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Jianying Wang  
*University of Missouri*

Greg Yeckel  
*University of Missouri*

Pramod K. Kandoth  
*University of Missouri*

Lakmini Wasala  
*University of Missouri*

Richard S. Hussey  
*University of Georgia*

*See next page for additional authors*

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Keywords
BAG6, cell death, cyst nematode, defence suppression, effector, Heterodera glycines, soybean

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Authors
Jianying Wang, Greg Yeckel, Pramod K. Kandoth, Lakmini Wasala, Richard S. Hussey, Eric L. Davis, Thomas J. Baum, and Melissa G. Mitchum

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TARGETED SUPPRESSION OF SOYBEAN BAG6-INDUCED CELL DEATH IN YEAST BY SOYBEAN CYST NEMATODE EFFECTORS

Jianying Wang | Greg Yeckel | Pramod K. Kandoth | Lakmini Wasala | Richard S. Hussey | Eric L. Davis | Thomas J. Baum | Melissa G. Mitchum

1Division of Plant Sciences and Bond Life Sciences Center, University of Missouri, Columbia, MO, USA
2Department of Plant Pathology, University of Georgia, Athens, GA, USA
3Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC, USA
4Department of Plant Pathology and Microbiology, Iowa State University, Ames, IA, USA
5Department of Plant Pathology and Institute of Plant Breeding, Genetics, and Genomics, University of Georgia, Athens, GA, USA

Correspondence
Melissa G. Mitchum, Department of Plant Pathology and Institute of Plant Breeding, Genetics, and Genomics, University of Georgia, Athens, GA, USA
Email: melissa.mitchum@uga.edu

Present address
Greg Yeckel, Corteva Agriscience, Johnston, IA, USA
Pramod K. Kandoth, National Agri-food Biotechnology Institute, Mohali, India
Lakmini Wasala, Department of Veterinary Pathobiology, University of Missouri, Columbia, MO, USA

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Abstract
While numerous effectors that suppress plant immunity have been identified from bacteria, fungi, and oomycete pathogens, relatively little is known for nematode effectors. Several dozen effectors have been reported from the soybean cyst nematode (SCN). Previous studies suggest that a hypersensitive response-like programmed cell death is triggered at nematode feeding sites in soybean during an incompatible interaction. However, virulent SCN populations overcome this incompatibility using unknown mechanisms. A soybean BAG6 (Bcl-2 associated anthanogene 6) gene previously reported by us to be highly up-regulated in degenerating feeding sites induced by SCN in a resistant soybean line was attenuated in response to a virulent SCN population. We show that GmBAG6-1 induces cell death in yeast like its Arabidopsis homolog AtBAG6 and also in soybean. This led us to hypothesize that virulent SCN may target GmBAG6-1 as part of their strategy to overcome soybean defence responses during infection. Thus, we used a yeast viability assay to screen SCN effector candidates for their ability to specifically suppress GmBAG6-1-induced cell death. We identified several effectors that strongly suppressed cell death mediated by GmBAG6-1. Two effectors identified as suppressors showed direct interaction with GmBAG6-1 in yeast, suggesting that one mechanism of cell death suppression may occur through an interaction with this host protein.

KEYWORDS
BAG6, cell death, cyst nematode, defence suppression, effector, Heterodera glycines, soybean
Soybean cyst nematode (SCN; Heterodera glycines) is a sedentary plant-parasitic roundworm that transforms root cells into a permanent feeding site (a syncytium) near the root vasculature to acquire nutrition for its growth and reproduction. When the nematode encounters a resistant soybean plant, a hypersensitive response (HR)-like programmed cell death (PCD) at the feeding site results in nutrient deprivation and cessation of nematode development (Endo, 1965). As with other pathogens, certain SCN populations are virulent on resistant soybean cultivars, that is, they can infect and reproduce (Niblack et al., 2002). Such virulent SCNs are able to modulate plant immunity through suppression or evasion of plant defences to establish a compatible interaction. Thus, the outcome of the plant–nematode interaction is largely controlled by effector proteins injected into plant cells using a hollow mouth spear, the stylet (Mitchum et al., 2013). To date research has identified several dozen such stylet-secreted effector (SSE) candidates from SCN (Gao et al., 2001a, 2003; Wang et al., 2001; Noon et al., 2015), of which only a few have been implicated in the suppression of stress and defence responses during feeding site establishment in susceptible plants (Mitchum et al., 2013; Gardner et al., 2015; Noon et al., 2016). Moreover, relatively little is known about the role of SCN effectors in interactions with resistant soybean plants.

To better understand the molecular basis of the resistance response to SCN, we previously coupled laser capture microdissection and microarray technology for a comparative analysis of syncytial gene expression profiles induced in soybean lines near-isogenic (NIL) for SCN resistance (Kandot et al., 2011). Of the 37,000 genes printed on the Affymetrix GeneChip Soybean Genome Array, 1,447 genes were differentially expressed between syncytia of NIL-Resistant (R) and NIL-Susceptible (S) lines. Genes involved in cellular stress processes such as autophagy, PCD, and the unfolded protein response were enriched among the differentially expressed genes, including genes at the Rhg1 gene cluster (Cook et al., 2012). A soybean transcript coding for a Bcl-2 (B cell lymphoma 2)-associated athanogene (BAG) domain protein with the highest homology to the Arabidopsis BAG6 protein (AtBAG6) was identified (Doukhanina et al., 2006). The proteins encoded by these AtBAG genes contain a BAG domain, which is partially conserved between plants and mammals. The domain organization of AtBAG1, AtBAG2, AtBAG3, and AtBAG4 is similar to AtBAG7 and was named (Doong et al., 2002). More recently, BAG genes were identified in plants (Doukhanina et al., 2006; Rana et al., 2012; Hu et al., 2013). In the model plant Arabidopsis thaliana, a family of seven genes, BAG-1 to BAG-7, was identified (Doukhanina et al., 2006). The proteins encoded by these AtBAG genes contain a BAG domain, which is partially conserved between plants and mammals. The domain organization of AtBAG1, AtBAG2, AtBAG3, and AtBAG4 was found to be similar to their animal homologs and contain a conserved BAG domain and an upstream ubiquitin domain. AtBAG5, AtBAG6, and AtBAG7 lack the ubiquitin domain, but contain a calmodulin (CaM)-binding domain (IQ) (Rhoads and Friedberg, 1997) upstream of a conserved BAG domain. The IQ domain was shown to be the site of interaction of these proteins with calcium and CaMs (Black et al., 2005). IQ domains are protein segments that contain an 11 amino acid consensus sequence defined as (I/L/V)QXXXRXXXX(R/K) (where X is any residue) (Van Petegem et al., 2005). The presence of an IQ domain is considered a unique feature found within plant BAG proteins reflecting possible divergent functions relative to their mammalian counterparts (Doukhanina et al., 2006). Interestingly, unlike the other AtBAG family members that function to promote cell survival, AtBAG6 to the contrary was shown to promote PCD of yeast cells (Kang et al., 2006) and more recently autophagy and plant defence against a fungal pathogen upon cleavage by an aspartyl protease (Li et al., 2016).

Although BAG genes encode an evolutionarily conserved family of proteins in animals, yeast, and plants, much less is known about the roles of BAG proteins in plants. To date, soybean BAG proteins have remained uncharacterized. Our finding that GmBAG6-1 was induced 87-fold in feeding sites of the SCN-resistant line undergoing HR-PCD, along with aforementioned studies indicating that AtBAG6 can trigger autophagy and PCD, led us to hypothesize that GmBAG6-1 may function in soybean immunity and hence be targeted for suppression by the nematode. We demonstrate that similar to AtBAG6, overexpression of the GmBAG6-1 protein or a truncated version spanning the IQ and BAG domains induced cell death in yeast and soybean, with the truncated form showing an enhanced cell death phenotype. We then screened 47 SCN SSEs originally isolated from a virulent SCN population for suppression of GmBAG6-1-induced cell death in yeast as a means to identify effectors that may be targeting GmBAG6-1 or conserved regulatory mechanisms underlying PCD. From this analysis, we identified several SSEs that can function as anti-PCD proteins, including two effectors that showed direct interaction with GmBAG6-1 in yeast two-hybrid experiments, suggesting that these effectors may play a central role in plant immune suppression and nematode virulence.

2 | RESULTS

2.1 | Cloning and sequence analysis of GmBAG6-1

A nucleotide BLAST search to the soybean genome (cv. Williams 82) Glyma1.0 assembly (http://phytozome.net; Goodstein et al., 2012)
using the consensus sequence for the corresponding Affymetrix proset Gm.7623.1.A1 previously found to be induced 87-fold in feeding cells of the SCN-resistant NIL-R undergoing HR-PCD (Kandoth et al., 2011) identified two best hits. Glyma07g06750 on chromosome 7 and Glyma16g03320 on chromosome 16. Reciprocal best BLAST hit analysis identified AtBAG6 (At2g46240) (50% amino acid sequence similarity) as the most closely related Arabidopsis protein to these two soybean protein sequences. Correspondingly, these two soybean genes were named GmBAG6-1 (Glyma07g06750) and GmBAG6-2 (Glyma16g03320). We were able to clone the full-length GmBAG6-1 cDNA sequence by reverse transcription (RT)-PCR using primers designed based on the Williams 82 genome sequence from nematode-infected root tissues of Williams 82 and NIL-R. No GmBAG6-2 clones could be recovered from nematode-infected root tissues in repeated attempts. These data indicate that GmBAG6-1, but not GmBAG6-2, is expressed in soybean root tissue. The full-length 3,387-bp GmBAG6-1 cDNA sequence from Williams 82 and NIL-R contained an open reading frame encoding a 1,129 amino acid protein with a predicted mass of 126.47 kDa. An alignment of GmBAG6-1 from NIL-R with that of Williams 82 identified 14 single nucleotide polymorphisms resulting in 11 amino acid changes. The predicted protein sequence for GmBAG6-1 shares a similar domain arrangement with AtBAG6, including conserved IQ and BAG domains (Figure 1a,b). A phylogenetic analysis including all Arabidopsis and rice BAG protein family members clustered the GmBAG6 proteins with AtBAG6 (Figure 1c).

2.2 GmBAG6-1 and GmIQBAG induce cell death in yeast

AtBAG6 was shown to induce PCD in yeast and the cell death phenotype was found to be more pronounced with a truncated version of the protein spanning the IQ and BAG domains (Kang et al., 2006). To investigate whether GmBAG6-1 functions similarly to AtBAG6 in causing cell death in yeast, GmBAG6-1 and a truncated version of GmBAG6-1 spanning the IQBAG domain were expressed in a Saccharomyces cerevisiae W303-1a. For these experiments, sequences corresponding to GmBAG6-1 and the IQBAG domain were cloned into the Gateway-compatible pYES2-DEST52 vector under control of the GAL1 promoter, which allows for the conditional expression of this protein when cells are plated on media containing galactose (Kang et al., 2006). AtBAG6 and AtIQBAG sequences cloned into pYES2-DEST52 were included as positive controls. Similar to AtBAG6 and AtIQBAG, both GmBAG6-1 and GmIQBAG induced cell death in yeast. The cell death phenotype was more pronounced with the IQBAG domains (Figure 2).

2.3 GmIQBAG induces cell death in soybean

We then used bean pod mottle virus to express the IQBAG domain in soybean leaves to test for a role in cell death induction in planta. The GmBAG6-1 cDNA of 3,387 bp exceeded the size limit for recombinant virus expression, and therefore was unable to be tested using this approach. The IQBAG domain was cloned into pBPMV-IA-V4 to generate pBPMV-IA-IQBAGOE (Figure 3a). Soybean leaves were rub-inoculated with pBPMV-IA-IQBAGOE or empty pBPMV and assessed for phenotypes. Plants inoculated with pBPMV-IA-IQBAGOE were severely stunted and displayed a lesion mimic phenotype on newly developed leaves (Figure 3b,d) when compared with newly developed leaves of plants inoculated with empty pBPMV (Figure 3b,c). These data indicated that GmIQBAG can induce cell death in soybean.

For a comparison with AtBAG6 and AtIQBAG, we also over-expressed GmBAG6-1 and GmIQBAG in Arabidopsis. Out of 53 T1 primary transformants for AtBAG6, 23% of plants showed an observable phenotype and were classified as an intermediate phenotype (15%) represented by plants with smaller rosettes and malformed leaves or a severe phenotype (8%) represented by dwarf plants displaying misshapen leaves, anthocyanin accumulation, lesion mimics, shoot apical meristem (SAM) termination, and even plant death (Figure 4a,c). In contrast, 67% of plants expressing AtIQBAG had an observable phenotype. Of the 133 primary T1 transformants observed, 21% were classified as an intermediate phenotype and 46% of the plants displayed a severe phenotype consistent with a prior study (Kang et al., 2006; Figure 4a,d). However, unlike AtBAG6, 95% of the plants expressing GmBAG6-1 were indistinguishable from wild-type control plants (Figure 4a,b). The other 5% of the plants were classified as having an intermediate phenotype (Figure 4a,e). In addition, only 10% of the plants expressing GmIQBAG had any observable phenotype. Of the 128 primary T1 transformants observed, 8% were classified as intermediate and only 2% as severely dwarfed, with misshapen leaves and premature SAM termination, but lacking anthocyanin accumulation and leaf lesions similar to that observed in plants expressing AtBAG6 and AtIQBAG (Figure 4a,f). All lines tested showed an increase in target transcript abundance by RT-qPCR compared to wild-type plants, with a relatively strong correlation between expression level and symptom severity (Figure S1). Interestingly, expression of AtBAG6 and GmBAG6-1 in transgenic lines of several independent insertion events was consistently lower than the expression level of AtIQBAG and GmIQBAG.

Finally, we tested the Arabidopsis and soybean BAG6 and the corresponding IQBAG domains for the ability to induce cell death in tobacco but no such ability was detectable in our assays, unlike the pro-apoptotic protein BAX, which induced cell death in tobacco leaves (Figure 5; Kawai-Yamada et al., 2001; Abramovitch et al., 2003).

2.4 SCN effectors suppress cell death in yeast mediated by GmBAG6-1 and GmIQBAG

The lack of a cell death phenotype in tobacco precluded high-throughput screening for GmBAG6-1- or GmIQBAG-mediated cell death suppression by SCN effectors in planta. We therefore tested for the ability of a set of 47 SCN effectors to suppress cell death triggered by GmBAG6-1 and GmIQBAG proteins in yeast (Table S1). Twelve of the 47 tested SCN effectors showed strong (S) suppression of cell death triggered by GmBAG6-1 (Figure 6a,b). Five of the
Eleven out of 47 tested SCN effector candidates showed strong suppression of cell death triggered by soybean GmBAG6-1, but not GmIQBAG, in yeast. This suggested that suppression of GmBAG6-1 triggered cell death might be dependent on protein sequences outside of the IQBAG domain, possibly through direct protein-protein interaction. Therefore, we tested for direct interaction between each of the 47 effectors and GmBAG6-1 in yeast two-hybrid assays.

Interestingly, two SCN effectors, 2A05 (Hg-VAP2; Gao et al., 2001b, 2003) and 7E05, both of which showed suppression of cell death by GmBAG6-1, but not GmIQBAG, showed a strong and specific interaction with GmBAG6-1, but not GmIQBAG, Hg-VAP1, or Lamin C (Figure 7), indicating that direct modulation of BAG6 activity by these effectors may represent a viable mechanism of defence suppression.
3 | DISCUSSION

Here, we describe structural and functional properties of a soybean BAG gene that is highly induced in SCN feeding sites of resistant soybean undergoing HR-PCD (Kandoth et al., 2011) and which shares homology with the Arabidopsis gene AtBAG6 (At2g46240), a known inducer of PCD and autophagy in yeast and plants (Kang et al., 2006; Li et al., 2016). Like AtBAG6, GmBAG6-1 contained an IQ motif immediately upstream of a conserved BAG domain. As a first test of GmBAG6-1 function, we carried out a yeast viability assay. A previous study demonstrated that AtBAG6 is a CaM-binding protein involved in plant PCD and is up-regulated by various stresses (Kang et al., 2006).

A 134 amino acid sequence spanning the IQ motif and BAG domain was sufficient to induce PCD. The IQ motif was shown to be required for Ca\textsuperscript{2+}-independent binding of BAG6 to CaM and PCD (Kang et al., 2006). Similar to AtBAG6, we found that expression of GmBAG6-1 in yeast cells induced cell death. In addition, expression of the GmIQBAG domain was not only sufficient for cell death, but also caused a more
robust cell death phenotype, indicating that GmBAG6-1 functions similarly to AtBAG6 in causing cell death in yeast.

We also demonstrate that GmIQBAG can induce cell death in soybean, but causes little or no cell death in Arabidopsis and tobacco. Interestingly, despite the similar cell death phenotypes of AtIQBAG and GmIQBAG in yeast cells, a much higher frequency of Arabidopsis plants overexpressing AtIQBAG exhibited lesion mimic phenotypes compared to GmIQBAG. One plausible explanation for this difference might be species-specific interactions of plant BAG6 proteins and corresponding proteases or binding partners. This idea is supported by our finding that soybean plants overexpressing GmIQBAG were severely stunted and displayed a lesion mimic phenotype on newly formed leaves consistent with phenotypes observed in Arabidopsis lines overexpressing AtIQBAG. Moreover, transient expression of BAG6 and IQBAG proteins from Arabidopsis and soybean failed to elicit cell death in tobacco leaves. Taken together, these results indicate that soybean and Arabidopsis BAG6 proteins function most efficiently in their native plant systems.

We also observed that transcript abundance correlated with symptom severity in Arabidopsis and, interestingly, that much higher levels of transgene expression were consistently observed in the IQBAG overexpression lines compared to the BAG6 overexpression lines. It has been reported that low and intermediate constitutive levels of AtBAG4 expression in tobacco plants correlated with tolerance to abiotic stresses (Doukhanina et al., 2006). It was also reported

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**FIGURE 4** Transgenic Arabidopsis expressing BAG6 and IQBAG. (a) Summary Table of BAG6 overexpression phenotypes in Arabidopsis. (b) Wild-type Col-0. (c) Expression of the full-length AtBAG6 protein in Arabidopsis induces intermediate developmental phenotypes including slightly misshapen leaves and smaller rosettes. (d) Expression of the AtIQBAG domain in Arabidopsis induces severe growth defects including shoot apical meristem termination, anthocyanin accumulation, chlorosis, lesion mimics, and plant death. (e) Expression of the GmBAG6-1 in Arabidopsis induces intermediate developmental phenotypes including slightly misshapen leaves. (f) Expression of the GmIQBAG domain in Arabidopsis induces intermediate developmental phenotypes including slightly misshapen leaves and smaller rosettes. Scale bars = 1 cm.
that low concentrations of the human Bag-1M activate the refolding activity of molecular chaperones Hsp70/Hsc70 under physiological conditions, whereas high concentrations of Bag-1M inhibited the refolding activity of the chaperones. This suggests that the concentration of a given BAG protein in the cell relative to the concentration of its binding partner may be critical for optimal activity (Gassler et al., 2001). The lower expression of the full-length BAG6 transgenes may suggest that elements present on the C- and/or N-terminus of the protein function in the regulation of its own transcription. If so, nematodes could conceivably counteract BAG6 via direct binding of its effectors to suppress the expression of BAG6. We exploited the robust cell death phenotype elicited by overexpression of either GmBAG6-1 or the GmIQBAG domain in yeast as a system to screen SCN effectors for specific suppression of GmBAG6-1 defense-related cell death or identify effectors that may be targeting conserved eukaryotic regulatory mechanisms underlying PCD. Although many SCN effectors have been reported (Gao et al., 2001a, 2003; Wang et al., 2001; Noon et al., 2015), a role for these effectors in plant defence suppression remains relatively unknown. It has been shown previously that effectors of bacteria (Abramovitch et al., 2003; Jamir et al., 2004) and oomycetes (Wang and Huang, 2011) can suppress BAX-induced PCD in yeast cells to varying degrees. Thus, we reasoned the specific targeting of PCD inducers or components of PCD regulatory mechanisms by SCN effectors may be an effective means to suppress plant defence against nematodes. The 47 effectors considered here were identified as members of the SCN parasitome (Gao et al., 2003). These gene sequences are expressed exclusively within the subventral or dorsal oesophageal gland cells of the nematode, harbour a predicted N-terminal secretion signal, and lack a transmembrane domain distinguishing them as putative SSEs. We found that Hg-VAP1 and Hg-VAP2 (2A05), 3H07, 4D06, 4D09, 4G12, 5D06, 7E05, 10A06, 13A06, 25G01, 27D09, 28B03, 32E03, 34B08, and 33A09 strongly suppressed GmBAG6-1- or GmIQBAG-induced PCD in yeast. The observed differences in suppression characteristics of the SCN effectors tested suggest they are functioning by different mechanisms.

While the functions of the majority of SCN SSEs tested remain unknown, homologous sequences of several of these SCN effectors in other cyst nematodes have been implicated in suppression of defence responses. For example, Hg-VAP1 and Hg-VAP2 (2A05) encode venom allergen-like proteins (VAPs) (Gao et al., 2001b). VAPs have been identified in many species of plant- and animal-parasitic nematodes (Cantacessi et al., 2009). Overexpression of VAPs from the potato cyst nematode Globodera rostochiensis, the beet cyst nematode Heterodera schachtii, and the root knot nematode Meloidogyne incognita in plants increased susceptibility to nematodes, bacteria, fungi, and oomycetes (Lozano-Torres et al., 2014). Transient expression of VAPs also selectively suppressed defence-related PCD in Nicotiana benthamiana leaves. In addition, overexpression of a homologous sequence of 10A06 from H. schachtii (Hs-10A06) increased the susceptibility of Arabidopsis to infection by nematodes, Pseudomonas syringae pv. tomato (Pst DC3000), and the yellow strain of Cucumber mosaic virus. PR-1, PR-2, and PR-5 genes were down-regulated in the overexpression lines, which may explain in part the result of defence suppression by 10A06 (Hewezi et al., 2010). In this study, Hg-VAP1, Hg-VAP2 (2A05), and Hg-10A06 showed strong suppression of GmBAG6-1-mediated cell death. In contrast, homologous sequences of SCN effectors 10A07 and 19C07 from H. schachtii have not been implicated in defence suppression, but are known to play roles in modulating auxin transport and signalling for feeding cell formation via direct interaction with an indole-3-acetic acid (IAA) transcription factor and auxin transporter protein, respectively (Lee et al., 2011; Hewezi et al., 2015). These effectors did not show any activity in suppression of GmBAG6-1-mediated cell death in yeast.

Lozano-Torres et al. (2012) presented evidence that G. rostochiensis Gr-VAP1 can modulate the activation of basal plant innate immunity by the cell surface receptor Cf-2 through direct interaction with an apoplastic protease Rcr3im. In nematode-infected Rcr3im tomato plants lacking Cf-2, a compatible interaction ensues and a tomato homolog of AtBAG6 was found to be down-regulated, suggesting that Gr-VAP1 or other VAPs from G. rostochiensis may modulate Bag6 defence-related PCD in tomato (Lozano-Torres, 2014). Although Gr-VAP1 can interact with an apoplastic target to modulate downstream plant defence responses, secretion of VAPs to the cytoplasm remains a plausible scenario. In fact, nematode genomes contain multiple VAP family members and although expression of Gr-VAP1 and Hg-VAPs is highest in motile stages, the genes are also expressed in the sedentary parasitic stages (Elling et al., 2009; Lozano-Torres et al., 2014). Here, we present evidence that Hg-VAP2 may suppress GmBAG6-1-triggered cell death in yeast through direct interaction with GmBAG6-1. In contrast, Hg-VAP1 did not interact with GmBAG6-1, despite suppressing GmBAG6-1 triggered cell death in yeast. Hg-VAP1 and Hg-VAP2 protein sequences are only 45% identical/58% similar (Figure S2), and
therefore the extensive sequence polymorphisms could account for the difference in binding to GmBAG6-1. While the function of Hg-VAPs remains unknown, our data suggest that SCN may deploy VAPs to modulate the plant defence signalling pathway on multiple levels warranting further investigation.

In summary, we demonstrate that the GmBAG6-1 protein induces cell death in yeast and plants similar to AtBAG6. Although the role of GmBAG6-1 in SCN resistance is not fully understood, our prior data indicate that it may be contributing to the HR-PCD response elicited in response to nematode infection in resistant cultivars and may serve as a target for suppression by nematodes during the establishment of a compatible interaction. One possibility is that GmBAG6-1 expression is misregulated or the protein is processed in feeding cells of the resistant cultivar, thereby exceeding the expression threshold or activating a unique function by truncation of the protein that contributes to HR-PCD. This is supported by a study demonstrating that the Arabidopsis BAG6 is cleaved in vivo in a caspase-1-like dependent manner to trigger autophagy and this promotes basal immunity to the fungal pathogen Botrytis cinerea (Li et al., 2016). Although the same caspase1 cleavage site is not predicted in the GmBAG6-1 protein described here, it may harbour a distinct protease cleavage site or these two proteins may be modulated differently. Further research will be needed to fully establish a role for GmBAG6-1 protein described here, it may harbour a distinct protease cleavage site or these two proteins may be modulated differently. Further research will be needed to fully establish a role for GmBAG6-1 in nematode resistance, especially as it relates to autophagy and vesicular trafficking (Amaya et al., 2015; Zhuang et al., 2018). In addition, we identified a suite of SCN effectors that strongly suppressed the activity of GmBAG6-1 and/or GmIQBAG-mediated cell death in yeast, suggesting that modulation of cell death pathways may be a key component of nematode pathogenesis. Indeed, four of the effectors showing GmBAG6-1 cell death suppression.
suppression (2A05, 5D06, 13A06, and 33A09) were also shown to suppress pathogen-associated molecular pattern (PAMP)-triggered immunity in a parallel screening study (Pogorelko et al., 2020), providing further support for these effectors in plant immune suppression. Remarkably, we demonstrated that in yeast two of these SCN effectors may directly modulate GmBAG6-1 activity by binding to this host target. The identification of cell death-suppressing *H. glycines* effectors will facilitate ongoing investigation of the underlying functions of these novel SSEs to ultimately uncover the mechanisms used by SCN to modulate plant immunity.

### EXPERIMENTAL PROCEDURES

#### 4.1 Plant and nematode material

Soybean (*Glycine max*) cv. Williams 82 (W82) and soybean near-isogenic line (NIL) 7923R (Li et al., 2004; Kandoth et al., 2011) were used in this study. *A. thaliana* ecotype Col-0 was employed for overexpression studies. *N. benthamiana* plants were used for agroinfiltration assays. Effector sequences were previously identified from soybean cyst nematode (SCN), *H. glycines* inbred line OP50 (Gao et al., 2003). OP50 is a virulent SCN population with an HG type 1.2.3− (Dong and Opperman, 1997; Niblack et al., 2002).

#### 4.2 GmBAG6 cloning

The AtBAG6 (At2g46240) and GmBAG6-1 (Glyma07g06750) cDNA sequences were PCR amplified from total RNA isolated from *Arabidopsis* leaf tissue and NIL-7923R root tissue 4 days post-SCN infection, respectively. The gene model found in the Glyma1.0 assembly on Phytozome.net was used to design primers for GmBAG6-1. Primers are described in Table S2. Amplified fragments were cloned into pGEM-T Easy plasmid (Promega) and verified by sequencing. The soybean NIL-7923R GmBAG6-1 cDNA sequence was deposited in GenBank under accession no. JX665043. It should be noted that the GmBAG6-1 gene region was recently reannotated in the Williams 82 Glyma1.1 and Glyma2.0 assembly (Wm82.a2.v1). Consequently, Glyma07g06750 was replaced with Glyma.07g061500 (alias Glyma07g06750. Glyma07g06750. v1.1, Glyma07g06750.2.v1.1). This new gene model is consistent
with the cDNA used in this study, but predicts a second intron immediately upstream of the original start site, which moves the predicted start site 99 nucleotides further upstream. This results in a predicted protein of 1,162 amino acids instead of the 1,129 amino acid protein used in this study. While this change does not impact the results and conclusions of this study, any future work to characterize GmBAG6-1 function in soybean should take this into consideration.

4.3 | Phylogenetic analysis

Phylogenetic tree construction and bootstrap analysis was performed using RAxML (Randomized Axelerated Maximum Likelihood) software comparing Arabidopsis (AtBAG1: At5g52060, AtBAG2: At5g62100, AtBAG3: At5g07220, AtBAG4: At3g51780, AtBAG5: At1g12060, AtBAG6: At2g46240, and AtBAG7: At5g62390), rice (OsBAG1: LOC_Os09g35630, OsBAG2: LOC_Os08g43270, OsBAG3: LOC_Os06g03640, OsBAG4: LOC_Os01g61500, OsBAG5: LOC_Os11g31060, and OsBAG6: LOC_Os02g48780), and soybean (GmBAG6-1: JX665043 and GmBAG6-2 Glyma16g03320) BAG protein sequences.

4.4 | Overexpression constructs

AtBAG6 and GmBAG6-1 cDNA sequences, as well as the corresponding IQBAG domains were amplified using PCR and cloned into the Gateway vector pDONR-Zeo (Invitrogen). The clones were sequence verified. The sequences were then cloned from the pDONR-Zeo donor plasmid into Gateway-compatible vector pAKK-2×35S (Wang et al., 2010) to generate 2×35S-GmBAG6-1, 2×35S-GmiIQBAG, 2×35S-AtBAG6, and 2×35S-AtIQBAG. To create the pAKK-2×35S Gateway vector, a ccdB cassette flanked by attR1 and attR2 sites was PCR amplified (pAKK Gateway vector primers; Table S2) and cloned into the CGT 2×35S vector digested with KpnI and HindIII, downstream of promoter 2×35S. A SbfI fragment from the resultant vector consisting of 2×35S promoter, ccdB cassette, and OCS terminator was then subcloned into binary vector pAKK using SbfI. Gene-specific primers flanked with partial attB sequences were used for PCR to amplify candidate genes, and the PCR products were further amplified to incorporate intact attB sequences into the amplified fragment for Gateway cloning. The primers used are described in Table S2.

4.5 | Arabidopsis transformation

Agrobacterium tumefaciens GV3101 was transformed with 2×35S-GmBAG6-1, 2×35S-AtBAG6, 2×35S-AtIQBAG, and 2×35S-GmiIQBAG. A. thaliana was transformed using the floral dip method (Clough and Bent, 1998). Transgenic Arabidopsis plants (T₁) were selected on 0.5 × Murashige and Skoog (MS) agar plates containing 20 μg/ml BASTA. T₁ transformants were transferred to soil and grown in a growth chamber at 22°C under long-day growth conditions to observe phenotypes.

4.6 | Virus-induced expression in soybean

The bean pod mottle virus (BPMV) virus-induced gene silencing (VIGS) vector pBPMV-IA-V4 (Zhang et al., 2010) was used in this study. Gene-specific primers (Table S2) were used to amplify a 312 bp sequence (spanning bp 1597-1908) corresponding to the IQBAG domain. The PCR product was digested with Xhol and Smal and ligated into pBPMV-IA-V4 digested with the same enzymes to generate pBPMV-IA-IQBAGOE. Plant inoculum for all viruses was produced as described Zhang et al. (2010). At 3–4 weeks postinoculation, BPMV-infected leaves were collected, lyophilized, and stored at −20°C for future experiments. Infected soybean leaf tissues were ground in a mortar and pestle with 0.05 M potassium phosphate buffer (pH 7.0) and used as viral inoculum for subsequent experiments. SCN-susceptible soybean cv. William 82 leaves were inoculated with pBPMV-IA-IQBAGOE. Control plants were infected with empty pBPMV. Each treatment consisted of at least 12 plants. Two independent experiments were conducted. For virus inoculation, uninoculated leaves of 9-day-old soybean plants were rub-inoculated using carborundum according to Zhang et al. (2010). Plants were grown in a growth chamber set to the following conditions: 20–21°C, 16 hr light/8 hr dark, and 100 μmol·m⁻²·s⁻¹ light intensity. Observations of plant growth were documented 16 days postvirus infection.

4.7 | RNA isolation and quantitative real-time PCR

RNA was extracted from 14-day old Arabidopsis seedlings using the RNeasy Plant mini kit (Qiagen) and treated with RNase-free DNase (Qiagen). cDNA was made from 1 μg of total RNA using the First Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics) according to the manufacturer’s instructions. RT-qPCR was carried out using the Applied Biosystems 7500 Real-Time PCR system. qPCR primers were designed for AtBAG6, GmBAG6-1, and an Arabidopsis polyubiquitin endogenous control gene, AtUBC10 (At4g05320) (Czechowski et al., 2005) using the Primer Express software (Applied Biosystems). Primers are described in Table S2. Triplicate qPCRs were set up and analysed as described in Wang et al. (2007).

4.8 | Yeast viability assay

Plasmids containing AtBAG6, GmBAG6-1 cDNA, and the truncated cDNA spanning the IQBAG domain for both genes were cloned into the p′RES2-DEST52 Gateway vector system (Invitrogen). The plasmids in yeast vectors were transformed into the wild-type S. cerevisiae W303-1a (Petrezselyova et al., 2010) (MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100). Strains were pregrown in
synthetic dropout (SD) medium lacking uracil in the presence of 2% glucose (SD–U/Glu) at 30°C to an optical density (OD600) of 0.17. After washing three times in sterile water to remove any excess glucose, aliquots (10 μl) of 10-fold serial dilutions were pipetted on plates of SD medium lacking uracil, in the presence of 2% glucose (SD–U/Glu) or 2% galactose (SD–U/Gal). Plates were incubated at 30°C and photographed after 4 days.

4.9 | Construction of SCN effector constructs and cell death suppression screening

Forty-seven SCN SSE effectors of the SCN parasitome (Gao et al., 2001b, 2003) were selected for cell death suppression in yeast. All selected genes were cloned into the pGBK7 Gateway vector to express the mature effector protein sequences without signal peptide sequences. To generate a pGBK7 Gateway vector, a ccdB cassette flanked by attR1 and attR2 sites was PCR amplified (pGBK7 Gateway primers; Table S2) and cloned into the pGADT7 vector (Clontech) digested with Ndel and XhoI to generate pGADT7 Gateway. The cloned ccdB gene was cut out from pGADT7 Gateway with Ndel and PstI and subcloned into pGBK7T7 to make the pGBK7T7 Gateway vector. Primer sequences are provided in Table S2. All constructs were confirmed by sequencing. Cell death suppression screening was conducted by coexpression of GmBAG6-1, and GmlQBAG with each of the effector proteins in S. cerevisiae W303-1a. Transformants were selected on SD–Ura–Trp plates. Viability of the yeast transformants was tested as described above.

4.10 | Agrobacterium-mediated transient assays

GmBAG6-1, AtBAG6, GmlQBAG, and AtIQBAG cDNA were cloned into the pAKK binary vector under control of the 2 × 35S promoter and transformed into A. tumefaciens C58C1 for expression in N. benthamiana via syringe infiltration. The GUS gene was used as a negative control. The pro-apoptotic protein BAX, which can induce cell death in tobacco leaves (Kawai-Yamada et al., 2003; Abramovitch et al., 2003), was cloned into the pAKK vector and used as a positive control. C58C1 was grown at 28°C in Luria Bertani broth with 50 μg/ml kanamycin. Bacteria were centrifuged at 3,795 × g at room temperature and resuspended in 10 mM MgCl2 to an OD600 of 0.3–0.4. Cells were then infiltrated into the abaxial air spaces of 3–4-week-old N. benthamiana plants using a needleless syringe. Agroinfiltrated plants were monitored for 4 days for cell death in leaf sections infiltrated with the constructs.

4.11 | Yeast two-hybrid analysis

GmBAG6-1 and GmlQBAG were cloned into the pGADT7 Gateway vector. GmBAG6-1:pGADT7 or GmlQBAG:pGADT7 was cotransformed with each effector gene cloned into pGBK7T7 in S. cerevisiae AH109. Yeast transformants were selected on SD–Trp–Leu plates. Transformants were cultured in SD–Trp–Leu medium at 30°C overnight. After washing in sterile water, the pellets were resuspended in sterile water to OD600 = 0.1. After serial dilution, 5 μl of yeast solution was spotted on SD–Trp–Leu + X-a-Gal and SD–Ade–Trp–Leu–His plates, separately. Coexpression of P53:pGBK and SV40:pGAD was used as a positive control. pGBK7T7 and pGADT7 empty vectors were used as negative control. Plates of yeast colonies were incubated at 30°C and photographed after 4 days.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

ORCID

Jianying Wang https://orcid.org/0000-0002-4991-8328
Richard S. Hussey https://orcid.org/0000-0002-2293-6067
Eric L. Davis https://orcid.org/0000-0001-8025-3742
Thomas J. Baum https://orcid.org/0000-0001-9241-3141
Melissa G. Mitchum https://orcid.org/0000-0002-9086-6312

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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