Molecular Genetic Evidence for the Role of SGT1 in the Intramolecular Complementation of Bs2 Protein Activity in *Nicotiana benthamiana*

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Pepper plants (*Capsicum annuum*) containing the Bs2 resistance gene are resistant to strains of *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) expressing the bacterial effector protein AvrBs2. AvrBs2 is delivered directly to the plant cell via the type III protein secretion system (TTSS) of *Xcv*. Upon recognition of AvrBs2 by plants expressing the Bs2 gene, a signal transduction cascade is activated leading to a bacterial disease resistance response. Here, we describe a novel pathosystem that consists of epitope-tagged Bs2-expressing transgenic *Nicotiana benthamiana* plants and engineered strains of *Pseudomonas syringae* pv *tabaci* that deliver the effector domain of the *Xcv* AvrBs2 protein via the TTSS of *P. syringae*. This pathosystem has allowed us to exploit *N. benthamiana* as a model host plant to use *Agrobacterium tumefaciens*–mediated transient protein expression in conjunction with virus-induced gene silencing to validate genes and to identify protein interactions required for the expression of plant host resistance. In this study, we demonstrate that two genes, *NbSGT1* and *NbNPK1*, are required for the Bs2/AvrBs2–mediated resistance responses but that *NbRAR1* is not. Protein localization studies in these plants indicate that full-length Bs2 is primarily localized in the plant cytoplasm. Three protein domains of Bs2 have been identified: the N terminus, a central nucleotide binding site, and a C-terminal Leu-rich repeat (LRR). Coimmunoprecipitation studies demonstrate that separate epitope-tagged Bs2 domain constructs interact in trans specifically in the plant cell. Coimmunoprecipitation studies also demonstrate that an NbSGT1-dependent intramolecular interaction is required for Bs2 function. Additionally, Bs2 has been shown to associate with SGT1 via the LRR domain of Bs2. These data suggest a role for SGT1 in the proper folding of Bs2 or the formation of a Bs2-SGT1–containing protein complex that is required for the expression of bacterial disease resistance.

INTRODUCTION

Bacterial spot disease of pepper and tomato is caused by the phytopathogen *Xanthomonas campestris* pv *vesicatoria* (*Xcv*). Pepper plants (*Capsicum annuum*) carrying the Bs2 resistance gene are resistant to strains of *Xcv* expressing the effector protein AvrBs2 (*Minsavage et al., 1990; Tai et al., 1999*), Bs2 belongs to the largest class of plant disease resistance genes, whose proteins are characterized by a central putative nucleotide binding site (NB) and a C-terminal leucine-rich repeat (LRR) region (*Tai et al., 1999*). Members of the NB-LRR class of resistance proteins are thought to either directly, or indirectly, recognize effector proteins within the plant cell and subsequently activate a signal transduction pathway, leading to the resistance response (*Staskawicz et al., 1995*). The NB-LRR class of resistances genes can be further subdivided by the N terminus, which influences the requirement for downstream defense response components (*Fey and Parker, 2000*). The homology of their N termini with known protein structures in existing databases (*Hammond-Kosack and Jones, 1997*) generally identifies two subclasses: (1) the TIR class, containing resistance genes with homology to the Toll protein of Drosophila and the Interleukin1 Receptor protein of mammals; (2) the non-TIR class, containing the remaining resistance proteins. The non-TIR class can be further divided into proteins containing putative coiled-coil (CC) regions at their N termini and those with no recognizable protein structure (NX) at their N termini. Although the Bs2 N terminus has some sequence similarity with the CC class Rx N terminus (*Moffett et al., 2002*), it lacks similarity to the CC consensus; therefore, we place it in the NX class, with no recognizable structure at its N terminus (*Tai et al., 1999*). Several *Nicotiana benthamiana* genes have been implicated in Bs2/AvrBs2–mediated disease resistance (*Jin et al., 2002; Peart et al., 2002a,b*). Virus-induced gene silencing (VIGS) of SGT1 inhibits the disease resistance–associated hypersensitive response (HR) mediated by numerous NB-LRR genes, including *Bs2*, *Rx* of potato, and *N* of tobacco (*Peart et al., 2002b*). An additional gene implicated in numerous disease resistance pathways is *NPK1* (*Jin et al., 2002*). Using VIGS and *Agrobacterium tumefaciens* transient expression, *NPK1* has also been shown to...
interfere with the resistance response mediated by Bs2, N, and Rx. In the absence of an effective Xanthomonas pathogen for evaluating Bs2 disease resistance in N. benthamiana, previous studies have focused on the resistance-associated HR elicited from Agrobacterium-mediated transient AvrBs2-Bs2 expression. To further define the contribution of genes required for Bs2-mediated disease resistance, we genetically engineered N. benthamiana and Pseudomonas syringae pv tabaci (Pstab) to create a Bs2-avrBs2 model pathosystem that allows us to quantitatively assess resistance by measuring the inhibition of pathogen growth. Stable transgenic Bs2 N. benthamiana lines confer resistance to the wild fire pathogen Pstab expressing a chimeric effector protein with an avrBs2 activator domain and a Pseudomonas secretion–translocation leader sequence. The shared modular structure of disease resistance proteins suggests that members of the NB-LRR class of resistance proteins may function in a similar manner. Recent work with the Rx protein from potato, a closely related NB-LRR protein, demonstrated specific intramolecular domain interactions related to the function of the Rx protein (Moffett et al., 2002). These intramolecular domain interactions were disrupted by the coexpression of the PVX coat protein, the known effector protein recognized by Rx. This disruption of the intramolecular interactions has been proposed to be the first step in the activation of the signal transduction pathway leading to the resistance response. It has yet to be determined whether other NB-LRR proteins function in a similar manner.

We present data evaluating the contribution of several genes involved in the Bs2/AvrBs2 resistance pathway and define the specific intramolecular domain interactions involved in the function of the Bs2 protein. Consistent with previous Agrobacterium-mediated transient HR assays (Jin et al., 2002), we find that SGT1 and NPK1 are both required for full resistance to pathogenic bacteria carrying avrBs2 but that Rar1 is not required. To extend the findings of Moffett et al. (2002) and further evaluate the functional requirements for Bs2 intramolecular interactions, we used Agrobacterium-mediated transient expression of epitope-tagged domain constructs of Bs2. Although similar specific domain constructs of Bs2 function in trans to complement the avrBs2-specific HR, we further demonstrate that these domain constructs interact specifically in the plant cell. However, unlike Rx, the Bs2 domain constructs were not disrupted in the presence of the effector protein AvrBs2. Surprisingly, the intramolecular interaction observed with the Bs2 domain constructs was disrupted in an SGT1-silenced background. This observation is supported by the fact that Bs2 appears to interact with SGT1 in communoprecipitation studies. These data suggest a possible role for SGT1 in the proper folding of Bs2 or the assembly of a Bs2-containing protein complex that is required for the expression of disease resistance.

RESULTS

Development of a Bs2/AvrBs2 Pathosystem in N. benthamiana

We have developed a model pathosystem in N. benthamiana to facilitate biochemical and molecular genetic studies of protein interactions that control Bs2-specific resistance. A C-terminal HA epitope tag was added to Bs2 to facilitate protein detection. To ensure that transgenic plants display a strong Bs2 resistance response, as well as detectable levels of Bs2 expression, we used a binary vector with a mannopine synthase (MAS) promoter plus triple chimeric octopine synthase (OCS) upstream activating sequence (UAS) and one MAS UAS (Ni et al., 1995). Stable transgenic Bs2:HA epitope-tagged plants were generated using Agrobacterium-mediated transformation. Bs2 expression with this promoter allows for functional resistance to a bacterial pathogen carrying the avrBs2 effector and immunoblot detection of epitope-tagged Bs2 but does not alter N. benthamiana growth. Protein gel blot analysis of these transgenic plants with the UAS-MAS-Bs2:HA construct demonstrates Bs2:HA protein expression in these lines (data not shown). These plants also exhibit the resistance-associated HR elicited from Agrobacterium-mediated transient avrBs2 expression (Table 1, Figures 1 and 2).

Because there is currently no virulent Xanthomonas leaf pathogen available for N. benthamiana, the wild fire pathogen Pstab was engineered with a chimeric effector that contains a Pseudomonas secretion–translocation domain engineered to deliver the effector domain of AvrBs2. The upstream promoter region and N-terminal secretion–translocation domain of AvrRpm1 (amino acids 1 to 89) (Guttman and Greenberg, 2001) were used with the effector domain of AvrBs2 (amino acids 62 to 714) (Mudgett et al., 2000) and a C-terminal HA epitope tag as a translational fusion and expressed in trans in the very stable broad host range plasmid pVSP61 (Mudgett et al., 2000) to make the avrBs2 construct p(VavrRpm1:avrBs2:HA). Subsequent analysis of the engineered Pstab strain with anti-HA antibody

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<th>Bs2 Domain Constructs</th>
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Agrobacterium-mediated coexpression of AvrBs2:GFP with either full-length, individual Bs2 domain constructs or two-domain construct combinations in N. benthamiana. For coinfiltration, each Agrobacterium construct strain was adjusted to 4 \times 10^6 cfu/mL. Plants were monitored for the timing of the appearance of a visible HR. No HR response was observed for domain construct control inoculations without AvrBs2 (data not shown). The four Bs2 domain constructs are the N terminus (NX:HA), NX-NB:HA, NB-LRR:Flag, and LRR:Flag, which contain amino acids 1 to 154, 1 to 500, 149 to 905, and 500 to 905, respectively. Each domain construct also has a C-terminal epitope tag for protein detection and communoprecipitation. Bs2 domain constructs were cloned in a binary vector with a chimeric UAS and MAS promoter. For AvrBs2 expression, a C-terminal translational fusion with GFP was constructed in a 35S promoter binary vector.
confirmed that the AvrRpm1:AvrBs2:HA protein was expressed in this strain (data not shown).

_N. benthamiana_ plants with and without Bs2:HA were hand infiltrated with 10^5 cfu/mL _Pstab_ expressing either the empty vector control or the avrBs2 construct, pV( 

Figure 1. AvrBs2-Elicited Bs2 Resistance in the _N. benthamiana_ Pathosystem Prevents _Pstab_ Wild Fire Symptoms.

N. benthamiana plants with and without Bs2:HA were hand infiltrated with 10^5 cfu/mL _Pstab_ expressing either the empty vector control or the avrBs2 construct, pV(.

VIGS Establishes That SGT1 and NPK1 Are Required for the Bs2/AvrBs2-Mediated Resistance Response

Although there is significant genetic evidence demonstrating the requirement for several genes in the signal transduction pathway leading to the activation of a resistance response in _N. benthamiana_, these studies have focused primarily on measuring the ability of these genes to block an HR associated with the AvrBs2-Bs2 Agrobacterium-mediated transient coexpression (Azevedo et al., 2002; Jin et al., 2002; Liu et al., 2002; Moffett et al., 2002). To demonstrate the biological significance of these genes in disease resistance, we combined VIGS with the newly generated model pathosystem described above to study the disruption of AvrBs2-Bs2 association as it relates to the specific inhibition of pathogen growth.

Transgenic _N. benthamiana_ Bs2:HA plants were silenced using a Gateway-compatible Tobacco rattle virus (TRV) two-component Agrobacterium-mediated expression system (Liu et al., 2002). VIGS of the _N. benthamiana_ orthologs of _PDS1_ was used as a visual photo-bleaching phenotype control for effective silencing (Liu et al., 2002). VIGS of Bs2 was used as a positive control for the disruption of Bs2 resistance in the pathosystem. VIGS of the nonplant prokaryotic gene _β-glucuronidase_ (GUS) was used to control for viral replication effects. VIGS of the _N. benthamiana_ orthologs of SGT1, NPK1, and RAR1 were evaluated for their contribution to Bs2 resistance within this pathosystem. A control assay for VIGS of specific target sequences was developed that uses Agrobacterium-mediated transient expression of a 35S green fluorescent protein (GFP) reporter that also has the specific VIGS target sequence located in the C-terminal untranslated region (UTR) before the translational terminaton. Immunoblot analysis reveals a reduction of detectible GFP resulting from co-silencing of the GFP-UTR reporter that contains the same target sequence used for VIGS (Figure 3). Additionally, anti-HA immunoblot analysis of the GUS-, Bs2-, SGT1-, NPK1-, and RAR1-silenced Bs2:HA transgenic plants detects reduced Bs2:HA protein in only the Bs2-silenced control (data not shown).

The silenced plants were inoculated with low levels of _Pstab_ (10^5 cfu/cm²) with or without the avrBs2 construct and assayed

Figure 2. VIGS of Bs2 Signal Transduction Genes Disrupts Bs2-Induced Resistance in the _N. benthamiana_ Pathosystem.

Bs2, SGT1, or NPK1 silencing interferes with Bs2/AvrBs2-mediated disease resistance, but RAR1 silencing does not. Plants tested were _N. benthamiana_ (Nb); Bs2:HA-expressing _N. benthamiana_ [Nb(Bs2:HA)]; Nb(Bs2:HA) silenced (3) for SGT1, NPK1, RAR1, or Bs2; and a GUS control for virus replication.

(A) Control in planta pathogen growth assay of _Pstab_ containing empty vector (pEV), lacking AvrBs2 expression, after low-inoculum infiltration. (B) In planta pathogen growth assay of _Pstab_ with the avrBs2 construct, pV( 

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for in planta pathogen growth. All silenced plants allowed normal growth of the Pstab strain lacking the avrBs2 construct (Figure 2A). As expected, genes shown to interfere with the HR in transient assays also were required for resistance to Pstab expressing the avrBs2 construct (Figure 2B). In planta pathogen growth assays with Pstab expressing the avrBs2 construct accumulated to $-2.0 \log \text{cfu/cm}^2$ leaf tissue greater population in plants silenced for SGT7 or Bs2. Similarly, in plants silenced for NPK1, intermediate bacterial growth reached $-1.0 \log \text{cfu/cm}^2$ leaf tissue greater than in the control plants. No disruption of AvrBs2-Bs2-mediated resistance to pathogen growth was observed in plants silenced for RAR1 or GUS. These observations are consistent with the ability of silencing of SGT7 and NPK1 to inhibit the Bs2-AvrBs2 HR associated with Agrobacterium-mediated transient expression (Jin et al., 2002).

**Bs2 Is Localized in the Plant Cytoplasm**

The subcellular localization of many NB-LRR resistance proteins has not been established. However, the RPM1 and RPS2 proteins of Arabidopsis thaliana have both been shown to be peripherally associated with the plant plasma membrane (Boyes et al., 1998; Axtell and Staskawicz, 2003). To determine the subcellular localization of Bs2, total protein was extracted from Bs2:HA-expressing plants and a total membrane fraction was isolated by ultracentrifugation. Bs2:HA and the cytoplasmically localized NptII and HSP90 (Milioni and Hatzopoulos, 1997) proteins remained in the supernatant after the high-speed spin used to pellet the plant membranes in plants inoculated with buffer alone (Figure 4). Conversely, a plasma membrane–localized H-ATPase (Lefebvre et al., 2004) was concentrated in the pellet after the high-speed spin.

To determine whether the localization of Bs2:HA changed during the resistance response, we followed the subcellular localization pattern of Bs2:HA during compatible and incompatible Pstab interactions. Interestingly, there was a similar cytoplasmic localization of the Bs2:HA protein during both compatible (Pstab) and incompatible (Pstab with the avrBs2 construct) bacterial infections (Figure 4). As an added control, anti-HSP90 antibody was used as a cytoplasmic control.

As seen in Figure 4, there appears to be an increased level of detectable Bs2:HA after Pstab inoculation. Although this increase in Bs2:HA expression does not alter Pstab symptoms or compatible pathogen growth (Figures 1 and 2A), it is nonetheless reproducible and represents a detectable change in the level of Bs2:HA.

**Intramolecular Interactions of the Bs2 Protein Domains in Planta**

In a previous study describing the NB-LRR resistance gene Rx (Moffett et al., 2002), Bs2 was used as a control, as it possesses a similar NB-LRR domain structure to that of Rx yet displays a different elicitor specificity. In this work, it was demonstrated that like Rx, the Bs2-specific domain constructs function in trans to complement the avrBs2-induced HR. To further evaluate the functional requirements for Bs2 intramolecular interactions, we also used Agrobacterium-mediated transient expression of epitope-tagged domain constructs of Bs2 (Tai et al., 1999). The four Bs2 domain constructs are the N terminus (NX:HA), NX-NB:HA, NB-LRR:Flag, and LRR:Flag, which contain amino acids 1 to 154, 1 to 500, 149 to 905, and 500 to 905, respectively. To

![Figure 3. Assay for VIGS.](image)

Reduction of detectible GFP resulting from cosilencing of the 35S-GFP-UTR reporter containing the target sequence used for VIGS of Bs2 signal transduction genes. All samples for a given VIGS treatment were infiltrated in the same leaf and collected 24 h after inoculation.

![Figure 4. Subcellular Localization of Bs2 in the Plant Cytoplasm.](image)

The first three columns show immunoblots of total protein extracts (T) from N. benthamiana Bs2:HA plants before ultracentrifugation at 100,000g for 1 h. Soluble (S) and membrane (M) fractions are indicated. The last six columns show immunoblots of protein extracts from N. benthamiana Bs2:HA plants at 24 h after inoculation with 10^8 cfu/mL of either Pstab containing empty vector (pVSP61) or Pstab with the avrBs2 construct, pV[avrRpm1-avrBs2]:HA. Samples were processed and analyzed as described above. Blots were probed with anti-HA antibody for detection of Bs2:HA, anti-NptII antibody as a soluble control protein, and anti-H-ATPase antibody as a membrane-localized control protein. As shown above, Bs2:HA localization was comparable with that observed for the soluble control protein NptII, indicating soluble (i.e., cytoplasmic) localization. The infection of Pstab with or without AvrBs2 did not alter Bs2 localization.
maintain consistency with Bs2 expression in the new pathosystem, the domain constructs were cloned into the identical binary expression vector with a chimeric UAS and MAS promoter (Ni et al., 1995). Each domain construct also has a C-terminal epitope tag (i.e., HA) for protein detection and coimmunoprecipitation.

For AvrBs2 expression, a C-terminal translational fusion with GFP was constructed in a 35S promoter binary vector. Agrobacterium-mediated transient expression of this AvrBs2:GFP construct induces the Bs2-specific HR, demonstrating that the addition of GFP does alter biological activity in the AvrBs2:GFP chimeric construct (Table 1). Immunoblot analysis of the transiently expressed AvrBs2:GFP construct 24 h after inoculation in N. benthamiana with anti-GFP antibody detects the predicted 107-kD fusion protein (data not shown).

Similar to previous observations described for Rx (Moffett et al., 2002), only the Bs2 protein domain construct combinations of NX plus NB-LRR and NX-NB plus LRR functioned in trans to complement the AvrBs2-specific HR phenotype (Table 1). Generally, the HR tissue collapse began to be visible ~24 h after infiltration of the Agrobacterium strains. None of the domain constructs alone was capable of generating an HR, with the exception of the NB-LRR construct of Bs2, which was able to partially complement the HR phenotype in the absence of the NX domain. However, the HR in this case generally did not appear until well beyond the 24 h time point at which the HR appeared when both the NX and NB-LRR constructs were expressed together (Table 1). None of the domain construct combinations was capable of generating an HR in the absence of the AvrBs2:GFP construct.

Having demonstrated that the domain constructs of Bs2 were biologically active, we used them in a series of coimmunoprecipitation experiments to test for the presence of specific intramolecular interactions in Bs2. As seen with Rx, the NX-NB domain of Bs2 interacts with the LRR (Figure 5A). The HA epitope-tagged NX-NB of Bs2 was able to coimmunoprecipitate the LRR-Flag domain after Agrobacterium-mediated transient expression in N. benthamiana. This interaction was confirmed with the reciprocal experiment, in which LRR-Flag coimmunoprecipitates the NX-NB:HA construct. However, unlike Rx, the NX domain of Bs2 does not appear to interact with the NB-LRR domain (Figure 5B). After transient expression and coimmunoprecipitation, no interaction was detected between these domains.

To determine whether the NX-NB and LRR interaction was involved in the activation of the Bs2 protein, this interaction was tested in the presence of AvrBs2. Unlike Rx, the NX-NB and LRR interaction was not only not disrupted in the presence of AvrBs2 when cotransient expression of these three constructs was tested by coimmunoprecipitation, but the interaction was more stable (Figure 5A). One significant difference between the immunoprecipitation experiments performed with Rx and those presented here with Bs2 is that the Rx experiments were performed in SGT1-silenced plants, whereas our Bs2 studies were conducted in wild-type plants at a time point at which visual HR symptoms begin to appear. Visual HR also confirms the cotransient expression of the AvrBs2:GFP, NX-NB:HA, and LRR:Flag constructs.

**Figure 5.** Intramolecular Interactions within the Bs2 Protein Domains. (A) The Bs2 NX-NB domain associates with the LRR but is not altered by the coexpression of AvrBs2. The Bs2 domain constructs NX-NB:HA and LRR:Flag were transiently expressed in N. benthamiana plants with and without AvrBs2:GFP. Total protein extracts were immunoprecipitated with anti-HA (left panels) and anti-Flag (right panels) antibodies. Immunoprecipitated proteins were detected by immunoblotting with anti-HA (top panels) and anti-Flag (bottom panels) antibodies.

(B) The Bs2 NX domain does not associate with the NB-LRR. The Bs2 domain constructs NX-HA and NB-LRR:Flag were transiently expressed for 24 h in N. benthamiana plants. Total protein extracts were immunoprecipitated (IP) with anti-HA (left panels) and anti-Flag (right panels) antibodies. Immunoprecipitated proteins were detected by immunoblotting with anti-HA (top panels) and anti-Flag (bottom panels) antibodies.
To reconcile some of the differences we observed with regard to what has been reported for Rx (Moffett et al., 2002), we tested the Bs2 NX-NB and LRR interaction in SGT1-silenced plants. Surprisingly, the NX-NB and LRR interaction was disrupted in the SGT1-silenced plants (Figure 6). After transient expression, the NX-NB:HA construct no longer coimmunoprecipitated the LRR:Flag construct. Likewise, the reciprocal experiment also gave negative results. In viral replication control experiments, the NX-NB:HA domain of Bs2 was able to coimmunoprecipitate the LRR:Flag construct in GUS-silenced plants.

BS2 Interacts with NbSGT1

Because silencing of SGT1 contributed to the disruption of the intramolecular interactions of Bs2 domains, we next sought to determine whether there is an interaction of Bs2 with SGT1 using transient expression and coimmunoprecipitation. Both the full-length Bs2:HA construct and the LRR:HA construct were able to immunoprecipitate N. benthamiana SGT1:Flag after Agrobacterium-mediated transient expression in N. benthamiana (Figure 7). In reciprocal experiments, SGT1:Flag also coimmunoprecipitated both the Bs2:HA and LRR:HA constructs. A weak interaction was observed between the NX-NB:HA domain and SGT1:Flag. As negative controls, the interactions between NX:HA, AvrBs2:HA, GFP:HA, and SGT1:Flag were also tested. In all cases, no interaction was detected between these various HA-tagged constructs and SGT1:Flag (data not shown).

To demonstrate the functionality of the SGT1:Flag construct, we used cotransient expression. Both tagged and untagged SGT1 constructs as well as an SGT1:Flag construct with a stop codon introduced at amino acid 80 were cotransiently expressed, with and without the AvrBs2:GFP construct, on Bs2:HA—expressing N. benthamiana silenced for SGT1 (Figure 8). With both tagged and untagged SGT1 constructs, transient overexpression was able to partially overcome the endogenous SGT1 silencing in Bs2:HA—expressing N. benthamiana to reactivate the AvrBs2-Bs2—specific HR. Consistent with this result, immunoblot analysis with both anti-SGT1 and anti-Flag antibodies detected transient expression of SGT1, although at reduced levels compared with non-SGT1-silenced control transient expression (data not shown). The stop codon mutant SGT1 did not reactivate AvrBs2-Bs2—specific HR (Figure 8) and was not detectable on immunoblots (data not shown).

**DISCUSSION**

To elucidate the signal transduction pathway leading to the activation of the resistance response, it is critical to understand how the protein–protein interactions, subcellular localization, and quantitative contribution of genes involved in the resistance response. Here, we present a model pathosystem for studying bacterial plant disease resistance and provide data quantifying the contribution of several genes involved in the Bs2/AvrBs2 disease resistance pathway. Additionally, we provide support for the hypothesis (Moffett et al., 2002) that intramolecular protein interactions are important for the function of Nb-LRR resistance proteins. We believe that AvrBs2-specific activation of Bs2 may occur in the plant cytoplasm, given the nature of its subcellular localization. Finally, we propose that SGT1 may be involved in the proper folding of the Bs2 protein as a result of its coimmunoprecipitation and the disruption of the Bs2 intramolecular interaction in SGT1-silenced plants.

The localization of Bs2 in the cytoplasm suggests that resistance proteins are not always associated with the plasma membrane of the plant cell. Of the three NB-LRR resistance proteins whose localization have been described previously, two are predicted to be plant plasma membrane–associated (Boyes et al., 1998; Axtell and Staskawicz, 2003) and one is both plasma membrane–associated and cytoplasm–associated (Bieri et al., 2004). Although Bs2 expression levels give effective resistance and cause no visual alterations in the normal growth of N. benthamiana, there may still be overexpression effects.

As the evidence supporting intramolecular interactions within NB-LRR resistance proteins grows, it becomes more important to understand how these interactions may regulate the activity of these proteins (Hwang et al., 2000; Moffett et al., 2002). N-terminal gain-of-function mutations of Mi were used to identify a mutation in the LRR region that results in specific loss of
nematode recognition while maintaining the constitutive gain-of-function activity, demonstrating intramolecular regulation and interaction (Hwang and Williamson, 2003). Similar to Rx, we demonstrate that in the case of Bs2, coexpression of the individual NB and LRR domains can interact and function to constitute resistance. Unlike Rx, the intramolecular interaction between the NX-NB domain of Bs2 and its LRR domain was not disrupted in the presence of the effector protein. One possible explanation for the lack of a disruption is that only a small percentage of the entire pool of Bs2 protein is activated by AvrBs2. Alternatively, the disruption of the intramolecular interaction seen with Rx may represent a negative feedback mechanism that turns off the resistance response once it has been activated. To determine whether this is the case, one could examine the intramolecular interactions of Rx during a Bs2/AvrBs2-mediated resistance response. If the disruption of the Rx intramolecular interaction is specific to its activation, then it should not be disrupted during a Bs2/AvrBs2-mediated resistance response. Alternatively, these intramolecular interactions may represent a transient state within a process that has different rate limitations for different R proteins. Herein, we demonstrate

**Figure 7.** The LRR of Bs2 Associates with *N. benthamiana* SGT1.

The Bs2:HA and Bs2 domain constructs NX-NB:HA and LRR:HA were transiently expressed with SGT1:Flag in *N. benthamiana* plants. Total protein extracts were immunoprecipitated with anti-HA (A) and anti-Flag (B) antibodies. Immunoprecipitated proteins were detected by immunoblotting with anti-HA and anti-Flag antibodies. Both Bs2:HA and LRR:HA coimmunoprecipitate with SGT1:Flag.

**Figure 8.** NbSGT1 Is Biologically Active.

Agrobacterium-mediated transient expression of SGT1 and SGT1:Flag reactivates Bs2:HA-AvrBs2–specific HR in SGT1-silenced *N. benthamiana* Bs2:HA plants. *N. benthamiana* SGT1 (*NbSGT1*), C-terminal epitope-tagged *NbSGT1*:Flag, and *NbSGT1*:Flagstop80 mutant, with the stop codon introduced at amino acid 80, were cloned in a binary vector with a chimeric UAS and MAS promoter for enough transient expression to overcome the effects of VIGS of SGT1. Agrobacterium coinfiltration mixtures were 5 × 10⁸ cfu/mL each, and HR reactions were photographed at 3 d after infiltration. Infiltrated plants were *N. benthamiana* viral replication control (*NbD Gus*), *N. benthamiana* Bs2:HA viral replication control [**Nb(Bs2:HA) ∆Gus**], and *N. benthamiana* Bs2:HA with VIGS for SGT1 [**Nb(Bs2:HA) ∆SGT1**].
the requirement of SGT1 for the intramolecular interaction(s) of the NB and LRR domains of a resistance protein.

One potential limitation of the coimmunoprecipitation of Bs2 and SGT1 data is that both proteins were overexpressed. However, because SGT1 did not coimmunoprecipitate the negative control proteins GFP and AvrBs2 (data not shown) and only weakly associated with the NX-NB domain of Bs2, we believe these data to be biologically significant. In addition, a recent article describes the interaction of SGT1p from yeast with the LRR domain of the adenylyl cyclase Cyr1p/Cdc35p (Dubacq et al., 2002). This article further describes a yeast two-hybrid screen using SGT1p as bait. One protein class shown to interact with SGT1p in the yeast two-hybrid screen contained proteins with well-defined or potential LRR domains. This result, in conjunction with the interaction of SGT1 and the LRR domain of Bs2, suggests a general role for SGT1 in NB-LRR regulation through indirect or direct interactions with the LRR domain. However, this SGT1–LRR interaction is not observed for barley (Hordeum vulgare) MLA6 (Bieri et al., 2004) or for Rx (Moffett et al., 2002). Our results with the N. benthamiana pathosystem suggest that Bs2 is RAR1-independent and interacts with SGT1, similar to barley RAR1-independent MLA1. This is in contrast with barley RAR1-dependent MLA6, which does not interact with SGT1 (Bieri et al., 2004). The Bs2 RAR1 independence may simply be the result of high Bs2 expression, similar to the RAR1 independence that results for high RPS2 expression in Arabidopsis (Tomoro et al., 2002).

SGT1 is a highly conserved eukaryotic protein involved in such diverse pathways as the regulation of cell division, SCF (Skp1p/Cdc53p-Cullin-F-box)-mediated ubiquitination of proteins, and the regulation of the cAMP pathway in yeast (Kitagawa et al., 1999; Schadick et al., 2002). One element these pathways seem to have in common is the involvement of multimeric protein complexes. Like these pathways, plant disease resistance is also thought to be regulated by resistance protein-containing complexes. This hypothesis has gained considerable support in recent data that suggest a role for Hsp90 in the resistance response of the heat shock proteins Hsp70 and Hsp90. Interestingly, both Sti1/Hop and p23 function as cochaperones that share homology with the TPR region of Sti1/Hop and p23, respectively; both Sti1/Hop and p23 function as cochaperones of the heat shock proteins Hsp70 and Hsp90. Interestingly, recent data suggest a role for Hsp90 in the resistance response (Holt et al., 2003; Lu et al., 2003; Takahashi et al., 2003). SGT1 may act as a cochaperone, delivering Bs2 to Hsp90 for proper folding and insertion into a multimeric protein complex.

METHODS

Generation of Stable Bs2-Expressing Plants

Nicotiana benthamiana leaf tissue was transformed and regenerated as previously described (Tai et al., 1999) with Agrobacterium tumefaciens strain LBA 4404 carrying the binary vector pATC940 with a MAS promoter plus triple chimeric OCS UAS and one MAS UAS (Ni et al., 1995) expressing the HA epitope-tagged Bs2 CDNA. Primary transformants were selected on tobacco medium (1× MS salts [Sigma-Aldrich, St. Louis, MO], 1× B5 vitamins, 3.0 mM Mes, pH 5.7, 3.0% sucrose, 0.1 mg/L naphthyl-acetic acid, 1.0 mg/L benzylaminopurine, and 7.0 g/L phytoagar) containing 300 mg/L kanamycin. Kanamycin-resistant primary transformants were allowed to self-pollinate, and lines segregating as single inserts on 1× MS medium containing kanamycin at 75 mg/L were selected for further study. Ten independent lines were allowed to self-pollinate, and a homozygous Bs2–HA-expressing line was selected from one line that segregated 3:1 for a positive HR with Agrobacterium-mediated transient expression of AvrBs2. Immunoblot analysis with anti-HA antibody confirmed the expression of the Bs2–HA protein.

Plasmid Construction

For transient expression, Bs2 and Bs2 domain constructs were expressed from the Gateway (Invitrogen, Carlsbad, CA)-modified binary vector pE1776 with a MAS promoter plus triple chimeric OCS UAS and one MAS UAS (Ni et al., 1995). The modified Gateway-compatible destination vector pE1776-attr1-attr2 was made by cloning the ccdB cassette B (Invitrogen) into the T4 DNA polymerase-filled in SacI and KpnI sites of pE1776. To add the appropriate epitope tags and incorporate bases required for entry into the Gateway system, the Bs2 CDNA was amplified by PCR with gene-specific primers designed to incorporate CACC at the 5′ end of each construct and the HA or Flag epitope tag immediately after the final C-terminal amino acid of each Bs2 construct. The PCR products were subcloned into pENTR/D TOPo (Invitrogen) and then transferred into the Gateway-compatible pE1776-attr1-attr2 vector by an LR Clonase (Invitrogen) reaction. Bs2 domain constructs NCX, NX-NB, NB-LRR, and LRR contain amino acids 1 to 154, 1 to 500, 149 to 905, and 500 to 905, respectively.

For delivery of a Xanthomonas campestris pv vescicatoria effector from Pseudomonas syringae pv tabaci (Pstav), constructs were expressed from a modified Gateway-compatible broad host range plasmid, pVSP61. First, a SalI site was incorporated one nucleotide before amino acid 62 of avrBs2 by PCR with an internal primer and subcloned. This modified part of avrBs2 was then recloned onto the rest of avrBs2 as a HindIII fragment into pVSP61 (promoterless avrBs2(97-714)-HA) (Mudgett et al., 2000). The ccdB cassette was cloned into the T4 DNA polymerase-filled SalI site to make pVSP61-attr1-attr2 (avrBs2(97-714)-HA) destination vector. The avrRpm1 promoter region, including 201 bp N terminal to the start codon, and amino acids 1 to 89 were amplified by PCR with gene-specific primers designed to incorporate CACC at the 5′ end for subcloning into pENTR/D TOPo and subsequent transfer into the Gateway-compatible pVSP61-attr1-attr2 (avrBs2(97-714)-HA) destination vector by an LR Clonase reaction to make the avrRpm1-avrBs2 reporter construct p(avrRpm1-avrBs2).

For transient expression of avrBs2, a C-terminal translational fusion with eGFP(U84737) was expressed from the 3SS binary pMD1 (Tai et al., 1999). PCR of avrBs2 was used to introduce an N-terminal SalI site and a C-terminal BamHI site with the stop codon removed. This construct was transfected as an XbaI–BamHI fragment from an intermediate cloning vector into pMD1. GFP was amplified by PCR with an N-terminal BamHI site and a C-terminal XhoI site and the cloned C terminus to avrBs2 in pMD1.

Using a partial SGT1 clone (Jin et al., 2002), 5′ and 3′ rapid amplification of cDNA ends PCR (Invitrogen) was performed, and a full-length N. benthamiana SGT1 cDNA sequence was generated. Internal nested primers were generated based on the partial SGT1 sequence and used with the 5′ and 3′ rapid amplification of cDNA ends primers and a pool of cDNA isolated from inoculated and un inoculated plants to amplify and sequence the 5′ and 3′ terminal ends of SGT1. Gateway-compatible 5′
and 3’ primers were used to PCR amplify a non-epitope-tagged, as well as a C-terminal Flag epitope-tagged, full-length cDNA that was cloned into pENTR/D TOPO. These clones were then transferred into the Gateway-compatible pE1776-attR1-attR2 vector by an LR Clonase reaction.

Primers for NbSGT1 were 5’-caccATGGGCTCCGATCTGGAGA-3’ and 5’-gaggctcTTAGATTCTCCCCATTCTTAGGT-3’; primers for NbSGT1: Flag were 5’-caccATGGGCTCCGATCTGGAGA-3’ and 5’-gaggctcttgcgtcgcgctctggtgatcTtGATTTCCCATTTCTTCAGCT-3’.

**Pseudomonas Strains and Pathogen Assay**

*Pst* isolates 11528 (Rommens et al., 1995) and *Pseudomonas syringae pv tomato* isolate DC3000 (Cuppels, 1986) were grown at 28°C on Pseudomonas Agar F plates (Difco, Piscataway, NJ) supplemented with Gateway-compatible pE1776-attR1-attR2 vector by an LR Clonase reaction. Agrobacterium strains were preincubated for 3 h in 10 mM MgCl2, 1.0 mM Mes, pH 5.6, 150 mM KCl, and inoculated at 10^5 cfu/mL by hand infiltration into leaves of *N. benthamiana* (this study) and *N. plumbaginifolia* (Tai et al., 1999) by standard methods. Pathogen growth in planta was measured as previously described (Tai et al., 1999).

**Agrobacterium-Mediated Transient Expression**

Agrobacterium strains and pathogen assay. Agrobacterium mediates the delivery of transferred DNA sequences from the vector to the host plant. The experiments involved transferring DNA into leaves of *N. benthamiana* (this study) and *N. plumbaginifolia* (Tai et al., 1999) by standard methods. Pathogen growth in planta was measured as previously described (Tai et al., 1999).

**TRV-Based VIGS**

The TRV-based VIGS system uses a bipartite sense RNA1 and RNA2 virus to silence genes expressed from a binary vector. Leaves of *N. benthamiana* were silenced by introducing the binary vector into leaves of *N. benthamiana* essentially as described previously (Tai et al., 1999). Before infiltration, Agrobacterium strains were preincubated for 3 h in 10 mM MgCl2, 1.0 mM Mes, pH 5.6, and 150 μM acetosyringone.

**Agrobacterium-Mediated Transient Expression**

Agrobacterium strain C58C1 (pCH32) carrying the gene of interest expressed from a binary vector was infiltrated into leaves of *N. benthamiana* essentially as described previously (Tai et al., 1999). Before infiltration, Agrobacterium strains were preincubated for 3 h in 10 mM MgCl2, 1.0 mM Mes, pH 5.6, and 150 μM acetosyringone.

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anti-Flag peroxidase (Sigma-Aldrich) to detect HA and Flag epitope-tagged proteins, respectively.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY899199 and U84737.

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