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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

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SHORT COMMUNICATION

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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

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,

Gennady Pogorelko and Jianying Wang contributed equally to this work.

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 defence 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 defence 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 defence-associated genes (Jones and Dangl, 2006; Schwessinger and Zipfel, 2008). To circumvent PTI responses and establish infection, pathogens have evolved effectors to suppress these defences. 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 defence response. In this second line of defence, 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 (Lauge *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 pathogen success relies on evolving effector repertoires that can deliver robust suppression of host defences.

Cyst nematodes also have been shown to elicit PTI responses in host roots (Manosalva *et al*., 2015; Mendy *et al*., 2017), and growing evidence suggests that the cyst nematodes deploy various effectors to attenuate such responses. For example, the potato cyst nematode *Globodera rostochiensis* secretes effector GrUBCEP12, which acts as a PTI inhibitor in planta (Chen *et al*., 2013; Chronis *et al*., 2013), while the effector SPRYSEC-19 secreted by the same pathogen suppresses programmed cell death in tomato by interacting with the SW5F protein (Postma *et al*., 2012). The soybean cyst nematode *H. glycines* is also known to elicit a strong HR-like response at the infection sites in resistant soybean cultivars (Endo, 1965; Riggs *et al*., 1973; Kandoth *et al*., 2011). *H. glycines* populations that can successfully parasitize these resistant soybeans have also been identified (Niblack *et al*., 2008; Mitchum, 2016), suggesting that *H. glycines* is also evolving effector repertoires that can overcome this robust host defence response. However, associated effectors are yet to be identified, and the underlying mechanisms remain unexplained. Identifying and investigating such mechanisms is essential for developing soybean cultivars that can combat virulent *H. glycines* populations. As a first step toward that goal, we conducted a comprehensive screen of *H. glycines* effectors to identify those capable of host defence suppression.

Over 70 bona fide *H. glycines* effectors have been identified and confirmed by mRNA in situ hybridization to the effector-producing gland cells to date (Wang *et al*., 2001; Gao, *et al*., 2003; Noon *et al*., 2015). Of these, we excluded for this study those effectors for which clear functions have already been elucidated, and selected the remaining effectors. 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 modelling 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 defence-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 defence 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 defences 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

TABLE 1 List of screened effectors

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	–
6EO7	Pioneer	MT125645	–
7EO5	Pioneer	AF500023	–
12H04	Pioneer	MT012338	–
13A06	Pioneer	MT125650	PTI suppressor
16B09	Pioneer	AF490246	–
18HO8	Pioneer	MT012333	–
19B10	Pioneer	MT012334	–
19C07	Pioneer	MT125652	–
20EO3	Pioneer	AF490251	–
21E12	Pioneer	MT125654	–
22C12	Pioneer	AF500029	–
23G12	Pioneer	MT012339	–
24A12	Pioneer	MT012336	–
30C02	Pioneer	MT125659	–
30D08	Pioneer	MT125660	–

(Continues)

TABLE 1 (Continued)

Effector ID	Highest protein similarity	Accession no.	ETI or PTI suppressor
30EO3	Pioneer	AF500035	–
32EO3	Pioneer	MT012335	–
33A09	Pioneer	MT125663	PTI suppressor
33EO5	Pioneer	AF502392	–
34B08	Pioneer	AF500037	–
45D07	Chorismate mutase—*Heterodera glycines*	MT012340	–

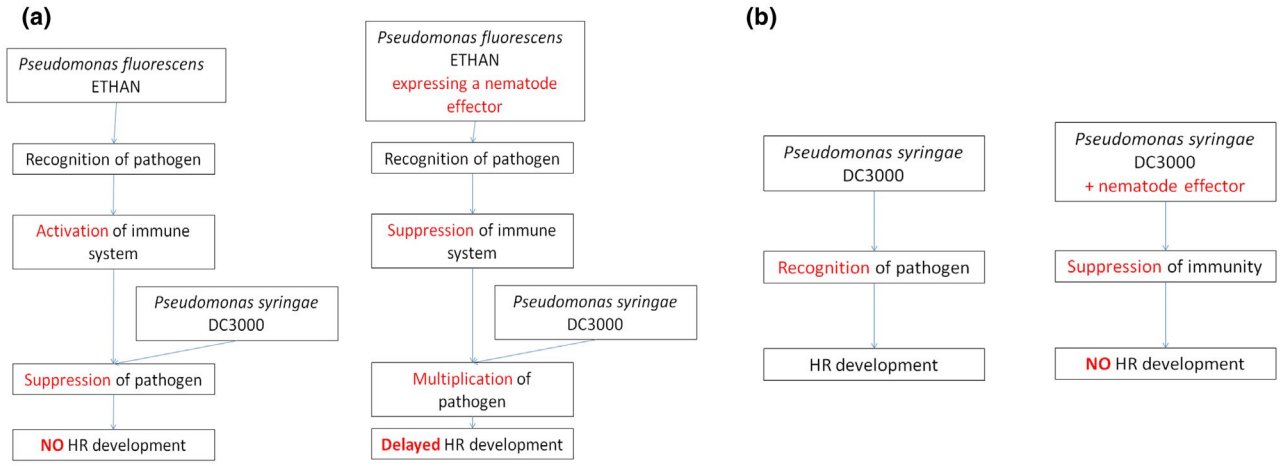

FIGURE 1 Outline of PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) suppression screening. (a) The screening for PTI suppression of *Heterodera glycines* effector proteins involves inoculation of *Pseudomonas fluorescens* EtHAn wild-type (left column) and recombinant strains (right column) expressing exportable nematode effectors into *Nicotiana benthamiana* leaves with subsequent additional infiltration of *Pseudomonas syringae* pv. *tomato* DC3000 wild-type cell culture. (b) The screening for ETI suppression of *H. glycines* effector proteins involves inoculation of *P. syringae* pv. *tomato* DC3000 wild-type (left column) and recombinant strains (right column) expressing exportable nematode effectors into *N. benthamiana* leaves

(Oh and Collmer, 2005; Figure 1a). If an effector expressed by *P. fluorescens* EtHAn suppresses PTI, HR caused by *P. syringae* DC3000 will appear earlier compared to the area infiltrated with EtHAn without an effector. To identify PTI-suppressing effectors, engineered *P. fluorescens* EtHAn strains containing a single *H. glycines* effector were used for inoculation of *N. benthamiana* leaves. Seven hours later, wild-type *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 *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 (*PR1* and *PR2*), WRKY transcription factor 12 (*WRKY12*), and proteinase inhibitor 1 (*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 *N. benthamiana* leaves was monitored after inoculation of *P. syringae* DC3000 with or without nematode effectors. In the control infiltration area, a quick and stable HR cell death was induced by DC3000, which served as the control for *P. syringae* DC3000 strains carrying individual nematode effector genes. Out of 51 effectors, only three (GLAND1, GLAND9, and 4F01) were able to inhibit HR activation (Figure 3a). To confirm these results, we also assessed expression of the chosen defence genes as a function of ETI-suppressing effectors and observed that all recombinant *P. syringae* DC3000 strains carrying ETI suppressors caused decreases in transcript levels of the defence-related marker genes compared with leaves infiltrated with the wild-type DC3000 (Figure 3b). As mentioned above, for comparative analysis,

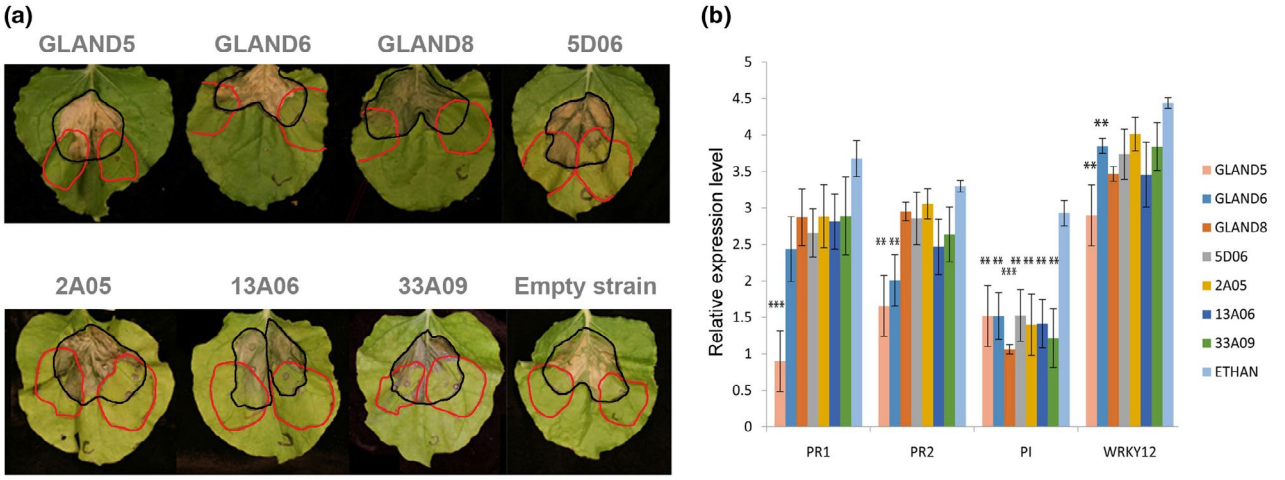

FIGURE 2 Identifying defence-suppressing *Heterodera glycines* effectors. (a) *Nicotiana benthamiana* leaves showing results from PAMP-triggered immunity (PTI) suppression assay. The red marker on the left side of each *N. benthamiana* leaf indicates the area inoculated with either wild-type *Pseudomonas fluorescens* EtHAn or *P. fluorescens* EtHAn harbouring a nematode effector. The red marker on the right side represents control infiltration of wild-type *P. fluorescens* EtHAn. The black marker indicates areas infected with *Pseudomonas syringae* wild-type culture. Hypersensitive response development was detected for strains expressing seven effectors: GLAND5, GLAND6, GLAND8, 5D06, 2A05, 13A06, and 33A09, indicating they are PTI suppressors. (b) Quantitative reverse transcription PCR (RT-qPCR) expression analysis of the salicylic acid-responsive defence marker genes during basal immune responses for both wild-type *P. fluorescens* EtHAn and *P. fluorescens* EtHAn harbouring selected PTI suppressors at 8 hr postinfiltration. All data are the average of three independent biological samples, each consisting of two technical replicates ± *SE*. Significant differences were analysed using Student's *t* test. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$. The mRNA abundance of these genes was quantified in leaf regions infected with either an empty EtHan strain or an EtHan strain expressing the requisite effector and both values were normalized using the mRNA abundance of the corresponding genes determined by RT-qPCR in noninfiltrated leaf regions

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 to suppress host defence responses. These effectors could be targeting multiple host factors to simultaneously modulate multiple defence networks. Identifying defence network-associated host factors targeted by effectors and investigating mechanisms adapted by pathogens to modulate host defence networks is crucial. For example, using this information, one can potentially tweak and modify these defence pathways to develop pathogen-resistant crop cultivars using genome-editing tools. We have taken the first steps toward that goal by producing a list of *H. glycines* effectors that are involved in host defence modulation. Studying each of these effectors independently to elucidate the underlying defence-suppressing mechanisms will lead the way in exploiting these discoveries in the future.

The experimental details of the work are as follows. *H. glycines* isolate OP50, which previously had been selected for virulence on resistant soybean cultivars, was cultivated on soybeans in Iowa State University greenhouses as previously described (de Boer *et al*., 1999). *N. benthamiana* was grown at 25°C with 16 hr light:8 hr dark cycles in growth chambers.

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.

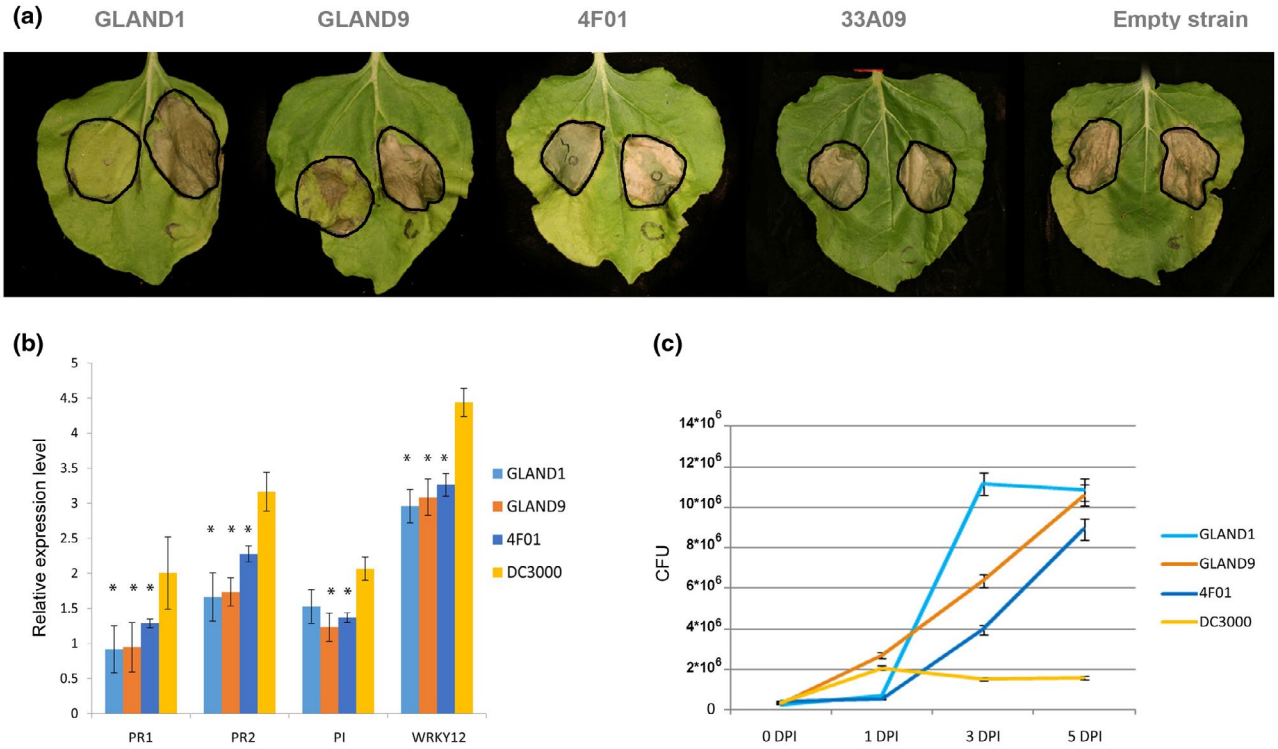

FIGURE 3 Identifying effector-triggered immunity (ETI)-suppressing *Heterodera glycines* effectors. (a) The left side of each *Nicotiana benthamiana* leaf was inoculated with either wild-type *Pseudomonas syringae* DC3000 or *P. syringae* DC3000 harbouring a nematode effector, and the right side represents control infiltration of wild-type *P. syringae* DC3000. Delayed hypersensitive response (HR) development was detected for strains expressing effectors GLAND1, GLAND9, and 4F01. (b) Quantitative reverse transcription PCR (RT-qPCR) expression analysis of the defence marker genes during HR for either wild-type *P. syringae* DC3000 or *P. syringae* DC3000 expressing selected ETI suppressors at 24 hr postinfiltration. All data are the average of three independent biological samples, each consisting of two technical replicates ± *SE*. Significant differences were analysed using Student's *t* test. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$. The mRNA abundance of these genes was quantified in leaf regions infected with either an empty DC3000 strain or a DC3000 strain expressing the requisite effector and both values were normalized using the mRNA abundance of the corresponding genes determined by RT-qPCR in noninfiltrated leaf regions. (c) Quantifying *P. syringae* growth in *N. benthamiana* leaves. The number of bacterial colony-forming units (with *SD*) recovered from 1 cm^2 of infected leaves is shown

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 $MgCl_2$ and infiltrated into *N. benthamiana* leaves at $OD_{600} = 0.2$ for EtHAn and $OD_{600} = 0.02$ 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 $2^{-\Delta\Delta Ct}$ [$\Delta C_t = C_{t,\ \text{gene of interest}} - C_{t,\ \text{Actin2}}$; $\Delta\Delta C_t = \Delta C_{t,\ \text{infiltrated}} - \Delta C_{t,\ \text{noninfiltrated}}$].

To determine bacterial growth, a piece of leaf disc (30 mm^2) in the infiltration area was ground in 500 μl of 10 mM $MgCl_2$ 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.

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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.

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