***Rsv1*-Mediated Resistance Against Soybean mosaic virus-N Is Hypersensitive Response-Independent at Inoculation Site, but Has the Potential to Initiate a Hypersensitive Response-like Mechanism**

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Accepted 23 January 2001.

***Rsv1*, a single dominant gene in soybean PI 96983, confers resistance to most strains of Soybean mosaic virus (SMV), including strain G2. The phenotypic response includes the lack of symptoms and virus recovery from mechanically inoculated leaves. To study the resistance mechanism, SMV-N (an isolate of strain G2) was introduced into PI 96983 by grafting. Hypersensitive response (HR)-like lesions occurred on the stems, petioles, and leaf veins, and virus was recovered from these lesions. The response demonstrated the cytological and histological characteristics of HR as well as elevated transcription of a soybean salicylic acid-inducible, pathogenesis-related (PR-1) protein gene. Mechanical inoculation of PI 96983 primary leaves with a high level of SMV-N virions caused no symptoms or up regulation of the PR-1 protein gene transcript. Furthermore, inoculation with infectious viral RNA did not alter the resistance phenotype. The data suggest that interaction of SMV-N with *Rsv1* has the potential to induce an HR-like defense reaction. *Rsv1*-mediated resistance in the inoculated leaf, however, is HR-independent and operates after virion disassembly.**

Additional keywords: extreme resistance, host defense response, microscopy, virus detection.

Plants have developed diverse mechanisms to resist viruses (Matthews 1991; Ratcliff et al. 1999; Whitham et al. 2000). These include the hypersensitive response (HR) and extreme resistance (ER), both of which have been subjects of more detailed studies (Bendahmane et al. 1999; Bendahmane et al. 2000; Whitham et al. 1994). However, 65% of viral resistance genes are not associated with either HR or ER (Fraser 1990) and, instead, affect virus multiplication or movement as a result of an incompatible combination of viral and host factors (Deom et al. 1997; Keller et al. 1998; Nicolas et al. 1997; Schaad et al. 1997). This type of resistance is most often controlled by recessive genes (Murphy et al. 1998; Schaad and Carrington 1996; Yoshii et al. 1998). Conversely, HR and ER are controlled by dominant host genes, which confer resis-

tance through matched specificity between a disease resistance gene (*R*) and a pathogen avirulence (*avr*) gene and operate on the basis of a “gene-for-gene” hypothesis (Keen 1990).

HR is characterized by rapid cell death at the primary infection site that arises from elicitation of a series of integrated responses following *R*-*avr* gene recognition (Dangl 1998). HR cell death is followed by an increase in the endogenous level of salicylic acid (SA), a major molecule in defense signaling (Delaney et al. 1994) and the concurrent expression of a large number of defense-associated genes, including those that encode pathogenesis-related (PR) protein genes (Van Loon and Van Strien 1999). Other known components of HR include pathogen arrest at or near necrotic lesions and induction of acquired resistance, local (LAR) or systemic (SAR) (Ryals et al. 1996).

In contrast, normal-appearing tissue following virus inoculation is characteristic of ER. In this case, the *avr* strain of the virus at the inoculation site is either undetectable or can be detected only with highly sensitive techniques (Saladrigas et al. 1990; Tozzini et al. 1991; Valkonen et al. 1996). Graft inoculation of plants expressing ER occasionally results in very limited systemic necrotic lesions (Bagnall 1961; Benson and Hooker 1960; Valkonen et al. 1996).

Currently, the relationship between ER and HR is not clear. ER expression, however, is epistatic to that of HR in the *Ry_{adg}*-*Potato virus Y*^o (PVY) and *Rx*-*avr* *Potato virus X* (PVX) pathosystems (Bendahmane et al. 1999; Valkonen et al. 1994). With the well-characterized *Rx*, a single gene controls the expression of ER and HR (Bendahmane et al. 1999; Bendahmane et al. 2000). Therefore, it has been proposed that ER and HR are components of the same defense response that is activated following *R*-*avr* recognition (Bendahmane et al. 1999). According to this view, ER is expressed when there is a high affinity between the elicitor and the receptor (assuming that they interact directly) or when they are produced early in the infection cycle, whereas the HR response occurs either as a result of a lower affinity of the interacting components or from their late expression. The concentration of the interacting components also can influence ER and HR, as demonstrated recently (Bendahmane et al. 2000; Cooley et al. 2000).

Among plant virus interactions, the *N*-*avr* *Tobacco mosaic virus* (TMV) and *Rx*-*avr* PVX represent typical HR and ER,

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respectively (Bendahmane et al. 1999; Bendahmane et al. 2000; Whitham et al. 1994). The *N-avr* TMV pathosystem is a widely studied model system used to dissect the components of HR and elucidate their role in TMV resistance (Klessig and Malamy 1994; Ryals et al. 1996). It has been demonstrated that localization of TMV is not affected by HR cell death (Mittler et al. 1996) and PR proteins lack antiviral activities (Beffa et al. 1996; Cutt et al. 1989; Linthorst et al. 1989). Although SA is essential for TMV localization to the vicinity of the necrotic lesions (Mur et al. 1997; Murphy et al. 1999), it remains unclear how HR suppresses *avr* TMV in inoculated leaves. In contrast to *N*, *Rx*-mediated *avr* PVX arrest in inoculated leaves does not involve HR cell death or other discernible symptoms (Bagnall 1961; Benson and Hooker 1960; Tozzini et al. 1991) and the response of plants in which SA accumulation has been compromised is unaffected (Bendahmane et al. 2000). These factors have led to the conclusion that *Rx*-mediated resistance is HR-independent (Bendahmane et al. 1999; Bendahmane et al. 2000). Nevertheless, graft or *Agrobacterium*-mediated inoculation of *Rx* plants with *avr* PVX or its elicitor has resulted in HR expression (Bagnall 1961; Bendahmane et al. 1999; Bendahmane et al. 2000; Benson and Hooker 1960). The *Rx-avr* PVX interaction induces resistance at the cellular level against taxonomically unrelated plant viruses (Köhm et al. 1993). Its mechanism and the involvement of SA-independent defense pathways (Pieterse and Van Loon 1999), however, have not been investigated. At present, *Rx* is the only reported *R* gene against plant viruses with these unusual features.

RsvI, a single dominant gene in soybean PI 96983 (Chen et al. 1991; Kiihl and Hartwig 1979), confers resistance against *Soybean mosaic virus* (SMV) strains G1 through G6 and C14 but not against SMV-G7 or G7a (Buzzell and Tu 1984; Kiihl and Hartwig 1979; Lim 1985). In *RsvI*-genotype plants, SMV-G7 induces systemic necrosis, whereas SMV-G7a causes systemic mosaic and stunting (Buzzell and Tu 1984; Jayaram et al. 1992). SMV is a single-stranded, positive-sense RNA virus belonging to the family *Potyviridae* (Jayaram et al. 1992). Strain designations are based on phenotypic reactions on soybean differential cultivars (Cho and Goodman 1979). Resistance to SMV is affected by a number of *RsvI* alleles or other genes present in different soybean lines (Buzzell and Tu 1984; Buzzell and Tu 1989; Chen et al. 1991; Ma et al. 1995), although *RsvI* is the only locus that is mapped genetically (Yu et al. 1994). An unusual feature of the *RsvI*-mediated resistance phenotype is the lack of visible symptoms and virus accumulation in the inoculated leaves (Buzzell and Tu 1984; Lim 1985). In this regard, *RsvI* is similar to *Rx* (Saladrigas et al. 1990) or an *R* gene present in the cowpea cultivar Arlington that confers resistance against *Cowpea mosaic virus* (CPMV) strain SB (Beier et al. 1979). Currently, the mechanism of *RsvI*-mediated resistance is not known. Thus, in this study, the nature of *RsvI*-mediated resistance in PI 96983 was investigated with SMV-N (Vance and Beachy 1984), a G2 isolate (Cho and Goodman 1979).

In this paper, we confirm previous findings that PI 96983 leaves containing *RsvI* remain symptomless after mechanical inoculation by using SMV-N (Buzzell and Tu 1984; Lim 1985). We further demonstrate that i) the resistance is not affected by inoculation with high virion concentration or viral genomic RNA, ii) SMV-N has the potential to trigger HR in

graft-inoculated PI 96983, iii) PI 96983 has defense responses characteristics of a classical HR, and iv) *RsvI*-mediated resistance to SMV-N in inoculated leaves does not correlate with accumulation of PR-1 mRNA. The data suggest that the *RsvI* mechanism of resistance against SMV-N in intact plants is a form of ER and is HR independent in inoculated leaves. Furthermore, they support the notion that HR cell death is not an essential component of the gene-for-gene resistance mechanism against plant viruses.

RESULTS

PI 96983 leaves mechanically inoculated with SMV-N lack symptoms and virus.

Mechanical inoculation of PI 96983 primary leaves with SMV-N infectious sap consistently lacked symptom expression, remained indistinguishable from mock-inoculated leaves (Fig. 1A), and the virus was not detected by back inoculation to Williams 82 (Bernard and Cromeens 1988), a SMV-susceptible soybean cultivar. These results are all in agreement with other reports (Buzzell and Tu 1984; Lim 1985). Moreover, the virus was not detected by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) from the inoculated tissues, and SMV-N RNA could not be detected in total nucleic acid extracts of mechanically inoculated apical portions of PI 96983 primary leaves by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 2, lanes 3 to 8). As expected, SMV-N was detected in inoculated Williams 82 (Fig. 2, lanes 11 and 12), and SMV-G7 was detected in inoculated PI 96983 leaves (Fig. 2, lanes 9 and 10). Similar results were obtained when the noninoculated basal portion of the primary leaves, petioles of inoculated primary leaves, and uninoculated trifoliate leaflets from the top of the same plants were tested (data not shown). Failure of SMV-N to infect PI 96983 was not the result of a low level of virus in the infectious sap used as inoculum because mechanical inoculation with 396 µg of purified SMV-N per plant also failed to alter the resistance phenotype, as demonstrated by symptom expression and DAS-ELISA. Under the same conditions, 4 µg of purified SMV-N per plant was sufficient to infect 80% of the inoculated Williams 82. Mechanical inoculation also was performed with infectious SMV-N RNA rather than virions. In two independent experiments, inoculation with 4.8 or 6.8 µg of SMV-N RNA per plant caused 80 and 100% infection in Williams 82, respectively. None of the 20 inoculated PI 96983, however, showed infection on the basis of symptom expression and DAS-ELISA.

SMV-N was transmitted from Williams 82-infected rootstocks to cleft- or approach-grafted Williams 82 scions (Table 1) and induced mosaic with slight leaf distortion. Efforts were made to recover SMV-N from PI 96983-inoculated rootstocks by grafting to susceptible Williams 82 scions, where the graft union was positioned above the inoculated primary leaves. No virus was evident in any of the susceptible Williams 82 scions grafted to SMV-N-inoculated PI 96983 rootstocks, however, as determined by symptom expression and DAS-ELISA (Table 1). Similar results were obtained by approach grafting after the graft union was positioned below the primary leaves and rootstocks were inoculated after the graft (Table 1). The transmission of SMV-G7 from infected PI 96983 rootstocks to susceptible scions (Table 1) suggests that the fusion of PI

96983 stem tissue with that of the susceptible cultivar was sufficient to allow SMV movement. Additionally, scions grafted above or below the inoculated leaves were expected to reveal any vascular entry of the virus from the inoculated leaves moving upward or downward in the stem. Therefore, failure of SMV-N transmission from the inoculated PI 96983 rootstocks to the susceptible Williams 82 scions indicates the apparent absence of the virus in PI 96983 vascular tissues.

To examine the potential that *Rsv1* could allow vascular transport of SMV-N, triple-grafted plants were made in which the susceptible Williams 82 served as rootstock and terminal scion. The middle interstem scion was either Williams 82 or PI 96983. When primary leaves of the rootstock were mechanically inoculated with SMV-N, virus was detected in the terminal scion by DAS-ELISA, irrespective of the genotype of the interstem scion (Table 2). The data suggest that *Rsv1* does not interfere with the long-distance transport of SMV-N.

SMV-N graft inoculation of PI 96983 induces HR-like lesions.

To bypass the effect of *Rsv1* on SMV-N replication and accumulation in mechanically inoculated leaves, the virus was introduced into PI 96983 plants by cleft grafting. SMV-N was transmitted from infected Williams 82 rootstocks to Williams 82 scions as well as from infected scions to rootstocks (Table 3). PI 96983 grafted to SMV-G7-infected Williams 82 rootstocks or scions showed mosaic symptoms that eventually developed into systemic necrosis (Table 3). These observations suggest that SMV transport occurs upward and downward in the susceptible soybean stem. Contrary to results obtained with mechanical inoculation, graft transmission of SMV-N to PI 96983 resulted in the appearance of macroscopically visible HR-like lesions on the stem, petiole, and leaf

veins approximately 3 to 4 weeks postgrafting (Fig. 1B). The lesions were limited to the lower third of the scions, and distribution on the PI 96983 stem was asymmetrical and appeared slowly along the sides of the stems. There was no petiole, leaf, or plant death. HR-like lesions in leaves were restricted to the abaxial and generally to the mid and secondary veins. The lesions occasionally extended to tertiary and quaternary minor veins, however, particularly in the young leaves adjacent to the graft union. When tertiary and quaternary minor veins showed HR-like lesions, mesophyll tissues surrounding the veins showed limited chlorosis (data not shown).

Fig. 2. Detection of *Soybean mosaic virus* (SMV) by reverse transcription-polymerase chain reaction (RT-PCR) in inoculated apical portion of primary leaves of the soybean genotypes Williams 82 or PI 96983. Following inoculation, the plants were maintained in the greenhouse until two disks (1-cm diameter) from the inoculated region of each leaf were collected 4 weeks postinoculation. Samples from three independent plants were mixed, and total nucleic acid from equivalent 4 mg of fresh tissue from each group was used in the RT-PCR reaction. Each lane represents 10 μ l of RT-PCR product. Arrow denotes position of 905-bp amplified SMV genomic fragment.

Fig. 1. Phenotypic response of PI 96983 to *Soybean mosaic virus* (SMV)-N inoculation. **A**, Adaxial (upper leaves) and abaxial (lower leaves) surface of primary leaves of PI 96983 inoculated with buffer (mock) or SMV-N infectious sap on the adaxial surface and photographed 3 weeks postinoculation. **B**, Hypersensitive response (HR)-like lesions on a portion of PI 96983 scion grafted to SMV-N-infected Williams 82 rootstock (not shown) photographed 8 weeks postgrafting. Arrowheads point to HR-like lesions.

Presence of the lesions was independent of time of inoculation (pre- or postgrafting), rootstock genotype, and position of PI 96983 as scion or rootstock (Table 3). The coat protein of SMV-N was consistently detected by squash-immunoblotting in tissues with HR-like lesions and not in those without such lesions (Fig. 3 and Table 3). Similarly, SMV particles were detected by serological-specific electron microscopy (SSEM) of leaf tissues with HR-like lesions (data not shown). SMV-N RNA also was detected by slot-blot hybridization of PI 96983 leaves with HR-like lesions (Fig. 4), although contrary to squash-immunoblotting results, a weak signal was detected in total RNA extracts from PI 96983 scion leaf tissues lacking the lesions (Fig. 4). The signal presumably represents SMV-N particles that are passively present in the phloem (Santa Cruz 1999) delivered through the vascular tissues of the infected rootstock. To confirm that the virus associated with the HR-like lesions was SMV-N rather than a mutant generated as a result of passage through PI 96983, a 905-bp fragment in the HC-Pro and the P3 regions of the SMV genome was amplified from three independent plants by immunocapture (IC)-RT-PCR and ligated into pGEM-T. One clone derived from each plant was sequenced and compared to the sequences of SMV-N (GenBank accession no. D00507). This region of the SMV genome is variable among SMV strains and differs by 36 nucleotides between SMV-N and the resistance-breaking strain G7 and 37 nucleotides between strains G2 and G7 (Eggenberger 1994; Jayaram et al. 1992). The sequence of one clone was identical to SMV-N, whereas one differed by a single and another by two mismatches. Furthermore, the virus from HR-like lesions recovered by inoculation to Williams 82 and its phenotype on soybean differential cultivars showed characteristics typical of a SMV-G2-type isolate (Cho and Goodman 1979).

Association of cytological and histological hallmarks of HR with SMV-N-induced HR-like lesions.

Abaxial leaf veins with SMV-N-induced HR-like lesions from PI 96983 (Fig. 5G and H) were examined for cellular

markers often associated with activation of a plant defense system (Koga et al. 1980; Mayama and Shishiyama 1976). Cross sections of PI 96983 leaf veins with HR-like lesions showed abnormal depositions in some of the phloem-associated cells, with some cells appearing totally filled with dense materials (Fig. 5J) and heavily lignified, as indicated by staining with phloroglucinol (Fig. 5K). In contrast, SMV-N-infected Williams 82 abaxial leaf veins obtained from scions grafted to inoculated rootstocks showed no abnormal dense material in the vascular tissues (Fig. 5D), and the intensity of staining with phloroglucinol (Fig. 5E) was similar to that of mock-inoculated leaves (data not shown).

PI 96983 abaxial leaf veins with SMV-N-induced HR-like lesions showed strong callose deposition when stained with aniline blue (Fig. 5I), and a cross section of veins showed strong autofluorescence in cells surrounding HR-like lesions (Fig. 5L). Callose and lignin deposition in or around SMV-N-infected Williams 82 abaxial veins (Fig. 5C and E) as well as the associated autofluorescence (Fig. 5F) were similar to that of Williams 82 and PI 96983 leaves from scions grafted on mock-inoculated rootstocks (data not shown).

SMV-N induced HR-like lesions in PI 96983 leaves that extended to tertiary and quaternary minor veins also were stained with trypan blue. The dye was retained only by the chlorotic mesophyll tissues surrounding minor veins with HR-like lesions and not by any other part of the leaf blade (data not shown). This suggests irreversible membrane damage in these cells, which is associated with HR (Keogh et al. 1980).

PR-1 gene expression in PI 96983 leaves with SMV-N-induced HR-like lesions.

Expression of PR-1, a SA-inducible SAR gene, is a common molecular marker of HR and SAR activation (Dangl 1998; Van Loon and Van Strien 1999). SA treatment of leaf tissues of Williams 82 and PI 96983 resulted in the up regulation of a soybean PR-1 protein gene (GenBank accession no. AI 930866) transcript, compared with H₂O treatment (Fig. 6). The probe was specific, as it hybridized weakly with tobacco PR-1a mRNA (Fig. 6). Therefore, both soybean genotypes are capable of expressing the PR-1 gene in response to SA.

Table 1. Recovery of *Soybean mosaic virus* (SMV)-N or G7 from inoculated Williams 82 and PI 96983 by grafting

Time of inoculation	Graft combination	Treatment		
		Mock	SMV-N	SMV-G7
Pregrafting ^a	Williams 82	5 ^d (0) ^c	13 (10)	8 (4)
	Williams 82 ^c	5 (0)	13 (10)	8 (4)
	Williams 82	8 (0)	42 (0)	8 (4)
	PI 96983 ^c	8 (0)	42 (0)	8 (4)
Postgrafting ^b	Williams 82 ^c	4 (0)	14 (14)	7 (6)
	Williams 82	4 (0)	14 (14)	7 (6)
	PI 96983 ^c	4 (0)	12 (0)	5 (5)
	Williams 82	4 (0)	12 (0)	5 (3)

^a Graft type = cleft.

^b Graft type = approach.

^c Indicates site of inoculation. Primary leaves were inoculated 3 weeks postplanting (a) or 3 days postgrafting (b). Inoculated and noninoculated leaves in (a) and (b) were tested for the presence of SMV-coat protein (CP) by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) 5 and 9 weeks postgrafting, respectively.

^d Total graft combinations examined for each treatment.

^e Total samples positive for SMV-CP by DAS-ELISA.

Table 2. Movement of *Soybean mosaic virus* (SMV)-N or G7 through interstem scions of Williams 82 and PI 96983

Time of inoculation	Terminal scion Interstem scion Rootstock	Treatment		
		Mock	SMV-N	SMV-G7
Pregrafting ^a	Williams 82	7 ^c (0) ^d	9 (5)	6 (3)
	Williams 82	7	9	6
	Williams 82 ^b	7 (0)	9 (5)	6 (3)
	Williams 82	6(0)	17 (14)	0 ^e
	PI 96982	6	17	5
	Williams 82 ^b	6 (0)	17 (15)	5 (2)

^a Graft type = interstem.

^b Indicates site of inoculation. Primary leaves were inoculated 2 weeks postplanting. A fully developed leaf from the rootstock and the top scion was tested for the presence of SMV-coat protein (CP) by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) 12 weeks postinoculation.

^c Total graft combinations examined for each treatment.

^d Total samples positive for presence of SMV-CP by DAS-ELISA.

^e Terminal scion was not established as a result of SMV-G7-induced systemic necrosis of *RsvI* interstem plant scions.

Expression of PR-1 transcript in PI 96983 leaf tissues with HR-like lesions was compared with leaves from the top of the same scion but without lesions or with leaves from PI 96983 scions cleft grafted on mock-inoculated rootstocks. A low level of PR-1 mRNA accumulation in mock-inoculated PI 96983 was detected (Fig. 4). Up regulation of PR-1 mRNA in PI 96983 leaves with HR-like lesions compared with mock-inoculated or PI 96983 leaves without lesions, however, was evident (Fig. 4). In contrast, expression of PR-1 mRNA in SMV-N-infected Williams 82 leaves from scions grafted on SMV-N-inoculated rootstocks remained low compared with mock-inoculated Williams 82 (Fig. 4). Up regulation of PR-1 in PI 96983 with HR-like lesions corresponded with accumulation of SMV-N RNA (Fig. 4). PI 96983 leaves originating from the top of the same scion but without HR-like lesions showed a weak signal for the presence of SMV-N RNA without elevation in PR-1 transcript (Fig. 4). This indicates that the presence of SMV-N in PI 96983 tissues is insufficient to trigger HR and, presumably, a threshold level is required.

SMV-N arrest in PI 96983-inoculated leaf is HR independent.

Despite the potential of SMV-N to trigger HR in graft-inoculated PI 96983 (Fig. 1B), no macroscopic HR cell death was evident in the mechanically inoculated leaves (Fig. 1A), even at high inoculum concentration. The possibility that *RsvI*-mediated resistance against SMV-N in inoculated leaves was either HR independent or the result of invisible micro-HR was examined by assessment of PR-1 mRNA accumulation. Micro-HR, although not detectable even microscopically, can lead to the activation of a battery of defense responses, including the expression of SAR genes (Alvarez et al. 1998; Tang et al. 1999). PI 96983 and Williams 82 attached primary leaves from at least five plants were mechanically inoculated with SMV-N or SMV-G7 (8.0- and 7.8- μ g purified virions per leaf, respectively), *Bean pod mottle virus* (BPMV) (infectious sap), or buffer. Total leaf RNA was assayed by slot-blot hybridization for PR-1 mRNA expression, and the presence of SMV sequences at 5, 14, 21, and 28 days postinoculation (dpi). The experiments were performed under greenhouse and growth chamber conditions. All PI 96983 SMV-N-inoculated primary leaves remained symptomless up to 28 dpi (data not shown). Furthermore, SMV-N did not induce PR-1 mRNA expression in any of the inoculated PI 96983 primary leaves compared with Williams 82 or mock-inoculated PI 96983 (Fig. 7). SMV-N viral RNA was detected (approximately 1 ng per 20 μ g of total host RNA) in the inoculated primary leaves (Fig. 7) but not in any of the noninoculated PI 96983 trifoliolate leaflets (Fig. 7), up to the 28 dpi tested (data not shown). Despite the presence of a low level of SMV-G7 in inoculated primary leaves of PI 96983 (Fig. 7), a slight elevation of PR-1 mRNA expression occurred compared with mock-inoculated leaves (Fig. 7). The expression, however, did not exceed that associated with BPMV infection, which causes systemic mosaic without necrosis in PI 96983 (Fig. 7). SMV-G7 caused systemic necrosis in trifoliolate leaflets of PI 96983, with associated high levels of viral RNA and PR-1 mRNA accumulation (Fig. 7). This suggests that the nature of systemic necrosis induced by SMV-G7 in *RsvI*-genotype plants is systemic HR (SHR).

DISCUSSION

The results presented here show that PI 96983 plants inoculated mechanically with SMV-N expressed no symptoms and DAS-ELISA or back inoculation could not detect the virus. These results were not influenced by the concentration or nature of the inoculum (i.e., infectious sap, purified virions, virion RNA) or by the environmental conditions used to maintain inoculated plants. RT-PCR also failed to detect the virus in the inoculated primary leaves. Furthermore, grafting of inoculated plants to a susceptible cultivar, which allows a dynamic rather than static analysis, also revealed no virus transmission.

SMV-N RNA, however, was detected by slot-blot hybridization in primary leaves of all PI 96983 plants inoculated with 80 μ g of virion per leaf and maintained in the greenhouse or growth chamber. The signal may reflect traces of remaining inoculum. This seems unlikely, however, because inoculated leaves were washed thoroughly postinoculation and the signal was detected in samples collected as late as 4 weeks postinoculation (data not shown). Alternatively, it may represent transcribed or replicated SMV-N RNA. One possible explanation is that high inoculum levels (80 μ g of virion per leaf) suppressed the inhibitory effect of *RsvI* on transcription or replication of SMV-N. High input of inoculum (1 ng of PVX particles per protoplast) has been reported to suppress the resistance effect mediated by *Rx* (Saladrigas et al. 1990). SMV-N CP was not detected by DAS-ELISA in these tissues, however, which may reflect either a lack of accumulation or the insensitivity of the technique.

At present, direct evidence for SMV-N replication in PI 96983 leaf tissue such as the presence of (–) strand or replication-associated proteins is lacking. Nevertheless, the association of SMV-N CP, virion RNA, and virus particles with HR-like lesions of graft-inoculated PI 96983 may reflect existence of a threshold level beyond which *RsvI* does not function suc-

Table 3. Graft inoculation of PI 96983 or Williams 82 with *Soybean mosaic virus* (SMV)-N or G7

Time of inoculation	Scion Rootstock	Treatment		
		Mock	SMV-N	SMV-G7
Pregrafting ^a	Williams 82	7 ^c (0) ^d	11 (11)	N. T. ^e
	Williams 82 ^b	7 (0)	11 (11)	
	PI 96983	11(0)	36 (25) ^f	9 (9)
	Williams 82 ^b	11(0)	36 (36)	9 (9)
	PI 96983	6 (0)	25 (23) ^f	5 (5)
	Pioneer 9413 ^b	6 (0)	25 (25)	5 (5)
Postgrafting ^a	Williams 82 ^b	7 (0)	20 (19)	N. T.
	Williams 82	7 (0)	20 (19)	
	Williams 82 ^b	6 (0)	18 (18)	9 (9)
	PI 96983	6 (0)	18 (15) ^f	9 (9)

^a Graft type = cleft.

^b Indicates site of inoculation. Primary leaves were inoculated 2–3 weeks pregrafting or 1 week postgrafting.

^c Total graft combinations examined for each treatment.

^d Total samples positive for SMV-coat protein (CP) by squash-immunoblotting 8–10 weeks postinoculation.

^e Not tested.

^f SMV-CP was detected only from PI 96983 tissues with hypersensitive response-like lesions and no SMV-CP was detected in tissues without lesions from the same plants.

cessfully to suppress virus replication or accumulation. Presumably, the graft-inoculated PI 96983 is subject to a continued high inoculum level supplied through virus replication from the systemically infected susceptible cultivar. The virus associated with HR-like lesions was not a newly emerged SMV mutant because cloned progeny of SMV-N were used in all experiments. Furthermore, the sequence of a 905-bp RT-PCR fragment isolated from the lesions provided evidence for SMV-N sequences and the phenotypic reactions of soybean differential cultivars to the recovered virus were typical of isolates of SMV-G2 (Cho and Goodman 1979).

HR-like lesions were obvious only in the abaxial midveins of PI 96983. This is not surprising because the location of soybean leaf phloem is abaxial (Lersten and Carlson 1987), and it is generally assumed that long-distance transport of viruses occurs in phloem tissues (Carrington et al. 1996; Nelson and Van Bel 1998; Santa Cruz 1999). The HR-like lesions rarely extended to minor veins or surrounding mesophyll tissues (data not shown). This may be the result of heavy callose deposition or the death of cells surrounding the veins.

Callose deposition has the potential to restrict virus movement through the plasmodesmata (Beffa et al. 1996), and cells surrounding the veins are known to be more susceptible to HR death-inducing signals (Pennell and Lamb 1997). Microscopic examination of cross sections of the abaxial midvein of PI 96983 containing HR-like lesions revealed abnormal cytoplasmic deposition in some phloem-associated cells, similar to that reported in the HR elicited during the *Ry_{sto}*-PVY interaction (Hinrichs-Berger et al. 1999). One likely explanation is that during long-distance movement, SMV-N initially infects these cells or accumulates to a higher level compared with other vascular-associated cells and, consequently, HR is induced. The route of vascular-dependent, long-distance transport of SMV in soybean or the vascular-associated cell type involved are not known. Studies with other viruses in potato (*Solanum tuberosum*) and pepper (*Capsicum annuum*) have shown that in resistant cultivars, virus accumulation is limited

to specific phloem cells compared with susceptible plants (Derrick and Barker 1997; Dufour et al. 1989; Guerini and Murphy 1999). It also is possible that *RsvI* is not expressed strongly in certain vascular-associated cells of PI 96983 and, therefore, the virus is allowed to replicate.

It is not known which stage(s) of SMV-N replication or infection is affected by *RsvI*. Virus replication, cell to cell, and long-distance movement are essential steps in the systemic infection of plant viruses (Carrington et al. 1996). The inability of SMV-N RNA inoculum to alter the PI 96983 resistance phenotype as well as the association of typical SMV particles with HR-like lesions of graft-inoculated plants suggest that *RsvI* allows virus disassembly and assembly. Furthermore, the PI 96983 interstem scion allows for the translocation of SMV-N from infected Williams 82 rootstocks to inoculated susceptible terminal scions. The data imply that *RsvI*-plants do not impede long-distance transport of SMV-N in phloem tissues. These features of *RsvI*-mediated resistance resemble those of *Rx* in potato (Adams et al. 1986; Bendahmane et al. 1999).

Successful systemic infection of a particular host with a plant virus requires, in addition to phloem translocation, entry into and exit from vascular tissues (Nelson and Van Bel 1998; Santa Cruz 1999). PI 96983 scions grafted onto SMV-N-infected rootstocks developed limited HR-like lesions, which were restricted to the lower third of scions close to the graft union. Tissues terminal to this region never developed lesions and did not show SMV-N accumulation or up regulation of the PR-1 transcript, which suggests that SAR is not involved in this region. This is inconclusive, however, because SAR can vary depending upon the plant genotype (Hämäläinen et al. 2000; Ross 1961; Ryals et al. 1996). Alternatively, a limited spread of lesions may reflect the inhibitory effect of *RsvI* on SMV-N entry into, movement through, or exit from particular phloem-associated cells of PI 96983 that are involved in the systemic spread of the virus.

Callose, lignin, deposition of autofluorescent material, cell death, and PR-1 mRNA up regulation are all associated with PI 96983 SMV-N-induced leaf veinal necrosis. These histological, cytological, and molecular markers are characteristics

Fig. 3. Detection of *Soybean mosaic virus* (SMV) coat protein (CP) by squash-immunoblotting of midvein leaf tissues. Rootstocks (Williams 82) were inoculated mechanically prior to grafting to scions (PI 96983). Midvein leaf tissues were removed from rootstocks and scions 5 weeks postgrafting, squashed on a nitrocellulose membrane, and probed with anti-SMV CP polyclonal immunoglobulin. + and – indicate the presence or absence of hypersensitive response-like lesions on PI 96983 midvein leaf tissues used for the analysis. Note that purple is indicative of a positive serological reaction.

Fig. 4. Slot-blot hybridization analyses of soybean PR-1 transcript up regulation and the presence of *Soybean mosaic virus* (SMV) in leaves of Williams 82 and PI 96983 scions cleft grafted on SMV-N or buffer (mock)-inoculated Williams 82 rootstocks. Total leaf RNA was isolated 7 weeks postgrafting, slot blotted onto a membrane, and hybridized with ³²P-labeled cDNA probes. Samples marked with (+) and (–) were from the same PI 96983 scion but with and without hypersensitive response-like lesions, respectively.

of pathogen-induced hypersensitive cell death (Dangl 1998; Dietrich et al. 1994; Tang et al. 1999; Vance et al. 1980). The association of these markers with HR-like lesions of PI 96983 indicates that this plant is capable of an HR-associated defense reaction (Dangl et al. 1996) against SMV-N. SHR induced by SMV-G7, which could occur as a result of mutation(s) in the elicitor as shown for other virus–host interactions (Culver et al. 1991; Kim and Palukaitis 1997) and may lead to a very low affinity interaction between the elicitor and receptor (Bendahmane et al. 1999; Culver et al. 1991), supports this conclusion.

The association of HR markers with SMV-N-induced HR-like lesions of PI 96983 suggests the virus possesses an *avr*

gene, which in interaction with *RsvI*, directly or indirectly, activates a host-defense system. Although it is unknown which of the 8 or 9 genes of SMV-N (Jayaram et al. 1992) serves as an elicitor of *RsvI*-mediated HR expression, a segment consisting of the 3' region of HC-Pro and the 5' region of the P3 coding domains has been identified as a resistance-breaking determinant (Eggenberger and Hill 1997). It is unknown, however, whether this region is the elicitor of *RsvI*-mediated HR expression or is involved in a counterdefense mechanism (Carrington and Whitham 1998).

Despite the potential of SMV-N to trigger HR in PI 96983 and the capability of PI 96983 to express an HR-like defense reaction against the virus, the *RsvI*–SMV-N interaction in

Fig. 5. Microscopic analysis of leaf tissues from A–F, Williams 82 and G–L, PI 96983 scions cleft grafted to *Soybean mosaic virus*-N-inoculated susceptible Williams 82 rootstocks. Fully expanded leaves were removed 7 weeks postgrafting. Abaxial leaf surfaces prior to (A and G) and after (B and H) lactophenol treatment. C and I, UV-stimulated fluorescence of a hand section of leaves stained with aniline blue. D and J, Cross sections of a leaf vein observed with bright field microscopy. E and K, Cross sections of leaf veins stained in 2% phloroglucinol and viewed with dark field microscopy. Note the staining of abnormal deposit in phloem-associated cells in K. F and L, UV-stimulated autofluorescence of lactophenol-treated cross sections of leaf veins. Scale bars = 100 μ m (C–E, I, and K); 300 μ m (F, J, and L); and 10 mm (A, B, G, and H). Hand and cross sections were prepared from the leaf tissues within boxes.

inoculated leaves failed to elicit detectable HR. SMV-N may replicate in initially infected PI 96983 cells, but its movement to adjacent cells may be blocked or its accumulation may rapidly be suppressed, as demonstrated for *Rx*-mediated resistance (Köhm et al. 1993). HR expression is known to be threshold dependent (Costel et al. 1999) and, in at least one virus–host interaction, requires virus movement outside of the initially infected cells (Canto and Palukaitis 1999). Alternatively, *RsvI*-mediated resistance may block SMV-N gene expression in infected cells and, consequently, viral elicitor is not produced.

Restriction of SMV-N in PI 96983 leaves inoculated mechanically with high inoculum concentration showed no correlation with PR-1 mRNA up regulation. The basal level of PR-1 in mock-inoculated PI 96983 was similar to that of SMV-N-susceptible Williams 82. This suggests that PR-1 is not expressed constitutively at high levels in PI 96983, as reported in a TMV-resistant tobacco line (Ahl and Gianinazzi 1982) or the *Tobacco ringspot virus*-resistant Arabidopsis *dnd1* mutant (Yu et al. 1998). Lack of PR-1 mRNA elevation in SMV-N-inoculated PI 96983 suggests that *RsvI*-mediated resistance at the point of inoculation is HR independent. This observation agrees with other reports that HR cell death is not essential for the gene-for-gene-mediated resistance mechanism against plant viruses (Baulcombe et al. 1994; Bendahmane et al. 1999; Bendahmane et al. 2000; Bi et al. 1995; Mittler et al. 1996; Mur et al. 1997). Induction of PR-1 mRNA expression requires SA accumulation that normally increases during classical *avr*–*R* gene interactions (Ryals et al. 1996). If any increase in the endogenous level of SA occurred during SMV-N arrest in the inoculated primary leaves of PI 96983, the level of PR-1 mRNA should have been elevated. Therefore, *RsvI*-mediated SMV-N arrest at the inoculation site is either SA-

independent or it does not require significant SA accumulation.

In addition to *RsvI* and *Rx*, the resistance gene in cowpea cultivar Arlington (Beier et al. 1979), *RTM1/RTM2* in Arabidopsis (Chisholm et al. 2000; Whitham et al. 2000), and *Ry* in potato (Valkonen et al. 1996) are other phenotypically unusual *R* genes against plant viruses that do not induce HR at the inoculation site. Interaction of CPMV-SB with the resistance gene in cowpea cultivar Arlington induces concurrent resistance (Bruening et al. 2000). Graft-inoculated tissues, however, do not express HR (Beier et al. 1979), and it is not known whether the pathosystem has such capability. *RTM1/RTM2*-mediated resistance against *Tobacco etch virus* in Arabidopsis allows virus replication and accumulation in the inoculated leaves, but restricts its long-distance movement without causing HR or SAR induction (Chisholm et al. 2000; Whitham et al. 2000). *Ry*-bearing potato leaflets exhibit macroscopically visible necrotic streaks along the vein after mechanical inoculation with high concentration of PVY (Hinrichs-Berger et al. 1999). Despite this phenotypic difference of *Ry*-mediated resistance with that of *Rx*, ER has been implicated as the primary mechanism of resistance (Mestre et al. 2000). The results presented here demonstrate that *RsvI*-mediated resistance shares a number of phenotypic similarities with those of *Rx*, including a two-tiered mechanism of resistance. Primary resistance or ER (HR independent) is operational at the point of inoculation. Secondary resistance (HR

Fig. 6. Salicylic acid (SA) induction of PR-1 gene transcripts from tobacco (*Nicotiana glutinosa*) and Williams 82 or PI 96983 soybean. Leaf disks were punched and floated on 0.01% SA, pH 6.8, or H₂O and incubated under continuous fluorescent illumination for 72 h. Total RNA was isolated, denatured, slot blotted onto the membrane, and hybridized with ³²P-labeled cDNA probes.

Fig. 7. Slot-blot hybridization analysis for PR-1 transcript up regulation and presence of *Soybean mosaic virus* (SMV) in leaves of the soybean genotypes Williams 82 or PI 96983. Total RNA was isolated from mechanically inoculated primary leaf (P) 14 days postinoculation (dpi) or a fully expanded noninoculated trifoliate leaflet of the inoculated plants (T) 28 dpi. Plants were mechanically inoculated with either 80 µg SMV-N or 7.8 µg SMV-G7 purified virions per primary leaf, sap from *Bean pod mottle virus* (BPMV)-infected soybean tissues or mock-inoculated with buffer. Lane marked with (+) was loaded with total RNA from mock-inoculated primary leaf, but with the addition of 1 ng SMV-N virion RNA. Total RNA was denatured, slot blotted onto the membrane, and hybridized with ³²P-labeled cDNA probes.

mediated) activates when primary resistance is bypassed and the HR elicitor is allowed to accumulate. The underlying mechanism of primary resistance and the relationship between ER and secondary resistance remains unknown.

MATERIALS AND METHODS

Viruses.

Subcultures of SMV strains used in this study were taken from cloned progenies of SMV-N (ATCC PV728) and G7 (ATCC PV722) (Eggenberger and Hill 1997). The viruses were purified according to Hill and Benner (1980), except the extraction buffer consisted of 0.1 M sodium phosphate (pH 7.5), 1% sodium sulfite, and 2 mM EDTA, and clarification was performed in the presence of 8% n-butanol. Following CsCl centrifugation, the purified virions were dialyzed against 0.05 M Na-borate, pH 8.0, and stored at 4°C. Virion concentration was determined spectrophotometrically. The BPMV isolate used in this study is a local isolate.

Plants, treatment, and maintenance.

Virus-free soybean seeds (*Glycine max*) obtained from virus-indexed greenhouse-grown cultivars Williams 82 (*rsv1*) and Pioneer 9341 (*rsv1*) and the soybean line PI 96983 (*Rsv1*) were grown in a greenhouse with supplemental lighting during winter at 22 to 25°C or in a growth chamber operating at 22°C with a photoperiod of 16 h. Carborundum-dusted (600 mesh) primary leaves of soybean were inoculated mechanically with purified virions or sap from infected tissues in 50 mM phosphate buffer, pH 7.0. For induction of PR gene expression by SA, soybean leaf disks were punched from fully developed trifoliate leaflets of 3- to 4-week-old plants and floated on H₂O or a solution of 0.01% SA (Sigma, St. Louis, MO, U.S.A.), adjusted to pH 6.5 (White 1979). Tissues were kept in a growth room under continuous fluorescent illumination at 22°C for 72 h. The tissues were harvested, frozen in liquid nitrogen, and stored at -85°C. Leaf disks from tobacco (*Nicotiana glutinosa*) were similarly treated.

Grafting procedure.

For cleft grafting, a 1- to 2-cm vertical cut was made above the primary leaves in the center of the stems of 2- to 3-week old rootstocks. Stems of 2- to 3-week-old plants, which served as scions, were trimmed below the primary leaves to the shape of wedges. After insertion of the scion into the rootstock, the scion-rootstock junction was wrapped with stretched Parafilm and the plants were covered for 7 to 10 days with plastic bags to avoid dehydration. Plants were then transferred to a mist chamber in the greenhouse for 1 to 2 weeks and were then maintained in the greenhouse. For interstem grafting, 2-week-old seedlings used as scions were cleft grafted on 5-week-old rootstocks, as described above. After 3 weeks, the leaves on the first scion were removed and the second scion was cleft grafted and maintained as above. For approach grafting, stems of 2-week-old seedlings were joined together after removing the epidermal surface 1 to 2 cm below the primary leaves. The graft was wrapped with stretched Parafilm, and the plants were maintained in the greenhouse.

Histochemistry and microscopy.

Leaf tissues were boiled for 2 min in alcoholic lactophenol (95% ethanol-lactophenol, 2:1, vol/vol), rinsed in 50% etha-

nol followed by H₂O (Dietrich et al. 1994), viewed under an Olympus SZH 10 zoom stereo microscope (Tokyo, Japan), and photographed with an Olympus photomicrographic System Model PM-10AK3. Cross sections of the cleared leaves were prepared with a tissue slicer OTS-400 (Electron Microscopy Science, Fort Washington, PA, U.S.A.). The sections were mounted on slides in 70% glycerin in water, and auto-fluorescence was observed with ultraviolet epifluorescence (excitation filter, 340 to 380 nm; dichroic mirror, RKP 400 nm; and barrier filter, LP430 nm) (Leitz Orthoplan Microscope, Wetzlar, Germany) and photographed with a Wild MPS 52 photomicrographic system from Leica (Heerbrugg, Switzerland). To visualize callose deposition, hand sections were stained for 1 h at room temperature in a 0.01% (wt/vol) solution of aniline blue in 0.15 M K₂HPO₄ (Eschrich and Currier 1964). Stained tissues were mounted in seven parts glycerol-three parts stain solution and examined with ultraviolet epifluorescence as above. For staining with trypan blue, leaves were boiled in lactophenol containing 0.01% trypan blue for 10 min (Tang et al. 1999). After incubation at room temperature for 1 h in the same solution, tissues were rinsed in H₂O, destained in 0.03% sodium hypochlorite for 3 days, and viewed under an Olympus SZH10 stereomicroscope. For lignin staining, cross sections of fresh leaf tissues were submerged in drops of a solution containing 2% phloroglucinol (EM Science, Gibbstown, NJ, U.S.A.) in 95% ethanol (Sass 1958). A drop of saturated hydrochloric acid was added to the tissue mount. After a 5-min incubation, the sample was viewed with a Leitz Orthoplan Microscope and photographed as above.

Immunoassay.

For squash-immunoblotting, soybean tissues were firmly pressed onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), which were then blocked in Tris saline buffer (TBS) (20 mM Tris-HCl, 500 mM NaCl), pH 7.5, containing 5% nonfat dried milk. The blots were probed with anti-SMV-specific polyclonal antibodies (McLaughlin et al. 1980) and processed as described (Hajimorad et al. 1996). SMV coat protein (CP) also was detected by DAS-ELISA, as described by Diaco et al. (1985), except polyclonal-biotinylated immunoglobulins (IgG) were used. To detect SMV virions, SSEM was performed as described by Derrick (1990). Tissue extract in 0.1 M sodium phosphate (pH 7.5), 1% sodium sulfite, and 2 mM EDTA was floated onto anti-SMV polyclonal IgG-coated grids, negatively stained with 1% phosphotungstic acid (pH 7.0), and viewed in a JEOL 1200EX II electron microscope (Tokyo, Japan).

RNA isolation.

Soybean total nucleic acid was isolated from fresh tissues (Langeveld et al. 1991) and, after ethanol precipitation, was dried and dissolved in H₂O (1:1, vol/wt of original tissue). Total plant RNA was isolated from liquid nitrogen-frozen leaf tissues kept at -85°C, as described by Pawlowski et al. (1994). Virion RNA was isolated in accordance with a modified procedure by Brakke and Van Pelt (1970). Purified virus was mixed with an equal volume of 0.2 M ammonium carbonate, pH 9.0, containing 2% sodium dodecyl sulfate, 0.002 M EDTA, and 5% washed bentonite (Fraenkel-Conrat and Tsugita 1961) and extracted twice with an equal volume of

phenol–chloroform–isoamyl alcohol. The RNA was ethanol precipitated, dried, dissolved in H₂O, and the concentration determined spectrophotometrically.

RT-PCR.

For IC-RT-PCR, Dynatech Immulon I Removawells (Dynatech Technologies, Chantilly, VA, U.S.A.) were coated with 1 µg per ml of anti-SMV monoclonal antibody S4 (Hill et al. 1989). Following washing and blocking with phosphate–saline buffer, pH 7.5, containing 5% nonfat dried milk, soybean sap extract was added to wells that were incubated overnight at 4°C and then washed thoroughly. First-strand cDNA was synthesized in the same wells with a SuperScript preamplification system (Life Technologies, Gaithersburg, MD, U.S.A.), according to the manufacturer's instructions, except the SMV-specific primer 5'-GCTTGAGGGCAATCCTCT-3', complementary to conserved nucleotides 2,758 to 2,741 of the SMV genome (Eggenberger 1994; Jayaram et al. 1992), was used. PCR amplification of the SMV sequences was performed with *Taq* polymerase (Life Technologies), the SMV primer used for the first-strand synthesis, and primer 5'-GGCAAGAGCGAC-AAGA-3' homologous to conserved nucleotides 1,854 to 1,871 of the SMV genome (Eggenberger 1994; Jayaram et al. 1992). PCR products were analyzed on 1% agarose gel stained with ethidium bromide (Sambrook et al. 1989). Gel-purified DNA (Sambrook et al. 1989) was ligated into pGEM-T (Promega, Madison, WI, U.S.A.) and sequenced with vector-specific T7 and SP6 primers at the Iowa State University DNA Analyses and Synthesis Facility. Sequence analysis was performed with the BLAST program (Altschul et al. 1990). SMV sequences in total soybean nucleic acid were amplified by RT-PCR in a similar fashion.

Probes and slot-blot hybridization.

A cloned fragment of a soybean PR-1 protein gene (GenBank accession no. AI930866) was PCR amplified with vector-specific T7 and T3 primers, gel-purified, and reamplified in the presence of ³²P-dCTP to generate a cDNA-labeled probe. A cloned fragment of the tobacco PR-1a protein gene (Cornelissen et al. 1986) was released by *Pst*I digestion, gel purified, and used as a DNA template to generate a labeled probe. Probes against tobacco PR-1a and soybean 18s ribosomal RNA (Eckenrode et al. 1984) were synthesized with a random-primed DNA labeling kit (Life Technologies), according to the manufacturer's instructions. The SMV probe was synthesized by PCR with gel-purified RT-PCR products as templates and the SMV-specific primers described previously. Unincorporated nucleotides were removed with Probe Quant TM G-50 microcolumns (Pharmacia, Piscataway, NJ, U.S.A.). RNA denaturation and slot-blot hybridization were performed as described (Bananej et al. 1998), except that the Gene Screen Plus transfer membrane (New Life Science Products, Boston, MA, U.S.A.) was used and prehybridization and hybridization were performed at 42°C. Hybridization signals were scanned and imaged with a Molecular Dynamics Storm 840 PhosphoImager (Sunnyvale, CA, U.S.A.). Images were reformatted for publication with Adobe PhotoShop (Adobe Systems, San Jose, CA, U.S.A.).

ACKNOWLEDGMENTS

We are grateful to A. L. Eggenberger for subcultures of cloned progenies of SMV-N and SMV-G7 isolates and advice on IC-RT-PCR; R. C.

Shoemaker and J. Erpelding (Agronomy Department, Iowa State University) for partial cloned sequences of a soybean PR-1 gene, and H. J. L. Linthorst (Leiden University, The Netherlands) for cloned sequences of tobacco PR-1a. We also thank M. J. Roossinck (The Noble Foundation, Ardmore, OK, U.S.A.), and T. J. Baum, R. P. Wise, and W. A. Miller for useful comments on the manuscript. H. T. Horner and T. M. Pepper of the Bessey Microscopy Center, Iowa State University, are acknowledged for advice with microscopy, and D. M. Volker for assistance with plant maintenance. This project was supported in part by the Iowa Soybean Promotion Board. Journal paper J-19075 of the Iowa Agriculture and Home Economic Experiment Station, Ames, IA, U.S.A., project 2428, and supported by funds from Hatch Act and the State of Iowa.

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