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Research Brief

A gene encoding a peptide with similarity to the plant IDA signaling peptide (AtIDA) is expressed most abundantly in the root-knot nematode (*Meloidogyne incognita*) soon after root infection

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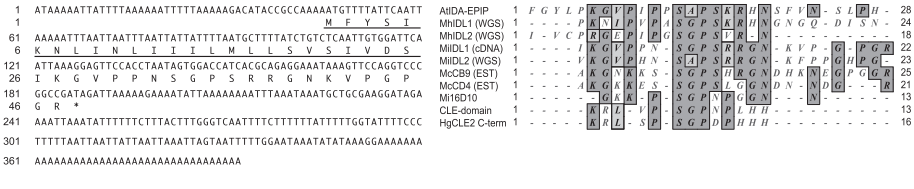
HIGHLIGHTS

- Root-knot nematode genes with similarity to the Arabidopsis IDA signaling peptide.
- The *Meloidogyne incognita* gene, *MiIDL1*, encodes a 47 aa protein with a 25 aa signal peptide.
- The *MiIDL1* gene is expressed early during gull development in the host.

GRAPHICAL ABSTRACT



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ABSTRACT

Small peptides play important roles in intercellular signaling. *Inflorescence deficient in abscission* (*ida*) is an Arabidopsis mutant that does not abscise (shed) its flower petals. The *IDA* gene encodes a small, secreted peptide that putatively binds to two redundant receptor-like kinases (HAESA and HAESA-like2) that initiate a signal transduction pathway. We identified *IDA*-like (*IDL*) genes in the genomic sequence for *Meloidogyne incognita* and *Meloidogyne hapla*. No orthologous sequences were found in any other genus of nematodes. Transcript for both *M. incognita* and *M. hapla IDLs* were found in total RNA isolated from infected root systems of tomato, *Solanum lycopersicum*. Five and three prime RACE of RNA from *M. incognita* infected tomato roots revealed a sequence of 392 nt that includes a poly (A) tail of 39 nt. The open reading frame encodes a 47 aa protein with a putative 25 aa N-terminal signal peptide. Expression of *MiIDL1* is very low in eggs and pre-parasitic J2 and rapidly increases in the first four days post inoculation (dpi) and then declines at approximately 14 dpi. A proposed role for the root-knot nematode IDL is discussed.

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1. Introduction

Numerous small bioactive peptides have been identified in animals and plants, and most are secreted (Matsubayashi and Sakagami, 2006). Secreted peptides function as defensins (a diverse group of antimicrobial peptides) and developmental signals (neuropeptides, systemin, CLAVAT3, phytosulfokines, IDA, etc.). Both defensins (Kato, 2007; Tucker et al., 2005) and neuropeptides (Johnston et al., 2010; Masler, 2012) have been identified in phytopathogenic nematodes. Another class of proteins, often referred to as effectors, are those that are secreted from the pathogen into the host to suppress host defenses or alter a variety of processes in the host that support the growth and development of the pathogen (Bellafiore and Briggs, 2010; Davis et al., 2008; Gheysen and Mitchum, 2011). Phytopathogenic root-knot nematodes (RKN) and cyst nematodes possess three specialized esophageal glands, one dorsal and two subventral, that secret proteins into the esophageal lumen where the proteins are secreted into the host through a protrusible stylet at the forward end of the nematode (Davis et al., 2008; Hussey, 1989). A large number of secreted proteins were identified by cloning RNA isolated from esophageal glands (Gao et al., 2003; Huang et al., 2003; Lambert et al., 1999; Wang et al., 2001) or characterization of proteins in stylet exudates (Bellafiore et al., 2008; Jaubert et al., 2002). Examples of stylet secreted proteins include cell wall hydrolases (e.g., endo-glucanases), which

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aid in the migration of the nematode to the site where it will establish its feeding structure (Smant et al., 1998; Wang et al., 1999), proteins that alter auxin synthesis and transport (Grunewald et al., 2009; Lambert et al., 1999; Lee et al., 2011), and proteins that regulate cell differentiation, e.g., CLAVAT3-like (Olsen and Skriver, 2003; Wang et al., 2011, 2005). Of particular interest here is that a large percentage of the clones identified in the esophageal gland libraries encode small proteins that were proposed to act as signal mimics in their respective hosts (Davis et al., 2008; Elling et al., 2007; Gheysen and Mitchum, 2011).

In an earlier report we demonstrated that several genes for cell wall modifying proteins that were up-regulated during soybean cyst nematode (SCN) colonization of soybean roots were also up-regulated during leaf abscission (organ separation) (Tucker et al., 2007, 2011). We hypothesized that regulation of gene expressed during SCN infection and abscission might in part utilize related signaling mechanisms. In this regard, an abscission mutant was discovered in Arabidopsis where the floral organs do not abscise, i.e., *inflorescence deficient in abscission* (*ida*) (Butenko et al., 2003). The *IDA* gene encodes a small, secreted protein that putatively binds to two redundant receptor-like kinases HAESA (HAE) and HAESA-like 2 (HSL2) (Cho et al., 2008; Stenvik et al., 2008) that signal a change in KNOX transcription factors that modulate floral organ separation (Shi et al., 2011). We hypothesized that expression of an *IDA*-like gene in soybean might play a signaling role in both abscission and SCN infection. We identified *IDA*-like genes in soybean and examined their expression in abscission and also in roots (Tucker and Yang, 2012). Although several *IDA*-like genes are expressed in roots we did not see any significant change in *IDA*-like gene expression during SCN infection (unpublished results). We then considered the possibility that the nematode might secrete an IDA-like peptide. We performed a tBLASTn search in all six reading frames for IDA-like protein sequences in the nr, EST and genomic sequence data for the phylum Nematoda at the National Center for Biotechnology Information (NCBI). No IDA-like translated sequence was found in SCN or any other cyst nematode, but we did find IDA-like sequences in root-knot nematodes, *Meloidogyne* spp.

2. Materials and methods

2.1. Plants and nematodes

Root-knot nematodes (RKN), *Meloidogyne incognita* Race 1, population LESREC (Lower Eastern Shore Research and Education Center) are maintained on the roots of pepper (*Capsicum annuum*) in a greenhouse at the Soybean Genomics and Improvement Laboratory, United States Department of Agriculture, Beltsville, MD, USA. *M. incognita* infected roots were harvested from pepper plants 3–4 months after inoculation and the eggs isolated using a method modified from those described by (Meyer et al., 2000; Nitao et al., 2002). Roots with gulls were shaken in 15% bleach for 3 min to release the eggs and the eggs isolated by successively passing the liquid through stainless steel sieves with pore sizes of 850, 150 and 25 μm (#20, #150 and #500 sieves from Newark Wire Cloth, Clifton, NJ, USA). Eggs were collected from inside the #500 sieve and hatched in 100 ml of water with 50 μg/ml of ampicillin shaken at 26 °C. After three days hatching, second stage juveniles (J2) were isolated from the eggs using the #500 sieve. Living J2 pass through the sieve while eggs and dead J2 remain inside. Tomato (*Solanum lycopersicum*, cv Ailsa Craig) plants were germinated and grown in a sandy soil in the greenhouse for 6 weeks and the roots inoculated with 2000 or 4000 J2 per plant in two replicate experiments.

2.2. RNA isolation and real-time Q-PCR

The root system from seven non-inoculate plants (0 days) and seven plants each at 1, 2, 4, 8, 14, and 20 days post-inoculation (dpi) were collected, frozen with liquid nitrogen and pulverized with a mortar and pestle. Total RNA was isolated from a sample of the frozen roots using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Procedures for quantitative real-time PCR have been described previously (Tucker et al., 2007). A single bulk cDNA synthesis reaction (5 μg of DNased RNA) was performed and the cDNA diluted to 2.0 ml to accommodate a large number of PCR reactions and thereby reduce differences that might occur between cDNA synthesis reactions. Real-time Q-PCR reactions were completed using a Brilliant II SYBR Green QPCR Master Mix in an Mx3000P instrument (Stratagene, La Jolla, CA). Primers used for Q-PCR are listed in Supplementary Table 1.

2.3. 5′ and 3′ RACE

5′ and 3′ RACE (Rapid Amplification of cDNA ends) were completed using the Life Technologies 5′ RACE kit (Life Technologies, Grand Island, NY). The protocol was modified slightly by using oligo dT for the first strand synthesis. Internal *MiIDL1* nested primers used for RACE reactions are listed in Supplementary Table 1. The 5′ and 3′ RACE products had 83 nt overlap. The two RACE products were joined into a single sequence by PCR, cloned and then sequenced.

3. Results and discussion

A tBLASTn search of the Nematoda nucleotide databases at NCBI identified a *M. incognita* and a *Meloidogyne hapla* genomic sequence (accessions CABB01008656 and ABLG01000143, respectively) with open reading frame (ORF) translations that had similarity to the plant IDA and IDL peptides (Fig. 1B). In addition, two small groups of *M. chitwoodi* EST sequences were identified in this search (representative accessions CB931567 and CD418743). No IDA-like sequence was found in any other genus of nematodes. To determine if the *M. hapla* and *M. incognita* genomic ORFs were expressed, we isolated RNA from tomato (*S. lycopersicum*) and pepper (*C. annuum*) roots infected for several months with *M. hapla* (supplied by Valerie Williamson, Univ. Calif. at Davis) and *M. incognita*, respectively. Real-time Q-PCR confirmed that these IDA-like ORFs were expressed at some stage during the development of these species in the root system. We then decided to focus our research on the *M. incognita IDA*-like gene we named *MiIDL1*.

To determine the approximate start of transcription and polyadenylation site, we performed 5′ and 3′ RACE using RNA isolated from 4 dpi tomato roots (Fig. 1A). Sequencing of the 5′ and 3′ RACE clones identified a 392 nt sequence that included a 39 nt poly (A) tail. The sequence 5′ to the poly (A) tail was identical to the genomic sequence deposited at NCBI; thus, the *MiIDL1* gene contains no introns. The ORF within the *MiIDL1* gene encodes a 47 aa protein that includes a predicted 25 aa N-terminal signal peptide (SignalP v 3.0).

The *MiIDL1* sequence was then used to again search the nematode databases. This search revealed one additional genomic sequence in *M. incognita* (accession CABB01003608), named *MiIDL2,* and another in *M. hapla* (accession ABLG01000076), named *MhIDL2*. The *MiIDL1* and *MiIDL2* ORF share 89% nt identity and 83% aa identity. All of the nematode IDA-like sequences identified included a contiguous ORF without introns and a putative N-terminal signal peptide. Fig. 1B shows the alignment of the nematode IDL protein sequences minus the signal peptide with the active, EPIP domain (Stenvik et al., 2008) of the Arabidopsis IDA and IDLs.

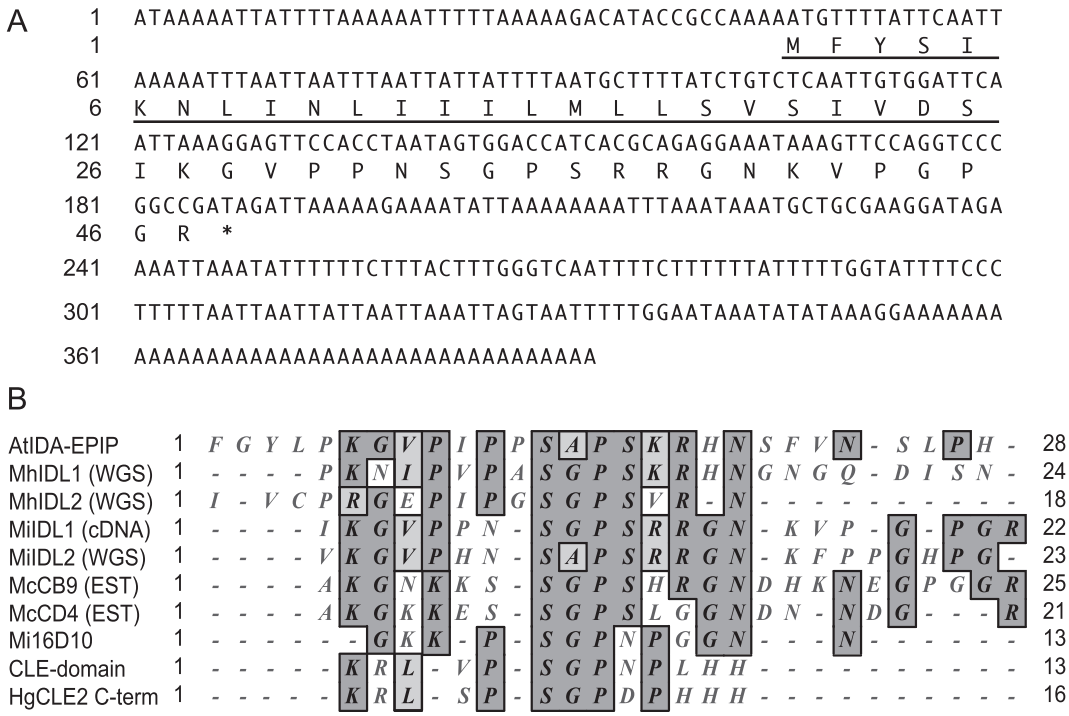

Fig. 1. Sequence of the *MiIDL1* cDNA (accession KC237722) and amino acid sequence alignment of nematode IDLs with Arabidopsis IDA EPIP domain, CLE-like peptides and Mi16D10. (A) Nucleotide and predicted protein sequence of joined *MiIDL1* 5′ and 3′ RACE products. A predicted N-terminal signal peptide is underlined and the stop codon for the ORF is indicated with an asterisk. (B) Peptide alignment of the Arabidopsis IDA active domain (EPIP) (Stenvik et al., 2008), RKN *IDA*-like ORFs minus the putative signal peptides including the *M. Chitwoodi* ESTs, CLAVATA3 domain (CLE-domain) (Olsen and Skriver, 2003), C-terminus of the *HgCLE2* ORF (Wang et al., 2010), and the *Mi16D10* ORF minus its signal peptide (Huang et al., 2006). Identical amino acids are enclosed in dark grey boxes and chemically similar amino acids in light grey boxes. Abbreviations: whole-genome shotgun contigs (WGS), complementary DNA (cDNA), and expressed sequence tag (EST).

Real-time Q-PCR was performed to monitor the expression of the *M. incognita IDLs*. Tomato plants were inoculated with 2000 or 4000 second-stage juveniles (J2) in two replicate experiments and RNA isolated at 0 (non-inoculated), 1, 2, 4, 8, 14, and 20 dpi. RNA was also isolated from purified nematode eggs and pre-parasitic juveniles, J2. To add perspective to the expression profiles for the *MiIDLs*, primers were prepared to amplify *MiENG1* (accession AF100549) and *Mi16D10* (accession AY134435), which are both synthesized in the subventral esophageal gland of the nematode (Huang et al., 2003). *MiENG1* encodes a beta-1,4-endoglucanase (cellulase) (Rosso et al., 1999) and *Mi16D10* encodes a protein that interacts with a host SCARECROW transcription factor (Huang et al., 2006). The coding sequence for *MiENG1* is highly similar (98% identity) to *MiENG3* (accession AY422836), which is also expressed in the subventral esophageal gland (Huang et al., 2004). The primers prepared for *MiENG1* do not distinguish between these two genes; however, based on sequence alignments and Q-PCR dissociation curves, it is unlikely that these primers amplify any other cellulase gene. Because the *MiENG1* primer pair amplifies two gene products, the expression of *MiENG* is referred to as *MiENG1**. Primer pairs for *MiIDL1* and *MiIDL2* (89% identity in the ORF), however, were designed to be gene specific. This was confirmed by Q-PCR using 0.5 pg of a full-length plasmid clone of the *MiIDL1* cDNA where the *MiIDL1* primer pair amplified a product with a Ct (cycles to threshold) of 22 and the *MiIDL2* primer pair did not amplify a product up to 45 cycles; however, the *MiIDL2* primer pair did amplify 5 ng of *M. incognita* genomic DNA with a Ct of 22 compared to a Ct of 23 for the *MiIDL1* primer pair.

To normalize the expression of the nematode genes, primers were prepared to monitor the expression of the tomato and nematode elongation factors 1b (*SlEF1b* and *MiEF1b*, respectively). The elongation factors are used in protein translation and are constitutively expressed in most tissues examined (Czechowski et al., 2005; Lilly et al., 2011; Tucker and Yang, 2012). Normalization of the Q-PCR results to the nematode *MiEF1b* transcript allows one to compare the mRNA expression level in eggs and *in vitro* hatched pre-parasitic J2 to nematodes growing in the tomato root. The expression of *MiIDL1*, *MiIDL2* and *Mi16D10* was low in eggs and pre-parasitic J2 and increased as the nematode developed in the root (Fig. 2). Conversely, *MiENG1** expression was low in eggs but very high in pre-parasitic J2 (Fig. 2). *MiENG1** expression was also high at 1 dpi.

Normalization to *MiEF1b* expression is useful but it doesn't give a good measure of the expression level per nematode throughout development in the root because the nematode increases greatly in size and mass as it grows in the root. The increase in mass of the nematode is presumably reflected in the amount of the elongation factor mRNA, *MiEF1b*, expressed in the nematode. Although the tomato root mass also increased over the 20 days of the experiment, the relative increase in root mass was less than the relative increase in mass for the nematodes. This was indicated by the large increase in expression of *MiEF1b* from 1 to 20 dpi in roots when the expression of *MiEF1b* was normalized to the tomato *SlEF1b* (Fig. 2). Thus, normalization to the tomato *SlEF1b* provides a better indication of expression per-nematode post inoculation. When expression is normalized to *SlEF1b*, the expression level of *MiIDL1* was readily detected after 1 day and peaked between 8 and 14 dpi (Fig. 2). In contrast to *MiIDL1*, expression of *MiIDL2* was very low. The expression profile for *Mi16D10* was similar to *MiIDL1* but appeared to lag slightly behind *MiIDL1* by approximately one day.

Expression of *MiENG1** in pre-parasitic J2 and very early post inoculation (1 dpi) fits well with expression results published previously where they proposed that these cellulases aided in breakdown of plant cell walls during migration of the nematode through the root to the vascular system near the root tip where it becomes sedentary and begins to feed (Huang et al., 2004; Rosso

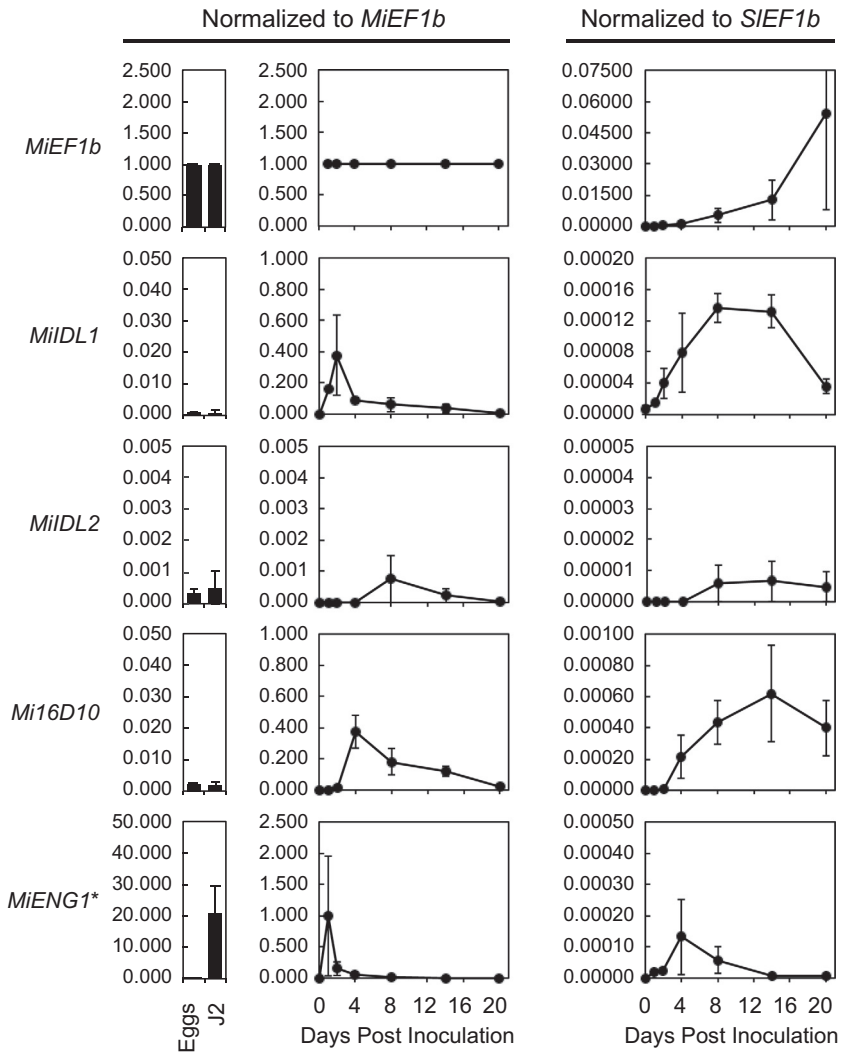

Fig. 2. Expression profiles (real-time Q-PCR) of selected *Meloidogyne incognita* genes normalized to the nematode elongation factor, *MiEF1b*, or the tomato elongation factor, *SlEF1b*. Total RNA was isolated from non-inoculated roots at 0 days post inoculation (dpi) and from inoculated tomato roots 1, 2, 4, 8, 14 and 20 dpi. Total RNA was also isolated from *M. incognita* eggs and pre-parasitic J2. The asterisk on *MiENG1** indicates that this primer pair most likely amplifies transcript from two genes (see text). Means and standard error bars are for two replicate experiments where each RNA sample was isolated from a fraction of the pulverized roots from seven plants collected at each time point.

et al., 1999; Vieira et al., 2011). Once sedentary, expression of the cellulases decrease as they are no longer required for development of the nematode. Expression of *MiENG1** steadily declined from a high at 1 dpi to a very low level at 8 dpi, which likely reflects continued but diminishing infection and migration of J2. Quite different from *MiENG1**, the expression profile for *Mi16D10* and *MiIDL1* appears to correlate best with the formation of giant cells and nematode feeding rather than migration of the nematode through the root (Jung and Wyss, 1999; Williamson and Gleason, 2003).

It was previously reported that the active domains of plant and nematode CLV3 (CLE domains) share similarity with IDA peptides (Stenvik et al., 2008); moreover, the *M. incognita* secreted peptide, 16D10, was also shown to share sequence similarity with plant CLE domains (Huang et al., 2006). Thus, these active domains are included in the sequence alignment displayed in Fig. 1B. The *M incognita and M. hapla* IDL peptides minus the predicted N-terminal signal peptides have more similarity to the Arabidopsis IDA EPIP domain (46%) than to the CLE domain (31%) or the Mi16D10 peptide (27%). However, after alignment of all the sequences, it became evident that the *M. chitwoodi* ESTs, CB931567 and CD418743, which were identified in our original tBLASTn search for IDA-like sequences, were more similar to the Mi16D10 protein sequence (40%) than the EPIP domain (27%) or the CLE domain (22%). Arabidopsis IDA and CLV3 both are thought to interact with leucine-rich repeat receptor-like kinases (LRR-RLK) in the plasma membrane (Cho et al., 2008; Fiers et al., 2005; Stenvik et al., 2008). The 16D10 protein, on the other hand, does not bind to an LRR-RLK but interacts with a SCARECROW transcription factor (Huang et al., 2006). In Arabidopsis, CLV3 interacts with the LRR-RLK CLAVATA2 (CLV2) in the meristem, which acts to set the boundary between the central zone and the peripheral zone within the meristem (Barton, 2010). Although the Arabidopsis IDA shares similarity with CLE proteins (Fig. 1), the Arabidopsis IDA does not bind to CLAVATA2 (Guo et al., 2010). The Arabidopsis IDA peptide putatively binds to the LRR-RLKs HAESA (HAE) and HAESA-like (HSL2), which signal a change in KNOX-like transcription factors in the abscission zone that in turn regulate the rate of abscission through an effect on cell enlargement (Shi et al., 2011).

CLE-like proteins have been identified in several different cyst nematodes (Mitchum et al., 2008; Wang et al., 2011) but not in RKN. The lack of CLE-like proteins in RKN may reflect differences in how the feeding structures form within the root. The cyst nematode forms its feeding structure, syncytium (multi-nucleated cell), by dissolution of the cell walls and membranes between adjacent

root cells (Jung and Wyss, 1999). As many as 200 root cells may be incorporated into the syncytium (Jung and Wyss, 1999). The cyst nematode CLE-like proteins may play a role in establishing the boundary between the syncytium and surrounding root cells. Formation of the RKN feeding structure, giant cells, which are also multinucleated cells, grow by induction of multiple cell divisions without cytokinesis followed by cell expansion (Williamson and Hussey, 1996). The observation that IDA signaling is involved in the regulation of KNOX expression in Arabidopsis abscission (Shi et al., 2011) might suggest a similar signaling path for regulation of KNOX expression in giant cells. In this regard, the *TKN2* KNOX transcription factor and *PHANTASTICA*, a myb like transcription factor that regulates expression of KNOX transcription factors, are up-regulated in giant cells (Koltai and Bird, 2000). The nematode signal that induces the up-regulation of *PHANTASTICA* and *TKN2* is currently unknown.

If the nematode IDLs act as IDA mimics, it is predicted that they bind to the apoplastic side of an LRR-RLK in the plasma membrane. Of interest in this regard is a comparison to the *Heterodera glycines* CLE-like peptide HgCLE2, which also putatively binds to the apoplastic side of an LRR-RLK. In the case of HgCLE2, after cleavage of the N-terminal signal peptide, the peptide is secreted into the host cytoplasm; however, an N-terminal variable region immediately downstream of the cleaved signal peptide somehow supports secretion of the peptide out of the host cell into the apoplast (Wang et al., 2010). The *M. incognita* IDLs do not have an N-terminal variable sequence. Nevertheless, there is evidence that some stylet secretions do not enter into the host cytoplasm of sedentary nematodes (Vieira et al., 2011), which implies that there is a mechanism that somehow differentiates between which stylet secreted proteins enter into the host cell cytoplasm and which remain in the apoplast.

4. Concluding remarks

The low expression level of the *MiIDLs* throughout development, the small size of the mRNA and the fact that the mRNAs are AT-rich outside the ORF may have contributed to their not being identified earlier. Although we predict that the RKN IDLs are secreted from the nematode into the host, these small peptides would be of interest even if they remained inside the nematode where they could modulate nematode development. Thus, there is much still to do to determine the function of the RKN IDL peptides.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.exppara.2013.03.019.

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