Comparison of transcript profiles in different life stages of the nematode *Globodera pallida* under different host potato genotypes

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SUMMARY

The potato cyst nematodes (PCNs) *Globodera pallida* and *Globodera rostochiensis* are important parasites of potato. PCNs undergo complex biotrophic interactions with their hosts that involve gene expression changes in both the nematode and the host plant. The aim of this study was to determine key genes that are differentially expressed in *Globodera pallida* life cycle stages and during the initiation of the feeding site in susceptible and partially resistant potato genotypes. For this purpose, two microarray experiments were designed: (i) a comparison of eggs, infective second-stage juveniles (J2s) and sedentary parasitic-stage J2s (SJ2); (ii) a comparison of SJ2s at 8 days after inoculation (DAI) in the susceptible cultivar (Desirée) and two partially resistant lines. The results showed differential expression of *G. pallida* genes during the stages studied, including previously characterized effectors. In addition, a large number of genes changed their expression between SJ2s in the susceptible cultivar and those infecting partially resistant lines; the number of genes with modified expression was lower when the two partially resistant lines were compared. Moreover, a histopathological study was performed at several time points (7, 14 and 30 DAI) and showed the similarities between both partially resistant lines with a delay and degeneration in the formation of the syncytia in comparison with the susceptible cultivar. Females at 30 DAI in partially resistant lines showed a delay in their development in comparison with those in the susceptible cultivar.

INTRODUCTION

The potato cyst nematodes (PCNs) *Globodera pallida* and *Globodera rostochiensis* are important parasites of potato (Grenier *et al.*, 2001). PCNs undergo complex biotrophic interactions with their hosts that involve gene expression changes in both the nematode and the host plant. Eggs of PCNs, containing second-stage juvenile

(J2) nematodes, remain dormant within the cysts in the soil until stimulated by the presence of a suitable host plant growing nearby (reviewed by Perry, 1989). The vermiform J2 then hatches and invades the root of the host plant. Each individual nematode feeds on a group of cells in the pericycle, cortex or endodermis, transforming them into a multinucleate, metabolically active syncytium. The sedentary juvenile nematode (SJ2) remains immobilized for the rest of its development, as it passes through two more juvenile stages to become either male or female. Males are active and leave the root to find and fertilize females. Females retain the fertilized eggs of the next generation inside their bodies, and the first moult from J1 to J2 occurs in the egg. When the females are fully mature, they die, and their cuticle hardens and turns brown to form a cyst around the eggs.

Some *G. rostochiensis* pathotypes present in Europe are controlled by the use of host cultivars containing the major resistance gene *H1*. However, similar major gene resistance for European *G. pallida* pathotypes has not yet been identified. Several genetic loci that provide quantitative (partial) resistance to *G. pallida* pathotypes Pa2 and Pa3 have been identified that are derived from diploid relatives of cultivated potato, including *Solanum vernei*, *S. tuberosum* ssp. *andigena* CPC1673 (only Pa2) and CPC 2802 (Pa2 and Pa3), *S. sparsipilum*, *S. spegazinii* and *S. tarijense* (Moloney *et al.*, 2009). Some of these sources, particularly those from *S. vernei* and *S. tuberosum* ssp. *andigena*, have been incorporated into commercially available cultivars, whereas others are still in earlier stages of the breeding process. Quantitative resistance against cyst nematodes often does not involve a classical hypersensitive response (HR); instead, the development of the feeding site is restricted by a response from the cells surrounding this structure (Rice *et al.*, 1985, 1987; Sobczak and Golinowski, 2011). As sex determination of PCN is determined by environmental factors (such as food availability), rather than the nematode genotype (Grundler *et al.*, 1991; Mugniéry and Fayet, 1981, 1984), a restricted feeding site results in a sex ratio bias towards males.

Although some studies have been performed on changes in gene expression in nematodes at various stages of their life cycles (Elling *et al.*, 2007; Ithal *et al.*, 2007; Klink *et al.*, 2009a), these

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studies have not examined either PCN species. In addition, little is known about the response of nematodes to different quantitative resistance sources.

Microarrays offer the possibility to simultaneously analyse the expression profiles of a large number of genes, and require less starting material than equivalent next-generation sequencing alternatives. This is important when studying an obligate biotroph, such as PCN, where scarcity of material is a major issue. Some microarray studies have been conducted on *Heterodera glycines* at different life stages and with different populations (Elling *et al.*, 2007; Ithal *et al.*, 2007; Klink *et al.*, 2009a, b). Arrays have also been used in the search for novel putative parasitism genes (effectors) (Elling *et al.*, 2009). However, for *G. pallida*, studies have focused more strongly on specific genes rather than on large-scale analysis of gene expression changes (Jones *et al.*, 2003, 2009; Prior *et al.*, 2001; Sacco *et al.*, 2009).

The aim of this project was to analyse the changes in gene expression in different *G. pallida* developmental stages and to identify genes whose expression profiles are modified in sedentary nematodes under susceptible or partially resistant potato plant genetic backgrounds. As part of this latter analysis, the histopathological response of host plants containing the resistance sources to nematode infection was examined.

RESULTS

Microarray design

Expressed sequence tags (ESTs) from life stage-specific (invasive second-stage juveniles, early parasitic-stage juveniles and adult female stage) cDNA libraries of *G. pallida* and the published mitochondrial genomes were used as the basis for microarray design. These were represented as 14 649 probes on the microarray. BLAST searching, BLAST2GO and PFAM domain searches were used to provide basic information for many of the putative genes represented on the array. This allowed some information about putative function to be applied to 45% of the target sequences (2564 of 5690). In addition, BLAST searching of the root-knot nematode genomes revealed that 1460 and 1514 target sequences matched proteins in *Meloidogyne hapla* and *M. incognita*, respectively. BLAST matches in the nonredundant (NR) and root-knot nematode databases are summarized in Table S1 and Fig. S1 (see Supporting Information). Sixty-three potentially novel secreted proteins (genes with a predicted signal peptide and lacking a transmembrane domain) identified in a previous study were represented on the microarray (Jones *et al.*, 2009).

Microarray analysis of nematode life cycle stages

The majority of the microarray target sequences, 4306 from 5690 (grouping targets with multiple probes), passed the quality control

(QC) filtering steps (see Experimental procedures). A comparison of gene expression at different life cycle stages [egg, invasive J2 and SJ2 at 8 days after inoculation (DAI)] identified 324 (7.5%) significantly differentially expressed putative genes (identified after taking an average of all probes for the same gene) after analysis of variance (ANOVA) using Bonferroni multiple testing correction (Tables S2–S5, see Supporting Information). Of these 324 putative genes, 147 (45.5%) showed sequence similarity to genes present in the National Center for Biotechnology Information (NCBI) NR database, and a further six and eight were similar to *M. hapla* and *M. incognita* predicted proteins, respectively. Only five of the 63 genes with a predicted signal peptide and lacking a transmembrane domain were found to be differentially expressed.

K-means clustering of putative genes with differential expression at different life cycle stages produced four main significant groups (Figs. 1 and S3, see Supporting Information). Some selected genes are shown in Table 1. The first cluster K1 stage (155 genes) represents the genes highly up-regulated in eggs and down-regulated in SJ2 nematodes. The majority of these genes were related to metabolism (Table S2). Cluster K2 stage (57 genes) represents genes with increased expression specifically in J2 compared with egg and SJ2 stages, and the majority are probably related to the first stages of penetration of the root and plant parasitism (Table S3). Numerous candidate effectors were present in this cluster, including several SPRYSEC proteins, which have previously been shown to be abundant in J2s compared with other life stages (Jones *et al.*, 2009), and the *G. pallida* effector IVG9 (Blanchard *et al.*, 2007). Cluster K3 stage (89 genes) represents genes up-regulated in SJ2 stages compared with egg and J2. Many of these genes reflect further development of the nematode and changes associated with the onset of feeding (Table S4). Cluster K4 stage (23 genes) represents genes up-regulated in J2 and down-regulated in the egg compared with the parasitic stage. These genes have more miscellaneous predicted functions (Table S5). Maximum differences between egg and parasitic stages compared with J2 were obtained in cluster K4 stage, with levels of down-regulated expression between eggs and J2 of 0.002 times, and an increase in expression of up to 7263 times between SJ2 and J2 in cluster K3 stage.

Gene ontology (GO) analysis of the differentially expressed genes showed 60 genes annotated with GO biological process (Table S6, see Supporting Information), 67 with GO molecular function (Table S7, see Supporting Information) and 41 with GO cellular compartment. Twenty-two molecular functions were modified in the stages studied, with the major molecular functions modified being binding activity and catalytic activity. K-means molecular functions and biological processes as GO annotations are shown in Tables S6 and S7. Some GO biological processes in the microarray were more represented by the genes differentially expressed in the nematode stages (i.e. GO:40029: regulation of gene expression, epigenetic; GO:9056: catabolism; GO:16032:

Fig. 1 K-means gene clustering based on expression profiles of *Globodera pallida* for egg, infective J2 (J2) and sedentary nematodes at 8 days after inoculation (DAI) (SJ2).

viral life cycle; GO:16049: cell growth). In addition to the analysis of the GO terms, simple manual functional annotation of uni-genes was performed with their top homologies to known genes in public databases, and subsequent bibliography searches (Tables S2–S5).

Histological response at 7, 14 and 30 DAI

At 7 DAI, a stronger hypersensitive reaction (with rapid death of cells near nematodes) was observed in the root of the partially resistant James Hutton Institute (JHI) potato lines (11305 and 11415) in comparison with the susceptible cultivar (Desirée). Fig. 2.1, 2.2 and 2.3 show the initial formation of the syncytial structure in cultivar Desirée in comparison with 11305 and 11415.

At 14 DAI, syncytial cells in 11305 and 11415 roots were smaller than the well-established syncytia in Desirée roots (Fig. 2.4, 2.5 and 2.6). At 30 DAI, the nematodes in 11305 and 11415 potato roots were still at the female stage and the corresponding syncytia had degenerated. In these roots, it is unlikely that any adult stage would be able to produce eggs (Fig. 2.7 and 2.8). By contrast, in Desirée roots, the newly developed cysts were full of eggs (Fig. 2.9).

Microarray analysis of sedentary nematodes in different potato genetic backgrounds

The majority of the genes, 4153 of 5690, passed the QC steps (see Experimental procedures). Comparisons of gene expression in SJ2

Table 1 Selected differentially expressed nematode genes grouped in different K-means groups during different stages of the nematode [eggs, invasive J2 (J2) and sedentary nematodes at 8 days after inoculation infecting susceptible cv. Desirée (SJ2)] in the stages microarray.

Array ID	Probe ID	Best hit ID	E-value	Best hit organism	Best hit description	Eggs/J2	SJ2/J2
K1 stage	contig28	000037, 000038, 000039	5.09E-12	<i>Bursaphelenchus xylophilus</i>	Small HSP21-like protein	3.21	0.34
	rc_contig745	013619, 013620, 013621	7.40E-53	<i>Caenorhabditis elegans</i>	Calpoin family member (cpn-3)	5.52	0.21
	Gpa_EST_P1_14-01M13F_H06_H06_017	001608, 001609, 001610	3.05E-19	<i>Simulium nigricornum</i>	Putative myosin light chain 1	2.14	0.37
	contig242	002740, 002741, 002742	6.60E-35	<i>Ditylenchus destructor</i>	Actin 1	1.10	0.35
	contig626	013222, 013223, 013224	5.85E-116	<i>Ascaris suum</i>	Synrophin-1	1.19	0.26
	gil5454762.1 gb CV578298.1 CV578298	006125, 006126	3.06E-18	<i>Brugia malayi</i>	α -Catulin	1.88	0.25
	Gpa-Est-05-1_H10_033	002974, 002975	1.25E-19	<i>Aureococcus anophagefferens</i>	β -Tubulin-like protein	0.99	0.19
	Gpa_EST_P1_17-01M13F_G01_G01_002	003858, 003859, 003860	2.15E-91	<i>Heterorhabditis bacteriophora</i>	ODoRant response abnormal family member	0.69	0.14
	Gpa-Est-05-4_E06_020	000282, 000283	1.23E-19	<i>Caenorhabditis elegans</i>	TYRAMine receptor family member (tyra-2)	1.80	0.06
	K2 stage						
K3 stage	Contig395	012419, 012420, 012421	2.31E-71	<i>Globodera pallida</i>	RBP-1 protein	0.03	0.02
	Gpa_EST_04_4_D10_037	003534, 003535, 003536	1.19E-76	<i>Globodera pallida</i>	RBP-2 protein	0.03	0.05
	Gpa_EST_06_2_A01_008	001762, 001763	1.50E-12	<i>Globodera mexicana</i>	IVG9	0.02	0.01
	gil5454574.1 gb CV577374.1 CV577374	008985, 008986, 008987	7.82E-16	<i>Ascaris suum</i>	Aquaporin-8	0.40	0.26
	J2contig169	007991, 007992, 007993	1.01E-09	<i>Caenorhabditis remanei</i>	Hypothetical protein CRE_02260	0.45	0.17
	K3 stage						
	Contig656	013326, 013327, 013328	1.55E-23	<i>Ascaris suum</i>	Cuticle collagen 13	0.74	235.00
	Gpa_EST_P1_17-01M13F_D06_D06_021	002281, 002282	4.74E-11	<i>Angiostrongylus cantonensis</i>	Cathepsin B-like cysteine proteinase 1	0.06	9.27
	Contig496	012787, 012788, 012789	8.39E-72	<i>Heterodera glycines</i>	Cathepsin S-like cysteine proteinase	0.57	79.37
	Contig666	013357, 013358	4.33E-28	<i>Heterodera glycines</i>	C-type lectin domain protein	0.90	5468.34
	gil54545190 gb CV577092.1 CV577092	000523, 000524	1.30E-32	<i>Heterodera schachtii</i>	Annexin 4F01	0.52	56.59
K4 stage	Gpa_EST_13_03_M13F_E01_004	008188, 008189, 008190	3.32E-34	<i>Ascaris suum</i>	Glutathione synthetase	0.61	60.89
	K4 stage						
	gil54544932 gb CV576963.1 CV576963	008585, 008586, 008587	1.96E-46	<i>Caenorhabditis elegans</i>	F54F11.2b	0.06	0.73
	Gpa_EST_P1_17-03M13F_F02_F02_003	004953, 004954, 004955	3.73E-44	<i>Globodera pallida</i>	RBP-4 protein	0.19	1.31
	Gpa-Est-05-3_D11_045	002381, 002382, 002383	3.55E-16	<i>Brugia malayi</i>	Phosphoglycerate mutase family protein	0.004	0.02
J2contig359	010349, 010350	XP_003115209	1.59E-30	<i>Caenorhabditis remanei</i>	CRE-CYP-33C11 protein	0.10	0.30

Fig. 2 Histological host responses of three potato genotypes to *Globodera pallida* infection stained with Safranin and Fast-green: 1, 2, 3, line 11305, line 11415 and Desirée at 7 days of exposure, respectively; 4, 5, 6, line 11305, line 11415 and Desirée at 14 days of exposure, respectively; 7, 8, 9, line 11305, line 11415 and Desirée at 30 days of exposure, respectively. c, newly developed cyst full of eggs; f, female; hr, hypersensitive reaction; n, nematode; s, syncytium. Scale bars: 1–6, 50 μ m; 7–9, 200 μ m.

within partially resistant JHI potato lines (11305 and 11415) and the susceptible cultivar (Desirée) identified 304 (7.3%) significantly differentially expressed genes (taking the average of all the probes for the same gene) from the 4153 genes after ANOVA using Bonferroni multiple testing correction (Tables S8 and S9, see Supporting Information). Of these, 136 (44.8%) genes were identified which had significant similarity to genes present in the NCBI NR database, and a further eight were similar to *M. hapla* and *M. incognita* predicted proteins, respectively. Only six of the 63 genes with a predicted signal peptide and lacking a transmembrane domain were differentially expressed in the general statistical analysis.

K-means clustering of genes with differential expression between host genotypes produced two main significant groups (Fig. S4, see Supporting Information). One cluster with 94 genes (K1 genotypes) showed higher expression in the resistant lines (Table S8), and 210 genes (K2 genotypes) had lower general

expression in resistant lines in comparison with the susceptible cultivar Desirée (Table S9). Manual functional annotation of genes was performed as for the life stages microarray (Tables S8 and S9). Comparisons of the two clusters of genes showed that different functions are regulated by the genetic background of the host plants. In addition, more putative effectors of plant-parasitic nematodes were modified in K2 genotypes than K1 genotypes (13 versus 2). Differences in the expression of genes in the K1 genotypes in comparison with cultivar Desirée ranged from 1.8 to 20 691.9 times and from 2.3 to 683.4 times in the lines 11305 and 11415, respectively, whereas probes in the K2 genotypes ranged from 0.01 to 0.5 times and from 0.009 to 0.7 times in the lines 11305 and 11415, respectively.

GO of the probes identified 63 genes annotated with GO biological process (Table S10, see Supporting Information), 66 with GO molecular function (Table S11, see Supporting Information)

and 48 with GO cellular component in the nematode genes regulated by the plant genotype. Twenty molecular functions were modified in the stages studied, and the major molecular functions identified were binding activity and catalytic activity. Different K-means biological processes and molecular functions GO annotations are shown in Tables S10 and S11, respectively. Some GO biological processes represented in the microarray were not modified by either genotype (i.e. GO:40029: regulation of gene expression, epigenetic; GO:16032: viral life cycle; GO:16049: cell growth). Several GO molecular functions did not seem to be modified by the genotype interaction with the nematodes (GO:5102: receptor binding; GO:3700: transcription factor activity; GO:8135: translation factor activity, nucleic acid binding; GO:3682: chromatin binding; GO:30234: enzyme regulator activity; GO:30528: transcription regulator activity; GO:45182: translation regulator activity).

A less restrictive statistical analysis using Student's *t*-test volcano plots of the genes differentially expressed between nematodes infecting susceptible and resistant genotypes (304 genes) showed that 24 genes were differentially expressed between the nematodes in the resistant lines (Table 2). Four of these were genes that were up-regulated in comparison with cultivar Desirée, whereas 20 of the genes were down-regulated in the initial comparison with Desirée. In total, three of the genes up-regulated were highly expressed in line 11305 and one in line 11415, whereas the rest of the genes were down-regulated less in line 11305. One of these highly regulated and differentially expressed genes matched a viral protein and is likely to be derived from a contaminant (Contig281). Although many genes important in the parasitic process of plant-parasitic nematodes are thought to have been acquired by horizontal gene transfer (e.g. Haegeman *et al.*, 2011), this sequence is not present in the current *G. pallida* genome assembly (<http://www.sanger.ac.uk/resources/downloads/helminths/globodera-pallida.html>), and is therefore more likely to be derived from contaminants. Differences in the down-regulated genes showed a wider range in number and function of genes, some of which were effectors secreted by the nematode (Table 2).

Genes affected in both experiments

Comparison of the genes changed significantly in their expression in both experiments identified 109 genes in common. These genes showed a clear pattern of association between K-means groups in both microarrays (Table 3). Only genes up-regulated in SJ2 nematodes (K3 stages, 36 genes) were up-regulated when nematodes were inoculated in partially resistant genotypes in comparison with the susceptible cultivar Desirée (K1 genotypes) (Table S12, see Supporting Information). The K2 genotype group contained genes from the K1 (44 genes), K2 (28 genes) and K4 (two genes) stages.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Seven genes expressed in both microarrays were selected for confirmation by quantitative real-time RT-PCR utilizing RNA that originated from the same experiments for hybridization to microarrays. Results of SJ2, J2, line 11315 and cultivar Desirée were used for comparisons in the microarray validation. These results are shown in Table 4. Patterns of expression correlated well between the microarray and the quantitative RT-PCR.

DISCUSSION

Microarray analysis identifies the differences in gene expression between treatments and provides insights into the complexity of the interactions between plants and nematodes. The comparison of several stages (egg, J2 and SJ2) in different potato genetic backgrounds (susceptible and partially resistant) reveals similar/different gene expression patterns between important developmental stages and during different phases of parasitism in the nematode life cycle. The majority of microarray studies involving plant-parasitic nematodes have used nematodes inside the roots (Elling *et al.*, 2007; Ithal *et al.*, 2007; Klink *et al.*, 2009a, 2009b). In our case, the sampling involved the maceration of the roots and collection of specific nematode stages by visual inspection in a restricted temporal period to improve the sensitivity and specificity of the microarray analyses. In addition, more than one probe per gene was printed on the microarray to increase the confidence and robustness of the analysis.

As expected, BLAST searches showed that the majority of genes had the closest matches to a range of nematode species, including animal parasites [*Ascaris suum* (741 BLAST top-hits), *Loa loa* (241 BLAST top-hits), *Brugia malayi* (218 BLAST top-hits), *Caenorhabditis elegans* (175 BLAST top-hits), *C. remanei* (170 BLAST top-hits), *C. briggsae* (146 BLAST top-hits)] and plant parasites including *H. glycines*, *G. pallida*, *G. rostochiensis*, *M. incognita* and *Bursaphelenchus xylophilus*. Searches were carried out against NCBI NR databases, and these matches reflect the sequence data deposited in these databases. In addition, we searched the *G. pallida* sequences on the array against the *M. incognita* and *M. hapla* predicted protein sets; predictably, many of the *G. pallida* sequences showed matches with this dataset, although limited annotation is present for these genome sequences (see Fig. S1). Interestingly, some genes were identified as having a plant origin, including *Solanum tuberosum* and *Vitis vinifera*, which matched with 23 and 18 genes, respectively (Fig. S2, see Supporting Information). A possible explanation for these matches could be from contamination in the ESTs, as the sedentary stages are difficult to separate from the pieces of plant root. The absence of these sequences from the *G. pallida* genome sequence provides evidence for this interpretation. However, some genes, which are

Table 2 Differentially expressed nematode genes in sedentary nematodes at 8 days after inoculation (SJ2) between partially resistant lines 11305 and 11415.

Array ID	Probe ID	Best hit ID	E-value	Best hit organism	Best hit description	11305/Desiree	11415/Desiree
Contig281	012010, 012011, 012012	ADJ93816	1.54E-112	<i>Potato virus S</i>	Coat protein	20691.92	15.97
Contig558	013009, 013010	ZP_08244251	6.06E-14	<i>Acetobacter pomorum</i>	Hypothetical protein APO_2582	13746.80	9.83
gil17969937 gb BM276574.1 BM276574	006309, 006310	–	–	–	–	142.41	36.61
rcJ2contig33	002804, 002805, 002806	XP_002592998	5.32E-19	<i>Branchiostoma floridae</i>	Hypothetical protein BRAFLDRAFT_142103	23.39	59.19
Gpa_EST_03_3_B01_007	002594, 002595, 002596	–	–	–	–	0.13	0.04
Gpa_EST_07_4_D12_045	000251, 000252, 000253	XP_001900590	2.43E-26	<i>Brugia malayi</i>	Small heat shock protein 12.6, identical	0.06	0.03
rcJ2contig298	001602, 001603, 001604	AAD56392	1.75E-39	<i>Globodera tabacum</i>	β -1,4-Endoglucanase 1 precursor	0.05	0.02
gil17969943 gb BM276580.1 BM276580	008027, 008028	AAC48325	1.84E-18	<i>Globodera rostochiensis</i>	β -1,4-Endoglucanase precursor	0.05	0.01
J2contig177	008204, 008205, 008206	–	–	–	–	0.05	0.02
Gpa_EST_P1_16_02-M13F_G02_G02_002	002591, 002592, 002593	YP_277429	4.38E-70	<i>Potato virus S</i>	25-kDa protein	0.04	0.02
gil1743514 gb AW505637.1 AW505637	008021, 008022, 008023	–	–	–	–	0.04	0.01
Gpa_EST_02_04_F08_027	003263, 003264	–	–	–	–	0.04	0.01
J2contig188	006883, 006884	ABF51007	1.46E-28	<i>Globodera mexicana</i>	IVG9	0.04	0.02
rcJ2contig172	004239, 004240, 004241	AAK60209	6.83E-68	<i>Heterodera glycines</i>	vap-1	0.04	0.02
Gpa_EST_13_03_M13F_D02_005	009132, 009133	–	–	–	–	0.03	0.01
Gpa_EST_03_3_D10_037	007609, 007610, 007611	–	–	–	–	0.03	0.01
Gpa_EST_02_test_B07_031	005236, 005237	–	–	–	–	0.03	0.01
Gpa_EST_03_4_A01_008	002624, 002625, 002626	–	–	–	–	0.03	0.02
Gpa_EST_07_2_C01_006	004340, 004341, 007752, 007753	–	–	–	–	0.03	0.01
J2contig53	003197, 003198	ABF51007	2.22E-24	<i>Globodera mexicana</i>	IVG9	0.03	0.01
Gpa-Est-05-2_E05_020	010613, 010614, 010615	NP_991205	1.91E-10	<i>Danio rerio</i>	Hypothetical protein LOC402939	0.03	0.01
J2contig110	001847, 001848	–	–	–	–	0.03	0.01
J2contig341	002156, 002157	–	–	–	–	0.02	0.01
J2contig296	005542, 005543, 005544	–	–	–	–	0.02	0.01

Table 3 Gene comparison between K-means groups in both microarrays (nematode development stages and different potato genotype backgrounds). J2, infective juveniles of *Globodera pallida*; SJ2, sedentary nematodes at 8 days after inoculation.

		K-means groups in different potato genotype backgrounds	
		K1 (genes up-regulated in comparison with susceptible genotype)	K2 (genes down-regulated in comparison with susceptible genotype)
K-means groups in different nematode stages	K1 (genes up-regulated in eggs and down-regulated in SJ2)	0	44
	K2 (genes up-regulated in J2)	0	27
	K3 (genes up-regulated in SJ2)	36	0
	K4 (genes up-regulated in J2 and down-regulated in eggs)	0	2

Table 4 Reverse transcription-polymerase chain reaction (RT-PCR) validation. Gene expression from sedentary nematodes expressed as the fold change referenced to J2 in the stages microarray experiment, and gene expression from sedentary nematodes in partially resistant line 11315 expressed as the fold change referenced to cv. Désirée in the genotypes microarray experiment.

Probes	Gene	Primers	BLAST hit	Stages*		Genotypes*	
				M	Q	M	Q
011903, 011904, 011905	4D08	F: 5'TCCCAATTGTGTGTTGCTG3' R: 5'CAGAGCAACCACATGCTGAC3' UPL-56: TGCTGTCC	gi 23451867 secretory protein 4D06 [<i>Heterodera glycines</i>]	383.5	87.7	105.7	134.7
013186, 013187, 013188	Cytc	F: 5'TGATCAAATACATCGAAGTGAG3' R: 5'GCGATCTGAACAACGCAATC3' UPL-135: GAAGCCAT	gi 196122398 putative cytochrome c [<i>Haemonchus contortus</i>]	0.3	0.4	0.3	0.2
000498, 000499, 000500	CytB	F: 5'GGTCACTACCAAAATGATGACTTCT3' R: 5'AAGTGCAATGCGTGAGGTC3' UPL-98: CTGTGCCT	gi 324519321 succinate dehydrogenase [ubiquinone] cytochrome b small subunit [<i>Ascaris suum</i>]	0.8	0.5	0.7	0.5
009550, 009551, 009552	Gluco	F: 5'CCCAGAGCAATACAAACGTG3' R: 5'CGGATAGCCGACACTTAGGA3' UPL-23: GGGCTGGG	gi 2494837 β -1,4-N-acetylglucosaminyl-transferase [<i>Lymnaea stagnalis</i>]	276.2	80	119.5	66.2
013218, 013219, 013220	29D09†	F: 5'ATTTCGGCCCCACAATTC3' R: 5'CATGGGAGGCCATCAGAG3' UPL-62: CAGCAGGT	gi 30315052 gb AAP30755.1 putative gland protein 29D09 [<i>Heterodera glycines</i>]	403.9	197	74.2	721.3
013089, 013090, 013091	Musc	F: 5'GAAGCTGTTCGGATGGATGT3' R: 5'ACACGTCGTCAAAGCGTTC3' UPL-39: AGGTGGAG	gi 71986236 G-protein-linked acetylcholine receptor family member (gar-2) [<i>Caenorhabditis elegans</i>]	0.2	0.2	0.2	0.2
003172, 003173, 003174	Glutsint	F: 5'GCCAGAAAATGATCGAACG3' R: 5'AAATCCAACAAAGCCGATG3' UPL-147: GCCATCAA	gi 324505313 glutathione synthetase [<i>Ascaris suum</i>]	548.5	489.5	197.3	210.3
Housekeeping genes							
001885, 001886, 001887	Eif	F: 5'GCTGAACCATCTCGAGCAGT3' R: 5'GCGGAAGCGACAGAAACTT3' UPL-53: CTCTGCCA	gi 312083156 ref XP_003143743.1 hypothetical protein LOAG_08163 [<i>Loa loa</i>]	—	—	—	—
011281, 011282, 011283	Tub	F: 5'ATTGGCATTTCGACCTG3' R: 5'GTCATTGGTCCGACTTTGGT3' UPL-5: CAGCCACA	gi 300797326 ref NP_001178004.1 tubulin γ -2 chain [<i>Rattus norvegicus</i>]	—	—	—	—
011570, 011571	40S-s21	F: 5'TGATCCCCGGTAAACTACG3' R: 5'CGTCTGACTCGCCCATGTA3' UPL-38: GGAAGCAG	gi 324539578 gb ADY49569.1 40S ribosomal protein S21 [<i>Ascaris suum</i>]	—	—	—	—

*M, microarray; Q, quantitative RT-PCR.

†Gene with expression not significantly modified in the microarray analysis after analysis of variance (ANOVA) on genes that passed the filtering criteria, with a *P*-value cut-off of ≤ 0.05 and Bonferroni multiple testing correction, and taking the average of all designed probes for the specified gene.

most similar to bacterial or fungal sequences, have been reported from a range of plant-parasitic nematodes and are thought to have been acquired by horizontal gene transfer (reviewed by Haegeman *et al.*, 2011). Both microarray analyses showed a number of differences between the stages and between the parasitic stage in different genetic backgrounds of the potato hosts.

These differentially expressed genes could be related to the size of the nematode in the major tissues in the developmental stages studied. However, this may be more important in the case of the life stages microarray, because the form and size of the nematodes differ between eggs, J2s and SJ2s (swollen J2 or J3), particularly in relation to the gland cells, but also the cuticle, muscles, digestive

and reproductive systems. No changes in nematode size were detected between the different potato genetic backgrounds (results not shown).

Different groups of genes are regulated in the nematode developmental stages

The identification of clusters of genes showing similar expression profiles in the K groups may allow biological function to be inferred for these genes. For the life stage microarray, the K-means clusters may infer the importance of certain genes at different stages in the nematode life cycle (Fig. 1). The K1-stage group is likely to include genes important for nematode survival in the environment, as they were more highly expressed in nematode eggs and mobile J2s and down-regulated in SJ2s. K2-stage genes were up-regulated specifically in J2s compared with other stages and are likely to be important for host location, invasion and the early stages of parasitism. K3-stage genes are likely to be important for sedentary stages of the nematode, including changes in metabolism and body structure associated with this period (loss of mobility and onset of digestion processes), as well as for the manipulation of the host defence responses and the induction and maintenance of the syncytium. Finally, K4-stage genes are activated in J2s and down-regulated in the egg stage. The reduced number of BLAST matches for this dataset could be because there are many pioneer genes that have not been described, and nematode proteins that enter the secretory pathway are known to evolve more rapidly than those that do not. For this reason, they could have produced fewer matches with sequences from other organisms (Harcus *et al.*, 2004; Jones *et al.*, 2009).

The K1-stage cluster contained genes implicated in diverse functions, but included genes that could encode proteins important for survival stages and protection against stresses (Table S1). These included three heat shock proteins (contig28, rc_gij7143511|gb|AW505634.1|AW505634, contig67) and one gene related to redox balance [glutathione S-transferase from *M. incognita* (Gpa_EST_P1_11_02_F08_F08_027)]. Surprisingly, the majority of these genes had similar expression levels in J2s. The other genes in the cluster that were down-regulated in SJ2s may reflect the host environment in which SJ2s develop. In addition, other genes show changes in expression profiles that are likely to reflect changes in body structure occurring as the nematode becomes sedentary and loses body wall musculature. In this respect, the data presented here are in agreement with the results obtained by Klink *et al.* (2007), in which a decline in transcript abundance for *H. glycines* homologues of *C. elegans* uncoordinated genes accompanies the development of its sedentary parasitic phase, including sarcopenia (wasting of muscles over time). Major decreases in levels of transcripts encoding three calponin isoforms (rc_contig745, rc_J2contig389 and gj|7143536|gb|AW505659.1|AW505659), myosin (Gpa_EST_P1_14-01M13F_

H06_H06_017), actin (contig242), syntrophin-1 (contig626), α -catulin (gj|54547621|gb|CV578298.1|CV578298) and β -tubulin (Gpa-Est-05-1___H10_033) were detected. Similarly, there were decreases in levels of transcripts related to the perception of environmental cues, including an ODoRant response abnormal family member (Gpa_EST_P1_17-01M13F_G01_G01_002), a G-protein-linked acetylcholine receptor family member (*gar-2*) (contig582) and TYRAmine receptor family member (*tyra-2*) (Gpa-Est-05-4___E06_020). All of these results suggest strong body modification and a reduction in sequences related to perception of the environment once the nematode becomes sedentary in the 'protected' environment of the root.

Genes up-regulated in mobile J2s are in cluster K2 and K4 stages, and many of the genes have a role in the parasitic process. This is in agreement with the results of Elling *et al.* (2009), who identified two distinct sets of putative effectors with differing expression patterns, with one set up-regulated at the early stages of parasitism and another set up-regulated at the later stages. One very large gene family of *G. pallida* effector proteins which fitted this pattern is encoded by the SPRYSEC genes. It has been suggested that SPRYSECs suppress host defences (Jones *et al.*, 2009; Rehman *et al.*, 2009), and different family members show different subcellular localization patterns in plants. All SPRYSEC genes investigated to date are expressed in the dorsal pharyngeal gland cell and have been reported to be down-regulated in adult females (Qin *et al.*, 2000). Interestingly, the majority of the effectors that are up-regulated in J2s are expressed in the dorsal gland, despite the dorsal gland cell being considerably larger and more active in parasite-stage nematodes when compared with J2s (Hussey and Mimms, 1990). However, a greater range of different SPRYSEC proteins have been detected in the J2 datasets (14 unigenes) than in the parasitic sedentary (three unigenes) or adult female (two unigenes) datasets, which were not proportional to the number of singletons and contigs across the cDNA libraries in the *G. pallida* EST study performed by Jones *et al.* (2009). It is possible that these proteins are important in the very early stage of the plant–nematode interaction. Another group of effector proteins which was highly upregulated in J2s was that encoding IVG9 proteins. These genes are expressed in the dorsal gland and do not show any obvious similarity with any other sequences in the database (Blanchard *et al.*, 2007). All IVG9 genes annotated on the array (four in total) were differentially expressed, but this was not true for other effectors annotated in the microarray (e.g. β -1,4-endoglucanase precursor, putative gland protein G7E05) using the stringent statistical analysis described here.

Genes present in the K3-stage cluster reflected the changes that occur in feeding parasitic nematodes, including changes in body morphology and the production of different effectors that presumably maintain the functionality of the feeding site. Seven genes that could encode collagens were up-regulated in SJ2s (gj|54549430, gj|54545050|gb|CV577022.1|CV577022,

Contig254, Contig266, Contig552, Contig656 and gil54546589[gb|CV577789.1|CV577789), presumably reflecting the onset of moulting and the need to synthesize collagen for a new cuticle. In addition to these structural genes, other changes reflected the start of the feeding process of SJ2s. Several digestive proteases were highly expressed in SJ2s, including cathepsin B-like cysteine proteinase 1 (Gpa_EST_P1_17-01M13F_D06_D06_021), cathepsin S-like cysteine proteinase (Contig496, Gpa_Est_04_02_B9_B09_039, Gpa_EST_P1_02_01_C4_C04_014) and a C-type lectin domain protein (Contig501, Contig666, gil7143534[gb|AW505657.1|AW505657), which has been shown to have a role in the function of the digestive system in other cyst nematodes (Lilley *et al.*, 1999). The other major group of highly expressed genes in SJ2s are putative effectors expressed in the dorsal gland that may be involved in the maintenance of the feeding site. These data are in agreement with those reported by Elling *et al.* (2009) for *H. glycines*. Some of these genes are pioneers and have not been functionally characterized (Gao *et al.*, 2003). A *G. pallida* homologue of the *H. schachtii* effector annexin 4F01 was one of the putative effectors up-regulated at this stage. This is thought to mimic the function of annexin in plants and has been shown to interact with an *Arabidopsis* oxidoreductase member of the 20G-Fe(II) oxygenase family, a type of plant enzyme demonstrated to promote susceptibility to oomycete pathogens (Patel *et al.*, 2010). Interestingly, other genes up-regulated in the SJ2s included glutathione synthetases (GSTs) (Gpa_EST_13_03_M13F_E01_004; Gpa_EST_P1_14-03M13F_H01_H01_001) and glutathione S-transferase-1 (Gpa_EST_P1_11_02_F08_F08_027). These proteins may manipulate the redox balance in plants in order to counter the induced defences by the plant. Glutathione S-transferase catalyses the addition of reduced glutathione (GSH) to electrophiles or the GSH-dependent reduction of hydroperoxides (Jasmer *et al.*, 2003; Sheehan *et al.*, 2001; Wilce and Parker, 1994) and, in plants, could also be bound to auxin and flavonoids, modulating their trafficking in the cell (Dixon *et al.*, 2002; Marrs, 1996; Moons, 2005). This protein from *M. incognita* has been shown to be synthesized in the oesophageal glands of infective juveniles, secreted via the stylet and up-regulated during parasitism (Dubreuil *et al.*, 2007).

Comparison of our results with those obtained by Klink *et al.* (2009a) in the susceptible temporal comparison between 12 h and 8 DAI showed similar results, with the increasing expression at 8 DAI of collagens, type lectin domain proteins and some oesophageal gland proteins.

Similarities in gene expression when nematodes are inoculated in different partially resistant potato genotypes

The clustering of genes differentially expressed in SJ2s inoculated on different potato genetic backgrounds produces a larger number

of genes down-regulated than up-regulated in the partial resistant genotypes than in the susceptible cultivar Desirée. These results have also been observed in microarrays studying the *H. glycines* response to resistant lines using two different populations differing in virulence. In this experiment, 13 genes were up-regulated and 1668 genes were down-regulated in the comparison between avirulent and virulent nematodes (Klink *et al.*, 2009a). However, the genes that were down-regulated followed a different pattern from that in our study comparing the same population of nematodes. In our case, β -1,4-endoglucanases (gil17969943[gb|BM276580.1|BM276580, Gpa_EST_03_4_A03_016, rc_J2contig298) and some putative effectors produced in the oesophageal glands were down-regulated in the partially resistant lines in comparison with the susceptible cultivar Desirée. These differences could be related to different rates of development within the susceptible and resistant lines. In our case, histopathological changes of the feeding site at 7 DAI showed a delay and degeneration in syncytia formation.

The K1 genotype group of genes showed an increase in metabolic enzymes, stress genes and some effectors in the nematodes inoculated on the partially resistant genotypes in comparison with the susceptible cultivar Desirée. Some of these genes suggested a role in overcoming plant defences and resisting a hostile host environment. These genes showed increased expression, as in the case of glutathione synthetase (Gpa_EST_13_04_M13F_G07_026, Gpa_EST_13_03_M13F_E01_004, Gpa_EST_P1_14-03M13F_H01_H01_001, Gpa_EST_13_01_M13F_A01_008, Gpa_EST_P1_17-04M13F_F01_F01_003) and sulphhydryl synthetase (Gpa_EST_P1_15-04M13F_H09_H09_033), which may be related to redox maintenance. Other highly expressed genes were the digestive enzymes (see above). Glyceraldehyde 3-phosphate dehydrogenase (Gpa_EST_13_02_M13F_E08_028) was highly expressed, consistent with increased nematode metabolic stress. The K2 genotype group showed the down-regulation of putative effector genes and a broad number of functions demonstrating the major impact of partial resistance on the parasitic-stage nematode.

The comparison of parasitic-stage gene expression for both partially resistant lines showed a major differential effect with line 11305 (Table 2). The up-regulated genes expressed in 11305 showed unexpected gene homologues, e.g. contig281 matched with the coat protein and a 25-kDa protein from *Potato virus S*, and contig558 matched with hypothetical proteins from *Acetobacter pomorum* and *Branchiostoma floridae*. These results are difficult to explain, but contamination during EST creation and the possibility of line 11305 infection by *Potato virus S* could explain these results.

The similarities in parasitic-stage gene expression with both partially resistant potato genotypes are closely related to the histopathological data in both partially resistant lines in the periods studied. Both partially resistant lines showed a similar reaction to the nematode, and these changes differed from those in the susceptible cultivar Desirée. A delay in syncytia formation at

7 days and the size of the syncytia at later stages in both partially resistant lines could explain these similarities in expression in both resistant lines. The observed interaction between *G. pallida* and Desirée shown here is similar to other susceptible interactions reported for this nematode.

Specific expression of genes differentially expressed in both microarrays showed a complete concordance between K-means clusters

The groups of genes differentially expressed when both microarrays were compared indicate that the genes highly expressed in J2s in comparison with the sedentary nematodes in the 'stages' array are also down-regulated in sedentary nematodes in the partially resistant lines, whereas genes highly expressed in SJ2s are also highly expressed in partially resistant lines in the comparison with the susceptible cultivar Desirée (Tables 3 and S12). These results suggest an increase in the metabolism and parasitism genes in order to obtain the same level of parasitism because of a delay in development in comparison with the susceptible host. As observed in the histopathological study, the delay and the smaller syncytia formed at 14 DAI in both partially resistant lines in comparison with Desirée could explain these similarities and differences. Interestingly, the expression pattern could be explained by the nematode adapting to the hostile environment of the partially resistant hosts in comparison with the susceptible host. These results could be related because similar transcription factors modified by the same cues could be involved. However, the majority of the genes that are differentially expressed in the genotype comparison have not been annotated. Many of the nematodes will develop into males in the partially resistant lines (Trudgill and Parrot, 1969), and the few females detected in the roots at 30 DAI were still young females without eggs. However, these lines could keep multiplying some part of the nematode population and could vary in resistant lines depending on the nematode population studied (Phillips and Trudgill, 1983, 1998).

Microarray validation

The genes studied by real-time PCR to validate the results obtained in the microarray hybridization showed a good correlation in their expression patterns with the microarray expression data. Although Gene *29D09* was up-regulated in both arrays and real-time PCR analysis, the up-regulation in the microarray study was not found to be statistically significant because of the stringent statistical approach applied here.

CONCLUSIONS

Microarray analyses are able to differentiate nematode gene expression between stages and different plant genetic back-

grounds in *G. pallida*. The major differences obtained in the different nematode stages showed a clear pattern of genes and functions of the nematodes in the different stages studied (eggs, J2s and SJ2s). The impact of the genetic background of the partially resistant lines on the gene expression pattern was similar, showing that lines 11305 and 11415 can be considered as histopathologically poor hosts and partially resistant to *G. pallida*.

One of the interesting observations in this study was the comparison between the different patterns of genes obtained in the K-means clustering. This showed that the specific responses were maintained between groups of genes. A future interesting point could be the study of longer periods of time in the nematode life cycle in order to identify effector expression profiles. This expression is interesting because the gene products maintain the feeding site, whereas the nematode is feeding in the root. From the plant resistance point of view, the partially resistant response is controlled by minor genes introduced in the genotypes selected, but a small part of the nematode population inoculated into these partially resistant lines could survive and multiply. These 'avirulent' interactions could be interesting in order to compare the expression of effectors or other genes involved in the survival from the defences generated by the plant in the resistance reaction.

EXPERIMENTAL PROCEDURES

Nematode inoculum and plant material

Globodera pallida (population Lindley from the JHI collection) was maintained in glasshouse conditions on the susceptible potato cultivar Desirée. Three potato genotypes were used for the microarray analyses. A susceptible reaction was obtained when nematodes infected the cultivar Desirée, which has no resistance against *G. pallida*. Two genotypes with different genetic backgrounds were used to study resistant interactions: the partially resistant line 11305, which contains *G. pallida* resistance derived from *Solanum vernei*, and the partially resistant line 11415, which contains partial resistance to *G. pallida* derived from *S. tuberosum* ssp. *andigena* CPC2802. The 11305 line also contains the *H1* gene which provides resistance to *G. rostochiensis* pathotypes Ro1 and Ro4.

Material was generated for two microarray experiments: (i) different stages of *G. pallida* (eggs hydrated for 24 h in H₂O; J2s and SJ2s in the susceptible cultivar Desirée); (ii) SJ2s under different host genetic backgrounds [Desirée and partially resistant (11305 and 11415)]. Experiments were conducted in a growth chamber adjusted to 20 ± 1 °C, 60%–90% relative humidity and a 14-h photoperiod of fluorescent light of 360 ± 25 µE/m²/s in a mixture of 2 : 1 of sand : loam in root-trainers (Ronaash, Kelso, Roxburghshire, UK). Plants were inoculated close to the roots with 3000 J2s suspended in 3 mL of sterile distilled water.

Eggs were released from cysts using a tissue homogenizer with a clearance of 0.46–0.54 mm between the glass pestle and the homogenizer tube. Cyst walls were removed from eggs by pouring the solution through a 100-µm-pore sieve nested over a 5-µm-pore sieve. Eggs were concentrated by decantation and centrifugation. J2s were obtained after soaking the cysts in sterile distilled H₂O and, for several days, in tomato root

diffusate (TRD). Hatched J2s were filtered through tissue paper in order to remove impurities and dead nematodes. Nematodes were concentrated by decantation and centrifugation. TRD was produced as described by Blair *et al.* (1999). SJ2s were obtained by removing the soil adhering to plant roots, followed by several 10-s periods of homogenization in sterile distilled H₂O using a blender at medium speed. The blended roots and nematodes were poured through a 1-mm-pore sieve nested over a 5- μ m-pore sieve. SJ2s were hand-picked into sterile distilled water and concentrated by centrifugation.

Histopathological study

Plant samples were inoculated following the same procedure as described for RNA sampling material. Soil adhering to the roots was carefully removed and roots were drained on tissue paper, cut into pieces and fixed using 4% formaldehyde. Fixed roots were dehydrated in a *tert*-butylalcohol series (40%–70%–85%–90%–100%), embedded in paraffin with a melting point of 58 °C and sectioned with a rotary microtome. Sections 10–12 μ m thick were placed on glass slides, stained with Safranin and Fast-green, mounted permanently in a 40% xylene solution of a polymethacrylic ester (Synocril 9122X, Cray Valley Products, Grimsby, South Humberside, UK), examined microscopically and photographed (Johansen, 1940).

Microarray design

A custom microarray was designed (SCRI_Gp_15k_v1, design ID 021218; Agilent Technologies, Santa Clara, CA, USA, A-MEXP-2180) using the 8 $\times$ 15k format. Probes were designed against a collection of EST unigenes from different cDNA libraries [invasive (second)-stage juveniles, early parasitic-stage juveniles and adult female-stage] (Jones *et al.*, 2009) and sequences from public databases, and the genes and intergenic regions of five published mitochondrial circles (accessions AJ249395, DQ631911, DQ631912, DQ631913 and DQ631914) (Armstrong *et al.*, 2000; Gibson *et al.*, 2007). In total, there were 14 311 unambiguous probes covering 5683 targets (5631 from the ESTs and 53 from mitochondria), and 338 ambiguous probes covering homologous mitochondrial target regions (not used in this study), giving, in total, 5690 target combinations.

The target sequence preparations and probe selection were scripted using Python and Biopython (Cock *et al.*, 2009). First, the 5604 EST sequences were polyA trimmed and assembled using CAP3 (Huang and Madan, 1999), giving 785 contigs and 3214 singletons, and thus 3999 candidate genes. Based on the polyA/polyT directionality and BLASTX searches against the NCBI NR database, the directionality of 2281 sequences was clear and probes were designed for their forward sequence only. For the remaining 1718 sequences with no or conflicting direction information, both the forward and reverse sequences were used, making, in total, 5717 targets from the EST sequences (although probes could only be found for 5631).

Agilent eArray (Agilent Technologies) was used with the 'Base Composition Methodology' to design sense probes between 40 and 60 bp. Five probes per EST target were requested with the 3' bias, whereas, for the mitochondrial targets, up to 10 probes were requested with the best distribution methodology. Separately, Array Designer 4 (PREMIER Biosoft International, Palo Alto, CA, USA) was used to generate seven sets of probes

trading specificity against probe length and melting temperature. The combined candidate probe list was screened against all the target sequences using BLASTN forward matches only to verify the matches and exclude potentially cross-hybridizing probes for EST. Redundant probes in which one was a subsequence of another were removed. Driven by the limit of 15k probes, the first three probes per EST target were selected (preferring the eArray probes and then most specific Array Designer probes), plus all the mitochondria probes, giving 14 649 probes. These were uploaded to Agilent eArray, and randomly laid out on the array using the default Agilent linker to extend the 3' end of the shorter probes to length 60 bp.

Annotation of microarray targets

Basic annotation of the microarray probe target sequences was performed as follows. BLASTX was used against the NCBI NR database with an expectation threshold of 1e-04, followed by BLAST2GO (version 2) (Conesa *et al.*, 2005) for the functional annotation of gene sequences using the default parameters. Separate BLASTX searches were performed against the predicted protein sets of *M. incognita* (20 359 Eugene predicted proteins with basic annotation; Abad *et al.*, 2008) and *M. hapla* (13 072 unannotated freeze 3 predicted proteins; Opperman *et al.*, 2008) with an expectation threshold of 1e-05, and then filtered for alignments of at least 30% identity and covering at least 50% of the query. For domain annotation, each forward open reading frame of at least 30 amino acids was searched using HMMER3 (Eddy, 2011) against the PFAM 26.0 annotated models (Punta *et al.*, 2012) with an expectation threshold of 1e-05. The PFAM results, the top three NR *M. incognita* and *M. hapla* BLAST matches and any GO terms are included in the tables.

RNA extraction and microarray hybridization

Total RNA was extracted using the RNeasy® Plus Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA digestion was conducted on-column during RNA extraction using the RNase-Free DNase Set (Qiagen) as recommended. Total RNA was quantified using a 2100 Bioanalyzer (Agilent Technologies) and the Agilent Small RNA Kit (Agilent Technologies) following the manufacturer's instructions.

In total, 16 hybridizations for each microarray experiment were performed (detailed in ArrayExpress accessions E-MTAB-999 and E-MTAB-1010 for stages microarray and genotypes microarray, respectively, together with all raw datasets). Hybridization consisted of an RNA reference sample for each array: J2s in the case of the stages experiment and SJ2s on cultivar Desirée for the experiment involving different host genotypes. Four biological replicates were performed for all individual microarray comparisons. Dye-swaps were incorporated within the biological replicates using cyanine 5 (Cy5) and cyanine 3 (Cy3) in both experiments.

Microarrays were hybridized and scanned by the Genome Technology Group at JHI using standard procedures. Approximately 10–20 ng of each total RNA extraction was used for RNA amplification and hybridization. Labelling of total RNA was carried out using the Low Input Quick Amp Labeling Kit (Agilent Technologies) as recommended. All microarray hybridization and washing procedures were performed according to the Two-Color Microarray-Based Gene Expression Analysis Guide (version 5.7; Agilent Technologies). Microarrays were imaged using a G2505B scanner (Agilent Technologies) at 5 μ m resolution with extended dynamic range (XDR).

Microarray data analysis

Data were extracted from microarray images using Feature Extraction software (version 9.5; Agilent Technologies) before being imported into Genespring (version 7.3; Agilent Technologies) for QC and data analysis. Normalization of raw data was performed using the Lowess algorithm, and dye-swaps were taken into account. Complete datasets were re-imported as single-colour data to allow comprehensive data analysis to be performed. Datasets were filtered to remove nonreliable data (intensity values ≥ 50 in four of 16 samples) and standard statistical tests were applied to identify significant differential gene expression.

One-way ANOVA was performed on genes which passed the filtering criteria, with a *P*-value cut-off of ≤ 0.05 and Bonferroni multiple testing correction, and taking the average of all designed probes for the specified gene. For comparison between partially resistant host genotypes, a less restrictive statistical analysis was also performed using volcano plots with a fold change cut-off of ≥ 2 between both genotypes, and a Student's *t*-test *P*-value cut-off of ≤ 0.05 . K-means clustering was used for gene clustering in both microarray experiments for probes significantly differentially expressed using default parameters.

Real-time RT-PCR

Six genes differentially expressed respective to the different life stages or host genotypes and one gene without differential expression were studied, together with three housekeeping reference genes (see Table 4). Housekeeping genes were chosen according to their expression in the microarray and other published studies (Hoogewijs *et al.*, 2008). Primer design was conducted on-line using the 'Universal Probe Library Assay Design Center' (Web Roche Applied Science, <https://www.roche-applied-science.com/sis/rtPCR/upl/index.jsp?id=>) employing the EST unigenes from which the microarray probes were designed. This uses the PRIMER3 program (Rozen and Skalka, 2000) in order to design primers close to the selected probe.

RNA samples used for microarray hybridization were also employed for the real-time experiment. cDNA generation was performed using a QuantiTect® Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. cDNAs were diluted 1:20 with PCR grade water and were used in real time employing FastStart TaqMan® Probe Master (ROX) (Roche, Burgess Hill, West Sussex, UK) with a primer concentration of 200 mM each and 5 µL of the diluted cDNA, in a final volume of 15 µL, with ROX as reference dye. Cycling conditions consisted of one cycle of denaturation at 94 °C for 10 min, followed by 40 cycles of 15 s of denaturation at 95 °C and 60 °C for 1 min. All real-time PCR assays were performed using Applied Biosystems StepOne™ (Applied Biosystems, Carlsbad, CA, USA). The final PCR products were verified by visualization of size PCR products on 2% agarose gels stained with SYBR® Safe DNA gel stain (Invitrogen Corporation, Carlsbad, CA, USA). All samples were run in triplicate and results were analysed using Gene Expression Macro™ version 1.1 (Bio-Rad, Hercules, CA, USA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Venn diagram using different species for identification. Mh, *Meloidogyne hapla*; Mi, *Meloidogyne incognita*; NR, National Center for Biotechnology Information (NCBI) nonredundant database.

Fig. S2 Top-hit species' distribution in the microarray used in this study.

Fig. S3 Heat map of K-means clusters from stages microarray.

Fig. S4 Heat map of K-means clusters from genotypes microarray.

Table S1 List of putative genes included in the microarray with the top three BLAST matches in *Meloidogyne hapla*, *M. incognita* and National Center for Biotechnology Information nonredundant (NCBI NR) database; gene ontology (GO) annotation and PFAM-A with HMMER3.

Table S2 K1-means cluster gene list from stages microarray with gene ontology (GO) annotation and the top three BLAST matches.

Table S3 K2-means cluster gene list from stages microarray with gene ontology (GO) annotation and the top three BLAST matches.

Table S4 K3-means cluster gene list from stages microarray with gene ontology (GO) annotation and the top three BLAST matches.

Table S5 K4-means cluster gene list from stages microarray with gene ontology (GO) annotation and the top three BLAST matches.

Table S6 Numbers and percentages of gene ontology (GO) annotations 'biological process' in K means from stages microarray.

Table S7 Numbers and percentages of gene ontology (GO) annotations 'molecular function' in K means from stages microarray.

Table S8 K1-means cluster gene list from genotypes microarray with gene ontology (GO) annotation and the top three BLAST matches.

Table S9 K2-means cluster gene list from genotypes microarray with gene ontology (GO) annotation and the top three BLAST matches.

Table S10 Numbers and percentages of gene ontology (GO) annotations 'biological process' in K means from genotypes microarray.

Table S11 Numbers and percentages of gene ontology (GO) annotations 'molecular function' in K means from genotypes microarray.

Table S12 Comparison between K-means clusters in genotypes (rows) and stages microarrays (columns) showing the putative gene identifications.

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