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A Root-Knot Nematode Secretory Peptide Functions as a Ligand for a Plant Transcription Factor

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Parasitism genes expressed in the esophageal gland cells of root-knot nematodes encode proteins that are secreted into host root cells to transform the recipient cells into enlarged multinucleate feeding cells called giant-cells. Expression of a root-knot nematode parasitism gene which encodes a novel 13-amino-acid secretory peptide in plant tissues stimulated root growth. Two SCARECROW-like transcription factors of the GRAS protein family were identified as the putative targets for this bioactive nematode peptide in yeast two-hybrid analyses and confirmed by *in vitro* and *in vivo* coimmunoprecipitations. This discovery is the first demonstration of a direct interaction of a nematode-secreted parasitism peptide with a plant-regulatory protein, which may represent an early signaling event in the root-knot nematode–host interaction.

Additional keywords: stylet secretion, transgenic plants.

Root-knot nematodes (RKNs; *Meloidogyne* spp.) are among nature's most successful parasites. These highly specialized biotrophic parasites attack more than 3,000 plant species from diverse plant families and represent a tremendous threat to crop production worldwide (Sasser 1980). A successful host–parasite relationship requires molecular signals from RKNs to transform, directly or indirectly, cells within the vascular tissue of susceptible plant roots into elaborate feeding cells, called giant-cells, that are required for RKN development and reproduction. Giant-cell formation represents one of the most complex responses elicited in plant tissue by any parasite or pathogen (Davis and Mitchum 2005). Infective RKN second-stage juveniles (J2) penetrate near the root tip and migrate intercellularly to a site near the differentiating vascular tissue. Secretory parasitism proteins are injected through the nematode's protrusible stylet (oral feeding spear) to transform five to seven root vascular cells into the metabolically active multinucleate giant-cells by inducing repeated nuclear divisions uncoupled from cytokinesis. The parasitism proteins are encoded by parasitism genes expressed in the nematode's esophageal secretory gland cells and developmental changes in the secreted proteins occur during the parasitic cycle (Davis et al. 2004; Huang et

al. 2003; Hussey 1989). The parasitism proteins mediate the dynamic interaction of the RKN with its plant hosts, but little is known about the molecular mechanism or mechanisms underlying these plant responses. The broad host range of this pathogen suggests that the RKN affects fundamental processes within plant cells. Understanding how infective RKN J2 regulate these essential plant responses is a critical area of study for limiting crop damage to RKNs in the future.

Low molecular weight secretions from potato cyst nematode J2 enhance division of plant protoplasts and mammalian cells, suggesting that an unknown mitogenic peptide is involved in feeding cell development (Goverse et al. 1999). Although peptide hormones are widely known to have key developmental roles in animal systems (Alberts et al. 1994), small peptides only recently have been recognized as a new group of plant-signaling molecules with diverse developmental functions (e.g., CLAVATA3 [CLV3] controls shoot meristem organization in *Arabidopsis*) (Fletcher 2002; Matsubayashi 2003; Ryan et al. 2002). Recent evidence suggests that plant-parasitic nematodes may have evolved a mechanism to mimic plant signaling peptides for parasitic modification of host plant cells. A parasitism gene, *Hg-SYV46*, encoding a secretory protein with a function similar to CLV3 of *Arabidopsis thaliana* has been characterized from the soybean cyst nematode, *Heterodera glycines* (Wang et al. 2005).

An RKN parasitism gene, designated as 16D10, encoding a putative secretory signaling peptide and expressed in the subventral esophageal gland cells, was isolated from a gland-cell-specific cDNA library of *Meloidogyne incognita* (Huang et al. 2003). Here, we describe that transgenic expression of the RKN 16D10 secretory peptide significantly stimulates host root proliferation with normal differentiation. This bioactive RKN peptide directly interacts with plant SCARECROW-like (SCL) transcription factors that, presumably, have important roles in plant growth and development. This is the first demonstration that a plant-parasitic nematode-secreted parasitism peptide functions as a signaling molecule to induce root proliferation by specifically targeting a host plant regulatory protein. This novel putative ligand-receptor pair may have a role in giant-cell induction in the RKN–plant interaction.

RESULTS

Analyses of the 16D10 gene.

The longest open reading frame (ORF) of the 16D10 cDNA (364 bp) encoded a deduced protein of 43 amino acids (aa) (Fig. 1A), including a 30-aa N-terminal hydrophobic signal peptide as predicted by Signal P (Nielsen et al. 1997). The ma-

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The genomic DNA sequence for 16D10 and the cDNA sequence for TmSCL are available in the GenBank database under accession numbers DQ087264 and DQ087265, respectively.

ture 16D10 peptide of 13 aa (GKKPSGPNPGGNN, molecular weight = 1,223 Da) had no significant BLASTP similarity. However, the peptide was similar to the C-terminal conserved motif of the plant CLE protein family (Cock and McCormick 2001; Olsen and Skriver 2003) (Fig. 1B) and contained a cAMP/cGMP-dependent protein kinase phosphorylation site (KKpS) as predicted by PROSITE (Hofmann et al. 1999). Sequencing of an 840-bp genomic DNA polymerase chain reaction (PCR) product amplified using 16D10-specific primers identified one intron of 476 bp (Fig. 1A). Furthermore, this 16D10 genomic DNA did not contain recognition sites for *EcoRI* or *BamHI* (i.e., those restriction endonucleases used for subsequent DNA blotting experiments to investigate the gene copy number of 16D10 in *M. incognita* and to assess the presence of homologues in other *Meloidogyne* spp.). A blot containing genomic DNA from *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla* hybridized with a 16D10 cDNA probe showed that 16D10 was present in each of the four agriculturally important *Meloidogyne* spp., with three to four copies or homologues (Fig. 2). No hybridization was detected with genomic DNAs from the soybean cyst nematode *H. glycines*, the nonparasitic free-living nematode *Caenorhabditis elegans*, and plants (tobacco and *Arabidopsis*) (data not shown).



Fig. 1. Gene 16D10. **A**, The cDNA and deduced protein sequences of 16D10. The position of intron is indicated by an arrowhead. The predicted signal peptide sequence and the putative polyadenylation signal are underlined. **B**, Alignment of the mature 16D10 peptide (GenBank accession no. AY134435) with CLAVATA3 (AF126009), CLE (the C-terminal conserved motif of the plant CLE protein family) (Olsen and Skriver 2003), and Hg-SYV46 (AF273728).

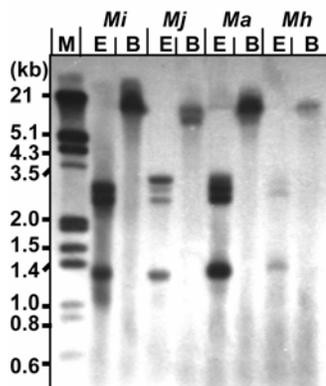


Fig. 2. DNA blot hybridization of restriction endonuclease-digested genomic DNA from four *Meloidogyne* spp. with a digoxigenin (DIG)-labeled 16D10 probe. *Mi*, *Meloidogyne incognita*; *Mj*, *M. javanica*; *Ma*, *M. arenaria*; *Mh*, *M. hapla*; E, *EcoRI*; B, *BamHI*; M, 80-ng DIG-labeled molecular weight marker in kilobases.

Immunodetection of 16D10 peptide.

Purified 16D10 antiserum bound to secretory granules within the subventral gland cells of preparasitic and parasitic J2 and their cytoplasmic extensions and expanded ampullae, which are located posterior to the pump chamber at the metacarpus (Fig. 3). These findings documented that the correct ORF of the 16D10 cDNA was identified. No specific labeling with the rabbit preimmune serum was observed in any nematode specimens.

Release of stylet secretions from the esophageal gland cells of *M. incognita* J2 was induced in vitro and stylet secretions were concentrated for use in enzyme-linked immunosorbent assay (ELISA) and immunoblotting analyses using the purified 16D10 antiserum. Both assays identified 16D10 peptide in the stylet secretions as well as total extracts of J2 and mixed parasitic stages of *M. incognita*. No signal was detected in the controls containing bovine serum albumen (BSA) or total protein extracts of the soybean cyst nematode, *H. glycines* (Fig. 4). None of the tested protein samples interacted with the preimmune serum.

Overexpression of 16D10 in tobacco hairy roots.

As a first assay to identify 16D10 function in host roots, the 16D10 cDNA was overexpressed in tobacco hairy roots with and without the nematode signal peptide sequence using the CaMV 35S promoter (Fig. 5A). Inclusion or exclusion of the signal peptide coding sequence should target the 16D10 peptide to the secretory pathway or the cytoplasm of transformed plant cells, respectively. Six hairy root lines with a single

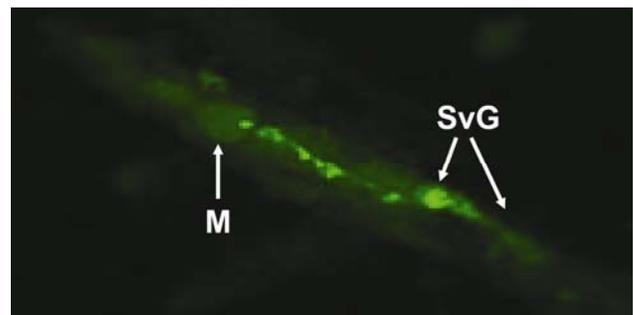


Fig. 3. Immunolocalization of 16D10 peptide within the subventral esophageal glands (SvG) of *Meloidogyne incognita* parasitic second-stage juveniles using 16D10 antiserum. M, metacarpal pump chamber.

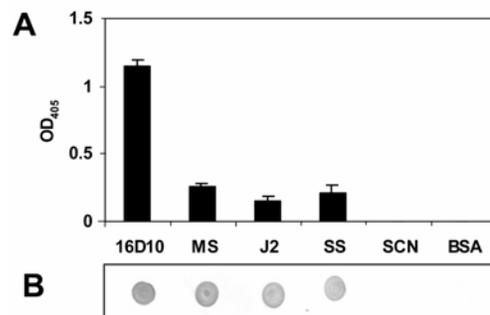


Fig. 4. **A**, Enzyme-linked immunosorbent assay and **B**, immunoblotting analyses of 16D10 in *Meloidogyne incognita* stylet secretions. 16D10, 100 ng of 16D10 mature peptide (>95% purity) synthesized from Sigma-Genosys as a positive control; J2/MS, 10 μ g of total extracts of second-stage juveniles (J2) or mixed parasitic stages of *M. incognita*; SS, 100 ng of concentrated *M. incognita* stylet secretions; SCN, 10 μ g of total extracts of the soybean cyst nematode *Heterodera glycines*; BSA, 10 μ g of bovine serum albumin as a negative control.

transgenic copy of 16D10 without its signal peptide sequence were established from independent transformations. We were unable to produce any hairy root lines harboring the 16D10 construct, including the nematode signal peptide coding sequence, despite extensive experimentation. Six independent control hairy root lines using the empty transformation vector also were generated. Strikingly, expression of 16D10 in the cytoplasm of hairy root cells increased root growth at the rate of approximately 65% (mean root length after 2 weeks of 5.20 ± 0.61 cm [$n = 90$] in 16D10 transgenic lines compared with 3.15 ± 0.34 cm [$n = 90$] in control lines), generated extensive lateral roots, and led to the formation of calli where root tips were cut for subculturing at 5 weeks (Fig. 5B). Reverse-transcriptase (RT)-PCR analysis of 16D10 expression showed that the steady-state mRNA levels in calli were higher than in the hairy roots (Fig. 5C). Immunoblotting analysis with the purified 16D10 antiserum revealed that 16D10 was produced in both hairy roots and calli (Fig. 5D). No expression of 16D10 was detected in the control vector-transformed hairy roots (Fig. 5C and D).

Overexpression of 16D10 in *Arabidopsis*.

In order to complement the tobacco hairy root data, four transgenic *Arabidopsis* T₂ homozygous lines (L-7, L-10, L-11, and L-17) containing a single-copy of 16D10 without a signal peptide under the control of the 35S promoter were generated. Two transgenic lines (L-2 and L-3) originating from the blank transformation vector also were generated as controls. RT-PCR and immunoblotting analyses confirmed that 16D10 was expressed in all of the 16D10 transgenic lines, but not in the control lines (Fig. 6A and B). As in tobacco hairy roots, expression of 16D10 in the cytoplasm of *Arabidopsis* cells significantly accelerated root growth, giving rise to a much-enlarged root system (Fig. 6C). No significant differences were found in the shoots of 16D10 transgenic lines and control lines. Further-

more, two transgenic lines expressing 16D10 with an *Arabidopsis* CLV3 signal peptide directing 16D10 into the apoplast demonstrated identical phenotypes to the control lines (data not shown). In the root-growth assay, significant differences ($P < 0.01$) in root length between 35S::16D10 transformants and control lines were observed at 12 days after germination. The length of primary root in 35S::16D10 transformants was increased by 84.97% (mean 54.01 ± 8.75 mm in four 16D10 transgenic lines [$n = 90$ /line] and 29.20 ± 4.50 mm in two control lines [$n = 90$ /line]) (Fig. 6D).

Two-hybrid screen for 16D10-interacting host proteins.

Reporter gene (*HIS3*, *ADE2*, and *lacZ*) confirmation of the two-hybrid interactions between the GAL4-BD-16D10 and GAL4-AD-tomato root-cDNA fusion proteins led to the identification of five tomato cDNA clones: one encoded an unknown protein, another encoded a ribosomal protein subunit, and three identical cDNA clones (544 bp, GenBank accession number DQ087265) encoded partial proteins with 30 to 78% identity to the C-termini of plant SCL transcription regulators, which control plant growth and development (Bolte 2004; Pysh et al. 1999).

For confirmation that 16D10 interacted with a plant SCL protein, 12 *Arabidopsis* SCL genes homologous to the tomato SCL gene identified here were cloned from an *Arabidopsis* root cDNA pool and expressed as GAL4-AD fusion proteins in a yeast two-hybrid assay. When tested, two *Arabidopsis* SCL proteins, AtSCL6 and AtSCL21, interacted with 16D10 in yeast. Domain analysis revealed the specific interaction of 16D10 with the SAW domain of AtSCL6 and AtSCL21 and no interaction of 16D10 with the rest of the domains of the SCL proteins (Fig. 7A), and indicated that the SCL transcription factors were two putative targets of the secreted 16D10 during RKN parasitism of plants. In vitro interaction of 16D10 with AtSCL6 and AtSCL21 was verified by coimmunoprecipitation

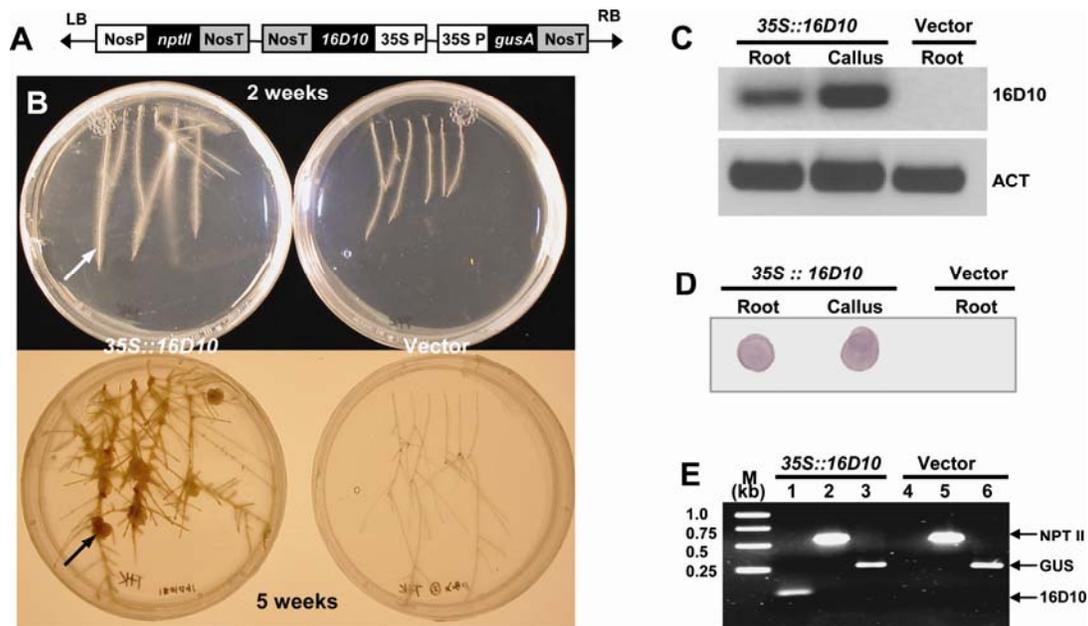


Fig. 5. Overexpression of 16D10 in tobacco hairy roots. **A**, Schematic T-DNA region of the binary vector pBIX containing 16D10 with or without a signal peptide sequence. **B**, Root growth assay of 16D10 constitutively expressing (35S::16D10) hairy roots (left plates) with control vector-transformed hairy roots (right plates). The white arrow indicates that a root tip was cut from a 2-week-old hairy root for subculture, and the black arrow indicates a callus formed at the cutting site 3 weeks after wounding. **C**, Relative reverse-transcriptase polymerase chain reaction (PCR) of 16D10 transcripts in transgenic hairy root lines. Expression of the tobacco actin *Tob104* gene (GenBank accession no U60494) was used as an internal control. **D**, Immunoblotting analysis of 16D10 expression in 5-week-old transgenic hairy roots assayed with the purified 16D10 antiserum. **E**, Representative PCR analysis for the presence (35S::16D10) or absence (vector) of 16D10 with *nptII* and *gusA* genes in transgenic hairy root lines, using *nptII*- or *gusA*-specific primers. M, 1-kb DNA molecular marker (Promega). *nptII*, neomycin phosphotransferase II gene; *gusA*, β -glucuronidase (GUS) gene.

(Co-IP) of ^{35}S -labeled, Myc-tagged 16D10 with ^{35}S -labeled, hemagglutinin (HA)-tagged AtSCL6 or AtSCL21 by using antigen-specific antibodies (Fig. 7B). In planta interaction of 16D10 with *Arabidopsis* root proteins also was confirmed by in vivo Co-IP analysis. A 61-kDa protein (the same size as AtSCL6) and a 31-kDa (possibly processed) protein (14 kDa smaller than AtSCL21) bound to the 16D10 peptide were pulled down from the total root-extracted proteins of 16D10 transgenic *Arabidopsis* line L-17 by using the purified 16D10 antiserum. Microsequencing of the Co-IP proteins revealed that two internal peptide fragments each from the CNBr-digested 61- and 31-kDa proteins had amino acid sequences of QKIERFLIQPEIEKLVLD RSRPIERP and AGFEPYPLSSIISA TIRALLRDYSNGYAIERD GALYLGW, which are identical to internal sequences of AtSCL6 and AtSCL21, respectively. In the control *Arabidopsis* line L-3, no protein was pulled down from the total root-extracted proteins with the purified 16D10 antiserum, nor did rabbit preimmune sera bind to any root protein in the transgenic *Arabidopsis* lines (Fig. 7C and D).

DISCUSSION

Parasitism proteins synthesized in the esophageal gland cells of plant-parasitic nematodes are secreted into host tissues to mediate nematode infection and parasitism of plants (Davis et al. 2004; Williamson and Hussey 1996). In this article, we report that a novel secretory peptide (16D10) secreted from the subventral esophageal gland cells of RKN specifically induces host root growth by directly interacting with a host intracellular SCL transcription regulator. Because 16D10 is conserved in RKN species, we infer that 16D10 is a fundamental signal for regulating RKN–host interactions by activating SCL-mediated signal-transduction mechanisms in parasitized cells.

In plants, small peptides represent a newly recognized group of signaling molecules with diverse functions, such as systemic wound response, cell proliferation and dedifferentiation, shoot meristem organization, root nodulation, and self-incompatibility (Matsubayashi 2003; Ryan et al. 2002). Some of these bioactive peptides (e.g., phytosulfokine [PSK] and CLV3) are synthesized through the secretory pathway and have been shown to act

extracellularly as peptide hormones, but others (e.g., ENOD40) apparently are synthesized in the cytosol on free ribosomes (Ryan et al. 2002). The extracellular peptides (systemin, PSK, and CLV3) act on either leucine-rich repeat transmembrane receptors or cysteine-rich receptor kinases (Matsubayashi 2003), whereas the cytoplasmic ENOD40 binds to a sucrose synthase (Rohrig et al. 2002).

Small secretory peptides also are involved in the interaction between parasitic nematodes and their host. For example, mitogenic peptides present in the excretory-secretory products of the animal parasitic nematode *Trichostrongylus colubriformis* are responsible for epithelial cell proliferation and can stimulate HT29-D4 cell growth in vitro (Hoste et al. 1995). A small, unknown peptide or peptides secreted by the potato cyst nematode *Globodera rostochiensis* stimulates the proliferation of both tobacco leaf protoplasts and human peripheral blood mononuclear cells in the presence of synthetic auxin and cytokinin analogues (Goverse et al. 1999). Although the amino acid sequence of the novel RKN 16D10 peptide is similar to the C-terminal conserved motif of the plant CLE protein family (Cock and McCormick 2001; Olsen and Skriver 2003), targeting of 16D10 to the apoplast or cytoplasm of *Arabidopsis clv3* mutants did not restore the wild-type phenotype (G. Z. Huang and R. S. Hussey, unpublished data). In contrast, a parasitism gene *Hg-SYV46* expressed in the dorsal esophageal gland cell of the soybean cyst nematode *H. glycines* encodes a secretory protein that is capable of functioning in a fashion similar to that of CLV3 by rescuing *clv3* mutants of *A. thaliana* (Wang et al. 2005).

Some plant peptides need a posttranslational modification for their stability or increased activity, such as glycosylation in systemins and sulfation in PSKs (Matsubayashi and Sakagami 1996; McGurl et al. 1992; Pearce et al. 1991). Immunoblotting and ELISA assays revealed that stable 16D10 peptide was present in the transgenic tobacco hairy roots and *Arabidopsis*. Whether a functional 16D10 in the transgenic roots is phosphorylated is under investigation, because 16D10 contains a predicted cAMP/cGMP-dependent protein kinase phosphorylation site at the serine residue.

SCL transcription factors (e.g., GAI, RGA, and SCR) are members of the GRAS protein family characterized by a vari-

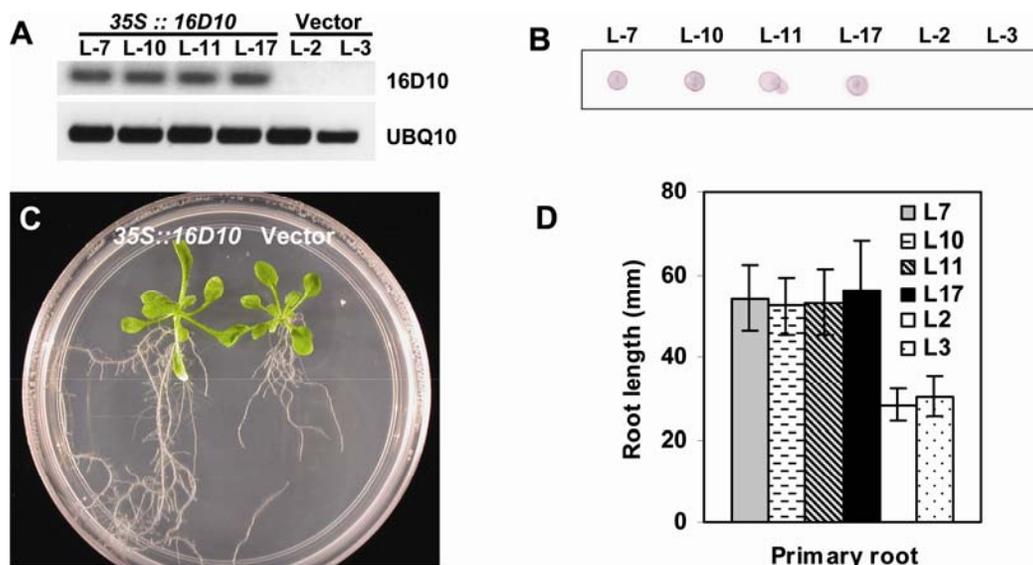


Fig. 6. Overexpression of 16D10 in *Arabidopsis*. **A** and **B**, Relative reverse-transcriptase polymerase chain reaction (RT-PCR) and immunoblotting analyses for the expression of 16D10 in four 16D10 transgenic homozygous T₂ lines (L-7, L-10, L-11, and L-17) and the absence of 16D10 expression in two vector-transformed homozygous T₂ lines (L-2 and L-3) 12 days after germination (dag). Expression of the *Arabidopsis UBQ10* gene was used as an internal control in RT-PCR. **C**, Seedlings of 16D10 constitutively expressing (*35S::16D10*) transgenic line (left) with control vector-transformed line (right) 18 dag. **D**, Primary root length 12 dag. Bars indicate \pm standard deviations; $n = 90$.

able N-terminus, leucine heptad repeat I (LHR I); a VHIID motif, leucine heptad repeat II (LHR II); a PFYRE motif; and a SAW motif (Fig. 7A). The LHR I-VHIID-LHR II region might function as a DNA binding and oligomerization domain, with the LHRs mediating protein-protein interactions and the VHIID motif mediating protein-DNA interaction (Pysh et al. 1999). The divergent N-terminal sequences are hypothesized to function as activating domains and the C-terminal region with the PFYRE- and SAW-conserved motifs may act as regulatory domains (Itoh et al. 2002; Pysh et al. 1999). Although more than 30 members of the GRAS protein family have been identified in plants (Bolle 2004; Pysh et al. 1999; Tian et al. 2004), the functions of only a limited number of GRAS family proteins have been elucidated. AtSCR and AtSHR are involved in root radial patterning, whereas AtGAI and AtRGA function as negative regulators controlling gibberellin signaling (Nakajima and Benfey 2002). Mutations in the SAW domains of AtSCR, AtRGA, and MtNSP1 proteins have strong mutant phenotypes, suggesting that the C-terminal SAW domain is important for protein function (Di Lorenzo et al. 1996; Silverstone et al. 1998; Smit et al. 2005). Inoculation of SCL6 and SCL21 *Arabidopsis* mutant lines, which had slightly retarded root growth, with *M. incognita* showed a 23 to 56% reduction in the number of nematode eggs per gram of root in these SCL mutants when compared with the wild-type control *Arabidopsis* (G. Z. Huang and R. S. Hussey, unpublished data). The specific interaction of RKN 16D10 with the SAW domain of a tomato SCL protein or *Arabidopsis* AtSCL6 and AtSCL21 in yeast, in vitro, and in transgenic plants indicates that an SCL protein is a putative target for the RKN 16D10 to function in parasitized host plant cells.

Most of the *AtSCL* genes are expressed predominantly in the roots (Pysh et al. 1999), and the 12 *SCL* genes analyzed herein were amplified from an *Arabidopsis* root cDNA library, suggesting that a subset of these *SCL* genes may play important roles in root biology. The functions of AtSCL6 and AtSCL21 are unknown, but homologues in the same group in the phylogenetic tree of GRAS proteins may provide some insight into the role of AtSCL6 and AtSCL21 in plant biology (Bolle 2004). AtSCL21 is grouped with AtPAT1 and OsCIGR1/OsCIGR2, which are involved in phytochrome signaling in *Arabidopsis* and chitin (N-acetylchitoooligosaccharide) elicitor perception in rice, respectively (Bolle 2004; Day et al. 2003). AtSCL6 is grouped with AtSCL22, which is catalogued by cDNA microarray analysis to function in mitotic cell cycle and cell cycle control (Yamada et al. 2003).

In host roots, vascular parenchyma cells near the primary xylem in the zone of elongation are preferred for RKN feeding cell initiation. The transition from parenchyma cell to a fully differentiated giant-cell occurs early in the parasitic association, indicating that secreted signaling molecules from the early parasitic J2 mediate giant-cell development (Hussey 1989; Williamson and Hussey 1996). In RKN-infected roots, cell cycle genes are upregulated in the feeding cells within the first hours of parasitism (De Almeida Engler et al. 1999; Niebel et al. 1996). This rapid reactivation of the cell cycle may be induced by an initial stimulus from the nematode (Goverse et al. 2000). In our study, we conclude that the RKN secretory peptide 16D10 may be involved in feeding cell formation, because 16D10 i) is conserved in RKN species and secreted from the stylet; ii) is strongly expressed in the parasitic J2 subventral esophageal gland cells, which become nonfunctional in later parasitic stages; and iii) binds to a specific plant transcription factor domain.

During feeding cell induction, the nematode inserts its stylet through the plant cell wall without penetrating the plasma membrane. Nematode stylet secretions may be deposited be-

tween the plasma membrane and the cell wall or released directly into the cytoplasm through a perforation in the plasma membrane at the stylet orifice (Williamson and Hussey 1996). Our transgenic expression data indicates that 16D10 only functions in the cytoplasm of root cells, which is supported by the direct interaction of 16D10 with a host intracellular regulatory protein.

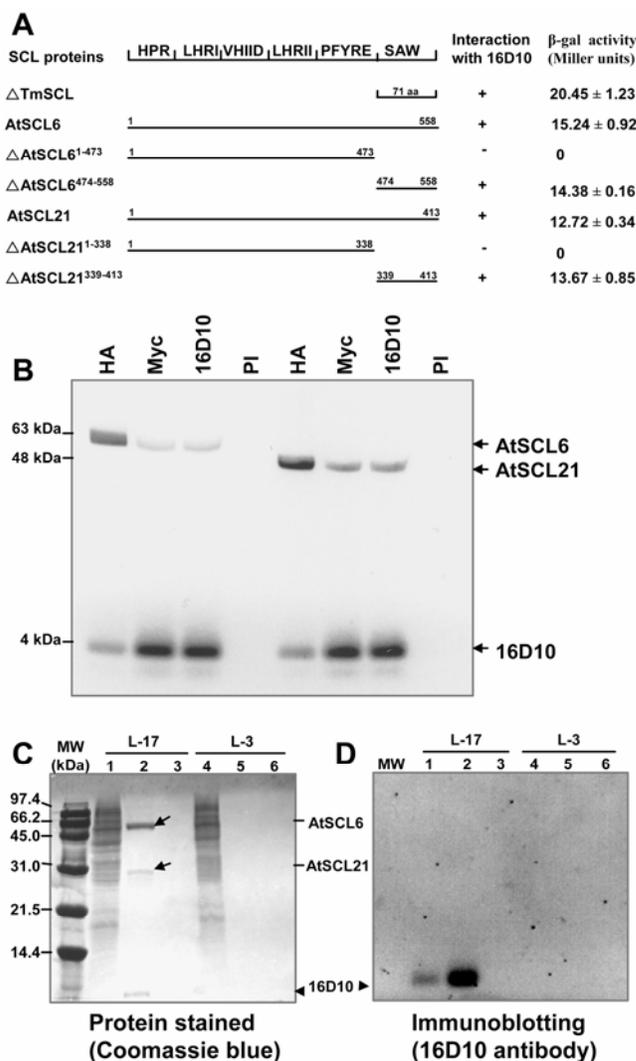


Fig. 7. Interaction of 16D10 with plant SCARECROW-like (SCL) transcription factors. **A**, Direct interaction of 16D10 with the SAW domain of tomato (TmSCL) and *Arabidopsis* (AtSCL6 and AtSCL21) proteins in yeast. The specific regions of these SCL proteins drawn schematically were tested to interact with 16D10. Positive interactions resulting in the activation of *HIS3*, *ADE2*, and *lacZ* genes were detected by growth in the absence of histidine and adenine, and β -galactosidase (β -gal) activity. β -Gal activity was measured from three independent β -gal liquid assays using *o*-nitrophenyl β -D-galactopyranoside as a substrate. **B**, In vitro co-immunoprecipitation (Co-IP) of 16D10 with AtSCL6 or AtSCL21. [³⁵S]-labeled 16D10 with HA-tag (4 kDa), AtSCL6 with c-Myc-tag (63 kDa), and AtSCL21 with c-Myc-tag (48 kDa) were translated from the corresponding cDNA in pGBKT7 or pGADT7. Antibodies used for Co-IP are indicated: HA, HA-tag polyclonal antibody; Myc, c-Myc monoclonal antibody; 16D10, the purified 16D10 antiserum; PI (control), rabbit pre-immune serum. **C** and **D**, In vivo Co-IP of 16D10 with *Arabidopsis* root proteins from the 16D10 transgenic line L-17 using the vector-transformed line L-3 as a control. Gels were stained with Coomassie blue R-250 and immunoblotted with the purified 16D10 antiserum. Lanes 1 and 4: total root extracts; lanes 2 and 5: products pulled down from the total root extracts by using the purified 16D10 antiserum; lanes 3 and 6 (controls): no product pulled down from the total root extracts by using rabbit pre-immune serum.

Transcription factors are known to direct gene expression patterns and the resulting cell differentiation, but a direct link between the transcription factors and the cell cycle has not yet been made. Although several transcription factors are among the upregulated genes in the nematode feeding cells, the changes in gene expression identified so far are likely to be several steps downstream from the initial plant responses to signals from the nematode (Gheysen and Fenoll 2002; Williamson and Hussey 1996). The regulation of SCL-transcription factors by 16D10 could affect the transcription of downstream root-specific genes by intervening in the signal-transduction pathways involved in root cell proliferation.

MATERIALS AND METHODS

Nematodes and plants.

Nematodes were cultured on host plant roots and RKN (*M. incognita*) parasitic J2 and parasitic stages were collected as described previously (Huang et al. 2005). Plants were grown under optimal conditions in growth chambers.

Analysis of the 16D10 gene.

Nematode genomic DNA was extracted from J2. *Eco*RI- or *Bam*HI-digested genomic DNA (10 µg) was electrophoresed on a 0.7% agarose gel, transferred to Hybond-N membrane (Amersham, Piscataway, NJ, U.S.A.), and hybridized with 15 ng of digoxigenin (DIG)-labeled 16D10 probe corresponding to the full-length cDNA sequence as described (Huang et al. 2005). Sequence of the *M. incognita* genomic DNA containing the coding region of 16D10 was obtained from the PCR product using the gene-specific primers 16D10GF (5'-GAGAAAATAAAATATAAATTATTCCTC-3') and 16D10GR (5'-CAGATATAATTTTATTCAG-3') and *M. incognita* genomic DNA template.

Immunodetection of 16D10.

Polyclonal antiserum to 16D10 was produced by immunizing rabbits with a synthetic mature (i.e., without the N-terminal signal peptide) 16D10 peptide (Eurogentec, Inc., San Diego, CA, U.S.A.). Peptide affinity-purified 16D10 polyclonal antiserum was used to localize 16D10 expression in specimens of *M. incognita* using immunofluorescence microscopy (Goverse et al. 1994) and for immunodetection of 16D10 in stylet secretions and transgenic plant-expressed or in vitro-translated 16D10.

Stylet secretions from *M. incognita* J2 were produced and collected in vitro as described previously (Davis et al. 1994) and concentrated with StrataClean resin (Stratagene, La Jolla, CA, U.S.A.). Nematode proteins were extracted from ground J2 and mixed parasitic stages of *M. incognita* and *H. glycines* as described (Ding et al. 1998). Plant proteins (0.5 g) were extracted by grinding transgenic seedlings or root tissues in 200 µl of extraction buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1× complete protease inhibitors) (Roche Applied Science, Indianapolis, IN, U.S.A.) in microcentrifuge tubes in liquid nitrogen. Supernatant was recovered from homogenates after centrifugation at 13,000 rpm for 10 min. All protein concentrations were estimated (with a Bio-Rad protein assay kit II; Bio-Rad, Hercules, CA, U.S.A.) with BSA as a standard, and ELISA and dot blots were performed with 2-µl protein samples and purified 16D10 antiserum (Ding et al. 1998).

Plasmid construction.

The coding regions of 16D10 with or without the nematode signal peptide sequence were amplified from the full-length cDNA clone with primers 16D10SF (5'-CGGGGTACCTAGATGTTTTACTAATTCAATTAA-3') or 16D10F (5'-CGGGGTAC

CTAGATGGGCAAAAAGCCTAGTG-3') and 16D10R (5'-GCTCTAGATCAATTATTTCTCCAGG-3') that introduced *Kpn*I or *Xba*I restriction sites (underlined) and the stop/start codons (in italics), cloned into the *Kpn*I and *Xba*I sites of binary vector pBIX (Fig. 5A) under the control of CaMV 35S promoter to generate pBIX(16D10SP) and pBIX(16D10), respectively, and confirmed by sequencing. pBIX was derived from pBI101 (BD Biosciences, San Jose, CA, U.S.A.) and contains a *nos* promoter-*nptII*-*nos* terminator cassette, a 35S promoter-*gusA*-*nos* terminator, and a second 35S promoter with a poly-linker having *Kpn*I and *Xba*I sites. Because we were unable to generate tobacco hairy root lines using the nematode 16D10 signal peptide (discussed above), we fused a plant signal peptide sequence (*clv3*) to the 16D10 sequence for targeting 16D10 to the apoplast in *Arabidopsis*. The fusion-expressed sequence of *clv3* signal peptide and 16D10 was generated by PCR amplifications from *Arabidopsis* genomic DNA using primers C3K (5'-GGGGTACCATGGATTCTAAAAGCTTTG-3') that introduced *Kpn*I restriction site (underlined) and C3R (5'-CCACTAGGCTTTTTGCCAAGGAACAAGAAGCAG-3') for the *clv3* signal peptide sequence, and from 16D10 cDNA using primers C3F (5'-CTTCTGCTTCTTGTTCCTTGCCAAAGGCCTAGTGG-3') and 16D10X (5'-GCTCTAGATCAATTATTTCTCCAGG-3') that introduced *Xba*I restriction site (underlined) for the mature peptide coding sequence using Vent polymerase (New England Biolabs, Ipswich, MA, U.S.A.). The two products then were used to prime each other in a fusion PCR reaction. The resulting fragment was cloned into pBIX to generate pBIX(CLV3SP-16D10) and verified by sequencing.

Expression of 16D10 in tobacco hairy roots.

The plasmids pBIX(16D10) and pBIX(16D10SP) and the empty vector pBIX as a control were transferred into *Agrobacterium rhizogenes* ATCC 15834 by electroporation (Shen and Forde 1989) and transformed into tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) using the *A. rhizogenes*-mediated cotyledon transformation (Christey 1997). Transformed hairy roots were generated from inoculated tobacco cotyledons on Gamborg's B-5 plates containing 0.8% Noble agar with kanamycin at 100 mg/liter and timentins (ticarcillin disodium at 230.8 mg/liter plus clavulanate potassium at 7.69 mg/liter). Individual hairy root tips (approximately 0.5 cm) were cultured for 3 weeks at 24°C in the dark, and two to three roots from individual hairy root system were subjected to β-glucuronidase (GUS)-staining selection (Jefferson et al. 1987). The kanamycin-resistant and GUS-positive root lines, confirmed by PCR analyses (Fig. 5E) and bacteria free, were used to establish hairy root lines. The root-tips were subcultured for root growth assay on Gamborg's B-5 plates without hormones every 2 weeks and the cut roots were kept in culture on the old plates at 24°C in the dark for assays. For root-growth assays, plates were cultured horizontally in the dark and five hairy roots from each transgenic line in each of the three repeats were investigated. Relative RT-PCR and immunoblotting analyses of transgenic hairy roots or calli with a single transgenic copy identified as described (Does et al. 1991) were carried out using the same procedures as in those of transgenic *Arabidopsis*.

Expression of 16D10 in *Arabidopsis*.

The plasmids pBIX(16D10) and pBIX(CLV3SP-16D10) and the empty vector pBIX as a control were introduced into *Agrobacterium tumefaciens* C58C1 by electroporation (Shen and Forde 1989) and transformed into *Arabidopsis thaliana* wild-type Col-0 plants by the floral dip method (Clough and Bent 1998). Segregation analysis of kanamycin resistance identified transgenic homozygous T₂ lines and PCR analysis was used to

confirm the presence of the transgene. Inverse PCR (Does et al. 1991) identified the homozygous lines with a single transgenic copy in the genome for molecular and root growth assays. Thirty plants from each transgenic line in each of the three repeats were in vitro cultured on Murashige-Skoog plates with 3% sucrose with cycles of 16 h of light (24°C) and 8 h of dark (20°C) and the plates were kept vertically for root growth assay.

mRNA was extracted and purified from ground plant tissues using Dynabeads mRNA DIRECT kit (DynaL, Lake Success, NY, U.S.A.) and converted into first-strand cDNA by a SMART PCR cDNA Synthesis kit (BD Biosciences), following the manufacturer's instructions. Relative RT-PCR was conducted on mRNA isolated from equivalent amounts of transgenic *Arabidopsis* seedlings to determine the transcript levels of 16D10. PCR reactions were carried out as described (Huang et al. 2005) with the first-strand cDNA and primers 16D10F and 16D10R as described above, using a 483-bp amplified fragment of the *Arabidopsis* uniformly expressed *UBQ10* gene (GenBank accession number NM_202787) as a control. Aliquots (10 µl) of each RT-PCR reaction were separated on a 2% agarose gel, blotted to nylon membranes, and probed with corresponding DIG-labeled DNA fragments.

Yeast two-hybrid screens.

The MATCHMAKER yeast two-hybrid system II (BD Biosciences) was used in the yeast two-hybrid screening. The cDNA encoding the mature peptide of 16D10 was cloned in frame into the GAL4-binding domain (BD) of pGBKT7 to generate pGBKT7(16D10) and expressed as bait to screen a tomato root cDNA library constructed from mRNA from tomato root tissues in the GAL4 activation domain (AD) of pGADT7. Twelve full-length SCL-encoding cDNAs (*AtSCL1*, *AtSCL3*, *AtSCL5*, *AtSCL6*, *AtSCL9*, *AtSCL13*, *AtSCL14*, *AtSCL21*, *AtSCR*, *AtSHR*, *AtRGA*, and *AtGAI*) were amplified from a root cDNA pool made from mRNA from *A. thaliana* root tissues with specific primers of each gene based on the corresponding sequences in GenBank databases (Bolle 2004) and cloned in frame into pGADT7. Each of the constructs was introduced with pGBKT7(16D10) into the yeast strain AH109. cDNAs encoding the specific regions of *AtSCL6* and *AtSCL21* (Fig. 6A) were cloned into pGADT7 and then cotransformed with pGBKT7(16D10) into the strain AH109. All procedures, including cDNA library screening, selection of positive clones, and the assay of β-galactosidase activity, were performed by following the protocol of MATCHMAKER yeast two-hybrid system II.

Co-IP.

The MATCHMAKER Co-IP kit (BD Biosciences) was used for Co-IP. T₇ promoters and either c-Myc or HA epitope tags were incorporated by PCR into *16D10*, *AtSCL6*, and *AtSCL21* cDNAs using the BD/AD Primer set. The products were in vitro translated and labeled with ³⁵S-methionine (Amersham) using a TNT T₇-coupled reticulocyte lysate system (Promega, Madison, WI, U.S.A.). The labeled proteins were incubated with antigen-specific antibodies and protein A agarose beads at room temperature for 1 h. After washing, the beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer (Bio-Rad). The proteins recovered were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. For the in vivo Co-IP experiment, total root protein extracts from the transgenic *Arabidopsis* line L-17 or L-3 were treated with the purified 16D10 antiserum in non-denaturing immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1× complete protease inhibitors) using the same procedures as described above. The Co-IP proteins, along with total root extracts, were separated on a Tricine-

SDS/15% PAGE, stained with Coomassie blue, transferred to nitrocellulose, and immunoblotted using standard procedures. The Coomassie blue-stained 61- and 31-kDa Co-IP proteins (Fig. 7C) also were excised from an SDS-PAGE gel, resolved, and immobilized on a polyvinylidene difluoride membrane for protein microsequencing of the major internal peptides release by in situ CNBr digestion by ProSeq, Inc. (Boxford, MA, U.S.A.).

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