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Sequence divergences between cyst nematode effector protein orthologs may contribute to host specificity

Tarek Hewezi and Thomas J. Baum*
Department of Plant Pathology; Iowa State University; Ames, IA USA

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*Correspondence to: Thomas J. Baum; Email: tbaum@iastate.edu

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The soybean cyst nematode (*Heterodera glycines*) and the closely related sugar beet cyst nematode (*Heterodera schachtii*) are devastating pathogens of plant roots that use secreted effector proteins to engage in sophisticated host-parasite interactions. While *H. schachtii* infects and reproduces readily on the roots of *Arabidopsis thaliana*, *H. glycines* rarely is able to infect this model plant. The molecular basis for differing host ranges remains obscure but likely involves differences between nematode effector proteins and the recognition of host factors. Recently we reported that constitutive expression of the *H. schachtii 10A06* effector protein gene (*Hs-10A06*) in Arabidopsis affected plant morphology and increased susceptibility to *H. schachtii* and that the 10A06 protein functions through its interaction with Arabidopsis spermidine synthase 2 (SPDS2). Therefore, we investigated whether differences between cyst nematode effector protein orthologs in two nematode species have a role in mediating host specificity. Here, we show that, similar to *Hs-10A06*, ectopic expression of *H. glycines 10A06* (*Hg-10A06*) in Arabidopsis affected leaf number and root length, however, to a much lesser extent. More importantly, no effect of *Hg-10A06* overexpression on Arabidopsis susceptibility to *H. schachtii* was observed. While we found that Hg-10A06 can weakly interact with Arabidopsis SPDS2 in yeast-two hybrid assays, this ability to interact with SPDS2 was decreased approximately five-fold compared with Hs-10A06. Collectively, these data suggest that sequence divergence between cyst nematode effector protein orthologs could contribute in determining cyst nematode host range.

Cyst nematodes are sedentary pathogens of roots of many economically important crop plants and induce the formation of specialized feeding cells, so-called syncytia, that provide the nematodes with nourishment. The infection process is mediated through secretion of an array of nematode effector proteins inside plant tissues and cells. One of these effector proteins is 10A06, which was initially identified from a gland cell cDNA library from *H. glycines*, the soybean cyst nematode.[1] The 927 bp full-length *H. glycines Hg-10A06* cDNA (GenBank Accession AF502391) encoded a predicted protein of 308 amino acids with an N-terminal signal peptide of 17 amino acids for secretion. Recently, we identified the orthologous 10A06 sequence from the sugar beet cyst nematode *H. schachtii* (*Hs-10A06*), which is able to infect the model plant *Arabidopsis thaliana*. The *Hs-10A06* cDNA (GenBank Accession GQ373256) contained an open reading frame of 858 bp encoding a 285-amino acid protein with an N-terminal signal peptide for secretion.[2] Sequence alignment of *H. glycines* and *H. schachtii* 10A06 proteins revealed a strong homology between both orthologues with 86% identity and 87% similarity. The largest difference between the two proteins is the lack of a stretch of 23 amino acids in Hs-10A06. Additionally, a region of 15 amino acid residues located between amino acid 167 and 181 exhibited a high degree of divergence between both proteins. Constitutive expression of

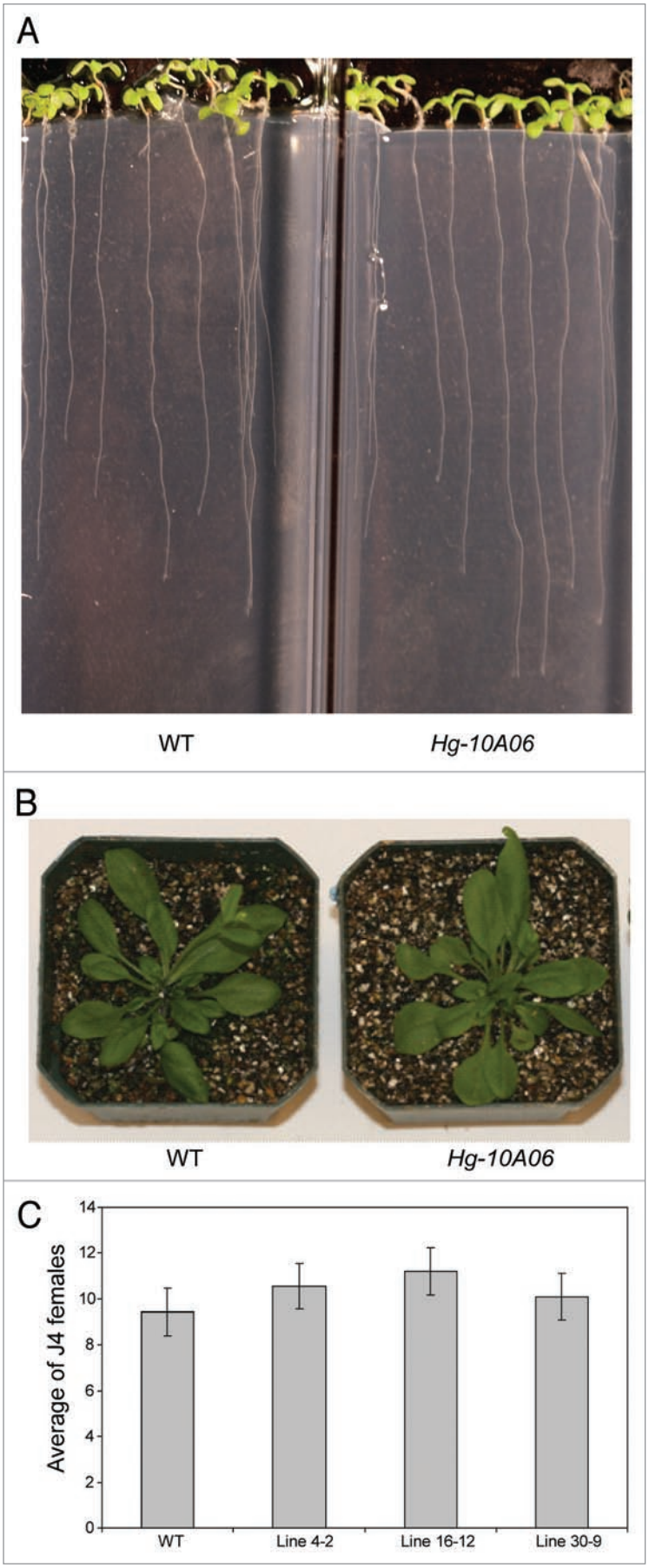

Figure 1. Characterization of *H. glycines 10A06*. Ectopic expression of *Hg-10A06* in Arabidopsis produced plants with significantly increased root lengths (A) and higher numbers of leaves than wild-type plants (B). Transgenic plants expressing *Hg-10A06* exhibited no significant changes in *H. schachtii* susceptibility (C). Homozygous T3 lines expressing *Hg-10A06* (lines 4-2, 16-12 and 30-9) were planted on modified Knop's medium, and 2-week-old seedlings were inoculated with approximately 250 surface-sterilized J2 *H. schachtii* nematodes. Three weeks after inoculation, the number of J4 female nematodes per root system was counted and used to quantify plant susceptibility. Data are presented as the mean ± SE.

Hs-10A06 in Arabidopsis affected plant morphology and increased susceptibility to *H. schachtii*.[2] We uncovered in yeast two-hybrid assays that the Hs-10A06 protein interacts with Arabidopsis SPDS2, a key enzyme involved in polyamine biosynthesis, to mediate susceptibility. Here, we assessed the effects of ectopic *Hg-10A06* expression in the non-host Arabidopsis on plant morphology and nematode susceptibility. Moreover, we assayed whether Hg-10A06 also is able to interact with SPDS2 from Arabidopsis.

Ectopic Expression of *Hg-10A06* in Arabidopis Alters Plant Morphology but not Susceptibility to *H. schachtii*

To investigate whether *Hg-10A06* has biological activity in Arabidopsis and can modulate susceptibility to *H. schachtii*, we used the CaMV 35S promoter to express *Hg-10A06* in Arabidopsis. Twenty four independent transgenic T1 lines were generated and three homozygous T3 lines (lines 4-2, 16-12 and 30-9) were selected for phenotypic analyses. Similar to *Hs-10A06*, ectopic expression of *Hg-10A06* in Arabidopsis affected root length and leaf number. A slight increase in root length of about 10% relative to the non-transgenic control was detected at 10 days after planting (**Fig. 1A**). Similarly, the total leaf number in the transgenic lines was increased and ranged between 22.55 ± 1.37 and 26.0 ± 1.39, whereas in wild-type plants the average leaf number was 16.6 ± 0.58 at the onset of flowering (**Fig. 1B**). As a comparison, expression

of *Hs-10A06* in Arabidopsis had much more substantial effects on these parameters.[2] These transgenic lines were used in a nematode susceptibility assay (**Fig. 1C**). Surprisingly, no statistically significant effects of transgene expression on nematode susceptibility were observed. These results show that the sequence differences between *H. schachtii* and *H. glycines* 10A06 are sufficiently large to alter biological activity in Arabidopsis. Taken one step further, these results suggest that effector protein divergence may be one mechanism of host range determination.

Hg-10A06 Interacts Specifically with Arabidopsis SPDS2 in Yeast Two-Hybrid Assay

To determine whether 10A06 from *H. glycines* also interacts with Arabidopsis SPDS2, the full-length *Hg-10A06* (minus the signal peptide coding sequence) was inserted into the bait vector and transformed into yeast cells along with the prey vector containing Arabidopsis *SPDS2*. The strength of binding between Hg-10A06 and SPDS2 was estimated by measuring *o*-nitrophenyl-α-D-galactoside concentrations as a function of the α-galactosidase activity produced by 10 yeast colonies. These α-galactosidase quantitative assays revealed that Hg-10A06 interacts with SPDS2 but its ability to interact with SPDS2 decreased by about five-fold when compared with Hs-10A06.

Conclusion and Perspective

Hg-10A06 binds Arabidopsis SPDS2, albeit weaker than the Hs-10A06 orthologue, which most likely results in similar to but weaker than the alterations of polyamine signaling found in *Hs-10A06* overexpressing Arabidopsis lines.[2] Consequently, the observed morphological changes in root length and leaf numbers that are less pronounced than observable in *Hs-10A06* lines comes as no surprise. However, unlike *Hs-10A06,* constitutive expression of *Hg-10A06* in Arabidopsis produced no significant differences in nematode susceptibility between the transgenic lines and wild-type plants despite the high sequence identity between both orthologs (86%). In other words, the *H. glycines* 10A06 protein does not have the same ability as Hs-10A06 to facilitate a compatible interaction between *H. schachtii* and Arabidopsis. I.e., Hg-10A06 does not work as a functioning effector in the non-host Arabidopsis. The basis for host range determination among Heterodera species is unknown, but our data now suggest a likely involvement of sequence differences between orthologous effector proteins. In *Pseudomonas syringae* pv. *phaseolicola*, DNA sequencing of *avrPphE* alleles has demonstrated that amino acid substitutions or an insertion of 104 bp are responsible for the difference between virulence and avirulence and lead to a gain in bean cultivar-specific virulence.[3] More recently, it has been reported that amino acid variation between flax rust fungus AvrL567 proteins alters host recognition.[4]

The finding that Hg-10A06 can only weakly interact with Arabidopsis SPDS2 suggests that this reduced protein-protein interaction may be responsible for a lack of altered susceptibility in the Hg-10A06 plants. Careful examination of the *10A06* sequence that shows strong variability between the two nematode species revealed that the Hs-10A06 cysteine residues in position 193 and 196 are replaced by valine in Hg-10A06. This change may prevent the formation of disulphide bonds in the Hg-10A06 protein, which may affect a protein structure necessary to bind to Arabidopsis SPDS2. In the fungal pathogen *Cladosporium fulvum*, sequence analysis of *avr4* alleles demonstrated single base changes leading to the loss of cysteine residues[5] and subsequent failure to act as an effective elicitor of the plant's resistance response.[6] Furthermore, as mentioned above, there is a 23-amino acid stretch missing in the *H. schachtii* 10A06 orthologue and in this region, Hg-10A06 is predicted to have two SV40-like nuclear localization signals (^{94}PVPKGKK^{100} and ^{96}PKGKKVE^{102}).[7] Reporter gene fusions of *Hg-10A06* sequences containing the predicted NLSs showed strong reporter gene activity in the nucleus with some minor accumulation in the cytoplasm. This suggests that Hg-10A06 may act as both a cytoplasmic and nuclear effector in soybean roots whereas Hs-10A06 may function only in the Arabidopsis cytoplasm. More research is needed to fully understand the different effector mechanisms in the two pathosystems.

H. glycines and *H. schachtii* are two closely related cyst nematode species that show very similar infection behavior on Arabidopsis roots up to the formation of feeding sites.[8] While *H. schachtii* infects and reproduces efficiently on Arabidopsis roots, *H. glycines* does so only very rarely. Our findings that Hg-10A06 only slightly affects root length and leaf numbers and not nematode susceptibility when expressed in Arabidopsis along with the observed weak Hg-10A06/SPDS2 interaction could partially explain the limited ability of *H. glycines* to parasitize Arabidopsis.

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