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An SXP/RAL-2 protein produced by the subventral pharyngeal glands in the plant parasitic root-knot nematode *Meloidogyne incognita*

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Abstract *Meloidogyne incognita* is a major parasite of numerous plant families, including many crop species. Upon infection of the plant root, it induces several multinucleate giant cells by the injection of pharyngeal gland secretions into the root cells. In order to obtain a better understanding of the nematode-plant interaction, characterization of the pharyngeal gland secretions is a necessity. By differential display, a nematode gene was identified that encodes a new member of the SXP/RAL-2 protein family. The gene is specifically expressed in the subventral pharyngeal glands and the protein is most likely secreted.

Introduction

Root-knot nematodes, such as *Meloidogyne incognita*, are obligate parasites on plant roots. Upon infection of the root, the second stage juveniles (J2) migrate

towards the vascular cylinder where they induce several multinucleate giant cells on which they feed until completion of their life cycle. Severe infections, as is often the case in agricultural systems, result in a disturbed solute transport in the vascular cylinder and stunted plant growth. Annual agricultural losses due to plant parasitic nematodes are estimated to be about \$77 billion (Sasser and Freckmann 1987); a major part of it caused by root-knot nematodes. Development of the giant cells involves a massive change of gene expression in the affected root cells, of which activation of cell cycle genes followed by acytokinetic mitosis is the most striking (Gheysen and Fenoll 2002). Induction of giant cell formation is performed by nematode pharyngeal gland secretions that are injected into the root cells via a hollow protrusible stylet. In response to the nematode stimulus, the initial feeding cell enlarges, the central vacuole disintegrates into several small vacuoles, and organelles, such as the endoplasmic reticulum, ribosomes, mitochondria, and dictyosomes, rapidly proliferate (Gheysen and Fenoll 2002).

In an attempt to better understand the nematode-plant interaction, an mRNA fingerprint analysis on *M. incognita*-infected *Arabidopsis thaliana* roots was performed at different time points. Therefore, nematode infection sites were harvested from the roots 2, 3, 4, 5 and 7 days post-inoculation. Total RNA was isolated from all samples and gene expression was compared using the differential display technique (Liang and Pardee 1992). Transcript derived fragments showing an altered expression were isolated from the polyacrylamide gel and cloned. The origin of the clones, either plant or nematode, was verified by southern hybridization. Analysis of the plant clones is described elsewhere (Vercauteren et al. 2001, 2002). One of the identified nematode genes encoded a secretory protein showing homology to the SXP/RAL-2 protein family. It was specifically expressed in the subventral pharyngeal glands.

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Materials and methods

Biological material

Meloidogyne incognita was grown on *Lycopersicon esculentum* cv. Marmande. For the hatching of second stage infective juveniles (J2), the plants were washed free of sand and placed in beakers with water that was aerated.

Differential display

Infection of *Arabidopsis thaliana* roots with *M. incognita* J2, and the differential display analysis of gene expression in the infection sites 2, 3, 4, 5 and 7 days after root inoculation, was performed as described by Vercauteren et al. (2001, 2002).

Full-length cDNA isolation

To isolate the 5'-end of the open reading frame, a nested PCR with two gene specific primers (primer1: 5'-AAC GAA TTG TAT TCT TAT GTG TTT ATT C-3' and primer2: 5'-GTT GTT GAT CAT CCA GAG TGT TGT C-3') and a vector primer (5'-GAC GGC CAG TGA ATT GTA ATA CGA C-3') was performed on the *M. incognita* pre-parasitic J2 plasmid cDNA library (constructed by Dautova et al. 2001 and kindly provided to us). The first PCR was done in a 50 µl reaction containing 100 ng cDNA library plasmid DNA, 0.2 µM of gene-specific primer1, 0.2 µM vector primer, 5 µl 10×PCR buffer (Perkin Elmer, Norwalk, Conn.), 1.5 mM MgCl₂ and 2 U Taq polymerase (Perkin Elmer). In the second PCR, 2 µl of a 1/100 dilution of the first PCR reaction was used as a template. Cycling conditions were: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 3 min. The amplification products were cloned into pGEM-T (Promega Benelux, Leiden, The Netherlands), and three separate clones having the largest insert were sequenced with SP6 and T7 primers.

In situ hybridization

Whole mount in situ hybridization of freshly hatched *M. incognita* second stage juveniles was performed with digoxigenin-labelled single-strand DNA probes as described previously (Vanholme et al. 2002) using slightly modified fixation conditions (2% paraformaldehyde for 16 h at 4°C and an additional 6 h at room temperature).

Bioinformatics

Sequence homology searches were done with the differential BLAST programs available at the National Centre

for Biotechnology Informatics web site (<http://www3.ncbi.nlm.nih.gov/BLAST/>). The 5' end of the cDNA was analysed for the presence of a signal peptide for secretion with the SignalP V2.0 program (Nielsen et al. 1999) available on the Centre for Biological Sequence Analysis web site (<http://www.cbs.dtu.dk/services/SignalP/>) or the PSORTII program (<http://psort.nibb.ac.jp/>). Intracellular sorting of proteins was predicted with the PSORT program (<http://psort.nibb.ac.jp/>). Multiple sequence alignments were done using the ClustalW Tool available in the BioEdit program (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

Results

did1 expression analysis

Differential display was used to identify alterations in gene expression in *Meloidogyne incognita* infection sites 2, 3, 4, 5 and 7 days after inoculation on *Arabidopsis thaliana* roots. One of them, *did1* (200 bp, GenBank accession number AJ286352), was specifically expressed in infected roots at all time points tested (Fig. 1). Southern hybridization with a *did1* probe on *A. thaliana* and *M. incognita* genomic DNA indicated that this was a nematode derived transcript fragment (data not shown).

Full-length open reading frame isolation

As the *did1* differential display fragment contained the 3' end of the gene, we determined the full-length open reading frame cDNA sequence by isolating the 5' end of the gene from a plasmid pre-parasitic J2 cDNA library. Therefore, two gene specific reverse primers were designed and used in a nested PCR together with a vector specific primer. The amplification products were cloned in pGEM-T and transformed to *Escherichia coli*. Several clones with the largest insert were further propagated, plasmid DNA was isolated, and the insert was sequenced. Out of the different DNA sequences obtained, a consensus sequence was generated, and based on the overlap with the original *did1* cDNA sequence, a full-length sequence was generated in silico. The cDNA sequence obtained contains an open reading

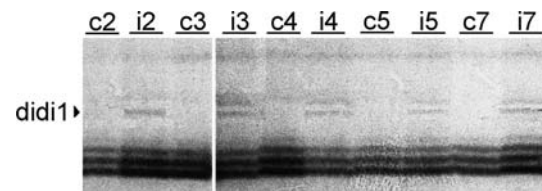


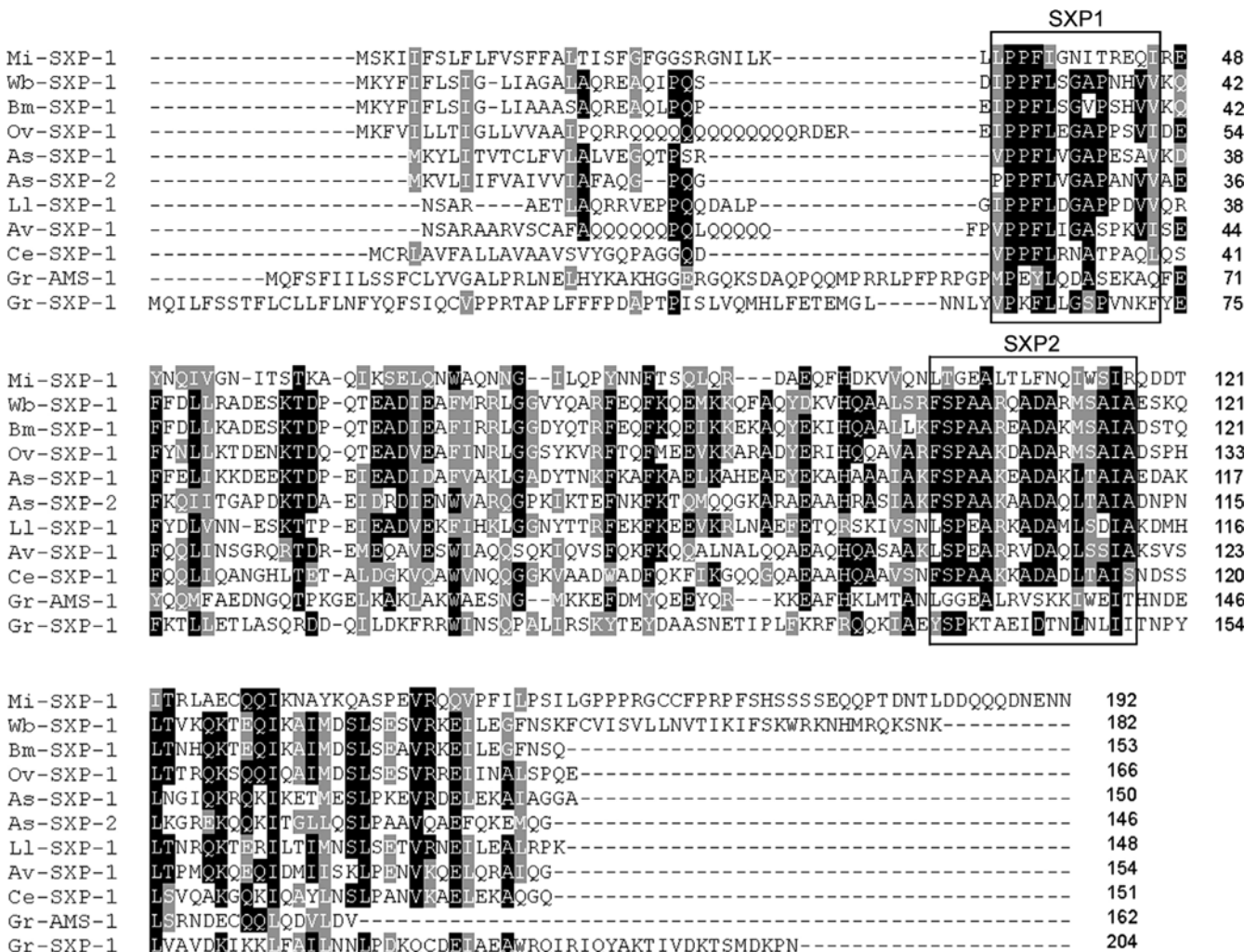
Fig. 1 Differential display analysis on control (c) and *Meloidogyne incognita* infection (i) sites of *Arabidopsis thaliana* roots 2, 3, 4, 5 and 7 days after inoculation. The *did1* fragment was specifically present in infected root segments at all tested stages

frame of 576 bp encoding a polypeptide of 192 amino acids. An in frame stop codon was found upstream of the identified start codon, and the start codon is surrounded by a partial Kozak sequence for the start of translation (Kozak 1991), indicating that the complete open reading frame was present.

did1 encodes an SXP/RAL-2 protein

Homology of full-length did1 cDNA (BLASTX, E-value 5e-11) was found with the amphid protein Gr-AMS-

Fig. 2 Multiple alignment of the Mi-SXP-1 amino acid sequence with those of several members of the SXP/RAL-2 protein family. *Wuchereria bancrofti* (Wb-SXP-1, accession no. AAC70783), *Brugia malayi* (Bm-SXP-1, accession no. AAA27864), *Onchocerca volvulus* (Ov-SXP-1, accession no. AI077025), *Ascaris suum* (As-SXP-1, accession no. BAC66614; As-SXP-2, accession no. BAB67769), *Loa loa* (Ll-SXP-1, accession no. AAG09181), *Acanthochoilonema vitae* (Av-RAL-2, accession no. AAB53809), *Caenorhabditis elegans* (Ce-SXP-1, accession no. AAA81053), *Globodera rostochiensis* (Gr-AMS-1, accession no. CAB66341; Gr-SXP-1, accession no. CAB75701). Black filled areas indicate identical amino acids, areas filled in grey are similar amino acids. Two conserved boxes, SXP motif 1 and 2, are indicated



1 (accession no. AJ271910) of *Globodera rostochiensis*, a plant parasitic cyst nematode. The did1 translation product also showed a significant homology with the SXP/RAL-2 protein family, characterized by the presence of the DUF148 (domain of unknown function) protein domain present in the NCBI database. Therefore, the name *Mi-SXP-1* was assigned to the did1 gene. Multiple alignment of the Mi-SXP-1 protein with several members of this SXP/RAL-2 protein family (derived from *Wuchereria bancrofti*, *Brugia malayi*, *Onchocerca volvulus*, *Ascaris suum*, *Loa loa*, *Acanthochoilonema vitae*, *Caenorhabditis elegans* and *Globodera rostochiensis*) showed that both characteristic boxes, SXP motif 1 and 2, were present (Fig. 2).

Mi-SXP-1 is expressed in the subventral pharyngeal glands

In situ hybridization with a DIG-labelled ssDNA probe of *Mi-SXP-1* showed specific staining in both subventral pharyngeal glands of pre-parasitic J2 (Fig. 3). The N-terminus of the Mi-SXP-1 protein was analysed with the SignalP and PSORTII program for the presence of a

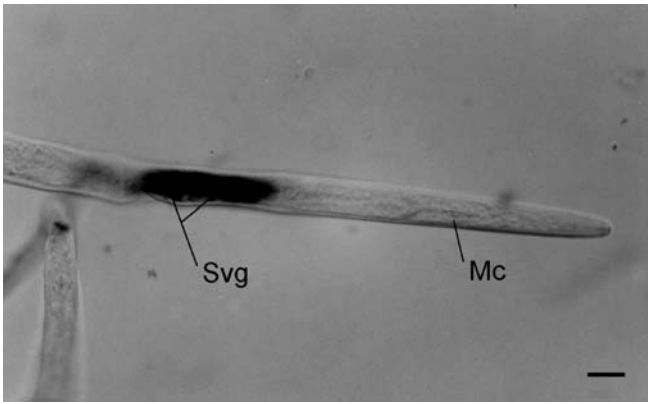


Fig. 3 Differential interference microscopy after in situ hybridization with the antisense probe of *Mi-SXP-1*. Specific staining was found in both subventral pharyngeal glands (*SVG*); *MC* metacorcal bulb. Scale bar 20 μ m

signal peptide for secretion. According to the neural networks protocol, *Mi-SXP-1* had a signal peptide for secretion with the most likely cleavage site between amino acids 24 and 25. A similar signal peptide was predicted with 97% certainty using the hidden Markov model, however with a most likely cleavage site between amino acids 22 and 23. The PSORTII program also detected a signal peptide for secretion in the first 20 amino acids. PSORT analysis of the *Mi-SXP-1* protein without the signal peptide for secretion resulted in a prediction of a cytoplasmic cellular sorting in the plant.

Discussion

A differential display analysis was performed on *Meloidogyne incognita* infected *Arabidopsis thaliana* root segments (Vercauteren et al. 2001). Several differentially expressed transcript-derived fragments were shown to be of nematode origin. Further characterization was done on an SXP/RAL-2 related gene.

The N-terminus of the translation product was analysed for the presence of a signal peptide for secretion with three different methods: the neural networks protocol and hidden Markov model, both available in the SignalP program, and the PSORTII program. None of these three can give absolute certainty. The signal peptide prediction in the PSORTII program is based on an old weight matrix which does not include all recently described secretory proteins (Von Heijne 1986). In contrast, the SignalP program is more recent and is based on much more data (Nielsen et al. 1997a, 1997b, 1999). It makes use of a combination of two methods, the first (neural networks protocol) to predict the location of the cleavage site and the second (hidden Markov model) to distinguish between actual signal peptides for secretion and signal anchors, the latter being present in transmembrane proteins. Signal anchors often have sites similar to signal peptide cleavage after their hydrophobic

(transmembrane) region, which makes them very difficult to distinguish. Because *Mi-SXP-1* is recognized as a secretory protein by the three different methods, it is most likely that it is indeed secreted. Since the neural networks protocol is considered to be the most accurate for cleavage site prediction, we assume that it is cleaved between amino acids 24 and 25. Although *Mi-SXP-1* showed only a small homology to the *G. rostochiensis* amphid protein Gr-AMS-1 described by Jones et al. (2000), it is a member of the same SXP/RAL-2 protein family, characterized by the presence of the DUF148 protein domain, comprising two conserved domains: the SXP1 and 2 motifs (Rao et al. 2000). This family of secreted proteins seems to be specific for nematodes, and several members are described in animal parasitic nematodes and in *C. elegans* (Gallin et al. 1989; Chandrashekar et al. 1994; Rao et al. 2000). SXP/RAL-2 proteins have been exploited as antigens for the development of serological diagnostic assays (Dissanayake et al. 1992; Harnett et al. 1998; Rao et al. 2000). They have also been identified as potential antimicrobial vaccines with the finding that gerbils immunized with recombinant *B. malayi* SXP protein showed significantly reduced worm burdens after challenge (Wang et al. 1997). However, nothing is known about the function of these proteins. Until now, in plant parasitic nematodes only two different SXP proteins, Gr-SXP-1 and Gr-AMS-1 have been identified (Jones et al. 2000). Gr-SXP-1 is specifically expressed in the epidermis, while the Gr-AMS-1 expression is restricted to the amphidial sheet cells. Both nematode organs are known to secrete a large amount of proteins. We identified a third SXP/RAL-2 protein, which is specifically expressed in the subventral pharyngeal glands. Until now, most characterized subventral gland secretions are involved in plant cell wall breakdown during intercellular migration of the infective J2 in the plant root prior to the feeding cell induction (Davis et al. 2000). The only subventral gland secretory proteins that are thought to be secreted into the feeding cell are chorismate mutase (Lambert et al. 1999) and calreticulin (Jaubert et al. 2002). Although SXP/RAL-2 proteins are also found in other nematodes which are not plant parasitic, it does not rule out that *Mi-SXP-1* could be involved in feeding cell induction. In case the *Mi-SXP-1* protein would indeed be injected via the stylet into the feeding cell, it is predicted to have a cytoplasmic localization.

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