williams plants were symptomless.

Plants considered to be nonhosts for BDMV were next examined. Studies with *Vigna unguiculata* cv. California Black-eye (cowpea) indicated that BDMV-GFP was competent to replicate in epidermal cells, and moved cell to cell in inoculated hypocotyls. In this situation, the actual rate of cell-to-cell movement was impaired relative to *P. vulgaris* (Table 3). Although BDMV-GFP eventually gained access to the phloem, in inoculated cowpea hypocotyls, the number of such cells containing GFP was limited and radial spread within the phloem was not observed (data not shown). Consistent with these results, neither BDMV-GFP nor wild-type BDMV were able to establish a systemic infection in cowpea (Table 2). For the remaining plants examined, *Cucurbita maxima* cv. Big Max (pumpkin), *Gossypium barbadense* cv. Pima S-7 (cotton), and *Zea mays* Pioneer Hybrid 3352 (corn), BDMV-GFP was restricted to individual epidermal cells, and wild-type BDMV was unable to establish a systemic infection in any of these species (Table 2). These results establish that BDMV-GFP cannot move cell to cell in these species. However, the presence of GFP in individual cells may reflect transient expression of the introduced viral DNA or viral replication. Experiments performed in which a BDMV-GFP *ACI* (replication-associated protein gene) frameshift mutant was coinoculated with wild-type BDMV-B into hypocotyls of common bean, cotton, and corn demonstrated that GFP expression was still observed in single cells of each host (data not shown). Thus, viral replication is not a prerequisite for GFP expression in these species.

Viral determinants responsible for induction of HR in cv. Othello.

To assess whether the capacity of BDMV to induce the HR in cv. Othello was virus specific in nature, bean seedlings were inoculated with three other begomoviruses: bean golden mosaic geminivirus (Mexican isolate, BGMV-MX), tomato

leaf crumple geminivirus (TLCrV), and tomato mottle geminivirus (ToMoV). As expected, BDMV induced the HR in cv. Othello, whereas it established a systemic infection in cv. Topcrop (Table 4). Experiments performed with BGMV-MX, which systemically infects a wide variety of bean germplasm, demonstrated its capacity to systemically infect both cvs. Topcrop and Othello. It is noteworthy that BGMV-MX did not induce an HR within the inoculated hypocotyl of either cultivar (Table 4).

Seedlings of cvs. Topcrop and Othello were inoculated with TlCrV, a geminivirus having a broader host range than BDMV or BGMV-MX. As shown in Table 4, a symptom phenotype similar to that of BDMV was observed, in that systemic infection developed in cv. Topcrop but not in cv. Othello. However, in contrast to BDMV, TlCrV did not induce an HR in the inoculated hypocotyl (Table 4). This result is similar to that observed for BDMV infection of *P. vulgaris* cv. Black Turtle Soup T-39 and cowpea (Table 2).

Experiments were conducted with ToMoV, for which beans represent a nonadapted host (Hou et al. 1998). Consistent with this observation, only two of nine cv. Topcrop seedlings inoculated with ToMoV developed a symptomless infection, and none of the inoculated cv. Othello seedlings became infected (Table 4). Taking advantage of the fact that infectious pseudorecombinants can be made by exchanging the cloned DNA components of BDMV and ToMoV (Gilbertson et al. 1993; Hou et al. 1998), experiments were performed to identify viral determinants involved in the induction of the HR in cv. Othello. As the HR was induced by ToMoV-A/BDMV-B, but not by BDMV-A/ToMoV-B or wild-type ToMoV, the viral determinant of the HR was mapped to BDMV DNA-B (Table 4).

DISCUSSION

In the present study, we report on an approach to identify virus-resistant plants and to define the sites, types, and potential underlying mechanisms of host resistance to viral infection through the use of a BDMV-GFP reporter (Sudarshana et al. 1998). A similar approach to investigating host-plant resistance to potyvirus infection has utilized the tobacco etch potyvirus- β glucuronidase reporter system (Hinrichs et al. 1998; Schaad and Carrington 1996; Valkonen and Somersalo 1996). The basic objective of these studies was to identify the cellular basis of host plant resistance as well as nonhost immunity, thereby extending our previous studies of the BDMV/susceptible host interaction (Sudarshana et al. 1998; Wang et al. 1996). Previous studies on the host range of

BDMV (Gilbertson et al. 1991; Hidayat 1991; Morales et al. 1990) allowed for the selection of resistant and susceptible bean cultivars, a putative nonadapted BDMV host (soybean), and nonhost plant species.

Parallel studies performed on the susceptible cv. Topcrop and the resistant cv. Othello demonstrated that BDMV was unable to establish a systemic infection in cv. Othello (Fig. 1, Table 1). Using BDMV-GFP, we clearly established that BDMV was able to replicate and move cell to cell in the epidermis and cortex of inoculated bean hypocotyls (Fig. 2). Furthermore, the block on systemic movement was found not to be due to an inability of BDMV to enter into and replicate within phloem tissues. Rather, in cv. Othello, viral infection activated a host defense response (HR) that was first detected 36 to 48 hpi within phloem cells and then further developed in cortical and phloem tissues (Figs. 2 and 3). Although the activation of this HR was closely correlated to the block in the efficient entry of BDMV into the long-distance translocation stream, the underlying event(s) responsible for this blockage may, or may not, involve the HR; i.e., an earlier event in the host response may be responsible for both the HR and the blockage of systemic infection. Interestingly, this block to systemic infection was not found to be absolute, in that a small number of cells expressing GFP were occasionally detected in bean roots. However, the presence of BDMV-GFP within the upper tissues of the plant was never detected, reflecting the natural direction of phloem translocation (within

Table 1. Comparative distribution of viral DNA and GFP (green fluorescent protein) fluorescence in *Phaseolus vulgaris* cvs. Topcrop and Othello seedlings inoculated with wild-type bean dwarf mosaic geminivirus (BDMV) or BDMV-GFP

Cultivar	Virus ^a	Probe ^b	Tissue ^c		
			Hypocotyl	Primary leaf	Shoot apex
Topcrop	BDMV	DNA	6/6 ^d	6/6	6/6
		DNA	6/6	6/6	6/6
		GFP	6/6	6/6	6/6
Othello	BDMV	DNA	6/6	0/6	0/6
		DNA	6/6	0/6	0/6
		GFP	6/6	0/6	0/6

^a BDMV, wild-type BDMV-A, and BDMV-B were cobombarded; BDMV-GFP, BDMV-A-mGFP4, and BDMV-B were cobombarded into hypocotyl tissues.

^b DNA, viral DNA detected with PCR (polymerase chain reaction); GFP, GFP fluorescence detected with a fluorescence microscope.

^c Tissues were assayed from plants 8 days post inoculation, hypocotyl tissue represents site of bombardment.

^d Number of plants in which DNA or GFP was detected / total seedlings inoculated.

Fig. 3. Comparison between the development of the resistance reaction in *Phaseolus vulgaris* cv. Othello to infection by bean dwarf mosaic geminivirus-green fluorescent protein (BDMV-GFP) and infection in the susceptible *P. vulgaris* cv. Topcrop. **A** and **G**, Transverse sections depicting GFP fluorescence in cvs. **(A)** Othello and **(G)** Topcrop hypocotyl tissues (4 days post inoculation [dpi]). **B** and **H**, bright field images of sections presented in **A** and **G**. **C** and **E**, Transverse sections of BDMV-GFP-infected (4 dpi) cv. Othello hypocotyls (cortex-vascular boundary) stained for **(C)** phenolics or **(E)** lignin. **D** and **F**, Equivalent sections to those in **C** and **E** taken from uninfected (control) cv. Othello hypocotyls. **I** and **K**, Transverse sections of BDMV-GFP-infected (4 dpi) cv. Topcrop hypocotyls (cortex-vascular boundary) stained for **(I)** phenolics or **(K)** lignin. **J** and **L**, Equivalent sections to those in **I** and **K** taken from uninfected (control) cv. Topcrop hypocotyls. **M** and **S**, Transverse sections depicting GFP fluorescence in cvs. **(M)** Othello and **(S)** Topcrop hypocotyl tissues (8 dpi). **N** and **T**, Bright-field images of sections presented in **M** and **S**. **O**, **P**, **U**, and **V**, Sections stained for phenolics as described for **C**, **D**, **I**, and **J**, respectively, except that tissues were taken from seedlings 8 dpi. **Q**, **R**, **W**, and **X**, Sections stained for lignin as described for **E**, **F**, **K**, and **L**, respectively, except that tissues were taken from seedlings at 8 dpi. All images were collected with a Nikon fluorescence microscope. Horizontal arrows identify the cortex-phloem (vascular) boundary. Darts in **A** and **B** identify initiation of necrotic sites within the phloem. Black darts identify regions of phenolic (blue-black color) or lignin (plum-colored) deposition. (Xy, xylem tissues). Scale bar in **A** = 100 μ m and is common to all images presented.

hypocotyl tissue) toward the roots. These results support our previous hypothesis that BDMV must move cell to cell (acropetally), in phloem cells, to reach the cotyledonary node (Sudarshana et al. 1998). At this site, BDMV can then gain entry into the phloem translocation stream that delivers nutrients (and the virus) to the vegetative apex. Thus, in the BDMV/cv. Othello interaction, the timing of the induction of the defense response acts to effectively contain the virus within the initial region of inoculation. The importance of timing, with respect to the host defense response interdicting viral systemic infection, was evident in the BDMV/cv. Topcrop (susceptible) interaction, where the response to viral infection was too slow to prevent systemic infection. However, previous studies have established that systemically infected cv. Topcrop plants do respond to BDMV infection, as evidenced by the later collapse and degradation of phloem tissues, which were associated with a general reduction in symptom severity (Wang et al. 1996; Sudarshana et al. 1998).

The involvement of an HR in this geminiviral plant defense response in cv. Othello is supported by the following. First, BDMV induced rapid, localized cell collapse (36 to 48 h), which was followed by necrosis and viral containment. Second, BDMV infection activated a series of biochemical processes not limited to but including the production of phenolic compounds (i.e., via the shikimic acid pathway), lignin, and suberin, which are typically associated with pathogen-induced HR (Goodman and Novacky 1994; Hammond-Kosack and Jones 1996; Lamb and Dixon 1997; Vance et al. 1980). In this regard, the finding that lignification was greater in phloem tissues of infected cv. Topcrop hypocotyls, compared with those of cv. Othello, was unexpected. This implies that lignification is likely to be a secondary response to viral infection, which is not involved in the resistance phenotype of cv. Othello. Third, there was a strong correlation between the location and timing of BDMV entry (here detected by GFP) and the induction of the HR within these same tissues. Finally, it is well established that Pinto bean cultivars have the capacity to respond to infection by certain RNA viruses, e.g., tobacco mosaic tobamovirus (TMV) and southern bean mosaic sobemovirus, with an HR (Mohamed and Sehgal 1997). It will be of

Table 3. Dynamics of bean dwarf mosaic geminivirus–green fluorescent protein (BDMV-GFP) cell-to-cell movement in inoculated hypocotyls of three leguminous species

Plant species	Cells showing GFP expression (no.) ^a		
	24 hpi	36 hpi	48 hpi
<i>Glycine max</i> cv. Haro 15	1 ^b	2.7 ± 0.4	12.4 ± 0.4 ^c
<i>Phaseolus vulgaris</i> cv. Othello	3.5 ± 0.2	9.9 ± 0.2 ^c	10.7 ± 0.1 ^c
<i>P. vulgaris</i> cv. Topcrop	3.6 ± 0.2	10.4 ± 0.3 ^c	10.9 ± 0.1 ^c
<i>Vigna unguiculata</i> cv. California Blackeye	1.9 ± 0.2	3.3 ± 0.4	6.2 ± 0.6

^a Hypocotyls were bombarded with BDMV-GFP; extent of cell-to-cell movement in this tissue was assessed based on detection of GFP fluorescence in transverse sections with a fluorescence microscope. Cells were scored for GFP in radial transects from the epidermis to the cortex/phloem boundary. Means were obtained from data collected from three infection foci in each of three inoculated plants for three replicate inoculation experiments. hpi = hours post inoculation.

^b GFP was restricted to the epidermis.

^c BDMV-GFP was detected in cells of the phloem.

Table 4. Response of *Phaseolus vulgaris* cvs. Topcrop and Othello to inoculation with four begomoviruses and infectious BDMV (bean dwarf mosaic geminivirus) / tomato mottle geminivirus (ToMoV) pseudorecombinants

Virus	Hypersensitive reaction in inoculated area ^a		Systemic infection ^b	
	Topcrop	Othello	Topcrop	Othello
BDMV	0/12 ^c	12/12	9/9*	0/9
BDMV-GFP	0/12	12/12	10/12*	0/9
BGMV-MX	0/6	0/6	11/11*	10/11*
TLCrV	0/9	0/9	9/9*	0/9
ToMoV	0/9	0/11	2/9	0/9
BDMV-A+ToMoV-B	0/12	0/12	4/9	0/9
ToMoV-A+BDMV-B	0/12	10/12	9/9*	0/9

^a Fresh hypocotyl sections at 4 days post inoculation were examined with a fluorescence microscope for necrosis in the inoculated area.

^b Systemic infection was determined based on symptom development (indicated by asterisks) or by polymerase chain reaction analysis of total genomic DNA extracted from the youngest leaf collected at 15 days post inoculation.

^c Number of plants in which either the hypersensitive reaction, viral DNA, or symptoms were detected / total seedlings inoculated.

Table 2. Host-BDMV (bean dwarf mosaic geminivirus) interactions restrict viral infection in different plant species

Plant species	Detection of GFP ^a	Cell-to-cell movement ^b			Systemic infection ^c	
		Epidermis	Cortex	Phloem	BDMV-GFP	BDMV
<i>Phaseolus vulgaris</i> cv. Topcrop	12/12 ^d	12/12	12/12	12/12	12/12**	12/12**
<i>P. vulgaris</i> cv. White Kidney Bean	12/12	12/12	12/12	12/12	12/12***	12/12***
<i>P. vulgaris</i> cv. Othello	12/12	12/12	12/12	12/12	0/12	0/12
<i>P. vulgaris</i> cv. Black Turtle Soup T-39	12/12	12/12	12/12	12/12	0/12	0/12
<i>Glycine max</i> cv. Haro 15	12/12	5/12	12/12	10/12	0/18	11/16*
<i>G. max</i> cv. Williams	12/12	5/12	12/12	9/12	0/12	6/12
<i>Vigna unguiculata</i> cv. California Blackeye	15/17	9/12	8/10	9/12	0/12	0/12
<i>Cucurbita maxima</i> cv. Big Max	6/18	0/18	0/18	0/18	NT ^e	0/6
<i>Gossypium barbadense</i> cv. Pima S-7	9/12	0/12	0/12	0/12	NT	0/6
<i>Zea mays</i> Pioneer Hybrid 3352	5/24	0/24	0/24	0/24	NT	0/12

^a GFP (green fluorescent protein) fluorescence detected in epidermal cells 2 days post inoculation (dpi) with a fluorescence microscope.

^b Cell-to-cell movement in these tissues was assessed based on detection of GFP fluorescence 4 dpi in transverse sections of hypocotyls with a fluorescence microscope.

^c Systemic infection was assessed based upon symptom development or polymerase chain reaction (PCR) analysis of total genomic DNA extracted from the youngest leaf collected at 15 dpi. *G. barbadense* plants were assayed for infection, by PCR, at 28 dpi. Relative degree of symptom development indicated by asterisks (* = mild; ** = moderate; *** = severe).

^d Number of plants in which either GFP, viral DNA, or symptoms were detected / total seedlings inoculated.

^e Not tested.

interest to determine whether the HR in Pinto bean cultivars to infection by DNA and RNA viruses involves similar pathways. Necrotic local lesions, induced in primary leaves of cv. Red Mexican 35 inoculated with BGMV, may represent another example of an HR to geminivirus infection but, in this case, this response is not associated with resistance (Morales and Niessen 1988). This is not the case in the BDMV/cv. Othello interaction, as cv. Othello was resistant to BDMV when it was mechanically inoculated onto primary leaves (Hidayat 1991).

The identification of the HR in cv. Othello led us to investigate whether this response provided the basis for BDMV resistance in other bean cultivars (Hidayat 1991). The absence of the HR in the BDMV-resistant cv. Black Turtle Soup T-39 must reflect the existence of non-HR-associated mechanisms of viral resistance (Goodrick et al. 1991; Schaad and Carrington 1996). The localization of viral infection, in the absence of necrosis, may be due to a reduced rate of viral cell-to-cell movement (Mize and Ahlquist 1995; Valkonen and Somersalo 1996) resulting in local containment of the virus by host defense responses, such as callose deposition (Goodman and Novacky 1994). Alternatively, viral containment could be due to an incompatibility between the viral movement proteins and the host factors required for phloem entry and/or exit (Gilbertson and Lucas 1996; Nelson and van Bel 1997; Schaad and Carrington 1996; Wang et al. 1998).

Nonadapted hosts of geminiviruses are characterized by mild or symptomless infections, low viral titer, and a requirement for the CP in systemic infection (Hou et al. 1998; Poona et al. 1996). The low incidence of systemic infection, as well as the absence or mild nature of the symptoms developed by soybean plants inoculated with BDMV, suggested that this species is a nonadapted host for the virus. The inability of BDMV-GFP, but not wild-type BDMV, to systemically infect soybean is consistent with a requirement for the CP in this process, and further supports the classification of this species as a nonadapted host. Because BDMV-GFP replicated and moved cell to cell in epidermal, cortical, and phloem cells of inoculated soybean hypocotyls (Tables 2 and 3), the CP must serve a critical function in facilitating long-distance transport in this nonadapted host.

Two different phenomena were observed in our studies conducted on nonhost plants. In cowpea, BDMV-GFP established a localized infection in inoculated hypocotyls that was similar to the situation observed for soybean. But, in contrast, neither wild-type BDMV nor BDMV-GFP was capable of establishing a systemic infection in cowpea. This indicates that cowpea is also a nonadapted host for BDMV and, furthermore, establishes another classification of a nonadapted geminivirus host; i.e., plants in which the virus can move locally but not long distance, irrespective of the CP. The basis for this nonadapted host status may well lie within an incompatibility between the CP and the host factor(s) required for long-distance transport in a nonadapted host such as soybean. In the remaining nonhost plants examined, the inability of BDMV-GFP to move cell to cell resulted in confinement to the initially bombarded epidermal cells. Because transient expression was observed in experiments conducted with a BDMV-A-GFP frameshift mutant, the mechanism of resistance in these nonhost species may involve inhibition of viral replication and/or cell-to-cell movement. Nonetheless, these species represent true nonhosts of BDMV.

In summary, our BDMV-GFP/host interaction studies revealed a complexity of virus/host interactions that further supports the emerging concept that viral host range is a continuum of responses rather than an all-or-none phenomenon. The following five classes of BDMV/host interaction were identified: (i) adapted hosts permissive for systemic infection (e.g., *P. vulgaris* cvs. Topcrop and White Kidney Bean); (ii) adapted hosts displaying the development of an HR that is associated with resistance to systemic infection (e.g., *P. vulgaris* cv. Othello); (iii) adapted (e.g., resistant *P. vulgaris* cv. Black Turtle Soup T-39) and nonadapted (e.g., *V. unguiculata* cv. California Blackeye) hosts in which cell-to-cell, but not long-distance, movement is permitted; (iv) nonadapted hosts in which systemic infection was CP dependent (e.g., *G. max* cvs. Williams and Haro 15); and (v) nonhosts in which the virus was confined to inoculated cells (e.g., *C. maxima* cv. Big Max, *G. barbadense* cv. Pima S-7, *Z. mays* Pioneer Hybrid 3352). These host responses show similarity to some of those previously described for the TMV host-plant interaction in the classic study of Holmes (1946).

Infection studies conducted with four different begomoviruses clearly established that induction of the HR in cv. Othello was specific to BDMV. Although TLCrV also systemically infects cv. Topcrop, cv. Othello was resistant to systemic infection without any detectable signs of an HR. This suggests that cv. Othello possesses a second type of resistance to geminiviral infection, which may be similar in nature to the resistance documented in cv. Black Turtle Soup T-39 for BDMV infection. As ToMoV did not induce an HR on either cvs. Othello or Topcrop, other host and/or viral factors must underlie the basis for bean serving as a nonadapted host for this geminivirus.

The use of BDMV-GFP and infectious pseudorecombinants formed between BDMV and ToMoV allowed for the initial identification of viral determinants involved in the induction of the HR in cv. Othello (Table 4). The BDMV CP can be eliminated as the viral elicitor, because this gene was replaced by GFP in BDMV-GFP (Sudarshana et al. 1998) without any detectable effect on HR induction. The role of additional BDMV DNA-A sequences and/or encoded proteins can similarly be discounted, as the BDMV-A/ToMoV-B pseudorecombinant failed to elicit an HR. Unambiguous evidence that the viral determinant for the HR maps to the BDMV-B component derives from the finding that the ToMoV-A/BDMV-B pseudorecombinant induced the HR in cv. Othello. Furthermore, the finding that this pseudorecombinant failed to establish a systemic infection in cv. Othello is consistent with the hypothesis that resistance to BDMV infection derives from an interaction between host factors and the BDMV-B component.

Future studies will focus on the fine-mapping of the BDMV DNA-B determinant(s) of the HR in cv. Othello. It will be important to elucidate whether the induction of the HR and the blockage to systemic infection map to the same region of the BDMV genome.

MATERIALS AND METHODS

Viral clones.

The construction and characterization of BDMV-GFP has been previously described (Sudarshana et al. 1998). Infectious 1.5-mers of DNA-A and DNA-B clones of an isolate of bean

golden mosaic geminivirus from Chiapas, Mexico (BGMV-MX; Garrido-Ramirez and Gilbertson 1996) were used in the present study. Infectious 1.5-mer DNA-A and DNA-B clones of BDMV, TLcRV, and ToMoV have been previously described (Hou et al. 1998; Paplomatas et al. 1994). A BDMV-A-GFP AC1 gene frameshift mutant was made by filling in a unique *Bgl*III site with the Klenow fragment according to the manufacturer's instructions (New England Biolabs, Beverly, MA). DNA for particle bombardment inoculation was prepared with a mega plasmid kit (Qiagen, Santa Clarita, CA) or a quantum midi-prep kit (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations.

Plant material and inoculation.

Seeds of *Phaseolus vulgaris* L. (cvs. Topcrop, Othello, White Kidney Bean, and Black Turtle Soup T-39), *Glycine max* (L.) Merr. (cvs. Haro 15 and Williams), *Cucurbita maxima* Duchesne (cv. Big Max), *Gossypium barbadense* L. (cv. Pima S-7), *Vigna unguiculata* (L.) Walp. (cv. California Blackeye), and *Zea mays* L. (Pioneer Hybrid 3352), were germinated for 2 to 3 days (at room temperature) on moist (dH₂O) Whatman No.1 filter paper (Fisher Scientific, Pittsburgh, PA) within plastic petri dishes. Particle bombardment of tissues was performed as previously described (Paplomatas et al. 1994; Sudarshana et al. 1998). Inoculated seedlings were planted in soil and maintained in a controlled environment chamber (250 $\mu\text{mol s}^{-1} \text{m}^{-2}$ photosynthetically active radiation, 16 h photoperiod, 30°C day, 22°C night). In some cases, plants were observed for symptoms, and GFP expression was detected with a hand-held, long-wavelength UV lamp (UVP, Upland, CA).

Detection of GFP expression and viral DNA.

Expression of GFP at the whole-plant level was studied with a hand-held UV lamp. At the tissue/cellular level, GFP expression was examined with either a Leica-MZ 12 fluorescent stereomicroscope coupled with an MP's 48/52 camera system (Leica, Heerbrugg, Switzerland), a Nikon Optiphot-2 microscope, or a Leica-TCS-4D confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). Image analysis was performed as previously described (Sudarshana et al. 1998).

For PCR analysis, DNA was extracted from plants inoculated with viral DNA or gold alone (control and infected) following the protocol described by Dellaporta et al. (1983). Detection of geminiviral DNA by PCR analysis was performed with 5 to 10 μl of extracted DNA, using primer PAL1v1978 paired with either PCRC1 or PAR1c496 (Rojas et al. 1993). PCR parameters were as follows: 94°C, 1 min; 50°C, 1 min; and 72°C, 2 min; for 30 cycles. To assess the sensitivity of the PCR method for detection of viral DNA, a dilution series experiment was conducted in which known amounts of pBDA1.5 DNA were added to water (control) or a DNA extract prepared from uninfected common bean leaf tissue. Based on these experiments, as little as 1 and 10 pg of DNA could be detected from water and plant tissues, respectively.

Histochemical analyses.

Free-hand transverse sections were prepared from virus-inoculated and control (gold alone) hypocotyls. Histochemical

staining procedures were employed to analyze the level of phenolics, lignin, and suberin in these tissues. Sections were stained with 0.05% (wt/vol) toluidine blue O in 20 mM sodium benzoate (pH 4.4) for 15 to 20 min, and then counterstained with a saturated phloroglucinol solution in 18% (vol/vol) HCl prior to analysis for phenolic compounds (Eastman et al. 1988). For lignin analysis, sections were mounted in an aliquot of saturated phloroglucinol solution in 20% (vol/vol) HCl (Jensen 1962). The presence of suberin was detected as follows: the sections were stained in 2% (wt/vol) Sudan Black B (dissolved in 70% ethanol) for 30 to 40 min, and briefly rinsed in 0.1% (vol/vol) acetic acid in 70% ethanol, followed by washing in 70% ethanol. For all histochemical assays, stained and unstained sections were viewed with a Nikon Optiphot-2 microscope in bright-field and fluorescence mode (excitation 450 to 495 nm, dichroic mirror 510 nm, barrier filter 520 nm).

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