Screening soybean cyst nematode effectors for their ability to suppress plant immunity

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Abstract
The soybean cyst nematode (SCN), *Heterodera glycines*, is one of the most destructive pathogens of soybeans. SCN is an obligate and sedentary parasite that transforms host plant root cells into an elaborate permanent feeding site, a syncytium. Formation and maintenance of a viable syncytium is an absolute requirement for nematode growth and reproduction. In turn, sensing pathogen attack, plants activate defence responses and may trigger programmed cell death at the sites of infection. For successful parasitism, *H. glycines* must suppress these host defence responses to establish and maintain viable syncytia. Similar to other pathogens, *H. glycines* engages in these molecular interactions with its host via effector proteins. The goal of this study was to conduct a comprehensive screen to identify *H. glycines* effectors that interfere with plant immune responses. We used *Nicotiana benthamiana* plants infected by *Pseudomonas syringae* and *Pseudomonas fluorescens* strains. Using these pathosystems, we screened 51 *H. glycines* effectors to identify candidates that could inhibit effector-triggered immunity (ETI) and/or pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). We identified three effectors as ETI suppressors and seven effectors as PTI suppressors. We also assessed expression modulation of plant immune marker genes as a function of these suppressors.

Keywords
cell death, cyst nematode, defence suppression, effector, ETI, PTI, soybean

Disciplines
Agricultural Science | Agriculture | Entomology | Plant Pathology

Comments
This article is published as Pogorelko, Gennady, Jianying Wang, Parijat S. Juvale, Melissa G. Mitchum, and Thomas J. Baum. "Screening soybean cyst nematode effectors for their ability to suppress plant immunity." *Molecular Plant Pathology* (2020). doi: 10.1111/mpp.12972.

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Nematodes are a group of animals occupying a broad range of ecological niches and consist of terrestrial and marine species as well as plant and animal parasites (Cobb, 1914). The most damaging plant-parasitic nematodes are the cyst and root-knot nematode species, which cause billions of dollars in yield and quality losses annually by infecting crop plants (Sasser and Freckman, 1987).

Cyst-forming nematodes, such as the soybean cyst nematode *Heterodera glycines*, invade plant roots and then migrate intracellularly to the vascular tissues, where they induce the development of syncytial feeding sites (Jones, 1981). *H. glycines* engages in complex molecular interactions with its host plants to accomplish this feat. In the past decade, a large body of research has shown that effectors,
a set of proteins secreted into the host plants by the nematodes on plant invasion, are important factors that facilitate the parasite's ability to parasitize its host (Mitchum et al., 2013; Mitchum, 2016; Juvale and Baum, 2018; Siddique and Grundler, 2018; Vieira and Gleason, 2019). Once inside the root tissue, nematode effector proteins interact with host factors and manipulate associated signal transduction pathways, causing complex morphological and physiological changes to selected host cells. These changes ultimately result in the formation of a syncytium composed of several hundred fused root cells. The syncytium acts as the nematode's sole source of nourishment throughout its life cycle. Therefore, establishing and maintaining the syncytium is an absolute necessity for the parasitic success of the cyst nematode. Naturally, plants have evolved counterstrategies to activate defense responses at the sites of infection to interfere with syncytium formation (Sato et al., 2019).

Pathogen-associated molecular pattern (PAMP)-triggered immune response (PTI) is the first line of defense activated by plants in the wake of pathogen attack. PTI is generally triggered when pattern recognition receptors localized on the plasma membrane detect conserved molecular features of pathogens such as flagellin or chitin. (Ayers et al., 1976; Felix et al., 1993, 1999). Processes induced in the plant by PTI responses include callose deposition, reactive oxygen species (ROS) production, and elevated expression of defense-associated genes (Jones and Dangl, 2006; Schwessinger and Zipfel, 2008). To circumvent PTI responses and establish infection, pathogens have evolved effectors to suppress these defenses. For example, the Ecp6 effector family, first identified from the fungal pathogen Cladosporium fulvum, specifically attenuates chitin-induced PTI responses in the host (de Jong et al., 2010). Similarly, effectors AvrPto and AvrPtoB from the bacterial pathogen Pseudomonas syringae suppress flagellin-induced PTI responses in hosts (Cunnac et al., 2009). In turn, plants have evolved mechanisms to detect pathogen effectors that circumvent PTI to trigger an even more robust defense response. In this second line of defense, the effector proteins delivered by the pathogen or their functions are recognized by host resistance (R) proteins, which then cause effector-triggered immunity (ETI). For example, C. fulvum-resistant tomato cultivars identify the Avr4 effector from the fungal pathogen and trigger ETI (Laugé et al., 2000). ETI usually culminates in a hypersensitive response (HR) resulting in localized plant cell death at the infected area, which halts pathogen infection (Glazebrook, 2005; Dangl et al., 2013). Thus, hosts and pathogens are engaged in a dynamic evolutionary interplay to gain superiority. Plants' survival relies on successful identification and mitigation of pathogens via PTI and ETI responses, while maintaining the syncytium is an absolute necessity for the parasitic success of the cyst nematode. Overall, we selected 51 effectors for this study (Table 1). Most of these effectors do not share any sequence homology with any known proteins in databases. Thus, computational analysis to predict effector function based on homology modeling could be misleading and direct experimental evidence is therefore more reliable. The traditional method of investigating functional properties of effectors is to stably express their coding sequences in plants and assess resultant phenotypes. However, in the case of investigating the functions of high numbers of effector proteins, this time- and labour-intensive approach becomes impractical. As an alternative, we employed an established high-throughput method to screen a large number of H. glycines effectors to identify those with defense-suppressing abilities in a quick, easy, and reproducible way.

Here, we used the bacterial type III secretion system (T3SS) for effector delivery to plant cells to facilitate rapid and reproducible testing for effector-like functions (Fabro et al., 2011). For this purpose, a specific T3SS signal peptide is translationally fused to the N-terminus of the candidate effector sequence (without the native signal peptide), which allows bacteria to secrete the nematode effector into plant cells. We used Nicotiana benthamiana and the plant pathogens P. syringae and Pseudomonas fluorescens to trigger defense responses and we performed such assays with the coding sequences of 51 total H. glycines effector proteins that were cloned into the pEDV6 plasmid (Sohn et al., 2007). All 51 recombinant pEDV6 plasmids harbouring single effectors were mobilized into either P. fluorescens EtHAn (a nonvirulent strain engineered to use the T3SS) for PTI suppression tests (Oh and Collmer, 2005) or P. syringae pv. tomato DC3000 bacterial cells to perform ETI suppression tests (Qi et al., 2016) in N. benthamiana.

The presence of nonpathogenic P. fluorescens EtHAn induces basal PAMP-triggered defenses in N. benthamiana tissues, which suppress the ability of pathogenic P. syringae DC3000 to cause a robust HR when subsequently infiltrated into the same leaf area 6–8 hr later.
| Effector ID | Highest protein similarity | Accession no. | ETI or PTI suppressor |
|------------|--------------------------|--------------|----------------------|
| GLAND1     | GNAT, Streptomyces       | KJ825712.1   | ETI suppressor       |
| GLAND2     | Pioneer                  | MT012314     | –                    |
| GLAND3     | G12H04, Heterodera glycines | MT012315     | –                    |
| GLAND4     | 1106_3E10, Globodera rostochiensis | MT012316 | – |
| GLAND5     | G11A06, Heterodera glycines | MT012317 | PTI suppressor |
| GLAND6     | 4D06, Heterodera glycines | MT012318     | PTI suppressor       |
| GLAND7     | G15A10, Heterodera glycines | MT012319 | – |
| GLAND8     | Pioneer                  | MT012320     | PTI suppressor       |
| GLAND9     | Pioneer                  | MT012321     | ETI suppressor       |
| GLAND10    | Cellulose binding protein, Heterodera schachtii | MT012322 | – |
| GLAND11    | Pioneer                  | MT012323     | –                    |
| GLAND12    | Pioneer                  | MT012324     | –                    |
| GLAND13    | Invertase, Rhizobium     | MT012325     | –                    |
| GLAND14    | Endopeptidase, Ascaris suum | MT012326 | – |
| GLAND15    | G23G11, Heterodera glycines | MT012327 | – |
| GLAND16    | Chorismate mutase, Heterodera glycines | MT012328 | – |
| GLAND17    | DUO-3, Caenorhabditis elegans | MT012329 | – |
| SY20       | Pioneer                  | AF273729     | –                    |
| 2A05       | HgVAP2                   | MT125638     | PTI suppressor       |
| 2B10       | HgCLE1                   | AF273728     | –                    |
| 2D01       | Pioneer                  | AF469057     | –                    |
| 3B05       | HgCBP                    | AF469058     | –                    |
| 3D11       | Chitinase—Caenorhabditis elegans | MT125639 | – |
| 4D09       | Pioneer                  | MT012330     | –                    |
| 4E02       | Pioneer                  | AF473826     | –                    |
| 4F01       | Annexin—Caenorhabditis elegans | MT012337 | ETI suppressor |
| 4G06       | Hexaubiquitin—Helianthus annuus | MT012331 | – |
| 4G12       | HgCLE2                   | AF473827     | –                    |
| 5D06       | Pioneer                  | MT012332     | PTI suppressor       |
| 5D08       | Pioneer                  | AF473828     | –                    |
| 6E07       | Pioneer                  | MT125645     | –                    |
| 7E05       | Pioneer                  | AF500023     | –                    |
| 12H04      | Pioneer                  | MT012338     | –                    |
| 13A06      | Pioneer                  | MT125650     | PTI suppressor       |
| 16B09      | Pioneer                  | AF490246     | –                    |
| 18H08      | Pioneer                  | MT012333     | –                    |
| 19B10      | Pioneer                  | MT012334     | –                    |
| 19C07      | Pioneer                  | MT125652     | –                    |
| 20E03      | Pioneer                  | AF490251     | –                    |
| 21E12      | Pioneer                  | MT125654     | –                    |
| 22C12      | Pioneer                  | AF500029     | –                    |
| 23G12      | Pioneer                  | MT012339     | –                    |
| 24A12      | Pioneer                  | MT012336     | –                    |
| 30C02      | Pioneer                  | MT125659     | –                    |
| 30D08      | Pioneer                  | MT125660     | –                    |

(Continues)
(Oh and Collmer, 2005; Figure 1a). If an effector expressed by \textit{P. fluorescens} EthAn suppresses PTI, HR caused by \textit{P. syringae} DC3000 will appear earlier compared to the area infiltrated with EthAn without an effector. To identify PTI-suppressing effectors, engineered \textit{P. fluorescens} EthAn strains containing a single \textit{H. glycines} effector were used for inoculation of \textit{N. benthamiana} leaves. Seven hours later, wild-type \textit{P. syringae} DC3000 cells were infiltrated into the same leaf area, partially overlapping with the area previously inoculated with EthAn. Of the 51 tested effectors, seven effectors enabled \textit{P. syringae}-induced HR development, thus showing their ability to suppress PTI: GLAND5, GLAND6, GLAND8, 5D06, 2A05, 13A06, and 33A09 (Figure 2a). Interestingly, effectors 5D06, 2A05, 13A06, and 33A09 were independently confirmed to strongly suppress BAG6 protein-mediated cell death in yeast (Wang et al., 2020).

To confirm the host defence-suppressing abilities of these PTI-suppressing effectors, we used quantitative reverse transcription PCR (RT-qPCR) to measure expression levels of selected defence-related genes. For this purpose, we chose the following genes whose activation is associated with salicylic acid signalling (Noon et al., 2016): pathogenesis-related 1 and 2 (\textit{PR1} and \textit{PR2}), WRKY transcription factor 12 (\textit{WRKY12}), and proteinase inhibitor 1 (\textit{PI}). We quantified the mRNA abundance of these genes from leaf regions infected with either an empty EthAn strain or an EthAn strain expressing the requisite effector and normalized both values using the mRNA abundance of the corresponding genes determined by RT-qPCR in noninfiltrated leaf regions. All seven PTI suppressors caused significant down-regulation of one or more defence genes in comparison with the empty EthAn strain during bacterial infection (Figure 2b).

The assay for the suppressors of ETI was conducted as shown in Figure 1b. Development of HR on \textit{N. benthamiana} leaves was monitored after inoculation of \textit{P. syringae} DC3000 wild-type (left column) and recombinant strains (right column) expressing exportable nematode effectors into \textit{N. benthamiana} leaves.
expression levels of the corresponding defence genes in noninfiltrated leaf regions were used to normalize expression.

Additionally, to discern whether these effectors in fact suppress plant defence responses rather than being directly detrimental to *P. syringae* viability, we monitored growth rates of bacteria in infected *N. benthamiana* leaves over the course of 5 days postinfection. Detection of significantly higher bacterial growth in the leaf areas infiltrated with *P. syringae* harbouring these effectors compared with the wild-type *P. syringae* growth confirmed that the effector expression is not detrimental to the bacterial viability and the effectors, in fact, are suppressing the host ETI responses (Figure 3c).

In summary, we used a relatively high-throughput system for screening a large number of effectors, and we successfully identified multiple soybean cyst nematode effectors with defence-suppressing abilities. The plant innate immune system is controlled by a network of sensors and receptors that recognize pathogen infection (Nürnberger et al., 2004). Thus, it is unsurprising that the pathogen secretes multiple effectors targeting host defense proteins after delivery into plant cells through T3SS (Sohn et al., 2016). The effector sequences were cloned into either pENTR D-TOPO or pDONRzeo entry vectors (Invitrogen). These clones were Gateway subcloned into pEDV6 (Fabro et al., 2011) with LR clonase (Invitrogen) according to the manufacturer’s recommendations. PCR products for the effector sequences were cloned into either pENTR D-TOPO or pDONRzeo entry vectors (Invitrogen). These clones were Gateway subcloned into pEDV6 (Fabro et al., 2011) with LR clonase (Invitrogen) and verified by sequencing. *Pseudomonas* expression vector pEDV6 is designed to express and deliver nonbacterial proteins fused to the *AvrRPS4* T3SS signal peptide into plant tissues where the N-terminus is designed to express and deliver nonbacterial proteins fused to the *AvrRPS4* T3SS signal peptide into plant tissues where the N-terminus

For the characterization of the 51 effectors reported by Gao et al. 2001 and Noon et al. (2015), we amplified and cloned the truncated coding sequences lacking the signal peptide either from cDNA generated from the mixed stage *H. glycines* population or from full-length cDNA clones using the primers listed in Table S1. RNA extraction from nematodes was performed as described previously (Noon et al., 2016). First-strand cDNAs were synthesized from 3 μg total RNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s recommendations. PCR products for the effector sequences were cloned into either pENTR D-TOPO or pDONRzeo entry vectors (Invitrogen). These clones were Gateway subcloned into pEDV6 (Fabro et al., 2011) with LR clonase (Invitrogen) and verified by sequencing. *Pseudomonas* expression vector pEDV6 is designed to express and deliver nonbacterial proteins fused to the *AvrRPS4* T3SS signal peptide into plant tissues where the N-terminus of *AvrRPS4* will be cleaved to release the mature nematode effector proteins after delivery into plant cells through T3SS (Sohn et al., 2007). Resequencing of obtained recombinant plasmids confirmed the absence of deviation.
Immunosuppression experiments were performed as described by Noon et al. (2016). Triparental mating was used for conjugation of pEDV6 vectors into *P. fluorescens* EtHAn and electroporation was used for *P. syringae* pv. *tomato* DC3000. Bacteria were suspended in 10 mM MgCl₂ and infiltrated into *N. benthamiana* leaves at OD₆₀₀ = 0.2 for EtHAn and OD₆₀₀ = 0.02 for DC3000. To quantify ETI suppression levels, *P. syringae* DC3000 strains carrying effectors were infiltrated into *N. benthamiana* leaves with 1-ml needleless syringes. Five different strains were infiltrated into each leaf, including the *P. syringae* DC3000 wild type on each leaf as a cell death control. Photographs were taken 3–4 days after infiltration. All experiments were repeated three times. For each biological sample, three of four fully expanded leaves from each plant and five 4–6-week-old *N. benthamiana* plants were used.

Expression levels of defence-related genes were quantified by RT-qPCR. qPCR was performed on an iCycler Real-Time system (Bio-Rad). Each reaction was performed in 20 μl final volume containing 10 μl of SYBR Green Master Mix reagent (Fermentas), 250 ng cDNA, and 200 nM primers (Table S1) designed to produce products of 150–200 bp using Vector NTI v. 9.0 software (InforMax). The qPCR conditions were as follows: 50°C for 2 min; 95°C for 10 min; 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 45 s. As the final step, a melting curve was analysed to confirm the specificity of the reactions. Each sample was tested in three biological replicates and two technical replicates. The *Actin-2* gene expression level was used as the internal control. The relative expression level was calculated as $2^{-\Delta\Delta CT} \left[ \Delta CT = CT_{\text{gene of interest}} - CT_{\text{Actin2}} \right]$.

To determine bacterial growth, a piece of leaf disc (30 mm²) in the infiltration area was ground in 500 μl of 10 mM MgCl₂ solution and a series of dilutions of 10⁻¹ to 10⁻⁸ were plated on Luria Bertani medium containing 50 mg/L rifampicin. Colony counts were performed from dilutions that gave approximately 50–70 colonies per plate.

**ACKNOWLEDGMENTS**

This work was supported by USDA-NIFA-AFRI award 2015-67013-23511 to T.J.B. and M.G.M., by Hatch Act and State of Iowa funds, and grants to T.J.B. from the Iowa Soybean Association. We thank Thomas R. Maier and Marena Henkle for their technical assistance.
DATA AVAILABILITY STATEMENT
The data that supports the findings of this study are available in the Supporting Information of this article.

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

How to cite this article: Pogorelko G, Wang J, Juvale PS, Mitchum MG, Baum TJ. Screening soybean cyst nematode effectors for their ability to suppress plant immunity. Molecular Plant Pathology. 2020;00:1–8. https://doi.org/10.1111/mpp.12972