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The use of DNA microarrays for the developmental expression analysis of cDNAs from the oesophageal gland cell region of *Heterodera glycines*

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SUMMARY

A microarray was printed containing cDNAs from a library made from cytoplasm microaspirated from the oesophageal gland cell region of parasitic stages of the soybean cyst nematode, *Heterodera glycines*. The array contained both previously described clones (Wang *et al*. *Mol. Plant-Microbe Interact.* 2001, **14**, 536–544) and uncharacterized cDNAs. Fluorescent probes for array hybridization were prepared using RNA polymerase amplification of nematode mRNA. Developmental expression profiles of the arrayed cDNAs were determined by hybridizing the microarray with probes from parasitic and non-parasitic *H. glycines* life stages. Distinct patterns of developmental expression were ascertained for the previously described gland expressed genes. In addition, four *H. glycines* cDNAs (SCN1018, SCN1020, SCN1028 and SCN1167) were identified that showed up-regulation in one or more parasitic life stages. Clone SCN1018 encodes a C-type lectin domain and is expressed in the hypodermis of females. Clone SCN1020 encodes a probable S-adenosylmethionine synthetase. Clone SCN1028 encodes a piwi protein with high similarity to the germ-line-specific protein R06C7.1 of *Caenorhabditis elegans*. The sequence of SCN1167 had no similarity to known genes. This paper describes the first use of cDNA microarrays to analyse genes of a plant-parasitic nematode and establishes a functional method to mine nematode cDNA libraries.

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INTRODUCTION

Cyst nematodes and root-knot nematodes are highly specialized endoparasites of economically important crops, which feed through the formation of a permanent feeding cell structure within the roots of their host plant (Davis *et al*., 2000; Williamson and Hussey, 1996). The identification of nematode secretions that play a role during the endoparasitic life cycle may offer a route to engineering resistance to these pests through the host-driven expression of molecules, such as inhibitory proteins, that interfere with the function of these nematode gene products. Of particular interest, in this respect, are secretory proteins produced in the subventral and dorsal oesophageal gland cells (Hussey, 1989), which are thought to play an important role in the induction and use of nematode feeding cells (Davis *et al*., 2000; Williamson and Hussey, 1996). However, other nematode secretory products, such as those released by the amphids or the hypodermis (Jones *et al*., 2000; Kaplan and Davis, 1987; Semblat *et al*., 2001) could also be suitable targets for parasite control.

The identification of genes for secretory proteins that are expressed specifically in the oesophageal gland cells of endoparasitic nematodes, termed parasitism genes (Davis *et al*., 2000), has made significant progress in the past few years. β-1,4-Endoglucanase and pectate lyase genes were shown to be active in the subventral gland cells of four nematode species, including the soybean cyst nematode, *Heterodera glycines* (De Boer *et al*., 2002; Goellner *et al*., 2000; Popeijus *et al*., 2000b; Rosso *et al*., 1999; Smant *et al*., 1998). Differential screening methods, either comparing nematode life stages or anterior and posterior nematode body fragments, have successfully identified additional oesophageal gland genes in both root-knot nematodes (Ding *et al*., 1998; Lambert *et al*., 1999) and the potato cyst nematode (Qin *et al*., 2000). As a more direct approach to gland gene

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identification, cytoplasm was microaspirated from the oesophageal gland cell region of parasitic juveniles of the soybean cyst nematode and used to construct a cDNA library (Wang *et al.*, 2001). Using selection for eukaryotic signal sequences, secretion genes were cloned from this library that were specifically expressed in the dorsal gland cell of parasitic life stages (Wang *et al.*, 2001). Finally, suppression subtractive hybridization has been used to enrich microaspirated cDNA pools for gland-expressed genes, which allowed the identification of additional *H. glycines* dorsal gland genes coding for secretory proteins (Gao *et al.*, 2001).

Determining the developmental expression pattern of newly identified gland and other secretion genes of plant-parasitic nematodes will assist in evaluating their possible roles in the host–parasite interaction. With the advent of cDNA microarray technology (Schena *et al.*, 1995) thousands of genes can now be tested simultaneously for their developmental or tissue-specific expression patterns. Such microarray hybridizations can further serve to characterize previously identified genes, but they can also be used to identify genes that warrant greater characterization.

Until now, the microarray expression analysis of nematode genes has been confined to the free-living species *Caenorhabditis elegans* (Jiang *et al.*, 2001; Reinke *et al.*, 2000). In the present paper, we have explored the use of microarray technology to study gene expression in a plant-parasitic nematode. A microarray was constructed containing cDNAs from a library made from cytoplasm microaspirated from the oesophageal gland cell region of *H. glycines* (Wang *et al.*, 2001). By employing a linear RNA amplification procedure, we were able to synthesize fluorescent probes from the mRNA of different life stages of *H. glycines* for array hybridizations. These hybridizations provided detailed developmental expression profiles of previously identified nematode genes (Wang *et al.*, 2001). In addition, these hybridizations were used to search among the uncharacterized library clones for additional genes with a developmentally regulated expression pattern.

RESULTS

Microarray construction and hybridizations

A random-primed cDNA library was previously made by RT-PCR from mRNA that was microaspirated from the oesophageal gland cell region of parasitic juveniles of *H. glycines* (Wang *et al.*, 2001). PCR-amplified cDNAs from this library were printed into an eight-subgrid microarray on glass slides (Fig. 1A). In addition to library clones that had previously been isolated through signal peptide selection (Wang *et al.*, 2001), the array contained 1358 uncharacterized cDNAs that were taken directly from the library. For fluorescence signal normalization, each of the eight array subgrids included a set of 10 plant cDNAs and samples of *H. glycines* genomic DNA (Fig. 2). All DNA samples were printed in duplicate as adjacent spots.

Messenger RNA samples were prepared from different nematode life stages and were subjected to a single round of amplification with T7 RNA polymerase (Salunga *et al.*, 1999). The amplified RNA (aRNA) was used for the synthesis of Cy3-dUTP and Cy5-dUTP labelled single strand cDNA probes. The probe synthesis reactions were spiked with a cocktail of control mRNAs from plant genes to allow for balancing of the Cy3 and Cy5 fluorescence signals. Five microarray slides were hybridized with pair-wise combinations of aRNA probes from *H. glycines* life stages, each time using the aRNA of pre-parasitic second-stage juveniles (J2) as the reference sample. In four of these five experiments, the J2 aRNA was labelled with the Cy3 dye. One hybridization experiment (the comparison of J3 vs. J2) was repeated with the dyes reversed so that the J2 aRNA was labelled with the Cy5 dye. Fluorescence signals of cDNA spots were used for data analysis only if their intensity exceeded a local threshold level that was recommended in the manual to the ScanAlyze image analysis software used (see Experimental procedures).

Evaluation of previously isolated, signal peptide-selected clones

Previously isolated cDNAs (Wang *et al.*, 2001) and their developmental expression profiles obtained in this study are shown in Table 1. Of two cDNAs that are expressed exclusively in the dorsal oesophageal gland, clone SYV46 (Fig. 1D) was present in higher concentrations in the parasitic life stages (J3, female J4, and females), whereas clone SY20 consistently gave a stronger expression signal in pre-parasitic J2. The secretory gene SYV80 was up-regulated in J3, female J4 and females, but could not be evaluated in eggs because of weak fluorescence signals. Four other cDNAs (SYS7, SYV52, SYS79 and SYV55) had low expression ratios in all array hybridizations, indicating that these genes are specifically up-regulated in the pre-parasitic J2 stage. Of these clones, SYV52 is identical to the subventral oesophageal gland-specific β-1,4-endoglucanase gene *Hg-eng-1* (Fig. 1B; Smant *et al.,* 1998), for which this exact expression profile has previously been determined through *in situ* hybridization (De Boer *et al.*, 1999).

Evaluation of uncharacterized gland library cDNAs

In the hybridized arrays, 25–63% of the uncharacterized library cDNAs produced fluorescence signals above the ScanAlyze threshold level. The signal scatter plots of the microarray hybridizations all presented the same two major clusters of cDNAs, as shown for the hybridization with J3 aRNA and J2 aRNA in Fig. 3. Sequencing of 110 cDNA clones, followed by additional array hybridization with gene-specific probes (data not shown),

Fig. 1 Overview of the soybean cyst nematode cDNA microarray and *in situ* hybridization patterns of selected nematode genes from the array. (A) Scanned image of an array hybridized with Cy3-labelled aRNA of J2 (green signal) and Cy5-labelled aRNA of J3 (red signal). cDNA spots hybridizing with both the Cy3 and the Cy5 probe are shown in various shades of brown or yellow. The marked cDNAs are described in the text. The blue box outlines a single subgrid of which the layout is shown in Fig. 2. (B) Subventral gland-specific expression (dark brown) of the *Hg-eng-1* gene in a pre-parasitic J2 of *H. glycines*. The arrow points at one of the many *Hg-eng-1* cDNA fragments present in the array. (C) *In situ* hybridization of an RNA probe of SCN1018 to a rear body section of a female of *H. glycines*. Staining (arrow) is localized to hypodermal tissue exposed along the cut edge of the cuticle. (D) Dorsal gland-specific expression (dark brown) of the SYV46 gene in a female of *H. glycines*.

Table 1 Microarray expression profiles of *H. glycines* cDNAs previously identified from an oesophageal gland cDNA library.

Clone name	GenBank accession no	Clone description	mRNA expression ratio* Eggs/J2	J3/J2	J3/J2 (dyes reversed)	J4F/J2	Fem/J2
SY20	AF273729	*H. glycines* dorsal gland secretory gene	0.12	0.35	0.24	—	0.11
SYS7	AF159590	Similar to hypothetical *Caenorhabditis elegans* protein	0.23	0.12	0.16	0.1	0.15
SYS16	AF273730	Similar to *Dirofilaria immitis* transglutaminase precursor	0.72	0.69	0.61	0.37	0.27
SYS79	AF273732	*H. glycines* secretory protein	0.05	0.07	0.17	0.06	0.04
SYS86	AF273733	Similar to *C. elegans* hypothetical protein	0.54	1.15	0.96	0.86	0.83
SYV42	AF273735	Similar to *Mus musculus* ERp99; expressed in dorsal gland and germ line of *H. glycines*	0.5	0.95	0.89	0.83	0.25
SYV46	AF273728	*H. glycines* dorsal gland secretory protein	0.41	13.32	5.32	3.36	6.84
SYV52	AF006052	*Hg-eng-1* precursor (*H. glycines*); expressed in subventral glands	0.07	0.07	0.08	0.06	0.04
SYV55	AF159591	Similar to *C. elegans* metalloproteinase inhibitor 2 precursor	0.11	0.35	0.24	0.26	0.14
SYV80	AF273736	*H. glycines* secretory protein	—	7.23	2.27	6.75	6.13

*J2, 2nd-stage juveniles; J3, 3rd-stage juveniles; J4F, female 4th stage juveniles; Fem, females. Each column represents the average of duplicate spots in a single array hybridization. J2 probes were labelled with Cy3, except for the array with dyes reversed where the J2 probe was labelled with Cy5. Ratios < 1 indicate higher expression in the J2; ratios > 1 indicate lower expression in the J2. '—' indicates a fluorescence signal below the evaluation threshold.

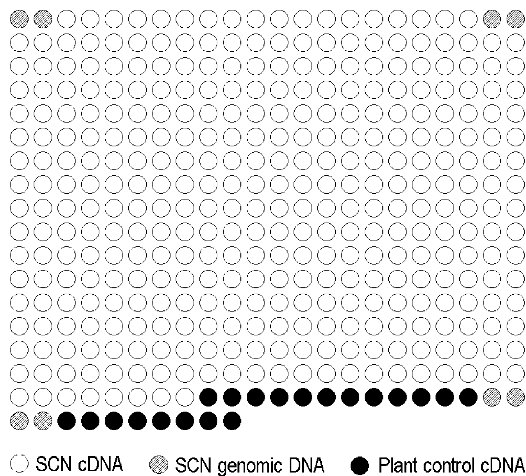

Fig. 2 Subgrid layout of the soybean cyst nematode cDNA microarray. Each sample was spotted as a pair of adjacent duplicate spots, using a 200 μm spacing between spots. The bottom two rows contained 10 plant cDNAs that were used for calibration of the fluorescence channels. Additionally, eight spots of soybean cyst nematode genomic DNA are included per subgrid.

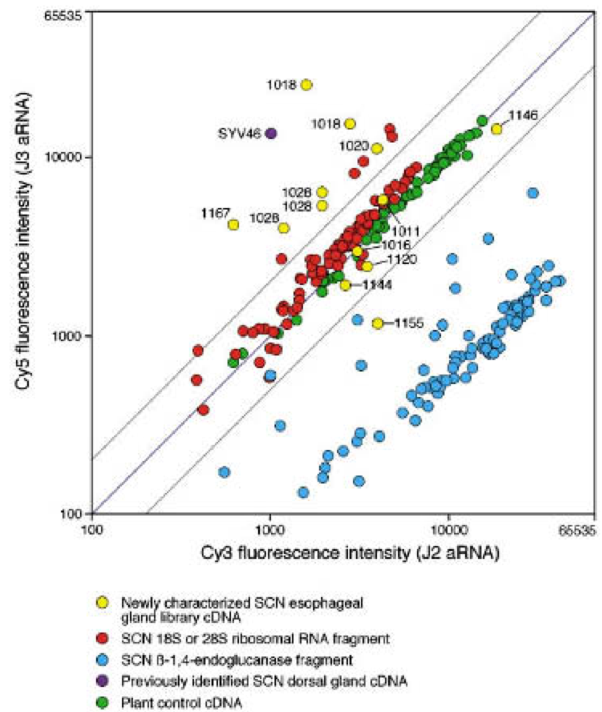

Fig. 3 Fluorescence intensity scatter plot of a microarray hybridization comparing J2 and J3. The averages of the background-subtracted fluorescence intensities of duplicate spots in the array are plotted. Black lines mark the 0.5× and 2.0× expression differences between J2 and J3. The library was found to consist predominantly of ribosomal RNA (red) and β-1,4-endoglucanase (blue) clones.

revealed the identity of the cDNAs in these clusters (Fig. 3). A group of J2 up-regulated spots consisted almost exclusively of fragments from the previously identified *Hg-eng-1* gene coding for a cellulase enzyme (Smant *et al.*, 1998). The other cluster of cDNAs, which invariably was positioned parallel to the 1 : 1 intensity diagonal, consisted mostly of 18S and 28S *H. glycines* ribosomal RNA fragments. These inadvertent cloning artefacts were used to assemble a partial sequence for the 18S rRNA of *H. glycines*, which was deposited in GenBank (accession no. AY043247). This occurrence of two major cDNA groups is explained by the low complexity of the cDNA library used.

A comparison of the scatter plots of all hybridized arrays identified four cDNAs that were up-regulated in one or more of the parasitic life stages examined (Fig. 3 and Table 2): SCN1018, SCN1020, SCN1028 and SCN1167. Southern blot hybridizations using these cDNAs as hybridization probes confirmed these clones to be of *H. glycines* origin (data not shown). Clone SCN1018 was found twice in the array, namely as a 580 bp and a 975 bp fragment (Figs 1A and 3), and was predicted by our analyses to be up-regulated in J3, female J4 and females. The larger cDNA fragment, which encompassed the smaller fragment, encoded the N-terminal 313 amino acids of a protein with a signal peptide and a C-type lectin domain (Fig. 4). *In situ* hybridization with digoxigenin-labelled antisense RNA and DNA probes, synthesized from the 580 bp fragment, indicated that SCN1018 is expressed in the hypodermis of females

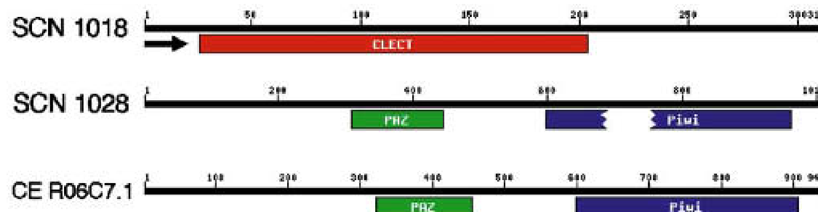

Fig. 4 Conserved domains present in predicted protein sequences of *H. glycines* (SCN1018 and SCN1028) and *Caenorhabditis elegans* (R06C7.1) cDNAs. The diagram of SCN1018 represents a partial sequence, showing only the N-terminal 313 amino acids. Clone SCN1018 has a C-type lectin domain (CLECT) that is preceded by a signal peptide (arrow). SCN1028 had high similarity to the hypothetical *C. elegans* protein R06C7.1. Both sequences possess a PAZ and a piwi domain (pfam database entries 02170 and 02171, respectively). The piwi domain of SCN1028 is split in two by a sequence from amino acids 689–752 that does not match the consensus piwi sequence.

Table 2 Microarray expression profiles of newly identified cDNAs from an oesophageal gland cDNA library of *H. glycines*.

Clone name	Size (bp)	GenBank accession no.	Homology search (BlastX) or conserved domain search (RPS-Blast)	Bits score/ *P*-value	Signal peptide†	mRNA expression ratio*				
						Eggs/J2	J3/J2	J3/J2 (dyes reversed)	J4F/J2	Fem/J2
SCN 1011	659	AY043248	Gluthathione reductase (*Onchocerca volvulus*)	227/8e-65	No	0.72	1.34	1.05	0.86	0.52
SCN 1016	1052	AY043249	Hypothetical ABC transporter W04C9.1 (*C. elegans*)	146/9e-67	Yes	0.37	0.95	0.48	0.46	0.3
SCN 1018	975	AY043250	C-type lectin domain	55/4e-9	Yes	0.23	9.22	4.22	9.66	6.53
SCN 1020	650	AY043251	Probable S-adenosylmethionine synthetase C06E7.3 (*C. elegans*)	212/3e-54	No	0.51	2.79	1.69	1.65	0.75
SCN 1028	3464‡	AY043252	Germ-line PAZ/Piwi protein R06C7.1 (*Caenorhabditis elegans*)	305/3e-81	No	0.35	3.09	2.34	1.49	1.61
SCN 1120	255	AY043253	Hypothetical protein F55A11.3 (*C. elegans*)	106/2e-22	Yes	0.35	0.69	0.53	0.45	0.33
SCN 1144	279	AY043254	Hypothetical protein Y48A6B.3 (*C. elegans*)	92/8e-18	—	0.68	0.72	0.58	0.96	0.71
SCN 1146	175	AY043255	DNA J-like heat shock protein (*Mus musculus*)	56/2e-7	—	0.68	0.77	0.42	0.45	0.44
SCN 1155	185	AY043256	Muscle-specific serine kinase 1 (*Homo sapiens*)	59/9e-8	—	0.28	0.29	0.23	0.26	0.17
SCN 1167	142	AY043257	No significant homologies	—	—	0.87	6.66	4.82	2.92	3.71

*J2, 2nd-stage juveniles; J3, 3rd-stage juveniles; J4F, female 4th-stage juveniles; Fem, females. Each column represents the average of duplicate spots in a single array hybridization; expression ratios for SCN1018 and SCN1028 are averages of, respectively, 2 and 3 independent samples in the array. J2 probes were labelled with Cy3, except for the array with dyes reversed where the J2 probe was labelled with Cy5. Ratios < 1 indicate higher expression in the J2; ratios > 1 indicate lower expression in the J2.
†Presence or absence of signal peptide as predicted from sequence (Nielsen *et al.*, 1997).
‡Isolated cDNA is full length; all other cDNAs are partial.

(Fig. 1C). No *in situ* hybridization signals were obtained in pre-parasitic J2, confirming the expression profile determined by our microarray analyses. Analysis of the (partial) protein sequence by Psort II (Nakai and Horton, 1999) predicted the SCN1018 protein to be targeted to the extracellular space.

Clone SCN1028 occurred three times in the array, with (near-) identical fragments of 437, 456 and 456 bp (Figs 1A and 3). Microarray analyses consistently predicted this gene to be up-regulated in J3. A cDNA library prepared from female J4 of *H. glycines* was screened for clones hybridizing with the 456 bp cDNA fragment. This procedure identified a 3464 bp full-length cDNA that encodes a predicted protein of 1014 amino acids. Sequence analysis with Psort II predicted this protein to be cytoplasmic. The full-length sequence of SCN1028 has a high similarity to the *Caenorhabditis elegans* proteins R06C7.1 and F55A12.1 (Blast *P*-values, respectively, 2e-72 and 3e-68). Both SCN1028 and these *C. elegans* proteins contain two hypothetical protein domains with an as-yet unknown function (Cerutti *et al.*, 2000): a PAZ domain and a piwi domain (Fig. 4). Clustal-W alignment of the protein sequences of SCN1028, R06C7.1 and F55A12.1 indicated that the piwi domain of SCN1028 is interrupted between amino acids 689 and 752 by a sequence unrelated to the consensus piwi domain (Fig. 4). *In situ* hybridizations with RNA and DNA probes synthesized from the 437 bp fragment of SCN1028 yielded no positive signals in J2, J3, female J4 or females.

Clone SCN1167 (142 bp), which our microarray analyses predicted to be up-regulated in J3, female J4 and females, yielded no homologies in database searches. Attempts to isolate a longer fragment of this cDNA from the female J4 cDNA library were unsuccessful. *In situ* hybridizations with RNA probes of SCN1067 on J2, J3 and J4 were unable to reveal a tissue-specific staining pattern.

Clone SCN1020, which only showed up-regulation in the J3 and J4 females, contains the first two of the three conserved domains of S-adenosyl-methionine synthetase (Takusagawa *et al.*, 1996). *In situ* hybridization with an antisense RNA probe of SCN1020 produced a strong general tissue stain in J4 females. In pre-parasitic J2, no *in situ* hybridization signal was obtained.

In the process of sequencing cDNAs from the major spot clusters in the hybridization scatter plots, six additional undescribed *H. glycines* genes (SCN1011, SCN1016, SCN1120, SCN1144, SCN1146 and SCN1155) were identified (Fig. 3; Table 2). All of these cDNAs hybridized to *H. glycines* DNA in Southern

blots. It is of note that clone SCN1155 showed a consistent up-regulation in J2. Its similarity to a human muscle-specific serine protein kinase suggests that this gene may be expressed predominantly in the body wall muscle cells, which are present and active only in J2 and males of *H. glycines.*

Calibration of array signals

In the present paper, the Cy3 and Cy5 fluorescence intensities of the hybridized arrays were balanced using a set of 10 different cDNAs of plant origin (Figs 2 and 3). Samples of *H. glycines* J2 genomic DNA were also included in the array (Fig. 2) to evaluate whether they could be used for signal balancing (Lashkari *et al.*, 1997). It was found, however, that the genomic DNA produced relatively weak fluorescence signals (Fig. 1A), with on average only 38% of the spots having an intensity above the threshold for data analysis. After balancing the signals with the plant control cDNAs, the genomic DNA spots consistently showed a 1.5–2-fold stronger hybridization signal with the J2 aRNA as compared to the aRNA of the other life stages. From these observations it was concluded that genomic DNA is less suitable for the signal normalization of microarrays containing nematode genes.

DISCUSSION

The present paper reports the first use of a DNA microarray to examine the expression of genes from a plant-parasitic nematode. The aim of this study was to determine the expression profiles of known parasitism genes and to identify additional genes with similar expression profiles, indicating a potential involvement in the interaction of *H. glycines* with its host plant. For this purpose, a microarray was printed containing clones from a cDNA library of the oesophageal gland region of *H. glycines*. To allow the detection of genes of which the expression is regulated during parasitism, the array was hybridized with aRNA probes from different life stages, each time using aRNA from pre-parasitic J2 as the reference sample. This procedure identified four undescribed SCN genes (SCN1018, SCN1020, SCN1028 and SCN1167) that showed an elevated expression in one or more of the parasitic life stages. Three of these cDNAs revealed similarities to known genes.

Clone SCN1018, which has an N-terminal C-type lectin domain (Drickamer, 1988), showed weak similarities to a variety of other genes possessing lectin domains. No similarity hits were found with the sequence outside this lectin domain area, suggesting that clone SCN1018 may represent a lectin that is unique to plant-parasitic nematodes. The *in situ* localization pattern of SCN1018 transcripts in the hypodermis of females, together with its predicted targeting to the extracellular space, indicate that this protein is secreted from the hypodermis and is therefore possibly a component of the cuticle. Both in a cockroach (Jomori and Natori, 1992) and a tunicate (Suzuki *et al.*, 1990), C-type lectins are involved in antibacterial defence. It is possible that SCN1018 has a similar defence function in *H. glycines.*

The presence of both a PAZ and a piwi domain in the SCN1028 protein places this gene's translation product in the family of piwi proteins (Cerutti *et al.*, 2000). The functions of the PAZ and piwi domains are as yet unknown, but several proteins belonging to the piwi family are involved in germ-line stem cell division (Cox *et al.*, 1998). In *C. elegans*, the expression of R06C7.1, which is highly similar to SCN1028, is strongly elevated in germ-line cells (Reinke *et al.*, 2000). It is therefore possible that SCN1028 is also expressed in germ-line cells. The observed up-regulation of SCN1028 in J3 is consistent with this gene being expressed in gonadal tissue, because at this life stage, cell divisions of the genital primordium are taking place that lead to the formation of the gonads.

The previously described *H. glycines* genes SYV46, SY20 and SYV52, which code for secretory proteins, were shown by *in situ* hybridization to be specifically expressed in the oesophageal gland cells (Wang *et al.*, 2001). The up-regulation of SYV46 in the parasitic life stages, as revealed by our microarray analyses, may indicate that its translation product is involved in late events of parasitism like nematode feeding or maintenance of feeding cells. Furthermore, this gene was also isolated from a different gland cDNA library from later parasitic stages (Gao *et al.*, 2001). On the other hand, the microarray analyses revealed gene SY20 to be up-regulated in J2, suggesting a function in the earlier events of parasitism. The SYV52 cDNA fragment, which corresponded to *Hg-eng-1* (Smant *et al.*, 1998), is known to be highly expressed in the subventral oesophageal gland cells of pre-parasitic J2 (Fig. 2B) and to become down-regulated in the feeding, parasitic life stages (De Boer *et al.*, 1999). This expression pattern was exactly confirmed by the microarray expression data.

By printing a microarray from uncharacterized cDNAs from the oesophageal gland cell region of parasitic juveniles of *H. glycines*, it was anticipated that additional undescribed gland secretion genes could be identified. However, the array hybridizations revealed that the library was of low complexity, consisting for a large part of cellulase and ribosomal RNA genes. Moreover, a cDNA was identified (SCN1018) for which the expression was localized in the hypodermis, demonstrating that the library also contained clones from non-gland tissues. These library properties are due to the technical difficulty of gland aspiration and library construction from extremely minute amounts of starting material. As a consequence, no novel gland secretion genes were identified among the library clones that were up-regulated in the parasitic stages. Nevertheless, from the distinct expression pattern of the dorsal gland-specific gene SYV46 it can be concluded that microarray analysis is able to identify similarly expressed gland genes among uncharacterized nematode cDNAs. A requirement

for such identifications will be the availability of better oesophageal gland cell cDNA libraries, in which clone redundancy has been decreased and tissue specificity has been increased. Such libraries are now available through (a) the use of suppressive subtractive hybridization and (b) more successful cloning strategies (Gao *et al.*, 2001).

The application of molecular techniques to plant-parasitic nematodes is traditionally hampered by the limited quantities of biomolecules that can be obtained from these microscopically sized obligate biotrophs. In particular, the collection of parasitic life stages from roots is an exceedingly labour-intensive process that effectively limits the direct synthesis of microarray probes from nematode mRNA. We were able to overcome this limitation by applying a linear mRNA amplification procedure with T7 RNA polymerase (Salunga *et al.*, 1999). This amplification procedure, for instance, allowed hybridizations to be performed with RNA from the J3 stage, which would otherwise have been impossible to do.

With the start of different EST sequencing projects (Dautova *et al.*, 2001; McCarter *et al.*, 2000; Popeijus *et al.*, 2000a; Hussey, Davis and Baum, unpublished results), unigene sets containing a large number of plant-parasitic nematode cDNAs will soon be available for microarray expression analysis. Equally, higher-quality gland-specific cDNA libraries are now available. Future microarrays containing such cDNAs will be powerful tools for identifying genes that are differentially expressed between the parasitic and non-parasitic life stages, allowing for more direct searches for genes that are important in the host–parasite interaction of plant-parasitic nematodes. The groundwork presented in this publication will be instrumental in realizing such endeavours.

EXPERIMENTAL PROCEDURES

Microarray sample preparation

A cDNA library was previously constructed in the vector pRK18 from microaspirated oesophageal gland cell contents of *H. glycines* by Wang *et al.* (2001). A total of 1440 single colony cultures of library clones were grown in 100 μL LB-ampicillin broth in 96-well PCR plates. After the addition of 100 μL of sterile water, the plates were heated for 20 min at 99 °C, and the *E. coli* cells were spun down. Five μL of supernatant was then used in 100 μL-volume PCR reactions in 96-well PCR plates. The cDNAs were amplified with the C6-amino-linked vector primers RK18.2 (5′-TCACTAACGAAGCTTATCGG-3′) and RK18.2B (5′-TTGGGTGTGAAGTGGACCAA-3′). The PCR products were cleaned by isopropanol precipitation, and the 70% ethanol-washed pellets were dissolved in 35 μL 2× SSC (20× SSC stock = 3.0 M NaCl, 0.3 M Na-citrate, pH 7.0). Three μL of each DNA sample were checked by electrophoresis for the presence of amplification product. The success rate of PCR amplification of these uncharacterized cDNAs was 94%. The size of the amplified cDNA fragments ranged from 200 to 1200 bp. The microarray included a series of 10 plant control cDNAs (explained below), which were PCR-amplified with the corresponding vector primers and processed as described for the gland library cDNAs. Genomic DNA was isolated from *H. glycines* pre-parasitic J2 using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) and was dissolved in 2× SSC to a concentration of 100 ng/μL.

Microarray design and printing

A 9 × 18 mm microarray consisting of eight subgrids (Figs 1A and 2) was printed from the prepared DNA samples on silylated (Cel Associates Inc., Houston, TX, USA) microscope slides using a PixSys PA 5500 arraying robot (Cartesian Technologies, Irvine, CA, USA) equipped with eight printing pins. All samples were spotted in duplicate within their respective subgrid. A set of 10 soybean and *Arabidopsis thaliana* cDNA fragments (D21.4, A1.1, A6.1, A10.1, A11.1, A20.2, A21.6, A31.4, A37.3 and A44.4) was included in each subgrid (Fig. 2) to allow for balancing of both fluorescence channels (Hermsmeier *et al.*, 1998, 2000). The size of these cDNA fragments ranged from 300 to 550 bp. Preliminary microarray hybridizations showed that Cy3- and Cy5-labelled probes prepared from these plant cDNAs did not cross hybridize to the arrayed nematode cDNAs. In addition, Cy3- or Cy5-labelled nematode mRNA from J2 or adult females did not show cross hybridization to the 10 plant cDNAs. Additionally, each subgrid contained eight spots with *H. glycines* genomic DNA, intended to assist with data normalization.

The dried microarrays were briefly hydrated above warm water and snap-dried on a hot plate. The DNA then was crosslinked to the slide surface by exposure to 120 mJ/cm^2 ultraviolet light. The arrays were washed twice for 2 min in 0.2% SDS and twice for 2 min in water with vigorous stirring. The arrays then were heated in boiling water for 5 min, air dried for 5 min, incubated in 0.25% sodium borohydride in 25% ethanol for 15 min, washed 3 × 1 min in 0.2% SDS and 1 min in water, heated in boiling water for 5 min, and air dried.

RNA isolation and synthesis

H. glycines was propagated on soybean in a greenhouse. Pre-parasitic second-stage juveniles (J2) were hatched from eggs, and the eggs were isolated from cysts as previously described (De Boer *et al.*, 1999). Parasitic life stages were isolated from infected roots as previously described (De Boer *et al.*, 1999). Total RNA was isolated from *H. glycines* life stages with a PureScript RNA isolation kit (Gentra Systems, Minneapolis, MN, USA), using approximately 300 mg eggs, 250 mg J2, 150 mg third-stage parasitic juveniles (J3), 200 mg fourth-stage female juveniles (J4) and 100 mg females per extraction. These tissue fresh weights

were determined after removing interstitial water from the nematodes with a fine glass capillary. Eggs and pre-parasitic J2 were frozen with liquid nitrogen in a mortar and powdered with a pestle before adding the cell lysis solution. Parasitic life stages and females were frozen in microfuge tubes, and cell lysis solution was added to the frozen nematodes before homogenizing them with a 3 mm diameter round-tipped steel pin. Yields ranged from 1.6 μg (J3) to 4.6 μg (J4) total RNA per mg nematode tissue. Poly(A) mRNA was purified from the total RNA samples using biotin-tagged oligo(dT) primer and streptavidin-coated paramagnetic particles (PolyATract kit; Promega Corp., Madison, WI, USA). The mRNA was eluted from the particles with 250 μL water and concentrated to 10–20 μL by centrifugation in a Microcon-30 centrifugation filter (Millipore Corp., Bedford, MA, USA). The mRNA was spectrophotometrically quantified and inspected for the absence of genomic DNA and ribosomal RNA by electrophoresing an aliquot on an agarose gel. The mRNA yields for eggs, J2, J3, J4 and females were respectively 17, 17, 18, 39 and 14 ng per mg tissue.

Unlabelled antisense RNA was synthesized in a single round of amplification from the mRNA of *H. glycines* life stages as described in detail by Salunga *et al.* (1999), with slight modifications. In short, 100 ng of nematode poly(A) mRNA were reverse transcribed using T7-oligo$(dT)_{21}$ primer and SuperScript II reverse transcriptase (Life Sciences, Rockville, MD, USA). The second-strand reaction was performed using EcoPol buffer (1× concentration is: 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 7.5 mM dithiothreitol). *E. coli* DNA ligase was omitted from the second-strand reaction. Following phenol/chloroform extraction, the cDNA was washed and concentrated using a Microcon-30 centrifugation filter (Millipore) and was further concentrated to approximately 8 μL by vacuum centrifugation. RNA was transcribed from the cDNA with T7 RNA polymerase in a 20 μL reaction using the MEGAscript High Yield Transcription Kit (Ambion Inc., Austin, TX, USA). The amplified RNA (aRNA) was purified with the PureScript kit (Gentra Systems) and quantified with a spectrophotometer. Typical yields were 40 μg aRNA per 100 ng mRNA starting material.

Unlabelled sense RNA was transcribed *in vitro* from each of the 10 plant control cDNAs using linearized plasmid as a template. The plant RNA samples were cleaned (PureScript RNA isolation kit) and quantified spectrophotometrically. The plant RNAs then were mixed to produce a cocktail in which the concentration (w/v) of each successive RNA decreased by a factor of two. The total RNA concentration of this cocktail was 10 ng/μL, with 10 pg/μL for the least abundant RNA and 5000 pg/μL for the most abundant RNA.

Microarray probe synthesis

Three μg of nematode aRNA was mixed with 5 ng of plant mRNA cocktail and 1 μL of hexanucleotide mix (Roche, Indianapolis, IN). The RNA/primer mixture then was reverse transcribed for 2 h at 37 °C in a 30 μL volume in the presence of 200 units of SuperScript II reverse trancriptase (Life Technologies), 1× first strand buffer (Life Technologies), 10 mM DTT, 0.5 mM d(ACG)TP, 0.2 mM dTTP, and 0.1 mM of either Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia Biotech, Piscataway, NJ). The labelled reverse transcription products were incubated with RNase A and RNase H at 37 °C for 30 min. For each microarray hybridization, the Cy3- and Cy5-labelling reactions (each equivalent to 3 μg aRNA) were pooled and cleaned using a spin column (QIAquick PCR Purification Kit, Qiagen Inc., Valencia, CA). The probe was eluted from the column with 50 μL 10 mM Tris-HCl pH 8.5 and concentrated to about 8 μL in a vacuum centrifuge.

Microarray hybridization and analysis

The arrays were hybridized and washed essentially as described by Hegde *et al.* (2000), using hybridization solution containing 1 μg/μL poly(A) RNA and 1 μg/μL yeast tRNA for blocking purposes and a humidity box for the hybridization. The arrays were scanned with a ScanArray 5000 laser scanner (GSI Luminomics, Watertown, MA, USA). Fluorescence signals were quantified from the scanned images using version 2.35 of the image analysis program SCANALYZE (Stanford University, USA). The Cy3 and Cy5 fluorescence signals were balanced by multiplying the Cy5 signals with a correction factor such that the average of the Cy5/Cy3 signal ratios of the plant control spots became one. Plant control cDNAs that presented saturated pixels in the array scans (typically 1–6 samples per subgrid) were not used for signal balancing. Cy5/Cy3 fluorescence ratios were calculated from the average spot fluorescence intensities after the background signal had been subtracted. The median (instead of average) background intensity of the area surrounding the spot was used for this background subtraction. The CH1GTB2 and CH2GTB2 parameters in the SCANALYZE data output file were used to remove low quality spots from the data analysis. For channel 1 and channel 2, respectively, these two variables give the percentage of pixels within a spot that have a fluorescence intensity of more than 1.5-fold the local background intensity. As recommended in the SCANALYZE manual, a threshold of 65% was used to select for spots with signals above the local background intensity.

CDNA library construction and screening

Poly(A) RNA from female J4 of *H. glycines* was cloned into the Stratagene Uni-ZAP XR vector system essentially following the cDNA Synthesis Kit Instruction Manual (Stratagene, La Jolla, CA, USA). Two μg of poly(A) RNA was converted to cDNA, which was then fractionated on a Sepharose CL-2B gel column. Fractions containing cDNA of > 500 bp were pooled and 100 ng of cDNA was ligated with 1 μg Lambda ZAP vector (Stratagene). The

ligation product was packaged using Gigapack III Gold packaging extract (Stratagene). After amplification, the library was estimated to contain 1.6×10^9 pfu/mL. An aliquot of the library was plated on four 15 cm NZCYM Petri dishes at a density of 3.0×10^4 pfu/plate and was screened by membrane hybridization for clone SCN1028, essentially as previously described (De Boer *et al.*, 2002). The radioactively labelled probe was prepared from the 456 bp fragment of SCN1028 by PCR using RK18 vector primers.

Southern blot hybridizations

Five micrograms of genomic DNA from pre-parasitic J2 of *H. glycines* were digested with *Dde*I, *Hin*fI and *Eco*RI (Gibco BRL, Rockville, MD, USA), separated by electophoresis on a 0.8% agarose gel, and transferred on to Nytran SuperCharge Nylon membrane (Schleicher and Schuell, Keene, NH, USA) using standard techniques (Sambrook and Russell, 2001). Membranes were hybridized with radioactively labelled probes synthesized by PCR from *H. glycines* cDNAs in the vector pRK18. Positive bands were identified by exposing BioMax MR film (Kodak, Rochester, NY, USA) to the membranes.

Sequence analysis

The template for sequencing of uncharacterized library cDNAs was prepared either by isolating plasmid or by PCR amplification of inserts from plasmid. The cDNA samples were sequenced at the DNA Sequencing and Synthesis Facility of Iowa State University. In about 6% of the sequence runs, evidence was presented that an arrayed cDNA actually consisted of a mixture of clones. Such mixed spots were omitted from the gene expression analysis. Database sequence similarity searches and protein conserved domain searches were performed at the National Center for Biotechnology Information <http://www.ncbi.nlm.nih.gov> using version 2.2 of the program BLASTX (Altschul *et al.*, 1997). Signal peptide-prediction was performed using the program SIGNALP 2.0 (Nielsen *et al.*, 1997) at <http://www.cbs.dtu.dk/services/SignalP>. Sequence alignments were performed with CLUSTALW (version 1.81) at the European Bioinformatics Institute <http://www.ebi.ac.uk/clustalw/> using default alignment parameters.

In situ hybridizations

Whole mount *in situ* hybridizations with antisense RNA or DNA probes were performed on formaldehyde-fixed, razor blade-cut nematode life stages, essentially as previously described (De Boer *et al.*, 1999). Specificity of hybridization was verified with the corresponding sense probes. The template for *in vitro* transcription of digoxigenin-labelled RNA probes was synthesized by PCR from *H. glyines* cDNAs in the vector pRK18 using extended RK18.2 and RK18.2b vector primers that contained, respectively, the T3 and T7 promoter sequence at their 5′ end. An aliquot (0.5–1.0 μL) of these PCR products was added to 20 μL probe synthesis reactions containing either T3 or T7 RNA polymerase. Single strand DNA probes were synthesized by asymmetric PCR with RK18 vector primers as previously described (Wang *et al.*, 2001).

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