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ORIGINAL ARTICLE

# A novel sugar beet cyst nematode effector 2D01 targets the *Arabidopsis* HAESA receptor-like kinase

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## Abstract

Plant-parasitic cyst nematodes use a stylet to deliver effector proteins produced in oesophageal gland cells into root cells to cause disease in plants. These effectors are deployed to modulate plant defence responses and developmental programmes for the formation of a specialized feeding site called a syncytium. The *Hg2D01* effector gene, coding for a novel 185-amino-acid secreted protein, was previously shown to be up-regulated in the dorsal gland of parasitic juveniles of the soybean cyst nematode *Heterodera glycines*, but its function has remained unknown. Genome analyses revealed that *Hg2D01* belongs to a highly diversified effector gene family in the genomes of *H. glycines* and the sugar beet cyst nematode *Heterodera schachtii*. For functional studies using the model *Arabidopsis thaliana–H. schachtii* pathosystem, we cloned the orthologous *Hs2D01* sequence from *H. schachtii*. We demonstrate that Hs2D01 is a cytoplasmic effector that interacts with the intracellular kinase domain of HAESA (HAE), a cell surface-associated leucine-rich repeat (LRR) receptor-like kinase (RLK) involved in signalling the activation of cell wall-remodelling enzymes important for cell separation during abscission and lateral root emergence. Furthermore, we show that *AtHAE* is expressed in the syncytium and, therefore, could serve as a viable host target for Hs2D01. Infective juveniles effectively penetrated the roots of *HAE* and *HAESA-LIKE2* (*HSL2*) double mutant plants; however, fewer nematodes developed on the roots, consistent with a role for this receptor family in nematode infection. Taken together, our results suggest that the Hs2D01–AtHAE interaction may play an important role in sugar beet cyst nematode parasitism.

**KEYWORDS**
cyst nematode, dorsal gland, effector, HAESA, *Heterodera*, syncytium

## 1 | INTRODUCTION

Cyst nematodes are sedentary endoparasites that maintain a close biotrophic relationship with their hosts. Second-stage juveniles (J2) hatch from eggs within the soil in response to host root exudates. Following root penetration, the infective juveniles migrate intracellularly through the cortex and select a cell near the plant vasculature for the establishment of a metabolically active feeding site called a syncytium (Sobczak & Golinowski, 2011). The nematode uses a stylet to inject effector proteins originating from two subventral and a single dorsal gland cell into root tissues to aid the infection process (Vieira & Gleason, 2019). The processes of penetration, migration and syncytium formation require the activity of cell wall-modifying proteins and enzymes originating in both the plant and the nematode (Goellner et al., 2001). Many stylet-secreted effector proteins have been shown to function in cell wall weakening to facilitate J2 penetration and migration intracellularly through the cortex (Golinowski et al., 1996; Wang et al., 1999). Following the selection of an initial syncytial cell, the J2 stops moving and enters the preparation phase with its stylet inserted through the cell wall of the selected host cell. During this sedentary phase, a suite of effector proteins is released to trigger host cell physiological and morphological changes involved in syncytium formation (Mitchum et al., 2013).

Both destructive and constructive modifications of the plant cell wall are necessary for syncytium formation. Cell walls undergo dissolution followed by digestion of the middle lamella between cells to allow for the fusion of the plasma membranes of adjacent protoplasts and subsequent fusion of several hundred root cells (Ohtsu et al., 2017; Sobczak & Golinowski, 2011). Outer cell walls are extended and thickened to withstand the increased turgor pressure inside the syncytium (Böckenhoff & Grundler, 1994). Other modifications of the syncytium cell wall include the production of cell wall ingrowths at the interface between xylem vessels and the outer syncytial cell wall to increase the plasma membrane surface area for solute uptake (Golinowski et al., 1996; Jones & Northcote, 1972). Although the exact molecular mechanisms remain unclear, there is evidence supporting a role for both direct and indirect modulation of the expression and activity of plant endo-1,4-β-glucanases, cellulases, pectate lyases, pectin methylesterases and expansins among others by the nematode in a tightly controlled process for cell wall modifications during syncytium formation (Goellner et al., 2001; Hewezi et al., 2008; Szakasits et al., 2009; Wieczorek et al., 2006, 2008, 2014).

In addition to cell wall remodelling, stylet-secreted effector proteins perturb intracellular signalling networks of the host cell to help counteract plant defence responses and alter host proteins that modulate developmental and metabolic pathways required for syncytium formation. Among the handful of cyst nematode effectors that have been characterized, several have been linked to the suppression of host defences (Diaz-Granados et al., 2016; Hewezi et al., 2010; Hu et al., 2019; Kud et al., 2019; Pogorelko et al., 2020; Wang et al., 2020). However, not all effectors directly undermine plant immunity. Several effectors are known to target key developmental programmes (Guo et al., 2017; Hewezi et al., 2015; Lee et al., 2011; Wang et al., 2021) or perturb transcriptional regulation (Barnes et al., 2018), mRNA splicing (Verma et al., 2018), and epigenetic modification of histones (Hewezi, 2020; Vijayapalani et al., 2018) to promote activities that benefit the nematode. Despite the significant progress in our understanding of cyst nematode effector protein function, the vast majority of stylet-secreted effectors discovered to date are novel proteins that lack sequence similarity or conserved sequence motifs with other known proteins and their functions are yet to be discovered.

Here, we conducted a series of experiments to further characterize the potential function of the novel 2D01 effector protein, first identified as a member of the soybean cyst nematode (SCN) *Heterodera glycines* parasitome (Gao et al., 2003). We show that 2D01 is a member of a highly diversified family of novel effectors present in the genomes of *H. glycines* and the closely related sugar beet cyst nematode (BCN) *Heterodera schachtii*. *Hs2D01* was cloned from *H. schachtii* for functional studies using the model plant *Arabidopsis thaliana*. Similar to Hg2D01 (Gao et al., 2003), Hs2D01 harbours a predicted N-terminal secretion signal and is highly upregulated in the dorsal gland of the nematode during parasitic life stages. We demonstrate that Hs2D01 interacts with the intracellular kinase domain of HAESA (HAE), an *Arabidopsis* leucine-rich repeat receptor-like kinase (LRR-RLK) known to play important roles in regulating floral abscission and lateral root emergence (LRE). We demonstrate that *HAE* is expressed in the developing syncytium and that *Arabidopsis* plants lacking functional members of this RLK family are less susceptible to BCN infection, implicating a role for the HAE signalling pathway in cyst nematode parasitism.

## 2 | RESULTS

### 2.1 | 2D01 belongs to a highly diversified family of novel nematode effectors

An early view on the SCN parasitome published by Gao et al. (2003) reported 53 candidate effector gene sequences identified from gland-enriched cDNA libraries. Six of the cDNA clones from the gland-enriched library shared significant sequence similarity, namely *Hg16B09*, *Hg2D01*, *Hg22C12*, *Hg30E03*, *Hg11A06* and *Hg24A12* (Figure 1a). In a subsequent report, an additional 18 candidate effector gene sequences named *HgGLAND1–GLAND18* (Noon et al., 2015) were identified. One of these, *HgGLAND5*, shared a high level of sequence similarity with these six clones. All of these effector proteins contained a predicted N-terminal secretion signal peptide (SP) of 25 amino acids, lacked a transmembrane domain, and shared conserved protein domains (Figure 1a). Figure 1b shows a phylogenetic tree including these sequences. Hg16B09, Hg22C12 and Hg30E03 share >93% amino acid (aa) and >95% nucleotide (nt) similarity with each other and clustered into one group, whereas Hg2D01, Hg11A06 and Hg24A12 share >89% aa and >92% nt similarity and clustered into a second group. HgGLAND5 is the most divergent member but shares

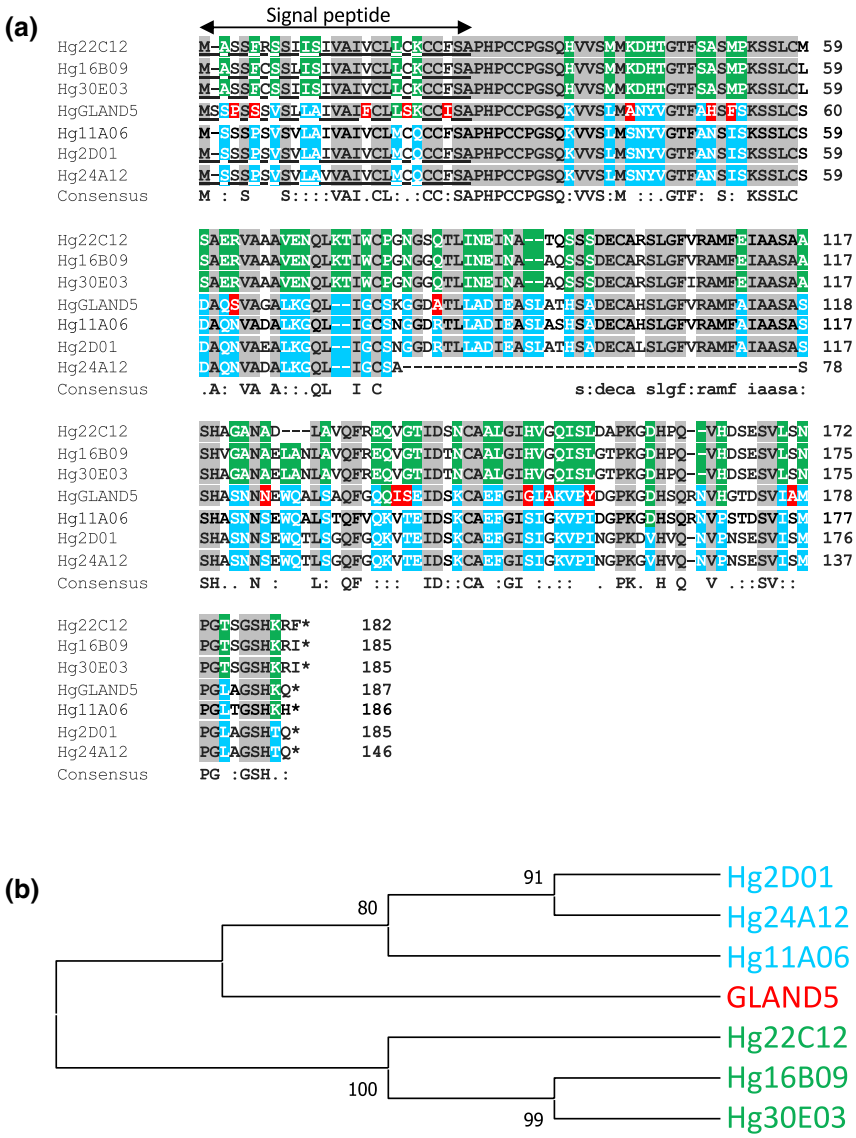
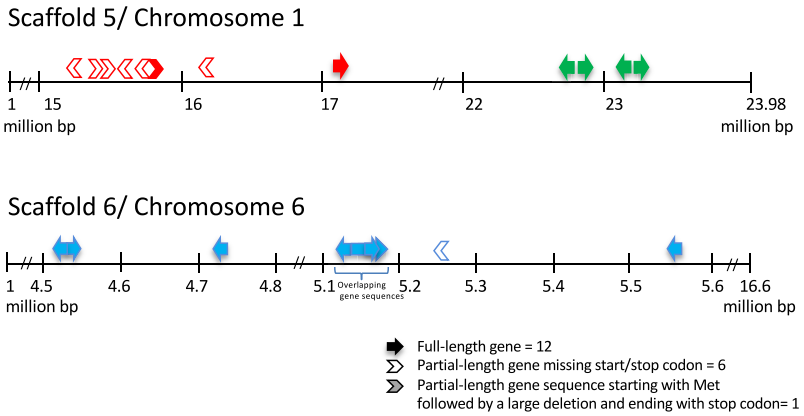



**FIGURE 1** Sequences representing members of a novel, highly diversified family of effector proteins found in the soybean cyst nematode *Heterodera glycines* (Hg) parasitome. (a) A multiple amino acid sequence alignment of members of this effector family generated using Clustal Omega shows conserved protein domains. Amino acids with 100% consensus are indicated by bold capitalized letters and highlighted in grey. Amino acids unique to a subgroup are highlighted in blue (Hg2D01, Hg24A12, Hg11A06), green (Hg16B09, Hg22C12, Hg30E03), and red (HgGLAND5). The black line with double arrow spanning the underlined amino acid sequences indicates the predicted signal peptides of the proteins using SignalP 5.0 software. (b) Protein sequences were aligned with Clustal Omega. Phylogenetic tree construction by the neighbour-joining method was performed using MEGA 11 software, with bootstrap values being calculated from 100 replicates

a higher percentage sequence similarity with the Hg2D01, Hg11A06 and Hg24A12 (>76%–83% aa and 87%–89% nt similarity) cluster compared with the Hg16B09, Hg22C12 and Hg30E03 (54%–55% aa and 61%–63% nt similarity) cluster.

A search of the nine-scaffold, 158-Mb pseudomolecule assembly of the *H. glycines* genome identified 20 full-length or partial copies (Table S1; Figure S1) of this effector gene family (Masonbrink et al., 2021). Twelve members of this family were present on scaffold 5/chromosome 1 and eight were present on scaffold 6/chromosome 6 (Figure 2a). A phylogenetic analysis including the aforementioned SCN parasitome sequences is shown in Figure 2b. The four sequences with similarity to Hg16B09 were all full-length and clustered together on one end of chromosome 1. Two of the four family members (Hetgly08775 and Hetgly08776) shared 100% identity and were arranged within the genome as inverted repeats or back-to-back repeats. Only one out of the eight sequences sharing similarity to HgGLAND5 was full-length and the sequences clustered on the end of chromosome 1 opposite from Hg16B09 sequences. The eight sequences similar to SCN parasitome members Hg2D01, Hg11A06 and Hg24A12 were clustered together on chromosome 6 and included seven full-length and one partial gene sequence. The sequence variation between the original SCN parasitome sequences and those identified in the SCN genome may reflect the natural variation across SCN populations consistent with the highly expanded and diversified nature of this effector protein family. The early SCN parasitome effector gene sequences were derived from the SCN OP50 inbred population whereas the SCN genome was derived from the SCN TN10 inbred population. These populations differ in virulence on resistant soybean (Niblack et al., 2008).

We also observed a high level of diversification of this effector family in *H. schachtii*. A search of the least fragmented version of the *H. schachtii* genome consisting of 395 contigs (accession number JAHGVF010000000; Siddique et al., 2021) identified 51 sequences spanning 16 contigs with >50% query coverage and >60% identity with Hg2D01, Hg16B09 or HgGLAND5. Of these, 18 were full-length and 33 were partial sequences. A phylogenetic



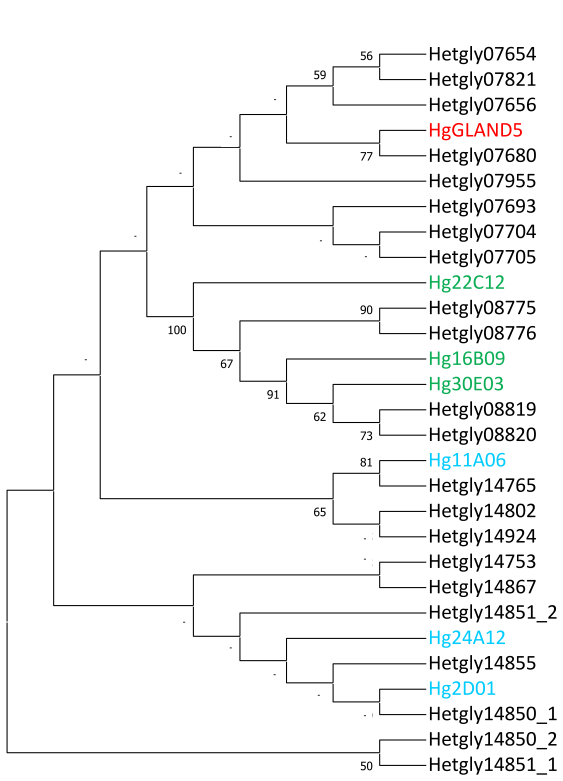




**FIGURE 2** Sequences representing members of a novel, highly diversified family of effector proteins found in the soybean cyst nematode *Heterodera glycines* (Hg or Hetgly) genome. (a) Genome organization of this effector gene family in the pseudomolecule genome assembly (Masonbrink et al., 2019). Twenty full-length and partial-length copies were physically clustered into three distinct subgroups on two chromosomes/scaffolds. (b) The protein sequences were aligned using the online tool Clustal Omega or MEGA 11 software and a phylogenetic neighbour-joining tree was generated using MEGA 11 (Tamura et al., 2021) with bootstrap values being calculated from 100 replicates. Bootstrap values of <50% are indicated by a dash

analysis including all identified SCN and BCN protein sequences belonging to this effector family is shown in Figure 3 with strong support for the 16B09 clade. Three *H. schachtii* contigs harboured the seven full-length sequences with >90% identity to Hg16B09, none of which overlapped with contigs matching Hg2D01 or HgGLAND5.

## 2.2 | Hg2D01 and Hs2D01 sequences are highly conserved

The Hg2D01 effector protein family member, for which no information was available when this study was initiated, was selected for further functional characterization. An orthologous sequence was isolated from the closely related BCN *H. schachtii* to facilitate functional studies using the model host plant *A. thaliana*. The *Hs2D01* cDNA encoded a 185-amino-acid protein with a predicted 25-amino-acid N-terminal SP for secretion. Hs2D01 shared 92% aa identity with Hg2D01 (Figure 4a).

## 2.3 | *Hs2D01* is up-regulated in the dorsal gland cell

Stylet-secreted effector proteins are typically produced either exclusively in the two subventral gland cells or the single dorsal gland cell. In situ hybridization demonstrated that *Hg2D01* transcripts re specifically expressed in the dorsal gland cell during the parasitic second-stage juvenile (J2) and third-stage juvenile (J3) life stages (Gao et al., 2003; Figure 4b,c). Consistent with this,

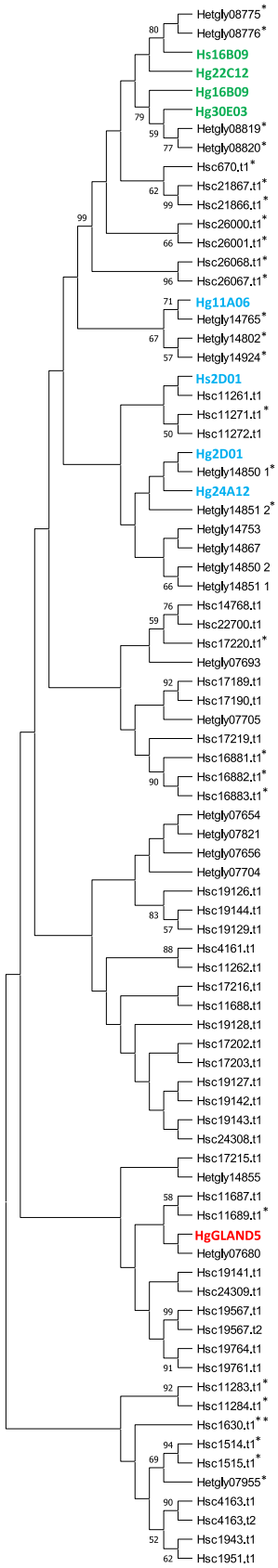



**FIGURE 3** Phylogenetic analysis of sequences representing members of a novel, highly diversified family of effector proteins found in the soybean cyst nematode *Heterodera glycines* (Hg or Hetgly) and the sugar beet cyst nematode *Heterodera schachtii* (Hsc). The protein sequences were aligned using the online tool Clustal Omega or MEGA 11 software and a phylogenetic neighbour-joining tree was generated using MEGA 11 (Tamura et al., 2021) with bootstrap values being calculated from 100 replicates. Bootstrap values of <50% are indicated by a dash

the developmental expression pattern of *Hg2D01* reported in a prior microarray study categorized the expression of this gene into cluster 2, which contained genes that were highly up-regulated in parasitic J2, peaked in J3, and remained stable into the adult female life stage (Elling et al., 2009). Similar to *Hg2D01*, in situ hybridization confirmed expression of *Hs2D01* in the dorsal gland of parasitic J2 and J3 life stages of *H. schachtii* (Figure 4d,e). No signal was detected in preparasitic J2 or specimens hybridized with a sense probe.

## 2.4 | *Hs16B09* is required for nematode parasitism

RNA interference (RNAi) by either soaking J2 in *Hg16B09*-double-stranded RNA (dsRNA) or host-induced gene silencing of *Hg16B09* in soybean hairy roots was previously used to demonstrate a role for *Hg16B09* in *H. glycines* parasitism (Hu et al., 2019). Here, we used a host-induced gene silencing approach to test for a role of *Hs2D01* in *H. schachtii* parasitism. For this, a full-length *Hs2D01* dsRNA hairpin construct was made (Figure 5a). We also included a full-length *Hs16B09* dsRNA hairpin construct for comparison. *Hs2D01* and *Hs16B09* share 66% nucleotide identity with only a single stretch of 25 nucleotides sharing 100% identity (Figure S2). RNAi constructs were transformed into *Arabidopsis* to generate multiple independent, single-insertion, homozygous lines for analysis. No off-target effects were observed in the aboveground or belowground growth and development of the *2D01i* and *16B09i* transgenic lines confirmed to be expressing dsRNA to the target genes (Figure 5b). The transgenic RNAi lines were tested in infection assays with *H. schachtii* to assess for any effects on parasitism. *H. schachtii* development was scored at 14 days postinoculation (dpi) by counting the number of J4 females. Consistent with prior studies on *Hg16B09* (Hu et al., 2019), all *Hs16B09* RNAi lines tested showed a significant reduction (30%–40%) in the number of J4 female nematodes developing on the roots at 14 dpi (Figure 5c). Reverse transcription-quantitative PCR (RT-qPCR) confirmed that the level of expression of *Hs16B09* in feeding nematodes at 4 dpi on the transgenic RNAi lines was reduced in comparison to nematodes feeding on wild-type Columbia-0 (Col-0) (Figure 5d), whereas the expression of the non-target effector gene *Hs19C07* remained unchanged (Figure 5e). In contrast, none of the *Hs2D01* RNAi lines tested showed a significant reduction in the number of J4 female nematodes developing on the roots at 14 dpi (Figure 5f) despite confirming the *Hs2D01* RNAi lines were expressing dsRNA (Figure 5b). In contrast to *Hs16B09*, only minimal silencing of *Hs2D01* was achieved in feeding nematodes at 4 dpi on the transgenic RNAi lines compared to nematodes feeding on Col-0 (Figure 5g). We also did not observe a significant reduction

(a)

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Hs2D01 MSSSPSVSVLAIVAIVLLVCQCCFSAPHPCCPGSQKVVSLMSNYVGTFAHSFSKSSLCSD 60
Hg2D01 MSSSPSVSVLAIVAIVCLMCQCCFSAPHPCCPGSQKVVSLMSNYVGTFANSISKSSLCSD 60
       *************** *:*******************************:*:********

Hs2D01 AQNVAGALKGQLIGCSNGGDRTLLAEIKASLATHSADECAHSLGFVRAMFAIAASASSHA 120
Hg2D01 AQNVAEALKGQLIGCSNGGDRTLLADIEASLATHSADECALSLGFVRAMFAIAASASSHA 120
       ***** ********************:*:************ ******************

Hs2D01 SNNSEWQTLSGQFGQKVTEIDLKCAEFGISIGKVPIDGSKGVHVQNVPNSESVIXMPGLA 180
Hg2D01 SNNSEWQTLSGQFGQKVTEIDSKCAEFGISIGKVPINGPKDVHVQNVPNSESVIXMPGLA 180
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Hs2D01 GSHTQ* 185
Hg2D01 GSHTQ* 185
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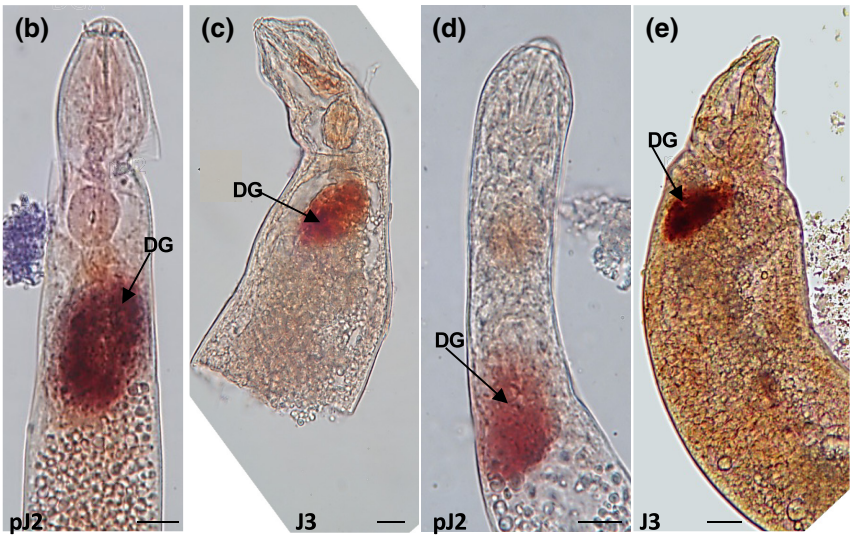


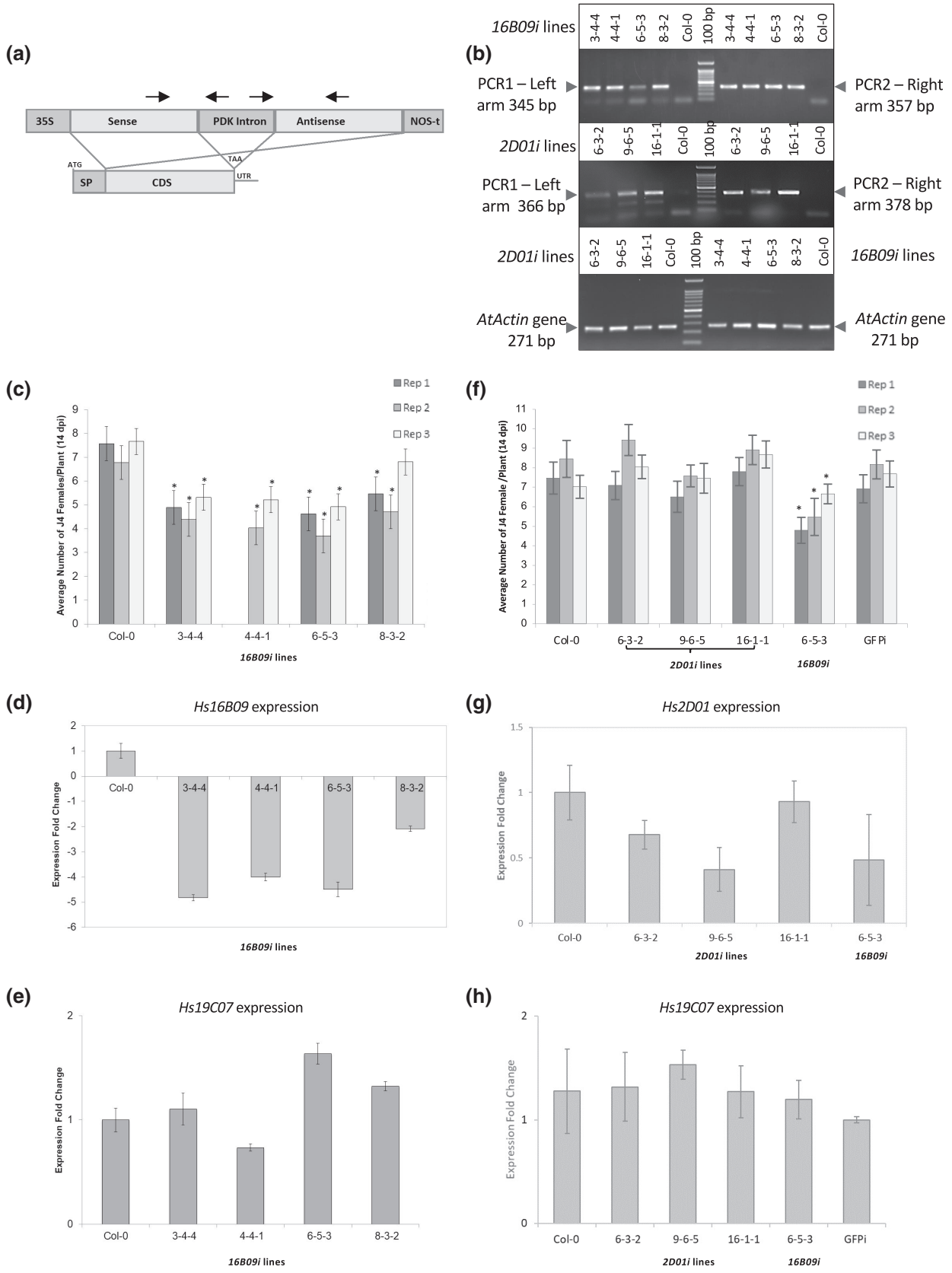

**FIGURE 4** Spatial expression pattern of *Hg2D01* and *Hs2D01*. (a) An amino acid sequence alignment of 2D01 from *Heterodera glycines* and *Heterodera schachtii* is shown. 2D01 is a novel 185-amino-acid protein with a predicted N-terminal 25-amino-acid secretion signal (highlighted in grey). In situ hybridization of digoxigenin-labelled antisense single-stranded DNA probes showed *2D01* expression exclusively in the dorsal oesophageal gland cell (DG of a parasitic second-stage juvenile (pJ2) and third-stage juvenile (J3) of *H. glycines* (b, c) and *H. schachtii* (d, e). Scale bars = 20 μm

in *Hs2D01* expression in the *Hs16B09* RNAi line tested, which reflects the high level of specificity of *Hs16B09i*. The expression of the nontarget effector gene *Hs19C07* also remained unchanged in the *Hs2D01i* lines (Figure 5h). Based on our genome analysis, the high copy number and complexity of the *2D01* gene family may have contributed to our inability to achieve a level of silencing sufficient to observe a robust infection phenotype.

## 2.5 | Hs2D01 is a cytoplasmic effector that interacts with the intracellular kinase domain of HAESA

To identify potential host targets of Hs2D01, a yeast two-hybrid approach was employed. Hs2D01ΔSP-pGBKT7 was used to screen approximately 32.2 million yeast colonies from an *H.*

**FIGURE 5** Effect of host-induced gene silencing of *Hs16B09* and *Hs2D01* on infection of *Arabidopsis* roots by the sugar beet cyst nematode *Heterodera schachtii*. (a) Schematic of the *Hs16B09* and *Hs2D01* RNA interference (RNAi) constructs. Arrows correspond to primer pairs in the coding sequence (CDS) and pyruvate dehydrogenase kinase (*PDK*) intron used for evaluating expression of dsRNA. (b) Reverse transcription (RT)-PCR of the CDS and *PDK* introns of the hairpin dsRNA to confirm dsRNA expression in roots of *Arabidopsis Hs16B09* and *Hs2D01* RNAi lines. (c) Infection assay of *Hs16B09* RNAi lines showed a statistically significant decrease in the number of J4 female nematodes compared to the control line (Col-0) at 14 days postinoculation (dpi). Data are presented as means ± *SE*. Mean values significantly different from the wild type are denoted by asterisks, as determined by unadjusted paired *t* tests ($p < 0.01$). Data from three biological replicates are shown. (d) RT-quantitative PCR (RT-qPCR) shows decreased *Hs16B09* expression in feeding nematodes on *16B09* RNAi lines at 4 dpi compared to those on wild-type plants. (e) RT-qPCR shows a similar level of expression of the nontarget gene *Hs19C07* in feeding nematodes on *16B09* RNAi lines at 4 dpi compared to those on wild-type plants. Data presented are from one biological replicate. (f) No significant difference in the number of J4 female nematodes compared to the control lines (Col-0 and GFPi) at 14 dpi on *Arabidopsis Hs2D01* RNAi lines was observed. The *Hs16B09i* line 6-5-3 was used for comparison. Data are presented as means ± *SE*. Mean values significantly different from the wild-type Col-0 are denoted by asterisks as determined by unadjusted paired *t* tests ($p < 0.01$). Data from three biological replicates are shown. (g) No significant reduction in *Hs2D01* expression was observed in feeding nematodes on *Hs2D01* RNAi lines at 4 dpi compared to those on wild-type plants. (h) RT-qPCR shows a similar level of expression of the nontarget gene *Hs19C07* in feeding nematodes on *2D01* RNAi lines at 4 dpi compared to those on wild-type plants. Data presented are from one biological replicate




*schachtii* nematode-infected *Arabidopsis* root (3 dpi) library (Hewezi et al., 2008). This screen had a mating efficiency of 5.675%. After restreaking, 82 clones selected on quadruple dropout synthetic defined (SD) medium (QDO) were 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal)-positive and one clone (S1-5) survived co-transformation testing. S1-5 did not autoactivate and was

specific in its interaction with Hs2D01 as it did not interact with Lamin C. We further tested the specificity of the interaction between the Hs2D01 effector and S1-5 by testing S1-5 for interaction with two additional unrelated *H. schachtii* effector proteins, Hs13A06ΔSP and Hs34B08ΔSP, orthologues of *H. glycines* effectors described previously by Gao et al. (2003). Neither of these effectors showed an interaction with S1-5 (Figure 6a). Sequencing of this prey clone revealed that S1-5 contained an in-frame portion of the kinase domain (spanning amino acids 783–999) of HAESA (At4g28490), an LRR-RLK (Figure 6b). The interaction was confirmed positive by reciprocal cloning of S1-5 into pGBKT7 and Hs2D01ΔSP into pGADT7 (data not shown). To examine this possible interaction further, several longer versions of the HAESA kinase domain were cloned into the prey vector pGADT7 and co-transformed with Hs2D01ΔSP-pGBKT7 in AH109 yeast cells to test for interaction. The regions of the kinase domain tested were (1) HAE-KD (aa 649–999), including the juxtamembrane region and glycine-rich loop; (2) HAE-KD (aa 689–999) without the juxtamembrane region, but including the glycine-rich loop; and (3) HAE-KD (aa 697–999), without the juxtamembrane region and glycine-rich loop. Blue colonies were scored on double dropout SD medium (DDO) + X-α-Gal and growth was measured on triple dropout SD medium (TDO) and QDO 3 dpi in a dilution series (Figure S3). HAE-KD (aa 697–999) confirmed the interaction with Hs2D01ΔSP; however, inclusion of either the glycine-rich loop or the juxtamembrane domain abolished this interaction, possibly due to membrane targeting, protein misfolding, or stability of the intracellular kinase domain in yeast when including sequences adjacent to the transmembrane domain.

Analysis of the Hs2D01 protein sequence with PSORT II revealed that this effector does not contain a putative nuclear localization signal or any other organelle-targeting motifs. To determine the subcellular localization of Hs2D01, Hs2D01ΔSP was fused with GUS:GFP and transiently expressed by agro-infiltration in *Nicotiana benthamiana* leaf epidermal cells. Confocal microscopy showed that Hs2D01ΔSP-GUS:GFP was mainly localized to the cytoplasm of epidermal cells with a weak signal observed overlapping with the coinfiltrated plasma membrane marker PEP116E (Park et al., 2017) (Figure 7a). HAE has previously been shown to be associated with the plasma membrane or closely associated membrane structures (Alexandersson et al., 2004; Jinn et al., 2000; Leslie et al., 2010). Burr et al. (2011) determined the localization profile of the HAE full-length protein-GFP fusion (HAE-FL-GFP) in transfected *Arabidopsis* leaf protoplasts and observed HAE at the plasma membrane and internally. Here, we also confirmed plasma membrane localization of HAE-FL-GFP by agro-infiltration in *N. benthamiana* leaves. We observed strong overlap of the HAE-FL-GFP signal with the coinfiltrated plasma membrane marker PEP116E (Figure 7b). Hs2D01 and HAE protein production was confirmed by western blot analysis (Figure S4a).

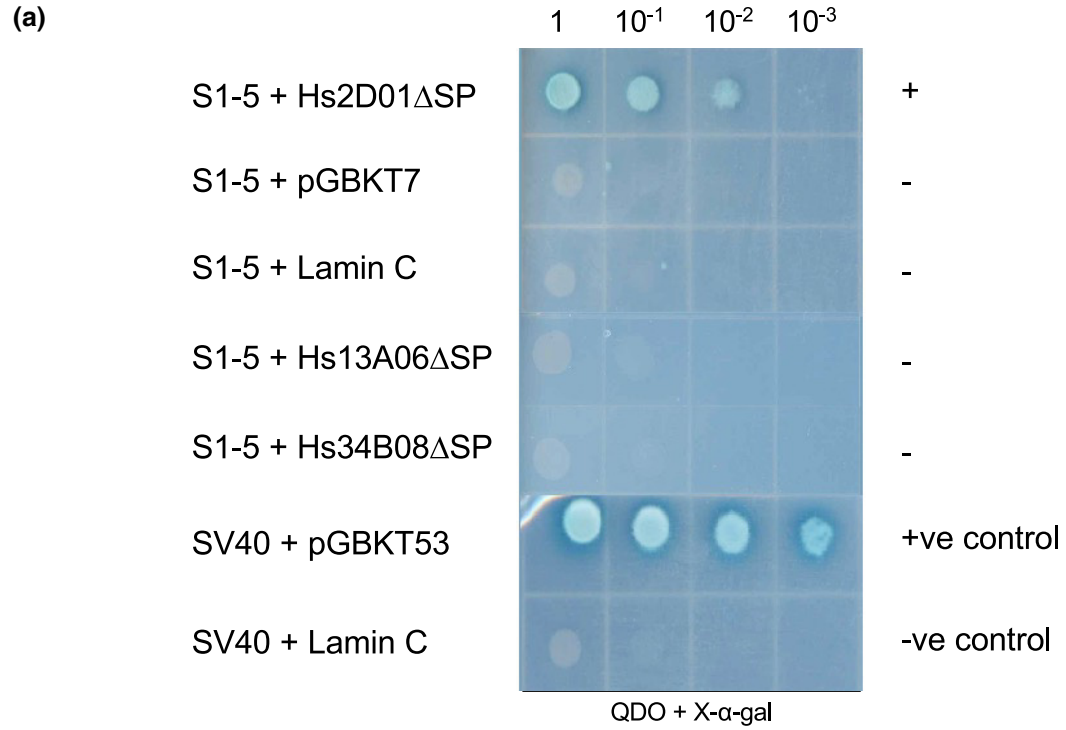
To verify the HAE–2D01 interaction in planta, bimolecular fluorescence complementation assays were conducted using the Hs2D01ΔSP effector protein and the full-length HAE protein sequence. Coexpression of HAE-FL-YFP$^N$ and Venus$^C$-Hs2D01ΔSP (Figure 7c) reconstituted a fluorescent protein signal in the plasma membrane and cytoplasm of the agro-infiltrated *N. benthamiana* epidermal cells. Hs16B09 and *Arabidopsis* HAE was used as an interaction control to test for specificity of the HAE–Hs2D01 interaction. No interaction was observed between HAE-FL-YFP$^N$ and Venus$^C$-Hs16B09ΔSP (Figure 7d). Protein production was confirmed by western blot analysis (Figure S4b). Empty vectors used as negative controls did not show fluorescence (Figure 7e). Together, these results provided additional evidence for a specific interaction between HAE and Hs2D01 in plant cells.

## 2.6 | *HAESA* expression is up-regulated at nematode feeding sites

Our finding that Hs2D01 is a cytoplasmic effector that can interact with the kinase domain of HAE led us to test whether *HAE* was expressed at nematode feeding sites where it could serve as a bona fide target of Hs2D01. Prior studies revealed *HAE* promoter activity at the base of the petioles and pedicels, abscission zones of the floral organs, and emerging lateral root primordia as assessed using a previously characterized transgenic *Arabidopsis HAE* promoter:β-glucuronidase (GUS) reporter line exhibiting an expression pattern consistent with *HAE* endogenous gene expression patterns (Jinn et al., 2000). Thus, to evaluate the spatial and temporal expression of *HAE* during cyst nematode infection, 10-day-old transgenic *HAEp:GUS* seedlings were inoculated with *H. schachtii* infective J2s and assayed for GUS activity at different time points following inoculation. *HAEp:GUS* seedlings exhibited specific induction of GUS activity during early syncytium development. Activity was detected in the initial syncytial cell and was then predominantly observed in adjacent cells at the borders and flanking regions of the developing syncytium during early J2 stages of parasitism (3–6 dpi; Figure 8a–e). At later stages of parasitism, *HAEp:GUS* expression was maintained, but expression was restricted to cells adjacent to the head during the J3–J4 (male) and J4 (female) nematode life stages and absent from the developed syncytium (11 dpi; Figure 8f–i). As previously reported, the uninfected roots of *HAEp:GUS* plants exhibited a low level of GUS activity in lateral root primordia (Figure 8j) with no detectable GUS activity observed in any other part of the root.

## 2.7 | *HAE* and *HSL2* are important for cyst nematode parasitism

To test HAE for a role in nematode parasitism, we conducted *H. schachtii* infection assays on *Arabidopsis* receptor mutant plants (Figure 9a). Due to the previously reported functional redundancy between HAE and HSL2 (Cho et al., 2008), we focused on *hae hsl2* double and *hae hsl1 hsl2* triple mutants. For this, we included two *hae hsl2* double mutant combinations and one *hae hsl1 hsl2* triple mutant. The *hsl2-1* mutant is a weak allele; thus, the *hae-1 hsl2-1* double mutant exhibits a weaker floral abscission phenotype due to



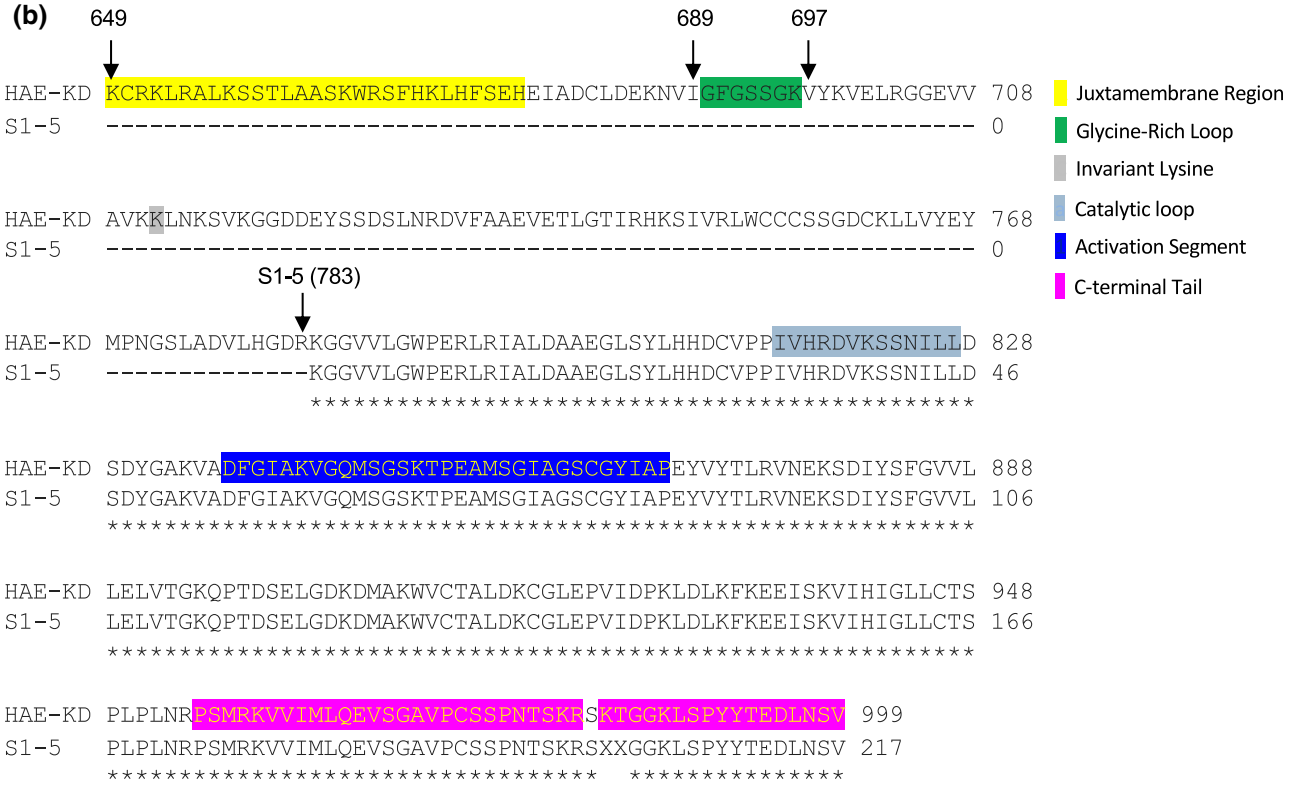




**FIGURE 6** Interaction of the *Heterodera schachtii* 2D01 effector protein with the kinase domain of the *Arabidopsis* HAESA receptor-like protein kinase in yeast. (a) Identification of a Hs2D01ΔSP-interacting clone by yeast two-hybrid screening. Clone S1-5 was isolated from the Hs2D01ΔSP bait screen of an *Arabidopsis* nematode-infected root prey library. S1-5 did not autoactivate and interacts specifically with Hs2D01ΔSP, but not Lamin C or two other unrelated dorsal gland effectors, Hs13A06 and Hs34B08, orthologues of *Heterodera glycines* effectors described previously by Gao et al. (2003). The positive control, SV40 + pGBKT53, and the negative control, SV40 + Lamin C, are also shown. (b) A schematic representation of the HAESA kinase domain showing the sequence and conserved structural elements of protein kinases, as well as widely conserved subdomains of protein kinases (adapted from Taylor et al., 2016). The region of the kinase domain contained in the S1-5 prey clone is shown. Arrows indicate the position of the kinase domain clones tested for interaction with Hs2D01ΔSP (Figure S3)

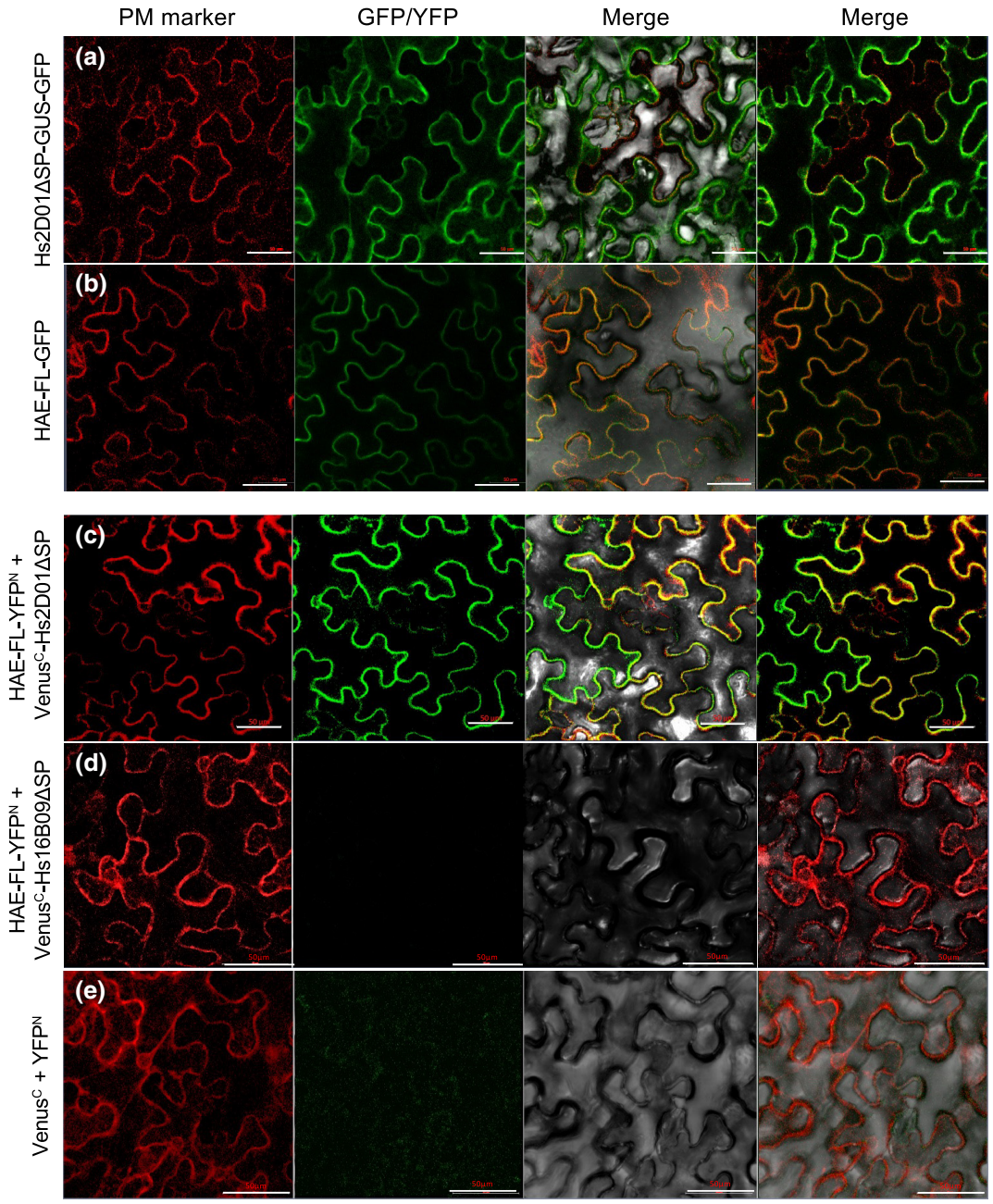



**FIGURE 7** Subcellular localization of Hs2D01ΔSP, AtHAE, and their interaction in planta. (a) Subcellular localization of Hs2D01ΔSP in *Nicotiana benthamiana* leaf epidermal cells. The Hs2D01ΔSP-GUS-GFP fusion construct was agro-infiltrated in *N. benthamiana* leaf cells and GFP was detected in the cytoplasm with a weak signal overlapping with the plasma membrane marker PEP116E (Park et al., 2017). (b) Subcellular localization of HAE-FL-GFP fusion protein in *N. benthamiana* mesophyll cells was localized to the plasma membrane overlapping with PEP116E. Fluorescence signals were observed by confocal microscopy using the following settings: GFP, 500–550 nm; mCherry, 600–650 nm. (c) In planta bimolecular immunofluorescence complementation of the interaction of Hs2D01ΔSP with AtHAE. Plasmids were agro-infiltrated in *N. benthamiana* leaves. pSPYNE-HAESA (HAE-FL-YFP$^{N}$) and KanII VYCE(R)-Hs2D01ΔSP (Venus$^{C}$-Hs2D01ΔSP) interacted in the plasma membrane and cytoplasm confirming interaction. (d, e) pSPYNE-HAESA (HAE-FL-YFP$^{N}$) and KanII VYCE(R)-Hs16B09ΔSP (Venus$^{C}$-Hs16B09ΔSP) demonstrated specificity of the Hs2D01ΔSP–AtHAE interaction; KanII VYCE(R) (Venus$^{C}$) and pSPYNE (YFP$^{N}$) were used as a negative control

the remaining HSL2 function in this mutant (Baer et al., 2016; Cho et al., 2008). For this reason, we also included the double loss-of-function *hae-3 hsl2-3* mutant, which is completely deficient in floral abscission and has delayed LRE (Baer et al., 2016). We also included the *ida* knockout mutant, which is floral abscission-deficient and has delayed LRE due to the loss of the IDA peptide ligand (Kumpf et al., 2013). No differences in root length in young seedlings or J2 penetration rate were observed for any of the mutants compared to wild-type Col-0 (Figure S5a,b). No cyst nematode infection phenotype was observed on the *ida* mutant, possibly due to expression

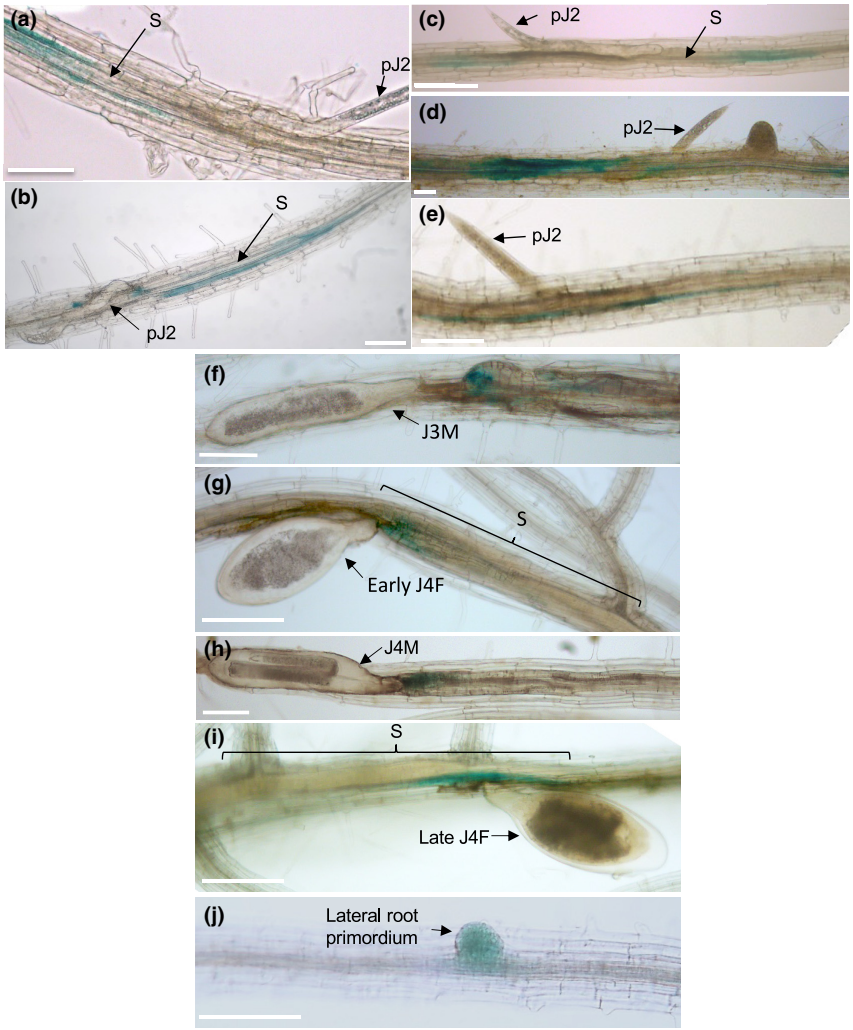



**FIGURE 8** Spatial and temporal analysis of *HAEp:GUS* expression in beet cyst nematode *Heterodera schachtii*-infected roots at 3–11 days postinoculation (dpi). Early (3 dpi, a–d) and late (6 dpi, e) parasitic second-stage juveniles (pJ2). (f–i) Male (M) and female (F) life stages (J3, J4) at 11 dpi. β-glucuronidase (GUS) activity was observed within the developing syncytium (S) during early stages of infection and then largely remained confined to the adjacent cells at the borders and flanking regions of the developing syncytium at later stages of infection. GUS activity was restricted to a region of cells adjacent to the head of the feeding nematode in fully expanded syncytia. (j) Uninfected root showing GUS activity in an emerging lateral root primordium. No GUS activity was visible in any other root tissues. Scale bars = 100 μm

of other IDA-like (*IDL*) gene family members sharing a common role with IDA (Stenvik et al., 2008) in response to nematode infection. However, a consistent and statistically significant ($p < 0.05$) reduction in the number of J4 females at 14 dpi was observed on the double loss-of-function *hae-3 hsl2-3* mutant compared with wild-type Col-0, providing evidence of an important role for these receptors in cyst nematode parasitism (Figure 9b).

## 3 | DISCUSSION

In this study, we carried out a series of experiments to further characterize the function of Hs2D01, a member of a highly diversified family of novel effector proteins found in cyst nematodes. The polymorphic nature of this family of effectors suggests they probably have co-evolved with their host plants to play important roles in parasitism. Although family members have some sequence similarity with each other and share conserved domains, there is sufficient divergence to suggest they may have unique roles in cyst nematode parasitism. Twenty copies of this family were clustered on two chromosomes in the *H. glycines* pseudomolecule (nine chromosomes) genome. The 20 sequences were present as segmental duplications and inverted repeats, which clustered into three subgroups. We observed a similar picture for this effector family in the current *H. schachtii* draft genome consistent with *H. schachtii* and *H. glycines* genomes sharing numerous orthologous regions, which are enriched in segmental duplications that probably share functional and structural similarity (Siddique et al., 2021). Interestingly, the genomes of the potato cyst nematodes *Globodera pallida* and *Globodera rostochiensis* have also been reported to harbour a highly diversified family of effectors sharing similarity to 16B09, 2D01 and GLAND5 (Cotton et al., 2014; van Steenbrugge et al., 2022), suggesting this family of effectors may play important roles across a wide range of cyst nematode species.

Clustering of genes offers an evolutionary advantage to an organism as it allows for tightly coordinated gene expression at the chromatin level and genetic linkage of functionally related genes, and ensures transcriptional regulation (Hurst et al., 2004; Wong & Wolfe, 2005; Yi et al., 2007). Inverted repeats may affect the stability of the genome due to their tendency to form secondary structures, such as palindromes and stem-loops, and they can also mediate gene amplification through homologous or illegitimate recombination causing changes in gene copy number (Wang & Leung, 2006). These amplifications can result in mutations due

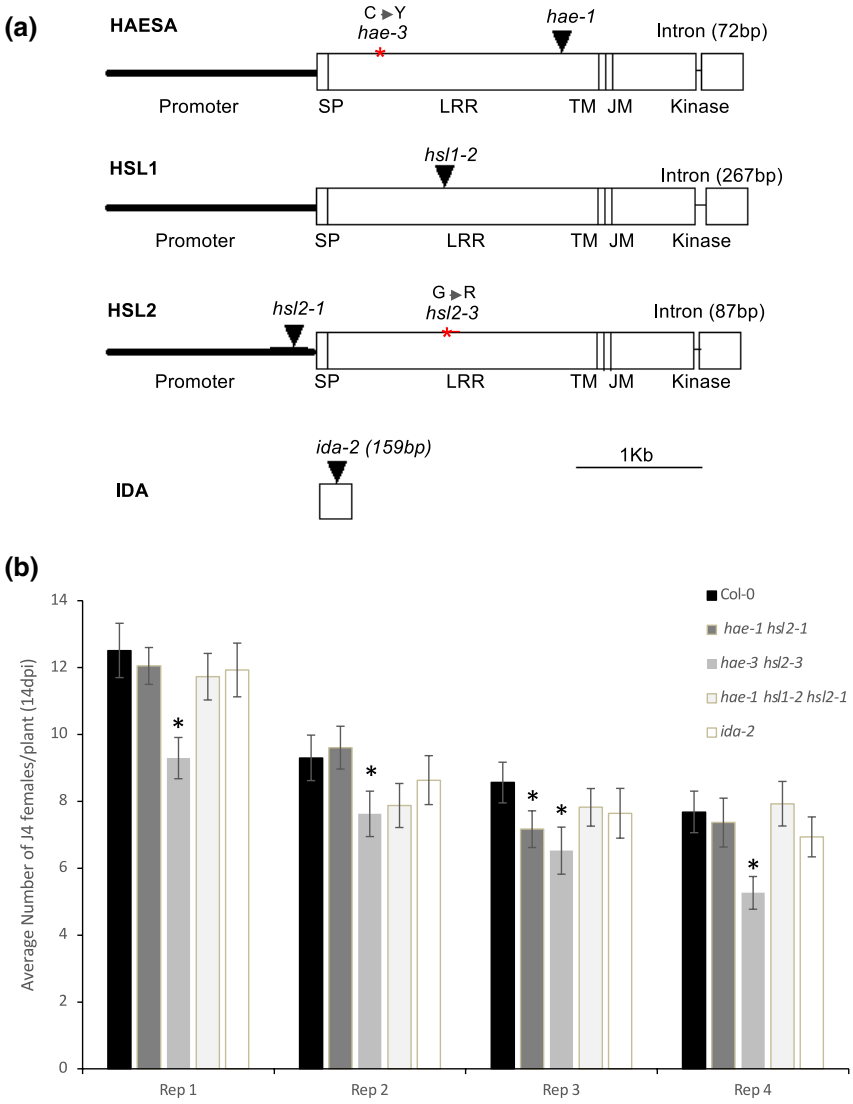



**FIGURE 9** (a) Schematic diagram of *HAESA/HSL1/HSL2/IDA* genes (adapted from Cho et al., 2008) showing *Arabidopsis* T-DNA insertion and EMS mutant alleles used in this study. (a) The *hae-1* line has a T-DNA insertion (SALK 105975) at nucleotide 1787 from the start codon. The T-DNA of the *hsl2-1* allele (SALK 057117) is located at nucleotide −205, upstream of the start codon. The *hsl1-2* line (SAIL-653-E08) has a T-DNA insertion in the coding sequence and *ida-2* (SALK 133209) has a T-DNA insertion 159 nucleotides from the start codon. EMS mutant alleles of HAESA and HSL2 are denoted by asterisks. SP, signal peptide; LRR, leucine-rich repeat; TM, transmembrane domain; JM, juxtamembrane region. (b) Effect of the different mutations on the susceptibility of *Arabidopsis* roots to the beet cyst nematode, *Heterodera schachtii*. *Arabidopsis* T-DNA insertion lines of *hae-1 hsl2-1*, *hae-1 hsl1-2 hsl2-1*, and *IDA-2* showed no reproducible significant differences in the number of female nematodes compared to the wild-type Col-0 line at 14 days postinoculation (dpi) in three biological replicates. Mean values significantly different from the wild-type Col-0 are denoted by asterisks as determined by unadjusted paired *t* tests ($p < 0.01$). The *hae-3 hsl2-3* mutant exhibited a significant decrease in the number of female J4 nematodes compared to the Col-0 line at 14 dpi

to misalignment of repeated sequences during DNA replication (Darmon & Leach, 2014). The density of inverted repeats can also enhance fragment rearrangement and recombination events (Gordenin et al., 1993; Wang & Leung, 2006). Thus, the clustering and inverted repeat structure of genes encoding this family of effector proteins could be beneficial for generating genetic variation within cyst nematode populations. In addition to causing greater variation in the population, the inverted repeats could potentially alter gene regulation through homology-dependent gene silencing (Billmyre et al., 2013; Kooter et al., 1999). Moreover, members of this effector family are tandemly duplicated. Transposable elements have been implicated in the duplication of genes in genomes (Muñoz-López & García-Pérez, 2010) and often sit within or in close proximity to effector genes (Bao et al., 2017), including members of this effector gene family (Masonbrink et al., 2019).

The low expression in preparasitic second-stage juveniles coupled with the significant up-regulation of these genes in the dorsal gland during parasitic stages suggests that these effectors are likely to play roles in the formation and/or maintenance of the syncytium. A potential role for 16B09 in suppression of plant defence responses in *H. glycines* and *Heterodera avenae* was previously reported (Hu et al., 2019; Yang et al., 2019). In a separate study, Hg16B09, Hg2D01 and HgGLAND5 were included among a larger group of *H. glycines* effectors tested for a potential function in plant immune suppression. HgGLAND5 was found to suppress pathogen-associated molecular pattern-triggered immunity in these assays (Noon et al., 2015; Pogorelko et al., 2020), but none of these effectors showed any evidence of suppression of effector-triggered immunity or cell death in the assays used (Pogorelko et al., 2020; Wang et al., 2020). Here, we demonstrated a 30%–45% reduction in parasitic success of the BCN *H. schachtii* on *Arabidopsis* roots expressing dsRNA targeted to *Hs16B09* to provide further evidence of a critical role of this effector in cyst nematode parasitism, although its function remains to be determined.

Family member Hg2D01 shares the same gene structure, localization and expression pattern with Hg16B09. However, their protein sequences, while related, have significantly diverged outside of conserved domains (Figure 1a). 16B09 sequences represent a strongly supported clade including four full-length genes clustered in the *H. glycines* genome and seven full-length gene sequences

spread over three contigs as tandem repeats in the *H. schachtii* genome. In contrast to 16B09, there were many more copies of 2D01 and closely related GLAND5 members. Thus, the potential for functional redundancy among these family members and the inability of host-derived RNAi to completely knock out gene function in nematodes may have contributed to our inability to detect a reduction in *H. schachtii* parasitism on *Arabidopsis Hs2D01* RNAi lines. However, key characteristics of Hs2D01, such as its lack of homology to any known protein, its high copy number in the genome, the presence of an N-terminal SP, and up-regulation in the dorsal gland of parasitic juveniles, are consistent with many known stylet-secreted effectors involved in parasitism. For this reason, we set out to identify potential host targets of this putative effector to gain functional insights. In a yeast two-hybrid screen, we identified an interaction of Hs2D01 with the intracellular kinase domain of HAESA (HAE), a receptor-like protein kinase (formerly RLK5) that belongs to a large family of cell surface LRR-RLKs (Walker, 1993). The biological relevance of the Hs2D01–HAE interaction was validated in planta using bimolecular fluorescence complementation and confirmed to be specific by lack of interaction with the related family member Hs16B09. We also demonstrated the increased activity of the *HAE* promoter:GUS reporter gene during establishment of the syncytium coinciding with the timing of *Hs2D01* expression in the nematode. Thus, based on these data HAE could serve as a bona fide target for Hs2D01. Interestingly, as the syncytium expanded, *HAE* expression shifted to the margins of the developing syncytium, and was ultimately restricted to a region of cells adjacent to the head of the feeding nematode in fully expanded syncytia. The observed reduction in nematode development on *hae-3 hsl2-3* loss-of-function mutant roots supports an important role for these receptors in syncytium formation and suggests that Hs2D01 may modulate the activity of the HAE signalling pathway to promote parasitism.

Considering HAE is known to play an important role in regulating the expression of cell wall-modifying proteins important in abscission and LRE, the interaction of 2D01 with HAE has biological relevance to the plant–nematode interaction. This is because, like abscission and LRE, the modulation of cell wall-modifying proteins that contribute to cell wall remodelling is a tightly controlled process critical for syncytium formation by cyst nematodes (Butenko et al., 2003). The HAE signalling pathway is triggered by binding of Inflorescence Deficient in Abscission (IDA), a small, secreted 14-amino-acid peptide, to the extracellular domains of HAE and HSL2, which activates KNOX transcription factors and the expression of target genes important for floral abscission and LRE in *Arabidopsis* (Aalen et al., 2013; Shi et al., 2011; Taylor et al., 2016). During abscission and LRE, the middle lamella between adjacent cells is broken down by cell wall-remodelling enzymes such as polygalacturonases and other hydrolytic enzymes (Kim, 2014; Kumpf et al., 2013; Merelo et al., 2017; Niederhuth et al., 2013). During LRE, *HAE* and *HSL2* are differentially involved in the regulation of genes encoding cell wall-remodelling enzymes in cells overlaying lateral root primordia (Cho et al., 2008; Kumpf et al., 2013; Stenvik et al., 2008). More recently, IDA signalling through HSL2 was found to regulate the interplay between cell wall separation processes and plant defence by promoting an interaction between HSL2 and RECEPTOR-LIKE KINASE 7 (RLK7), leading to the release of cytosolic calcium and reactive oxygen species to activate defence gene expression (Olsson, Joos, et al., 2019; Olsson, Smakowska-Luzan, et al., 2019). Our finding that HAE/HSL2 loss-of-function mutants were less susceptible to nematode infection suggests that the interaction of Hs2D01 with the intracellular kinase domain of HAE may be perturbing the HAE signalling pathway to promote parasitism.

Interestingly, IDL peptides have been identified from the root-knot nematode (RKN) *Meloidogyne incognita* and *Meloidogyne hapla* genome sequences but have yet to be identified in cyst nematodes (Mitchum & Liu, 2022; Tucker & Yang, 2013). In *M. incognita*, two *IDL* genes, *MiIDL1* and *MiIDL2*, code for small proteins with predicted N-terminal secretion signals that are expressed during the early stages of RKN parasitism (Tucker & Yang, 2013). Indirect evidence suggests that these IDL peptides are secreted by RKNs directly to the apoplast of host cells where they undergo proteolytic cleavage to 14-amino-aci bioactive peptides. Exogenous application of synthetic MiIDL1 peptide to the *Arabidopsis ida* mutant rescued the floral abscission and lateral root phenotypes in a HAE/HSL2-dependent manner (Kim et al., 2018). Consistent with this, constitutive expression of *MiIDL1* in the *ida* mutant reverted to wild-type floral abscission. Host-derived RNAi targeting of *MiIDL1* resulted in approximately 40% fewer and smaller galls on roots, indicating a critical role in parasitism (Kim et al., 2018), although the exact role remains unclear.

Taken together with our results, HAE may represent a common target of cyst nematode and RKN effectors to regulate similar or different aspects of nematode feeding site formation. RKNs induce a feeding site composed of five to seven giant cells that remain as individual cells, but expand to hundreds of times the size of a normal root cell. Thus, RKNs may secrete IDA-like peptides directly to the apoplast of giant cells to interact with the extracellular LRR domain of HAE, whereas cyst nematodes may be targeting the intracellular kinase domain of HAE by secreting 2D01-type effectors directly into the cytoplasm of the developing syncytium. This suggests that these two different plant-parasitic nematodes may have evolved to use two different types of effectors that converge on the same host target to regulate aspects of host cell wall remodelling and defence for feeding site formation. Future work directed at how 2D01 and other members of this family of novel effectors impact the function of their binding partners and downstream signalling pathways will no doubt provide a clearer understanding of their roles in molecular plant–nematode interactions.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Plant and nematode material

*A. thaliana* ecotype Col-0 was used in these studies. *HAE* belongs to a small gene family that includes two other closely related family members, *HAE-like 1* (*HSL1*) and *HSL2*. *HAE* and *HSL2* are functionally

redundant in floral abscission and differentially regulated during LRE, while *HSL1* expression decreases just before the onset of floral organ abscission (Cho et al., 2008; Kumpf et al., 2013; Stenvik et al., 2008; Stø et al., 2015). The *ida-2* (SALK_133,209) mutant (Cho et al., 2008), T-DNA insertion double (*hae-1 hsl2-1*) and triple (*hae-1 hsl1-2 hsl2-1*) mutant lines (Niederhuth et al., 2013), an ethyl methanesulfonate (EMS) double mutant line (*hae-3 hsl2-3*; Baer et al., 2016), and a *HAE* promoter:GUS reporter line (Jinn et al., 2000) were either obtained from the ABRC collection or kindly provided by Dr John Walker (Division of Biological Sciences, University of Missouri, USA). PCR genotyping and/or sequencing was performed to confirm homozygosity of all lines using PCR primers described by Cho et al. (2008). *H. schachtii*, the BCN, was propagated on greenhouse-grown sugar beets (*Beta vulgaris*), and *H. glycines*, the SCN, was propagated on greenhouse-grown soybean (*Glycine max* 'Williams 82').

## 4.2 | Genome analysis and phylogenetic tree construction

*Hg16B09*, *HgGLAND5* and *Hg2D01* cDNA sequences were used to search the SCN pseudomolecule genome assembly (Masonbrink et al., 2021) and current *H. schachtii* assembly consisting of 395 contigs (accession number JAHGVF010000000; Siddique et al., 2021) using BLAST. Transcript/gene IDs were procured from Wormbase Parasite. Sequences within each contig were mapped and an alignment with reference sequences was carried out using MEGA 11 software (Tamura et al., 2021). The amino acid sequences were used to construct phylogenetic trees using MEGA 11 software by the neighbour-joining method (Saitou & Nei, 1987; Tamura et al., 2021). The evolutionary history of the analysed taxa is represented by the bootstrap consensus tree drawn from 100 replicates (Felsenstein, 1985). The matrix for pairwise distances was estimated by using the JTT model and by selecting the topology with superior log likelihood value.

## 4.3 | Cloning and sequence analysis of *H. schachtii 16B09* and *2D01*

The *Hs16B09* and *Hs2D01* sequences were obtained by designing primers to amplify the full-length coding sequence from *H. schachtii* cDNA with 5′ untranslated region (UTR) and 3′ UTR primers designed based on the *H. glycines 16B09* and *2D01* cDNA sequences (Gao et al., 2003). Amplified PCR products were cloned into the pCR4TOPO vector and sequenced. The clones were verified in two independent PCR experiments and named Hs16B09MC1 and Hs2D01MC1. Primer sequences are listed in Table S2.

## 4.4 | In situ hybridization

In situ hybridization was conducted according to De Boer et al. (1998) to determine the spatiotemporal expression pattern of *2D01* as previously described by Gao et al. (2003). Gene-specific primers (Table S2) were used to generate a 250-bp DNA template for generating *2D01* sense and antisense digoxigenin-labelled single-stranded DNA probes.

## 4.5 | *Hs16B09* and *Hs2D01* RNAi constructs

A 483-bp fragment of *Hs16B09* minus the sequence corresponding to the secretion SP was cloned in the sense and antisense orientation at the *Xho*I–*Kpn*I (fragment amplified using primers Hs16B09XhoIBamHIiF and Hs16B09ClaIKpnIiR) and *Bam*HI–*Cla*I (fragment amplified using Hs16B09BamHIDSPF and Hs16B09ClaIKpnIiR) restriction sites, respectively. Similarly, Hs2D01 full-length (558-bp) coding sequences were amplified using primers 2D01iEcoRI and 2D01iKpnI for the sense strand and 2D01iXbaI and 2D01iClaI for the antisense strand. The amplicons were first cloned into pHANNIBAL/pKANNIBAL vectors (Wesley et al., 2001) in the sense direction followed by cloning in the antisense direction on the opposite side of the pyruvate dehydrogenase kinase (*PDK*) intron resulting in a hairpin construct expressed under the control of the CaMV 35S promoter and with the *Arabidopsis PDK* gene intron as a hairpin spacer. The resulting *Hs16B09* and *Hs2D01* constructs were digested with *Not*I and ligated into the pART27 binary vector. Successful cloning was confirmed by Sanger sequencing. Expression of both the sense and antisense fragments was confirmed by RT-PCR in the $T_2$ transgenic lines, and single insertion lines were advanced to the $T_3$ generation.

To investigate RNAi efficiency in the *Hs16B09* and *Hs2D01i* RNAi and control lines (Col-0 and GFPi line) and to confirm expression of a nontarget effector gene, *Hs19C07*, 10-day old *Arabidopsis* seedlings were inoculated with 50 preparasitic *H. schachtii* per root. At 4 dpi, root segments containing nematodes were collected and total RNA was isolated using the MN Nucleospin miRNA kit (Macherey-Nagel Inc.). cDNA was prepared using the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio USA, Inc.). Transcript abundance of nematode target genes was analysed by RT-qPCR. Primer pairs are listed in Table S2.

## 4.6 | *Arabidopsis* transformation

Constructs were introduced into wild-type *Arabidopsis* (ecotype Col-0) by *Agrobacterium*-mediated floral dip transformation (Clough & Bent, 1998). The resulting transgenic plants ($T_1$) were selected on 0.5× Murashige and Skoog (MS) agar plates containing appropriate antibiotics. The $T_1$ transformants were then transferred to soil, observed for phenotypes, and grown to maturity at 22°C under long-day growth conditions. $T_2$ seed were checked for a segregation ratio of 3:1 for a one-locus insertion on 0.5× MS agar plates containing appropriate antibiotics. Plants segregating at a 3:1 ratio were selfed and $T_3$ seed was harvested. $T_3$ seeds were then plated on 0.5× MS agar plates containing appropriate antibiotics to identify homozygous lines.

Hs16B09ΔSP pART27 RNAi lines 3-4-4, 4-4-1, 6-5-3 and 8-3-2, and Hs2D01FL pART27 RNAi lines 6-3-2, 9-6-5 and 16-1-1 were used for infection assays.

## 4.7 | Root length, penetration and infection assays

*Arabidopsis* seeds were sterilized using the chlorine gas method (Wang et al., 2011) for 6 h and stratified by incubation at 4°C for 2 days. For root length measurements, seeds were plated on modified Knop's medium (Sijmons et al., 1991) in square Petri plates. Five seeds each of a homozygous line and Col-0 were plated on a single plate and each line was planted twice to negate the potential effects of well position on growth. Plates were incubated at 23°C under 16 h light/8 h dark conditions and plants were allowed to grow vertically for 10 days in a growth chamber. Root length (distance between the crown and the tip of the main root) was measured in three independent experiments. The data were analysed for significant differences in mean root length between each transgenic line and wild-type controls using a two-tailed Student's *t* test. For spatiotemporal expression analysis of *HAE* during cyst nematode infection, 10-day-old transgenic *HAEp:GUS Arabidopsis* seedlings grown on square Petri plates were inoculated with 50 infective J2s of *H. schachtii* and assayed for GUS activity at different time points following inoculation.

For infection assays, single sterilized seeds were planted in individual wells of 12-well culture plates (Falcon) containing 2 ml modified Knop's medium in a randomly blocked experiment. The seedlings were allowed to grow in a growth chamber at 25°C under 16 h light/8 h dark conditions for 14 days. Nematodes were incubated in sterilizing solution (0.004% wt/vol mercuric chloride, 0.004% wt/vol sodium azide, 0.002% vol/vol Triton X-100) for 8 min followed by five or six washes with sterile water and resuspended in 0.1% (wt/vol) agarose. For infection assays, 14-day-old seedlings were inoculated with approximately 200 surface-sterilized BCN J2s. Female nematodes were counted at both 14 and 30 dpi. Three biological replicates were conducted, and statistical significance was analysed using Student's *t* test.

For the penetration assay, seedlings at 4 dpi were stained with acid fuchsin. The inoculated seedlings were treated with 10% bleach for 1 min, rinsed with water, and then placed into a boiling acid fuchsin (1:100 diluted) solution (3.5% acid fuchsin in 25% acetic acid in 75 ml distilled water) for 2 min. The stained plants were rinsed in distilled water and the number of nematodes inside the roots was counted using a stereomicroscope.

## 4.8 | Subcellular localization

The *Hs2D01* coding sequence without the secretory SP was cloned under the control of the 35S promoter as a GFP-GUS fusion reporter gene in the pMDC43/83 vector series (Curtis & Grossniklaus, 2003). The *AtHAE* full-length sequence was PCR-amplified from *Arabidopsis* cDNA and cloned as a GFP fusion in the pMDC83 vector under the control of the CaMV 35S promoter. The constructs were transiently expressed in *N. benthamiana* leaves by agro-infiltration. Tobacco plants were grown in a growth chamber at 25°C under 16 h light/8 h dark conditions for 4 weeks and infiltrated with transformed *Agrobacterium tumefaciens* GV3101 resuspended in infiltration buffer (10 mM $MgCl_2$ in 10 mM MES pH 5.2, 0.1 mM acetosyringone) to an $OD_{600}$ of 0.2. The infiltrated plants were incubated at 24°C for 72 h in a growth chamber before visualization using a Zeiss LSM 880 confocal microscope.

## 4.9 | Yeast two-hybrid

The BD Matchmaker Library Construction and Screening Kit (Clontech) was used for the yeast two-hybrid assay. The *Hs16B09ΔSP* and *Hs2D01ΔSP* cDNAs sequence were amplified and cloned into the pGEM-T Easy vector (Promega). The resulting plasmids were digested to release the insert and cloned into the pGBKT7 bait vector to produce fusion constructs with the GAL4 DNA-binding domain (BD) to generate the bait constructs. Bait constructs were verified by sequencing and transformed into *Saccharomyces cerevisiae* Y187 using the lithium acetate yeast transformation procedure (Clontech). Each bait construct was tested for transcriptional activation and toxicity as per the manufacturer's instructions. The bait clone was mated with a previously generated prey library of an *Arabidopsis* nematode-infected root (3 dpi) prey library (Hewezi et al., 2008). The colonies from the mating were plated directly onto QDO medium. Each colony that was at least 1 mm at 5 days after mating was restreaked onto QDO. Yeast plasmid DNA was obtained using a Plasmid Miniprep kit (Qiagen) from each colony, and PCR using primers pGAD-F and pGAD-R was performed to determine the number of prey plasmids. The yeast DNA plasmid was transformed into *Escherichia coli* DH5α. The resulting DNA was cotransformed to confirm interaction, no autoactivation of the prey, and specific interaction. Once all three were confirmed, the prey clone was sequenced. The HAE kinase domain (aa 649–999), HAE-KD (aa 689–999), and HAE-KD (aa 697–999) were obtained by using the corresponding primers, ligated into pGADT7, and cotransformed to check for interaction with the bait.

## 4.10 | Bimolecular fluorescence complementat assay

The full-length cDNA fragment (without stop codon) of *AtHAE* was amplified from wild-type Col-0 cDNA and cloned into pSPYNE (Schütze et al., 2009) to generate HAE-FL-$YFP^N$. Hs2D01ΔSP/Hs16B09ΔSP cDNA sequences were cloned into KanII VYCE (R) (Schütze et al., 2009) to generate the $Venus^C$ Hs2D01ΔSP/Hs16B09ΔSP constructs, respectively. The constructs were introduced into *A. tumefaciens* GV3101 and infiltrated into *N. benthamiana* leaves.

Fluorescence of the epidermal cell layer of the lower leaf surface was examined at 3 dpi. Images were captured using an LSM 880 confocal microscope (Zeiss). All constructs were checked for protein expression by western blot analysis.

## AUTHOR CONTRIBUTIONS

A.V., M.L. and D.S. performed experiments and analysed and interpreted the data; T.H., J.W., E.L.D., R.S. and T.J.B. provided materials and assisted with data analysis and interpretation; M.G.M. supervised the experimental work and assisted with data analysis and interpretation; M.G.M. cowrote the article with A.V. and M.L. All authors reviewed and commented on the manuscript.

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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.

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## SUPPORTING INFORMATION

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