***Pseudomonas syringae* Effector *avrB* Confers Soybean Cultivar-Specific Avirulence on *Soybean mosaic virus* Adapted for Transgene Expression but Effector *avrPto* Does Not**

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Soybean mosaic virus (SMV) was adapted for transgene expression in soybean and used to examine the function of avirulence genes *avrB* and *avrPto* of *Pseudomonas syringae* pvs. *glycinea* and *tomato*, respectively. A cloning site was introduced between the *P1* and *HC-Pro* genes in 35S-driven infectious cDNAs of strains SMV-N and SMV-G7. Insertion of the *uidA* gene or the green fluorescent protein gene into either modified cDNA and bombardment into primary leaves resulted in systemic expression that reflected the pattern of viral movement into uninoculated leaves. Insertion of *avrB* blocked symptom development and detectable viral movement in cv. Harosoy, which carries the *Rpg1-b* resistance gene corresponding to *avrB*, but not in cvs. Keburi or Hurrelbrink, which lack *Rpg1-b*. In Keburi and Hurrelbrink, symptoms caused by SMV carrying *avrB* appeared more quickly and were more severe than those caused by the virus without *avrB*. Insertion of *avrPto* enhanced symptoms in Harosoy, Hurrelbrink, and Keburi. This result was unexpected because *avrPto* was reported to confer avirulence on *P. syringae* pv. *glycinea* inoculated to Harosoy. We inoculated Harosoy with *P. syringae* pv. *glycinea* expressing *avrPto*, but observed no hypersensitive reaction, *avrPto*-dependent induction of pathogenesis-related protein 1a, or limitation of bacterial population growth. In Hurrelbrink, *avrPto* enhanced bacterial multiplication and exacerbated symptoms. Our results establish SMV as an expression vector for soybean. They demonstrate that resistance triggered by *avrB* is effective against SMV, and that *avrB* and *avrPto* have general virulence effects in soybean. The results also led to a reevaluation of the reported avirulence activity of *avrPto* in this plant.

Additional keywords: bacterial blight, GFP, GUS, transient expression.

To establish conditions favorable for colonization of plants, gram-negative phytopathogenic bacteria depend on the type III secretion system, encoded by *hrp* genes, to deliver suites of effector proteins into host cells. Collectively, effectors are re-

quired for pathogenesis; however, their individual functions are only beginning to be understood. Many effectors were first identified as avirulence proteins by virtue of their ability to trigger plant defense in host varieties expressing corresponding resistance proteins (White et al. 2000). Except for a handful of these, virulence functions have been difficult to discern genetically due to their quantitative and probably redundant nature (Chang et al. 2004).

Transient expression of bacterial effector genes in plants has been a fruitful approach to understanding their functions (Abramovitch et al. 2003; Hotson et al. 2003; Shao et al. 2003). In fact, transient expression experiments first demonstrated that effectors function within plant cells, establishing the role of type III secretion in plant pathogenesis (Bonas and Van den Ackervaken 1997). Pathogens of different plants rely on distinct inventories of effectors (Lindeberg et al. 2005). Dissecting the roles that different effectors play in diseases of different plants will advance our understanding of plant molecular processes and potentially reveal targets for new approaches to disease control. However, transient expression techniques are limited for many crop plant species.

Methods for transient expression in plants include transformation mediated by *Agrobacterium* spp. infiltrated into plant tissues (Janssen and Gardner 1990), introduction of DNA into plant cells by particle bombardment (Sanford et al. 1993a), introduction of expression constructs into plant protoplasts by electroporation (Guerche et al. 1987), and inoculation of plants with recombinant viruses or infectious derivatives carrying transgenes (Scholthof et al. 1996). Transient expression takes less time and generally is less costly than stable transformation. Also, in contrast to stable transformation, which relies on regeneration of whole plants from individual, transformed cells, transient expression can accommodate gene expression that results in developmental defects or cell death. Compared with other approaches, virus-mediated methods uniquely allow for systemic expression in whole plants, and often achieve expression to higher levels and of greater duration (Porta and Lomonosoff 1996).

Soybean is a major crop species for which transient expression methods are limited. *Agrobacterium*-mediated transient expression has not proven to be robust. Particle bombardment has been used for transient expression in soybean (Ponappa et al. 1999; Wang et al. 1988); however, it is costly and results in expression only in a small number of cells. *Clover yellow vein virus* (ClyVV) was developed as an expression vector for clover and reported to function in soybean (Masuta et al. 2000), but has not seen widespread use.

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We adapted *Soybean mosaic virus* (SMV) for transgene expression in soybean and used it to examine the function of avirulence genes *avrB* and *avrPto* of *Pseudomonas syringae* pvs. *glycinea* and *tomato*, respectively. A Potyvirus, SMV consists of a single positive-sense RNA in a filamentous virion. The RNA is translated to yield a polyprotein that is self-processed by the proteinase components P1, HC-Pro, and NIa to yield eight mature proteins, two of which undergo partial further processing (Urcuqui-Inchima et al. 2001). Potyviruses are transmitted by aphids, but aphid transmissibility is readily lost upon serial mechanical passage (Sako 1980). *avrB* governs race- and cultivar-specific resistance to bacterial blight of soybean in conjunction with its corresponding resistance (*R*) gene *Rpg1-b* (Ashfield et al. 2004; Tamaki et al. 1988). *avrB* also is recognized by the *Arabidopsis* gene *RPM1* (Bisgrove et al. 1994). Typical of most gene-for-gene interactions, the interaction of *avrB* and *Rpg1-b* (or *avrB* and *RPM1*) elicits the defense-associated localized plant cell death known as the hypersensitive reaction (HR) and enhanced expression of pathogenesis-related (PR) proteins (Hammond-Kosack and Jones 1996). In soybean cultivars lacking *Rpg1-b*, *avrB* contributes to pathogen virulence (Ashfield et al. 1995). *avrPto* triggers resistance to bacterial speck of tomato mediated by the corresponding *Pto* gene (Ronald et al. 1992). As a transgene in *Potato virus X* (PVX), *avrPto* prevented systemic infection by the virus in tomato plants carrying *Pto* (Tobias et al. 1999). *AvrPto* was reported to elicit resistance in several soybean cultivars when expressed in *P. syringae* pv. *glycinea* (Lorang et al. 1994; Ronald et al. 1992). In addition to its avirulence function, *AvrPto* was observed to enhance the virulence of *P. syringae* pv. *tomato* in tomato plants lacking the *Pto* gene (Shan et al. 2000a) and, more recently, shown to suppress cell-wall-based defense responses in *Arabidopsis* (Hauck et al. 2003).

RESULTS

Biologically active cDNA clones of SMV-N and SMV-G7.

SMV strain SMV-N causes mosaic, rugosity, and stunting on susceptible cultivars, whereas SMV-G7 causes mild mosaic or no symptoms (Cho and Goodman 1979). Therefore, SMV-N was considered a good candidate for use as a vector to examine the effects of transgenes on the virus–host interaction, and SMV-G7 potentially useful as a general expression vector. Plasmids containing 35S promoter-driven cDNAs of non-aphid-transmissible isolates of SMV strains SMV-N and SMV-G7 (Table 1) were constructed and designated as pSMV-N and pSMV-G7 (Fig. 1) (discussed below). Introduction of the plasmids by particle bombardment into primary leaves of intact soybean plants of cv. Williams 82 resulted in systemic viral infection within 2 weeks, equivalent to that following inoculation with virions.

SMV-mediated systemic expression

of β -glucuronidase and green fluorescent protein in soybean.

pSMV-N and pSMV-G7 were modified to permit insertion of a transgene between the P1 and HC-Pro coding regions and to allow subsequent processing of the product from the SMV polypeptide (discussed below). The resulting constructs, pSMV-Nv and pSMV-G7v, were confirmed to be infectious. Insertion of the *uidA* gene into pSMV-G7v (Fig. 2A) or pSMV-Nv (not shown) and introduction of the resulting plasmids into primary leaves of Williams 82 plants by particle bombardment resulted in systemic expression of β -glucuronidase (GUS) activity (Fig. 2) that reflected the general pattern of viral replication and movement (Carrington et al. 1996). Expression of GUS was evident along the veins in young expanding leaves, spreading into the interveinal tissue in newly ex-

Table 1. Bacterial strains and plasmids used in this study

Viral or bacterial strain or plasmid	Relevant characteristics ^a	Source or reference
SMV-G7 (ATCC PV-722)	...	Cho and Goodman 1979
SMV-N (ATCC PV-728)	...	Vance and Beachy 1984
<i>Pseudomonas syringae</i> pv. <i>glycinea</i> PsgR4	Race 4, Rf ^r	Kobayashi et al. 1989
<i>Escherichia coli</i> DH5 α	F' <i>lndA1 hsdR17(rK– mK+)</i> <i>supE44 thi-1 recA1 gyrA (Nalr) relA1 D(lacIZYA-argF)U169 deoR (F80dlacD(lacZ)M15)</i>	Invitrogen, Carlsbad, CA, U.S.A.
Plasmids		
pBR322	Cloning vector, Ap ^r	Bolivar et al. 1977
pAGUS1	Binary vector, Ap ^r	Skuzeski et al. 1990
pBR322-35S	pBR322 containing the CaMV 35S expression cassette from pAGUS1 with an expanded multiple cloning site, Ap ^r	This work
pSMV-G7	pBR322-35S containing full-length cDNA to SMV-G7, Ap ^r	This work
pSMV-G7v	pSMV-G7 containing a transgene cloning site, Ap ^r	This work
pRAJ275	Source of <i>uidA</i> used in this study	Jefferson et al. 1986
psmGFP	Source of <i>GFP</i> used in this study	Davis and Vierstra 1998
pSMV-G7v:: <i>uidA</i>	pSMV-G7v containing <i>uidA</i> , Ap ^r	This work
pSMV-G7v:: <i>GFP</i>	pSMV-G7v containing <i>GFP</i> , Ap ^r	This work
pSMV-N	pBR322-35S containing full-length cDNA to SMV-N, Ap ^r	This work
pSMV-Nv	pSMV-N containing a transgene cloning site, Ap ^r	This work
pSMV-Nv:: <i>uidA</i>	pSMV-Nv containing <i>uidA</i> , Ap ^r	This work
pSMV-Nv:: <i>GFP</i>	pSMV-Nv containing <i>GFP</i> , Ap ^r	This work
pSMV-Nv:: <i>avrB</i>	pSMV-Nv containing <i>avrB</i> , Ap ^r	This work
pSMV-Nv:: <i>avrPto</i>	pSMV-Nv containing <i>avrPto</i> , Ap ^r	This work
pPtE2	pRK404 containing a 2.6-kb insert with <i>avrPto</i> , Tc ^r	Ronald et al. 1992
pPtE2 Ω	pPtE2 carrying an Ω fragment substitution in <i>avrPto</i> , Tc ^r , Sm ^r , Sp ^r	Ronald et al. 1992
pDSK519	Broad-host-range vector, Km ^r	Keen et al. 1988
pPtE6	pDSK519 containing a 0.6-kb fragment with <i>avrPto</i>	Ronald et al. 1992
pAVRB (our designation)	pDSK519 containing <i>avrB</i> , Km ^r	Tamaki et al. 1988
pRK2013	Helper plasmid, Km ^r	Ditta et al. 1980

^a Rf^r, rifampicin resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Sp^r, spectinomycin resistance; and Sm^r, streptomycin resistance.

panded leaves, and, finally, in fully expanded leaves, confluent throughout interveinal tissue and reduced along the veins. Insertion of the green fluorescent protein (*GFP*) gene resulted in similar, systemic expression of GFP. Fluorescence in systemic leaves was greater for pSMV-Nv::GFP than pSMV-G7v::GFP (Fig. 2B), consistent with the greater replication of SMV-N in Williams 82 relative to SMV-G7 (A. Eggenberger and J. Hill, unpublished observation). As determined by immunocapture reverse-transcriptase polymerase chain reaction (RT-PCR) (discussed below), the *GFP* gene, 0.7 kb long, was stable in both pSMV-Nv (Fig. 2C) and pSMV-G7v (not shown), with no detectable deletion of the gene in the eighth trifoliate approximately 6 weeks following bombardment. Subpopulations of virus having undergone spontaneous deletion of the larger, 1.8-kb *uidA* gene were apparent in second and later trifoliate leaves of plants bombarded with either pSMV-Nv::uidA (Fig. 2C) or pSMV-G7v::uidA (not shown), but GUS activity was maintained substantially through the eighth trifoliate leaf (Fig. 2).

Functions in soybean of *avrB* and *avrPto* in pSMV-Nv.

avrB and *avrPto* were amplified by PCR and cloned into pSMV-Nv using primers designed such that processing would preserve the native N-termini (Fig. 1) (discussed below), because activities of AvrB and AvrPto depend on an N-terminal myristylation motif (Nimchuk et al. 2000; Shan et al. 2000b). Introduction of pSMV-Nv::avrB into primary leaves of soybean cv. Hurrelbrink (Fig. 3) or cv. Keburi (not shown), which are susceptible to *P. syringae* pv. *glycinia* strains containing *avrB* (Fig. 4; data not shown), resulted in systemic symptoms. In contrast to the mild mosaic that became apparent approximately 2 weeks following introduction of pSMV-Nv (not shown) or pSMV-Nv::GFP (Fig. 3), symptoms following introduction of pSMV-Nv::avrB first became apparent at 10 days and developed into relatively severe veinal chlorosis and interveinal necrosis (Fig. 3). The presence of virus containing *avrB* in upper, noninoculated leaves was confirmed by immunocapture RT-PCR (Fig. 3). Introduction of pSMV-Nv::avrB into cv. Harosoy, which carries *Rpg1-b*, did not result in sys-

temic symptoms of any kind, and virus was undetectable in upper, noninoculated leaves. This result contrasts with pSMV-Nv::GFP in cv. Harosoy, which caused mild systemic mosaic accompanied by detectable amounts of virus in upper leaves. *avrPto* in pSMV-Nv, pSMV-Nv::avrPto, enhanced virus-associated symptoms in cvs. Hurrelbrink (Fig. 3) and Keburi (not shown) which were reported to be susceptible to *P. syringae* pv. *glycinia* expressing this gene, but also in cv. Harosoy (Fig. 3), which was reported to be resistant (Lorang et al. 1994). Black necrosis of veins and adjacent tissue, as well as rugosity, were apparent in cv. Hurrelbrink, and brown and spotty necrosis developed along veins and within interveinal tissues in cv. Harosoy (Fig. 3). Immunocapture RT-PCR indicated the presence of the virus containing *avrPto* in upper, noninoculated leaves of both Hurrelbrink and Harosoy (Fig. 3). Thus, *avrB* but not *avrPto* confers corresponding soybean cultivar-specific avirulence on SMV, and both *avrB* and *avrPto* in pSMV-Nv enhance virus-associated symptoms in susceptible plants, albeit differently.

Lack of observed avirulence activity

in soybean of *avrPto* expressed in *P. syringae* pv. *glycinia*.

Given the *avrB* result and the ability of *avrPto* to block PVX infection in tomato (Tobias et al. 1999), failure of *avrPto* to prevent SMV-associated symptom development and viral movement in cv. Harosoy was puzzling in light of the reported resistance of Harosoy to *P. syringae* pv. *glycinia* containing cloned *avrPto* (Lorang et al. 1994). Therefore, the effects of *avrPto* on *P. syringae* pv. *glycinia* interaction with soybean were reexamined. Plasmids pPtE2, a 2.6-kb clone containing *avrPto* downstream of the *lacZ* promoter in the vector pRK404 (Table 1), and pPtE2Ω, containing an omega fragment in place of *avrPto* (Table 1), were introduced into the *P. syringae* pv. *glycinia* race 4 strain PsgR4 (Table 1). PsgR4 is virulent on most soybean cultivars, including Harosoy. Neither of the resulting strains, PsgR4(pPtE2) or PsgR4(pPtE2Ω), elicited the HR in cvs. Acme, Centennial, Faribault, Harosoy, or Norchief at any of several bacterial titers ranging as high as approxi-

Fig. 1. Soybean mosaic virus (SMV) expression constructs used in this study. **A**, Schematic representation of the modified SMV genome. Open boxes represent the indicated cistrons. The shaded box represents sequence modified for transgene insertion. Arrows indicate P1 and NIa proteinase cleavage sites within this sequence. **B**, Nucleotide and deduced amino acid sequences of the transgene cloning site. The AvrII site is underlined, and the proteinase cleavage sites are represented by a forward slash mark. **C**, Deduced terminal amino acid sequences following release from the polypeptide by the P1 and NIa proteinases for the products of each of the transgenes cloned into the AvrII site in this study. Terminal sequences are separated by ellipses, with the N-terminal sequence on the left. Non-native residues are indicated by italics. The NIa proteinase site added to AvrB and AvrPto is shown in bold. Cleavage at this site results in N-termini for both proteins that correspond to their native sequence beginning at a glycine residue at position two.

mately 5×10^8 CFU/ml (optical density at 600 nm [OD₆₀₀] = 1.0) (data not shown). Population growth over 6 days of PsgR4(pPtE2) and PsgR4(pPtE2Ω) inoculated at a lower titer to Harosoy plants, as determined with selection for the plasmids, was highly variable within and across four replicate experiments. In two of these, the mean population growth of PsgR4(pPtE2) was 10- to 20-fold less than that of PsgR4(pPtE2Ω); however, in the other two, roughly the opposite was true, and in no experiment were these differences statistically significant. Plating without selection for the plasmids greatly reduced variability and resulted in higher values for the populations that were not significantly different from one another. These patterns suggested that the results were influenced by plasmid instability.

Therefore, the effects of *avrPto* were reexamined using different plasmids: pPtE6, a 0.6-kb subclone of the pPtE2 insert containing the *avrPto* open reading frame, oriented with the

lacZ promoter in the vector pDSK519 (Table 1), and pDSK519 without any insert. pPtE6 failed to confer HR-eliciting activity on PsgR4 in Harosoy (Fig. 3) or Acme, Centennial, Faribault, or Norchief. In cv. Harosoy inoculated with a low titer of bacterial cells, population growth was not statistically different for the transformant with pPtE6, PsgR4(pPtE6), and the transformant with pDSK519, PsgR4(pDSK519) (Fig. 4). Comparison of populations measured with and without selection for these plasmids revealed no indication of plasmid loss in planta over 6 days. On Harosoy leaves inoculated with PsgR4(pPtE6), bacterial blight symptoms developed that were equivalent to those caused by PsgR4(pDSK519) (Fig. 4) or the parental strain (not shown).

Northern blot analysis of Harosoy leaves sampled over a time course following inoculation with PsgR4(pAVRB), PsgR4(pPtE6), or PsgR4(pDSK519) revealed no effect of *avrPto* on the pattern of expression of the *PR1a* gene, in contrast to *avrB*, which triggered rapid, enhanced accumulation of *PR1a* transcript (Fig. 5).

Western blotting of proteins in PsgR4(pPtE2) and PsgR4(pPtE6) and immunodetection of AvrPto confirmed that AvrPto was expressed in both strains. Plasmid rescue and testing in *P. syringae* pv. *tomato* inoculated to tomato plants with or without *Pto* confirmed that *avrPto* in both plasmids was functional (not shown). PsgR4 transformed with the pDSK519 derivative pAVRB containing the *avrB* gene, PsgR4(pAVRB), elicited a robust HR in cvs. Harosoy (Fig. 4), Faribault, and Norchief. In Harosoy, PsgR4(pAVRB) multiplied approximately 10^4 -fold less than PsgR4(pDSK519) over 4 days and caused no bacterial blight symptoms (Fig. 4).

In summary, no evidence of avirulence function of *avrPto* in soybean was observed.

Virulence activity in soybean of *avrPto* expressed in *P. syringae* pv. *glycinea*.

PsgR4(pAVRB), PsgR4(pPtE6), and PsgR4(pDSK519) were inoculated to cv. Hurrelbrink and populations monitored over 4 days (Fig. 4). PsgR4(pDSK519) grew to approximately 10-fold lower levels after 4 days in this cultivar relative to Harosoy. A

Fig. 2. Soybean mosaic virus (SMV)-mediated expression of *uidA* and green fluorescent protein (GFP) in soybean cv. Williams 82. **A**, Leaflets of the indicated trifoliate leaves, ranging from expanding to fully mature (left to right), stained for β -glucuronidase activity and cleared 6 weeks following bombardment of primary leaves with pSMV-G7v::*uidA*. **B**, Leaflets of the eighth trifoliate leaf, photographed under UV light 6 weeks following bombardment of primary leaves with pSMV-G7v::GFP or pSMV-G7v (left panel) and pSMV-Nv::GFP or pSMV-Nv. **C**, Products of immunocapture reverse-transcriptase polymerase chain reaction (PCR) amplification of the transgene site for leaflets of the second, fifth, and eighth trifoliate leaves of plants 6 weeks following bombardment of primary leaves with pSMV-Nv::GFP (lanes 1, 2, and 3) or pSMV-Nv::uidA (lanes 4, 5, and 6). The relative amounts of PCR products do not necessarily reflect the relative amounts of viral RNA, because smaller templates may be preferentially amplified.

Fig. 3. Effects of *avrB* and *avrPto* as transgenes in plasmid of Soybean mosaic virus Nv (pSMV-Nv). Shown are **A**, the appearance of leaflets and **B**, the products of immunocapture reverse-transcriptase polymerase chain reaction (PCR) amplification of the transgene site for the third trifoliate leaf of Harosoy (Ha) or Hurrelbrink (Hu) plants 2 weeks following bombardment of primary leaves with pSMV-Nv carrying the indicated transgenes. The leftmost lane in B contains the pooled products of PCR amplification using the indicated plasmids as templates individually.

small but significant difference in bacterial population growth was observed between PsgR4(pAVRB) and PsgR4(pDSK519), as demonstrated previously in other susceptible cultivars (Ashfield et al. 1995): the population levels for PsgR4(pAVRB) in Hurrelbrink were higher and similar to those of PsgR4(pDSK519) in Harosoy. AvrPto caused a small but significant ($t = 3.3845$, $P = 0.0277$ on day four) increase in bacterial population growth as well. Also, by 2 weeks after inoculation, plants inoculated with PsgR4(pAVRB) or PsgR4(pPtE6) had developed more extensive necrosis and more severe chlorosis than plants inoculated with PsgR4(pDSK519) (representative leaflets are shown in Figure 4). Thus, like *avrB*, *avrPto* can contribute to virulence in bacterial blight of soybean.

Fig. 4. Avirulence and virulence activity of *avrB* and *avrPto* in *Pseudomonas syringae* pv. *glycinea* in soybean. **A**, Hypersensitive reaction assay of strain PsgR4 transformants with the indicated plasmids in primary leaves of Harosoy and Hurrelbrink plants. The minus sign indicates no plasmid. Bacterial suspensions of approximately 10^8 CFU/ml were introduced into leaves by using a needleless syringe, and leaves were photographed after 48 h. **B**, Bacterial population growth in Harosoy and Hurrelbrink leaves over 4 days post inoculation (DPI) of whole plants by vacuum infiltration with PsgR4 transformants carrying the indicated plasmids, at a concentration of approximately 10^5 CFU/ml. Values are means of three samples per time point. Vertical bars represent standard deviation. **C**, Bacterial blight symptoms on leaflets of Harosoy (Ha) and Hurrelbrink (Hu) plants 2 weeks following inoculation as in B. Results were essentially identical in five replicate experiments.

DISCUSSION

In this study, SMV was adapted for transgene expression in soybean and used to examine the function of avirulence genes *avrB* and *avrPto* of *P. syringae* pvs. *glycinea* and *tomato*, respectively. The results establish SMV as an efficient expression vector for soybean. They demonstrate that *avrB* triggers *R*-gene-mediated resistance that is effective against SMV, and that *avrB* and *avrPto* have general virulence effects in soybean. Finally, the results led to a reassessment of previously reported avirulence activity of *avrPto* in soybean.

Expression of *uidA* and *GFP* via SMV reflected the pattern of viral replication and movement in plants, resulting in confluent, homogenous activity in fully expanded trifoliate leaves following transfection by bombardment of primary leaves. In addition to enabling rapid, systemic expression of transgenes, SMV has other important advantages. The availability of non-aphid-transmissible strains, such as those used here, mitigates the risk of unintended transmission, as does the fact that SMV is not readily transmitted accidentally by standard practices used in a greenhouse due to its relative instability outside of a host (University of Idaho Plant Viruses Online database). At the same time, simple methods for (intentional) mechanical transmission (Hajimorad and Hill 2001) would permit use of SMV for transgene expression in large-scale experiments, using sap from a plant transfected by particle bombardment to inoculate others. Strains such as SMV-G7 which cause mild or no symptoms can be useful as general expression vectors, whereas more virulent strains, such as SMV-N, can be useful for assaying the effect of transgenes on the virus–host interaction. The modifications necessary to adapt SMV as a vector were straightforward and could be used with other strains as needed for studies in different soybean cultivars.

Another advantage of SMV is that, because it is a filamentous virus, there is no strict limit on the size of an inserted gene. Nevertheless, as observed of *uidA* in this study, some transgenes may suffer spontaneous deletion. Spontaneous deletions can be a function not only of insert size but also of structural characteristics such as GC content (Lee et al. 2002). Therefore, stability will be difficult to predict for specific transgenes, but may be manipulable (Lee et al. 2002).

Gene silencing would not be expected to occur to a significant extent with SMV because it encodes HC-Pro, a strong suppressor of silencing (Voinnet 2001).

Finally, incorporation of an N1a proteinase site at the 5' end of a transgene in SMV permits expression of the product with an unmodified or minimally modified N-terminus. The heptapeptide sequence recognized by N1a is (E or N)xxVxxQ/(A, G, or S), with the cleavage site denoted by the forward slash mark (Ghabrial et al. 1990; Riechmann et al. 1992). If the N-terminal amino acid of the protein of interest is A, G, or S, which was the case for AvrPto and AvrPtoB, the first six codons for the site can be added and the protein will be released without additional amino acids at its N-terminus. If not,

Fig. 5. Northern blot analysis of *PR1-a* expression in leaves of soybean cv. Harosoy in response to *avrB* and *avrPto* expressed in *Pseudomonas syringae* pv. *glycinea*. Ten-day-old plants were inoculated by vacuum infiltration with PsgR4 transformants carrying the indicated plasmids at a concentration of 5×10^7 CFU/ml. RNA was isolated from whole primary leaves sampled at the times shown following inoculation, and hybridized to probes for *PR1-a* and actin transcript.

the complete N1a site can be added, and the protein will have an additional (A, G, or S) residue at its N-terminus.

Insertion of *avrB* into the adapted SMV genome blocked symptom development and detectable viral movement in cv. Harosoy, which carries *Rpg1-b*. The efficacy of *avrB*- and *Rpg1-b*-mediated resistance against SMV, like *Pto*-dependent resistance of tomato to PVX containing *avrPto* (Tobias et al. 1999), bolsters the notion that, despite specific triggers, *R*-gene-mediated responses are general and effective against a diversity of pathogens. This notion derives also from observations that some *R* genes have multiple specificities, overexpression of *R* genes or components of *R*-gene-mediated defense signaling confers broad-spectrum disease resistance, and *R* genes to viral, bacterial, and fungal pathogens share downstream signaling components (Martin et al. 2003). Even *R* genes that rely on distinct signaling pathways generate largely identical output (Eulgem et al. 2004), though timing and intensity of responses may differ for different *R* genes (Eulgem et al. 2004; Ritter and Dangl 1996).

The mechanism whereby SMV-associated symptoms and movement were blocked by *avrB* and *Rpg1-b* interaction is not clear. Very rarely, mild mosaic developed on emerging trifoliate leaves 3 to 4 weeks following bombardment of primary leaves. By immunocapture RT-PCR, virus usually was undetectable, but occasionally could be detected at a size consistent with it having undergone partial deletion of *avrB* (data not shown). It is tempting to speculate based on the infrequency of these escapes that resistance is mediated at the level of replication, perhaps by rapid cell death triggered by AvrB. Analysis of the effect of *avrB*- and *Rpg1-b*-mediated resistance compared with responses mediated by other *R* genes that differ in timing or intensity will shed light on the viral processes that are disrupted.

In susceptible cultivars transfected with SMV containing *avrB*, symptoms appeared more quickly and were more severe than those caused by the virus without *avrB*. *avrB* contributes to multiplication of *P. syringae* pv. *glycinea* in susceptible soybean plants (Ashfield et al. 1995; this study) and enhances bacterial blight symptoms (this study). Enhanced bacterial blight symptoms could be a consequence of greater bacterial multiplication, but a cytotoxic effect of AvrB has been noted in *Arabidopsis* (Nimchuk et al. 2000). AvrB also induces phosphorylation of RIN4, a negative regulator of basal defense in *Arabidopsis* (Mackey et al. 2002). RIN4 is not required for the cytotoxicity of AvrB; therefore, AvrB likely targets other plant proteins as well. However, targeting, possibly stabilization, of RIN4 by AvrB suggests a role for the effector in foiling plant defense (Belkhadir et al. 2004). Either a cytotoxic effect of AvrB (resulting directly in necrosis) or a defense-suppressive effect (leading to greater viral activity), or both, might explain the enhancement of SMV-associated symptoms. The more rapid spread of symptoms caused by SMV carrying *avrB* relative to SMV carrying *GFP* suggests that *avrB*-dependent symptoms were not a consequence of AvrB triggering delayed or incompletely effective defense response (i.e., lethal systemic HR) (Hajimorad et al. 2003) but, rather, that *avrB* enhanced spread of the virus itself. AvrB-enhancement of bacterial virulence in susceptible plants also weighs in favor of this interpretation. An indirect effect due to structural alteration of the virus by insertion of *avrB* cannot formally be ruled out; however, insertion of *uidA* or *GFP* had no such effect.

Insertion of *avrPto* into SMV caused necrosis in cvs. Harosoy, Hurrelbrink, and Keburi. As for *avrB*, this effect of *avrPto* may be due to necrogenic activity of the effector, or a dampening of defense that permitted greater viral activity. In previous studies, *avrPto* rendered PVX necrogenic (Tobias et al. 1999) and increased the number of lesions caused by *P. syringae* pv.

tomato (Chang et al. 2000; Shan et al. 2000a) in tomato plants lacking *Pto* or the *Prf* gene required for *Pto*-mediated resistance. Similarly, however, whether these effects occurred because AvrPto is necrogenic or because it prevents or counteracts plant defense could not be distinguished. Suppression of cell-wall-based basal defenses by AvrPto in *Arabidopsis* (Hauck et al. 2003) and the lack of necrogenesis by *avrPto* transiently expressed in *prf* plants (Chang et al. 2000) support the latter conclusion.

Like *avrB*, in *P. syringae* pv. *glycinea* inoculated to cv. Hurrelbrink, *avrPto* also enhanced bacterial multiplication and exacerbated symptoms. Taken together with the enhancement of virus-associated symptoms, the results suggest that both *avrB* and *avrPto* play general roles in virulence in soybean (notably, however, *avrPto* has not been detected in any of several races of *P. syringae* pv. *glycinea*) (Ronald et al. 1992). This notion, combined with the virulence and virulence-related effects of these proteins in *Arabidopsis* and tomato, further suggests that the respective plant targets are broadly conserved across diverse plant taxa.

Enhancement rather than preclusion of SMV-associated symptoms by *avrPto* in cv. Harosoy prompted a reexamination of the avirulence function of *avrPto* in soybean that was reported by Ronald and associates (1992) and Lorang and associates (1994). In their article demonstrating that the cloned gene triggers HR on tomato, Ronald and associates reported (but did not show) that *avrPto* (in pPtE2) transferred to PsGR4 elicited HR also in soybean cv. Centennial and, in one experiment presented, caused a reduction in bacterial multiplication of approximately 100-fold relative to PsGR4(pPtE2 Ω) in this cultivar over 4 days. Lorang and associates, examining the possibility that the four then-known *avr* genes of *P. syringae* pv. *tomato* accounted for nonhost resistance in soybean, tested whether these genes individually would trigger HR in several soybean cultivars when expressed in PsGR4 (using pPtE2 as the *avrPto* clone), and reported their results in a table, indicating that *avrPto* elicited HR in Harosoy and several other cultivars, but not in Hurrelbrink or Keburi. We obtained no evidence of avirulence function for *avrPto* in soybean using strain PsGR4 and either of two *avrPto*-containing plasmids, including pPtE2: no *avrPto*-mediated HR occurred in Harosoy, Centennial, or any of several other cultivars and, tested in Harosoy, no induction of *PR1a* or limitation of bacterial population growth due to *avrPto* was observed. Expression of AvrPto in the PsGR4 transformants used in each of these experiments and integrity of the gene following transformation were confirmed by Western blot analysis and testing of reisolated plasmid in *P. syringae* pv. *tomato* inoculated to tomato, respectively (not shown).

We found that instability of pPtE2 can result in stochastic, apparent limitation of bacterial populations when measured with selection for the plasmid. This fact may have contributed to the discrepancy between our and the prior results, but cannot explain it completely. The SMV result however, agrees with a lack of avirulence activity of *avrPto* in *P. syringae* pv. *glycinea*.

Though there was no limitation of bacterial growth in Harosoy due to *avrPto*, there also was no augmentation, as there was in Hurrelbrink. The population of PsGR4 without *avrPto* (carrying pDSK519) was approximately 10-fold higher in Harosoy than in Hurrelbrink 4 days after inoculation (Fig. 4), suggesting lower basal defense in Harosoy. If AvrPto contributes to virulence by suppressing defense, already low basal defense in Harosoy might explain the lack of an observable effect of *avrPto* in this cultivar.

Apparent failure of soybean to mount resistance in response to *avrPto* supports interesting speculation regarding the poten-

tial functional conservation of *Pto* outside of the family Solanaceae. *Pto* encodes a serine-threonine protein kinase, and *Pto* homologs are present in many plant species, including soybean (Martin et al. 1993). Soybean expresses at least two homologs of Pti1 (Staswick 2000). Pti1 is a Pto-like Pto substrate thought to be involved in signaling downstream of AvrPto recognition (Zhou et al. 1995). Both Pti1 homologs are phosphorylated by Pto but, in contrast to Pti1, neither were active in vitro (Staswick 2000). Thus, soybean may contain a functionally conserved homolog of Pto that is not effective due to the lack of an active Pti1 homolog. SMV offers an efficient means to test whether expression of Pti1 in soybean might confer (or restore) the ability to resist *P. syringae* pv. *glycinea* expressing *avrPto*.

Adaptation of SMV as an expression vector for soybean provides a powerful new tool for understanding gene function in this important crop plant. As shown here, it also allows tagging of the virus to better understand its biology in planta, and offers a fruitful approach to determining the roles of effectors of other plant pathogens. SMV will be an effective means to continue exploring the functions of AvrB and AvrPto. Expressing *avrB* and *avrPto* in soybean in isolation from other bacterial proteins, combined with mutagenesis and genetic analyses of both the effectors and the plant, promises further elucidation of effector activity and identification of targets that will lead to a better understanding of soybean resistance and susceptibility to disease.

MATERIALS AND METHODS

Viral and bacterial strains and plasmids used, growth conditions, and transformation.

Viral and bacterial strains and plasmids used are presented in Table 1. *Escherichia coli* was cultured in Luria-Bertani (LB) medium at 37°C, or, for strains containing viral cDNA, in LB supplemented with 20 mM glucose at 30°C. *P. syringae* was grown in King's B medium at 28°C. Ampicillin (100 µg/ml), kanamycin (50 µg/ml), rifampicin (750 µg/ml), spectinomycin (25 µg/ml), and tetracycline (10 µg/ml) were added to growth media as appropriate for selection. Plasmids were introduced into *E. coli* by electroporation and into *P. syringae* by electroporation or triparental mating.

Construction of pSMV-N, pSMV-G7, and derivatives.

For expression of SMV cDNA in planta following particle bombardment (discussed below), an *EcoRI-SphI* fragment containing the 35S promoter and NOS terminator from pAGUS1 (Table 1) was inserted between these sites in pBR322 (Table 1), and oligonucleotide-directed mutagenesis was used to expand the multiple cloning site within this fragment to include *StuI*, *BamHI*, *DraIII*, and *ClaI* sites. The resulting plasmid was designated as pBR322-35S. The *StuI* site in pBR322-35S is proximal to the 35S promoter.

SMV-N and SMV-G7 were purified from infected soybean leaves as described previously (Vance and Beachy 1984), and viral RNA was isolated using an RNeasy RNA purification kit (Qiagen, Valencia, CA, U.S.A.). cDNA to each strain was generated using AMV reverse transcriptase (Seikagaku, Falmouth, MA, U.S.A.) or Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD, U.S.A.) according to the manufacturer's instructions, followed by PCR using virus-specific primers. PCR was carried out using an LA PCR kit (Panvera, Madison, WI, U.S.A.). Complete nucleotide sequences are available for SMV-G7 (GenBank accession number AY216010) (Hajimorad et al. 2003) and SMV-N (GenBank accession number NC_002634) (*unpublished results*). cDNAs were cloned into pBR322-35S such that transcription from the 35S pro-

motor would be initiated at the first nucleotide. The resultant plasmids were designated as pSMV-N and pSMV-G7. Next, to allow for transgene insertion and subsequent release of the transgene product from the SMV polypeptide, an *AvrII* site followed by a sequence encoding an Nla proteinase cleavage site were added immediately downstream of the P1 proteinase cleavage site following the *P1* cistron in these plasmids, yielding pSMV-Nv and pSMV-G7v.

uidA and *GFP* were amplified from pRAJ275 and psmGFP (Table 1), respectively, using primers designed to introduce an *AvrII* site at each end, and the resulting products each were inserted into the *AvrII* site in pSMV-Nv and pSMV-G7v. *avrB* and *avrPto* were amplified from pAVRB and pPte6 (Table 1) and inserted in pSMV-Nv similarly, except that an Nla proteinase recognition site (Ghabrial et al. 1990) was added that includes and directs cleavage immediately preceding the glycine at position 2 in the AvrB and AvrPto proteins (Fig. 1).

Introduction of SMV constructs into plants.

SMV constructs were introduced into the primary leaves of 10- to 12-day-old seedlings by particle bombardment as described (Sanford et al. 1993b) using a Biolistic PDS-1000/He system (Bio-Rad Laboratories, Hercules, CA, U.S.A.), 1.0- or 1.6-µm gold particles, and 1,100-psi rupture disks at a distance of 6 cm. Following bombardment, plants were maintained in a greenhouse.

Detection of SMV and visualization of GUS activity and GFP.

Accumulation of SMV in bombarded and systemic leaves was confirmed using enzyme-linked immunosorbent assay (Diao et al. 1986). GUS activity was visualized as described previously (Jefferson 1987). GFP was visualized by illumination with a high-intensity long-wave UV lamp (Ultraviolet Products, Upland, CA, U.S.A.) and photography through a Kodak #58 Wratten Gel Filter.

Immunocapture RT-PCR.

Immunocapture RT-PCR was performed using anti-SMV monoclonal antibody S4 as described previously (Hajimorad and Hill 2001), except that reactions were carried out directly in standard PCR tubes. The primers 5'-GAG CGA TCA TCT TTA ACC AC-3' and 5'-GTG CAT TCA TGA TTC TCC AC-3', which correspond to sequences flanking the transgene cloning site in pSMV-Nv and pSMV-G7v, were used for amplification.

Bacterial HR and virulence assays.

Plants were grown in a growth chamber under a cycle of 16 h of light at 25°C and 8 h of dark at 22°C and the HR assay was performed as described previously (Bogdanove et al. 1998), with bacterial suspensions in 10 mM MgCl₂ at approximately 1×10^8 CFU/ml ($OD_{600} = 0.2$) unless noted otherwise. For virulence assays, 3-week-old seedlings were vacuum infiltrated with bacterial suspensions in 10 mM MgCl₂ plus 0.04% Silwet L-77 at approximately 1×10^5 CFU/ml and, following inoculation, plants were returned to the growth chamber with humidity added to 80%. Bacterial populations were measured by dilution plating of homogenized leaf disks excised using a 1-cm cork borer. For each time point per inoculum, the first trifoliate leaves of three inoculated plants were sampled individually and the mean and standard deviation calculated. Experiments were repeated five times.

Western blot assay.

Bacteria were grown to $OD_{600} = 0.5$, and cells in 1 ml were resuspended in 50 µl of 1× Laemmli buffer and heated to

100°C for 3 min to obtain total cellular proteins. Polyacrylamide (15%) gel electrophoresis and Western blotting of samples were performed using standard protocols. Rabbit anti-*avrPto* antiserum diluted 1:20,000 (kindly provided by G. Martin, Boyce Thompson Institute) followed by goat anti-rabbit peroxidase-conjugated antibody (Pierce, Rockford, IL, U.S.A.) and the ECL Plus Western Blotting Detection System (Amersham, Piscataway, NJ, U.S.A.) with Blue Sensitive Autoradiography film (Midwest Scientific, St. Louis) were used according to manufacturer protocols to detect AvrPto.

Northern blot assay.

Seedlings of cv. Harosoy (10 days old) were inoculated as described above for the virulence assay, with bacterial suspensions of approximately 5×10^7 CFU/ml. RNA was extracted from primary leaves using a modified hot trizol protocol (Huang et al. 2005) and subjected to standard Northern blot analysis using approximately 20 µg of total RNA per lane and NYTRAN Supercharge nylon membranes (Schleicher & Schuell Bioscience, Inc., Keene, NH, U.S.A.) with Rapid-Hyb buffer (Amersham, Piscataway, NJ, U.S.A.) according to the manufacturer's instructions. The PR1-a probe was generated by RT-PCR amplification and cloning of a 200-bp fragment of the gene (GenBank accession AF136636) from RNA of cv. Williams using the primers 5'-CGTGAATGCACACAATGCAG-3' and 5'-GCCACTTAGTTCACCACTGC-3'. The probe was labeled using the Prime-A-Gene labeling system (Promega Corp., Madison, WI, U.S.A.). A probe for actin message (provided by T. Baum, Iowa State University) was labeled in the same way and used as a control. Signal was detected using a Molecular Dynamics (Sunnyvale, CA, U.S.A.) phosphorimaging system.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

University of Idaho Plant Viruses Online database:
image.fs.uidaho.edu/vide/