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# A broad characterization of the transcriptional profile of the compatible tomato response to the plant parasitic root knot nematode *Meloidogyne javanica*

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**Abstract**

The root knot nematode *Meloidogyne javanica,* a plant parasite that is an agricultural pest, establishes and maintains a permanent feeding site within plant roots. In order to gain a broad view of gene expression in nematode feeding sites during the compatible response of tomato to root knot nematodes, we used the tomato spotted microarray chip, followed by real-time reverse transcription polymerase chain reaction (RT-PCR) validation. Cluster analysis and determination of the amplitude of gene expression suggested that the plant response at 5 days post-inoculation was not as vigorous as that at 10 days post-inoculation. In addition, by filtering the data for genes that were significantly up- or down-regulated during the interaction, we identified both the quantitative and qualitative differences between the two time points. Our microarray experiments demonstrated significant changes in the steady-state levels of transcripts of several functional categories, including pathogenesis-related genes, hormone-associated genes and development-associated transcription factors.

*Abbreviations:* d.p.i – days post-inoculation; EREBP – ethylene-responsive element binding protein; GA – gibberellin; PR – pathogenesis related; qPCR – real-time quantitative RT-PCR; RKN – root knot nematode; RT-PCR – reverse transcription-polymerase chain reaction; SAR – systemic acquired resistance.

## Introduction

The emergence of plant parasitism forced pathogens to change the development path and metabolism of the plant cell, in order to establish and maintain a compatible interaction. The root knot nematode (RKN) *Meloidogyne javanica,* a plant parasite, establishes a permanent feeding site in the differentiation zone of the root by inducing nuclear division without cytokinesis in the host cells. This process gives rise to large multinucleate cells, termed giant cells, which serve to direct plant resources to the parasitic nematode. The plant cells around the feeding site divide and swell, causing the formation of galls (Williamson and Hussey, 1996).

The complex changes in plant gene expression elicited by nematode infection have been studied for a long time, and the molecular mechanisms that produce feeding sites have begun to be elucidated (reviewed by Gheysen and Fenoll, 2002). Many plant genes associated with plant development are involved in the parasitic process, therefore the processes that lead to nematode feeding-site formation involve alterations to some fundamental aspects of programmed plant development (Bird, 1996; Hussey and Grundler, 1998; de Almeida Engler et al., 1999; Bird and Koltai, 2000; Goellner

et al., 2001; Gheysen and Fenoll, 2002; Vercauteren et al., 2002; Williamson and Gleason, 2003). These fundamental aspects are also likely to involve phytohormones; studies indicated increased levels of cytokinin in nematode feeding sites (Bird and Loveys, 1980; Lohar et al., 2004), and auxin accumulation and reduction, basipetally and acropetally, respectively, to the forming gall (Hutangura et al., 1999). In addition, pathogen-responsive genes (Lambert et al., 1999; Gheysen and Fenoll, 2002; Vercauteren et al., 2002; Puthoff et al., 2003) and several nodulation-associated genes (Koltai et al., 2001; Favery et al., 2002; Gheysen and Fenoll, 2002) were up-regulated during the compatible RKN–plant interaction.

Large-scale microarray analysis has proved to be a useful tool for discovering new genes and genetic pathways and for studying gene expression in numerous systems, including the incompatible and compatible interactions between plants and pathogens. The latter include bacterial, fungal and cyst nematode pathogens (e.g., review by Wan et al., 2002; Puthoff et al., 2003; Dowd et al., 2004; Lee et al., 2004).

In the present study, we adopted a genomic approach and used microarray analysis to gain a broader view of the transcriptional changes in tomato during the compatible response to RKN. We used spotted tomato microarrays, followed by quantitative polymerase chain reaction validation, to measure changes in the transcription abundance of tomato genes. We characterized the kinetics of nematode parasitism at two time points and identified genes that were differentially expressed during the compatible interaction.

## Materials and methods

### *Plant growth and nematode infection*

Eggs of RKN *(M. javanica)* were extracted from greenhouse cultures, and second-stage juveniles (J2) were hatched (Hussey and Barker, 1973). Tomato *(Lycopersicon esculentum,* VF36 spp.) plants, susceptible to *M. javanica,* were maintained in pots containing autoclaved quartz sand at 16 h light and 60% humidity. The plants were inoculated 40–60 days post-germination with 5000 J2 of *M. javanica* per plant or with water, as experimental or mock-inoculations. In order to minimize dilution of RNA extracted from RKN-infected tissue with that of the healthy tissue, galls were dissected from nematode-infected roots 5 and 10 days postinoculation (d.p.i) and immediately frozen in liquid nitrogen. In a similar manner, the mock-inoculated roots systems were dissected and immediately frozen in liquid nitrogen. To verify the level and stage of infection at these time points, nematodes and giant cells were detected in some of the collected roots by clearing and staining with acid fuschin (not shown; Daykin and Hussey, 1985). Each biological replicate was pooled for eight infected plants and eight mock-inoculated plants.

### *Microarray chip description*

The tomato microarray chip was developed and printed by Cornell University (Ithaca, NY, USA); it contains 12,500 clones, selected at random from a number of different cDNA libraries derived from a range of tissues including leaf, root, fruit and flower (http://bti.cornell.edu/CGEP/CGEP.html).

### *Microarray experiment design*

In order to compare transcription patterns of numerous genes in a tissue at various time points by means of cDNA microarrays, a reference RNA sample design (Churchill, 2002) was used. In this experimental design, RNA test samples (i.e., RNA samples extracted from a nematode-infected or mock-inoculated tissue at specific time points) were co-hybridized to the cDNA microarray with a RNA reference sample. The RNA reference sample was pooled from all test RNA samples that were involved in the experiment (Churchill, 2002). Thus, the reference sample provided a hybridization signal for each microarray spot that was hybridized with any of the test samples, which facilitated comparison among all test samples. In addition, since all hybridization used the same orientation of dye labeling, i e., the RNA reference sample was labeled with Cy3-dUTP and all RNA test samples were labeled with Cy5-dUTP, no dye-swap was needed (Yang and Speed, 2002).

### *RNA sample preparations*

RNA test samples were extracted, as total RNA, from mock-inoculated and infected tissues 5 and 10 d.p.i, by means of the RNeasy Kit (Qiagen,

Valencia, CA, USA), and were treated with DNase (Qiagen). Twenty micrograms of total RNA were taken from each of the RNA test samples and were pooled to form the RNA reference sample. Thus, all test samples were equally represented in the reference sample. For each microarray hybridization, 5 $\mu$g of total RNA test sample and 5 $\mu$g of total RNA reference sample were taken for mRNA amplification with the MessageAmp aRNA kit (Ambion, Austin, TX, USA).

*RNA labeling*

Five micrograms of amplified mRNA (aRNA) were labeled by means of the LabelStar Array Kit (Qiagen). Labeling was performed, according to the manufacturer's instructions, by reverse transcription using Cy3-/Cy5-dUTP (Amersham, Uppsala, Sweden) and random hexamers (Amersham). The aRNA reference sample was labeled with Cy3-dUTP, whereas all aRNA test samples were labeled with Cy5-dUTP. The frequency of incorporation (FOI) of Cy3 or Cy5 was calculated for each sample (35.1 × A650 /A260 and 58.5 × A550/A260 for Cy5 and Cy3, respectively; A260, A550 and A650 are the absorbance values of a labeled sample at wavelengths of 260, 550 and 650 nm, respectively; http://www.wi.mit.edu/CMT/protocols/Direct%20Labelingv.WICMT4.pdf). The amounts in picomoles of dye incorporated was calculated for each sample ($\mu$l × A550/0.15 and $\mu$l × A650/0.25 for Cy3 and Cy5, respectively; http://www.wi.mit.edu/CMT/protocols/Direct%20 Labelingv. WICMT 4. pdf).

*Microarray hybridizations*

Prehybridization and hybridization were performed as described in http://132.183.243.28/assets/pdf/protocols_niddk_oligo_cdna_microarray.pdf; supplemented by the Keck Biotechnology Resource Laboratory (Yale University, CT, USA), with modifications. For pre-hybridization, microarray slides were incubated at 76 °C for 2 min, overlaid with 60 $\mu$l of pre-hybridization buffer (48% formamide, 3.2× SSPE, 0.4% SDS, 2× Denhardts and 0.177 mg ml$^{-1}$ salmon sperm), covered with an RNase-free hybridslip (Shleicher & Schuell, Dassel, Germany) and incubated for an additional 1 h at 50 °C. The slides were washed with sterile double-distilled water, then with 70% ethanol followed by 100% ethanol, and dried. For each hybridization to the chip, a probe was prepared by mixing 100 pM of Cy5-labeled test sample and 100 pM of Cy3-labeled reference sample (in each the FOI was greater than 10). The probe was concentrated to 8 $\mu$l with a Microcon YM30 filter (Millipore, Cambridge, UK). A probe solution was prepared by mixing 16 $\mu$l of hybridization buffer (62.8% formamide, 0.8% SDS, 4× Denhardts and 5× SSPE) with the probe. The probe solution was denaturized by heating to 90 °C for 5 min and applied immediately to the microarray slide. The slide was covered with an RNase-free hybridslip and incubated overnight at 42 °C in the dark. Slides were washed twice with 2× SSC/0.1× SDS, followed by washing once with 0.2× SSC/0.1× SDS, and dried.

The hybridized microarray slides were scanned immediately for fluorescence emission. Separate images for each fluorescence were acquired by using the ScanArray 4000 software (Packard BioScience, Meridan, CT, USA) at a resolution of 10 $\mu$m pixel$^{-1}$, with the photomultiplier and laser power adjusted to achieve an optimal distribution of signals with minimal saturation. Initial image analysis was performed by using the histogram method of QuantArray version 3 software (Packard BioScience).

*Data analysis*

Data normalization was performed using the overall background by applying per-spot and per-chip normalization (GeneSpring 5.1; Silicon Genetics, Redwood City, CA, USA), for each of the two co-hybridized samples, i.e., RNA test (infected or mock-inoculated) and RNA reference samples. The normalized microarray data was clustered and visualized with the CLUSTER and the TREEVIEW software, respectively (Eisen et al., 1998). The infected:reference or mock-inoculated:reference ratios were determined from each microarray slide. The infected:reference ratio was divided by the mock-inoculated:reference ratio for each clone in each biological replicate, to determine the infected:mock-inoculated expression ratio. These ratios were divided by their median value determined for each biological replicate. For each time point (5 and 10 d.p.i) the values of the infected:mock-inoculated expression ratios of each individual clones were averaged from the biological replicates. The

amplitude of the averages of the infected:mock-inoculated expression ratios was calculated for each time point examined. The average of the infected:mock-inoculated expression ratios was used for filtering the data for significantly differentially expressed genes at a significance level of $P = 0.05$ (Jain et al., 2003; Reiner et al., 2003; van Wees et al., 2003). These significantly differentially expressed genes were clustered by experiment (chip), according to their nematode-inoculated:reference or mock-inoculated:reference expression ratios. Genes that were significantly differentially expressed at both time points were clustered according to their infected:mock-inoculated expression ratios. Annotations of significantly regulated genes were based on the post-re-sequencing tomato microarray data (http://bti.cornell.edu/CGEP/CGEP.html).

*Validation of hybridization results by quantitative real-time RT-PCR*

The expression patterns of genes of interest that were significantly regulated during nematode infection were validated by quantitative real-time, reverse transcription PCR (qPCR). Gene-specific primers were designed with the Primer Express software (Applied Biosystems, Foster City, CA, USA). Four micrograms of the RNA test samples, which were taken for amplification followed by chip hybridization, were subjected to reverse transcription followed by qPCR. The Sybr Green I qPCR Mastermix (Eurogentec, Seraing, Belgium) was used to perform the qPCR, and the ABI 7000 detection system (Applied Biosystems) was used for detection and analysis. To minimize mRNA quantification errors and to correct for inter-sample variations, expression levels of genes of interest (target genes) in infected and mock-inoculated tissues were determined relative to that of *Lycopersicon* actin (accession no. BF096262) mRNA, and three technical replicates were performed. Infected:mock-inoculated expression ratios of target genes were determined as 2 ([average actin mock-inoculated − average gene of interest mock inoculated] − [average actin infected − average gene of interest infected]); average actin and gene of interest (either in nematode-infected or mock-inoculated roots) values of expression are the average of the threshold cycles for each of the three technical replicates (Ginzinger, 2002). Means and standard deviations of infected:mock-inoculated gene expression ratios were determined from two biological replicates for each time point (5 and 10 d.p.i). The primers used for amplification of genes of interest and of *Lycopersicon* actin are detailed in Table 1.

## Results

To gain a broad profile of gene expression involved in the tomato response to RKN we used

*Table 1*. Sequences of primers used for amplification of genes of interest and *Lycopersicon* actin (accession no. BF096262) for quantitative real-time RT-PCR (qPCR) analysis

| Clone ID/ accession no. | Forward primer | Reverse primer |
|---|---|---|
| BF096262 | 5′-GAGCAGGAACCTTGAAACCG | 5′-AACGGAACCTCTCAGCACCA |
| cLEC-32-J20 | 5′-ATTTCATGATGGGTTGCCCA | 5′-CATCCTGACAAGGGCAGCA |
| cLEC-5-O21 | 5′-TCCTGCTGGTGCTTAACAACC | 5′-GATTTGCGCGATGAACACAA |
| cLES-18-B2 | 5′-TGATAGAAGATGCCGATGCG | 5′-GGATCCTTCAGGTCTTGGGC |
| cLEC-31-O18 | 5′-TCTCATCCAAAAACGACCACAC | 5′- TTAAGCTACCCTTCGTCTCCGA |
| cLEN-12-I17 | 5′-CCGAGATTTGCGTTTATGACAA | 5′-TGGGAAAGGACTGTTTTTGACAG |
| cLED-4- I8 | 5′-TCTCAACCAGCAGCCAGATG | 5′-CGTTGTGAAAGTGTTGGCTGA |
| cLED-11-A3 | 5′-GCACGAGCTATATATGGTGGTCAG | 5′-CCTTTAAACACCGGATGCAAAA |
| cLER-1-P6 | 5′-AATGGGACCAACGAGAATCG | 5′-CGCAAACCGAGGCACAAT |
| cLEC-25-H1 | 5′-GCGTCCGAAGTGATTGTGAA | 5′-GCTTCCCTTTCCATTCTCGC |

Genes of interest putatively encode peroxidase (cLEC-32-J20), homeotic protein VAHOX1 (cLEC-5-O21), ethylene-responsive transcriptional coactivator (cLES-18B2) ethylene responsive element binding factor (cLEC-31-O18), WRKY family transcription factor (cLED-4-18), hin1-like protein (cLed-11-A3), plant defensin protein (cLER-1-P6) and gibberellin 2-oxidase-like protein (cLEC-25-H1).

the tomato microarray chip. To focus on the events that are specifically related to nematode infection we compared nematode-infected and mock-inoculated tissues at two time points (5 and 10 d.p.i) during nematode parasitism. Three biological replicates were performed for 5 d.p.i, two for 10 d.p.i. Thus, a total of 10 chips was used (six for the 5-d.p.i and four for the 10-d.p.i samples).

*The plant response to RKN is more vigorous at 10 than at 5 d p.i*

We determined, for each gene represented on the tomato microarray chip, its expression levels in nematode-infected tissue and in the corresponding mock-inoculated control. Following filtration of the data by GeneSpring normalization, 10,327 genes of the tomato chip were subjected to hierarchical clustering analysis. Clustering was applied to the log 2-transformed values of the ratios between experimental samples (nematode infected or mock-inoculated) and reference samples (containing equal amounts of all the RNA test samples) (Figure 1).

Five days after nematode inoculation, each of the three biological replicates was clustered, whereas 10 d.p.i the nematode-infected samples in all biological replicates were clustered and the corresponding mock-inoculated samples fell into a separate cluster (Figure 1). Thus, at 5 d.p.i the expressional changes conferred by the nematode were minor compared with the biological variation between replicates (biological replicates, rather than nematode inoculated samples were clustered together; Figure 1). On the other hand, at 10 d.p.i the effect of nematode parasitism on plant gene expression dominated that of the biological variation between replicates, resulting in clustering of nematode-infected samples separately from the mock-inoculated samples (Figure 1). A full list of normalized expression data for all microarray experiments, including clone identifications (IDs) of all the genes are presented in the Supplemental Data S1.

*Quantitative and qualitative differences are evident between expression patterns of significantly regulated genes at 5 and 10 d.p.i*

For each time point (5 or 10 d.p.i), 516 (out of 10,327) genes were determined to be significantly

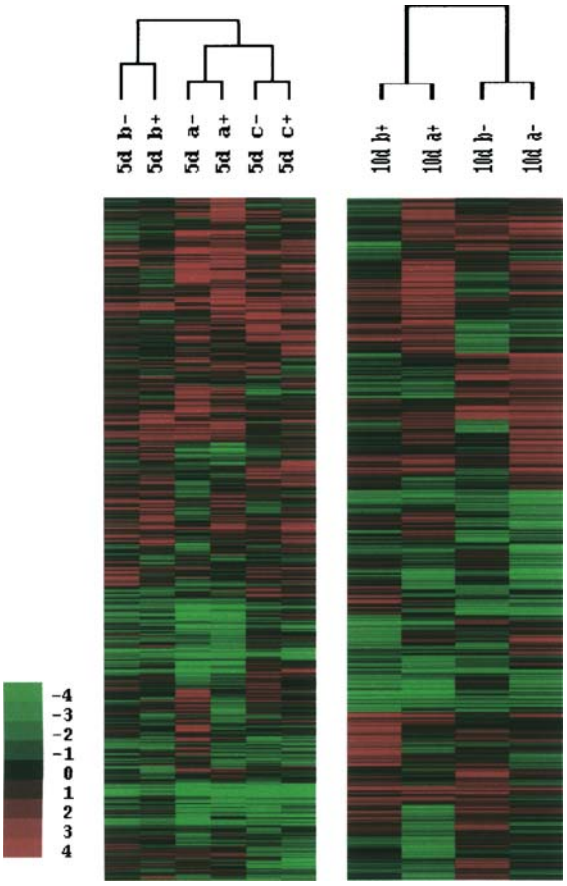


*Figure 1.* Cluster by array with 10,327 genes of the tomato chip, using expression data of *Meloidogyne javanica*-infected (+) and mock-inoculated (−) tomato *(Lycopersicon esculentum)* roots at 5 and 10 days post-inoculation. Test sample:reference expression ratios were transformed to log 2 and subjected to hierarchical cluster analysis. a, b, c denote the biological replicates. Color legend: log 2 of expression ratios.

differentially expressed, at a significance level of $P = 0.05$. As a result, at 5 d.p.i, induction and reduction of transcription by expression ratios of 1.58 and 0.68 in infected:mock-inoculated tissue was determined to be significant ($P = 0.05$). Whereas at 10 d.p.i the corresponding expression ratios for significance at $P = 0.05$ were 2.43 and 0.49, respectively. In addition, the amplitudes of expression ratios were greater after 10 days than after 5 days (16.19 and 3.79, respectively). Thus, most quantitative changes in the steady-state levels of gene transcription products in the infected compared with the mock-inoculated tissue were observed 10 days after RKN inoculation and not

5 days after it, which suggests that there were quantitative differences between the overall patterns of gene expression at the two examined time points. In addition, only a minor proportion (11.4%; 118 out of 1032) of all the genes that were found to be significantly regulated at the two time points examined, were common to the two, which suggests that there were qualitative differences in gene expression between 5 and 10 d.p.i.

*The significantly regulated genes are associated with nematode infection*

Hierarchical cluster analysis for arrays of the 516 significantly regulated genes was performed based on the nematode-infected: reference and mock-inoculated:reference ratios at 5 or 10 d.p.i (log 2-transformed). This clustering demonstrated that at both 5 and 10 d.p.i, the infected plants in all biological replicates clustered together, and that the corresponding mock-inoculated plants fell into a separate cluster (Figure 2). These results suggest that filtering the data for genes that are differentially expressed at a significance level of $P = 0.05$ enabled reduction of the effect of the biological replicates, so revealing the nematode-associated biological process.

*Most of the significantly expressed genes that are common to both examined time points exhibit similar tendencies of expression regulation*

Nematode-infected:mock-inoculated expression ratios of the significantly regulated genes, which are common to the two examined time points and had similarity of patterns (of up- or down-regulation) in at least two biological replicates, were clustered by gene (log 2-transformed). This procedure resulted in four major clusters of genes, the two biggest clusters (A and C) exhibited similar tendencies of expression regulation, i.e., down- or up-regulation, respectively, at both time points (Figure 3). Cluster A comprised 41 genes that were down regulated at both time points (5 and 10 d.p.i), cluster B included two genes that were up-regulated at 5 and down-regulated at 10 d.p.i, cluster C comprised 49 genes that were significantly induced at both 5 and 10 days and cluster D included two genes that were down-regulated at 5 days and up-regulated at 10 days. A full list of the significantly regulated genes in both time

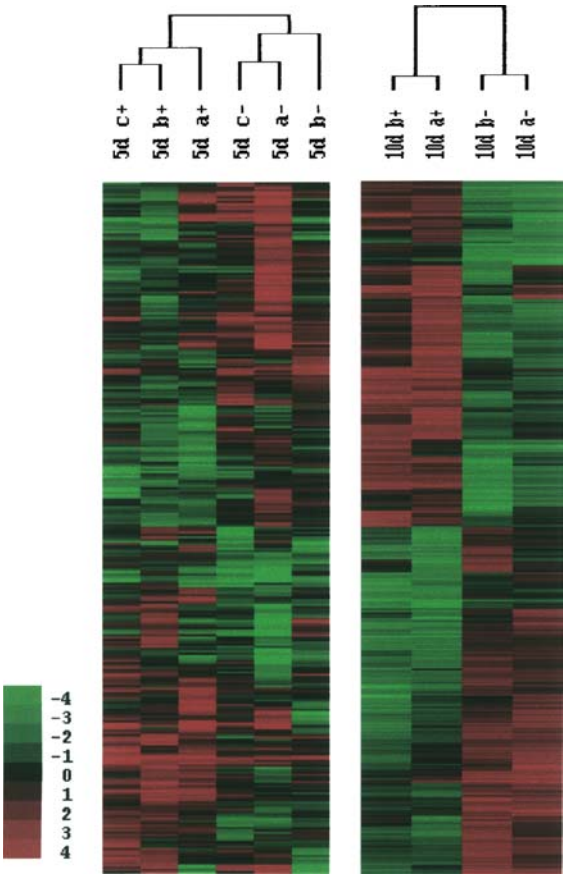


*Figure 2.* Cluster by array of significantly ($P = 0.05$) regulated genes of the tomato chip at 5 or 10 days post-inoculation, using expression data of *Meloidogyne javanica*-infected (+) and mock-inoculated (−) tomato *(Lycopersicon esculentum)* roots. Test sample:reference expression ratios were transformed to log 2 and were subjected to hierarchical cluster analysis. a, b, c denote the biological replicates. Color legend: log 2 of expression ratios.

point, including their clone ID, the revised Cornell BLAST-based annotation, the test sample to reference hybridization ratios in each biological replicate, the nematode-infected:mock-inoculated ratios in each biological replicate and their averages are presented in the Supplemental Data (S2).

*Real-time quantitative RT-PCR confirms or resolves gene expression pattern inferred from chip results*

A qPCR approach was used to confirm the expression patterns, as inferred from the chip results, of genes of interest. The values determined by qPCR

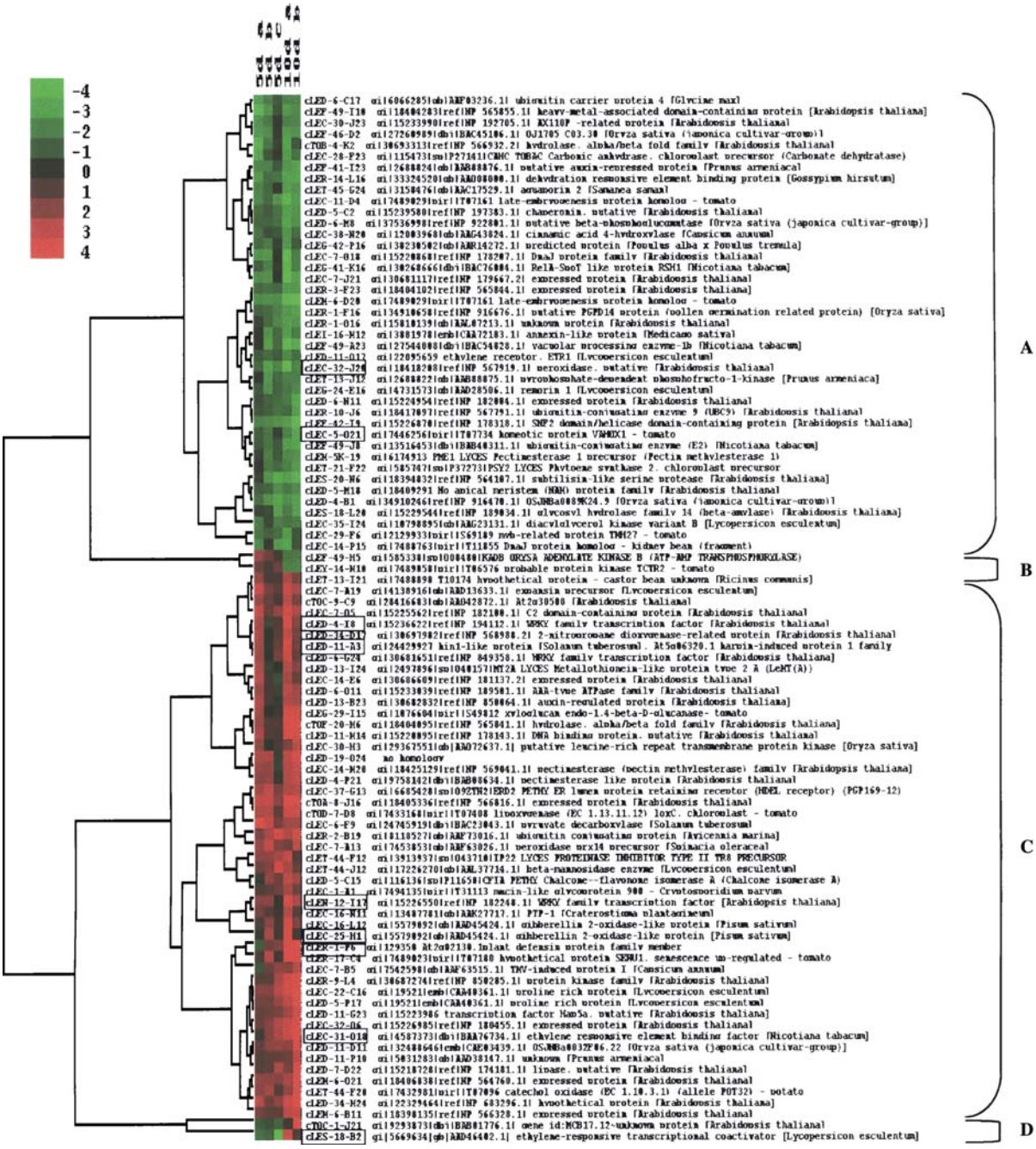


*Figure 3*. Cluster by gene of significantly ($P = 0.05$) regulated genes of the tomato chip at both 5 and 10 days postnematode-inoculation, using expression data of *Meloidogyne javanica*-infected (+) and mock-inoculated (−) tomato *(Lycopersicon esculentum)* roots. Nematode-infected:mock-inoculated expression ratios were transformed to log 2 and subjected to hierarchical cluster analysis. A, B, C, D denote gene clusters; a, b, c denote the biological replicates; clone ID and their BLAST -based annotation are indicated. Genes that their expression pattern was confirmed by qPCR are framed. Color legend: log 2 of expression ratios.

are presented in Figure 4 as log 2 of the ratio of gene expression between nematode-infected and mock-inoculated roots. Expression of genes encoding peroxidase (clone ID cLEC-32-J20) and homeotic protein VAHOX1 (cLEC- 5-021) was reduced in the chip results at both 5 and 10 d.p.i (Figure 3). This

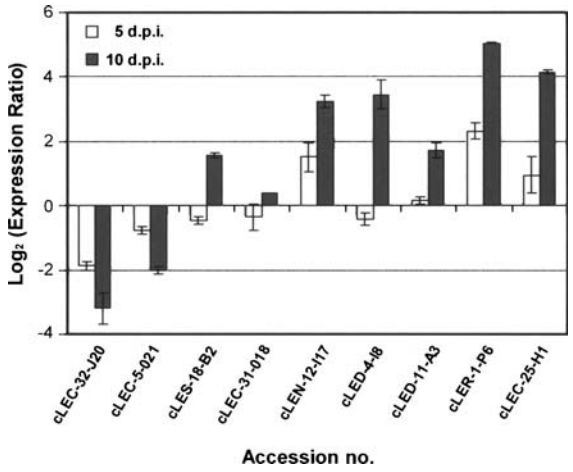


*Figure 4*. Quantitative real-time RT-PCR (qPCR) results presented as log 2 of the ratio between gene expressions in nematode-infected roots and those in mock-inoculated roots. Genes putatively encode peroxidase (cLEC-32-J20), homeotic protein VAHOXI (cLEC-5-O2I), ethylen-responsive transcriptional coactivator (cLES-18-B2), ethylene responsive element binding factor (cLEC- 31-O18), WRKY family transcription factors (cLEN-12-I17 and cLED-4-I8), hin1-like protein (cLED-11-A3), plant defensin protein (cLER-1-P6) and gibberellin 2-oxidase-like protein (cLEC-25-H1). White columns signify the expression ratio at 5 days post-nematode or mock inoculation; black columns signify the expression ratio at 10 days. Expression ratios (in either nematode-infected or mock-inoculated roots) were calculated as the averages of three technical replicates. Error bars represent standard deviations calculated for the qPCR results of two biological replicates of each experiment.

down-regulation was validated by qPCR (Figure 4). According to the chip results, expression of an ethylene-responsive transcriptional coactivator gene (cLES-18-B2) was down-regulated at 5 d.p.i and up-regulated at 10 d.p.i (Figure 3). Once again, this pattern was validated by qPCR (Figure 4). As inferred from chip results, the expression of an ethylene-responsive element binding factor gene (cLEC-31-O18) was induced at both 5 and 10 d.p.i (Figure 3), whereas qPCR validated its induction at 10 d.p.i but indicated no significance changes in its expression at 5 d.p.i (Figure 4). According to the chip results, expression of a WRKY family transcription factor gene (cLEN-12-I17) was induced at both 5 and 10 d.p.i This pattern was confirmed by qPCR (Figure 4).

In addition, we used qPCR to resolve the expression pattern of other genes of interest. According to the chip results, expression of another WRKY family transcription factor gene (cLED-4-I8) varied among the biological replicates at 5 d.p.i (Figure 3). The qPCR results indicated its repression (Figure 4). At 10 d.p.i, both chip and qPCR results demonstrated its induction (Figures 3 and 4). As inferred from the chip results, expression of *hinl*-like gene (cLED-11-A3), plant defensin gene (cLER-1-P6) and gibberellin 2-oxidase-like gene (cLEC-25-H1) was induced at 5 d.p.i, in only two of the three biological replicates (Figure 3). Their induction was demonstrated by qPCR at 5 d.p.i (Figure 4), and was established by both chip and qPCR results at 10 d.p.i (Figures 3 and 4).

## Discussion

In the present study, a genomics approach has highlighted some of the aspects of the parasitic association between the RKN *M. javanica* and susceptible tomato. Quantitative and qualitative differences were observed between the tomato response to RKN at 5 and 10 d.p.i. Only a minority of the significantly regulated genes was common to both time points. Nevertheless, the two biggest clusters of genes that were significantly regulated at both 5 and 10 d.p.i exhibited similar tendencies of expression regulation (up- or down-regulation), at both time points. These patterns probably reflect a progressive process of plant response to the parasitic nematode, i.e., acceleration of up- or down-regulation of gene expression.

Studies on the compatible and incompatible interactions between *Arabidopsis* and *Pseudomonas syringae* suggested that the level of biological variation between biological repeats observed in large-scale expression profiles depends heavily on the robustness of the plant response to the microbe (Tao et al., 2003). In our study, the clustering of the unfiltered gene expression data demonstrated that 5 d.p.i the variations between biological replicates were higher than that at 10 d.p.i. Thus, the tomato response to RKN at 10 d.p.i may be more robust than that at 5 d.p.i. Moreover, the amplitude of the tomato response to nematodes at 10 d.p.i was greater than that at 5 days, which again indicates a more vigorous response at 10 than at 5 d.p.i.

The events in the formation and maintenance of feeding sites (especially, giant cells) are tightly coupled to the developmental status of the nematode, and giant cells reach their maximal size and

activity at the onset of egg laying (Bird, 1971; Bird and Koltai, 2000). On the other hand, studies of cell cycle progression in giant cells suggested that cell cycle inhibition at early stages of infection resulted in arrested giant cell development, whereas at a later stage (9 d.p.i) feeding sites were apparently sufficiently developed to allow nematode maturation despite cell cycle arrest (de Almeida Engler et al., 1999). Our microarray results suggest progressive and accelerating gene expression pattern, which may reflect an acceleration of the parasitic process. This acceleration may include a growing need on the part of the plant (presumably forced by the presence of the nematode) for the maintenance of the parasitic association and the continuation of feeding site development, excluding perhaps a need for cell cycle progression (de Almeida Engler et al., 1999), even at later stages of nematode infection.

Some previously known and several newly recognized components associated with the host response to RKN were identified. The former include auxin-responsive genes (clone IDs cLED-13-B23 and cLEF-41-I23; Hutangura et al., 1999; Goverse et al., 2000), and cell wall components such as endoglucanase (clone ID cLEG-29-I15; Goellner et al., 2001). Some of the newly recognized components of the tomato response to the parasitic process are detailed below.

*Pathogenesis-related genes*

In agreement with the hypothesis that nematodes elicit the expression of pathogenesis-related (PR) genes during compatible interactions (Lambert et al., 1999; Gheysen and Fenoll, 2002; Vercauteren et al., 2002; Puthoff et al., 2003) we have identified several PR elements that were significantly up-regulated during nematode infection (as established by integration of the chip and qPCR results). They included a defensin homolog, which putatively encodes a secreted antimicrobial peptide protein that is also locally up-regulated after infection with bacteria or fungi (Moreno et al., 1994; Vignutelli et al., 1998; de Castro et al., 2002) and a harpin-induced *hin1*-like gene. The potato *hin1* and a tomato *hin1*-related gene were previously found to be induced by bacterial harpins (Gopalan et al., 1996). Harpins are members of a class of bacterial virulent/avirulent proteins, produced and secreted by hrp genes (reviewed by Cornelis and van Gijsegem, 2000). Interestingly, the induction of *hin1* by RKN may suggest a virulence/avirulence mechanism similar to the one elicited by bacterial pathogens, induced perhaps by the secretion of a nematode-originated virulent/avirulent (avr) protein. However, a nematode avr protein has yet to be identified (Williamson and Gleason, 2003).

The induction of toxins, PR genes and, especially, the *hin1*-like gene, suggest that a plant can and indeed does identify the RKN as a pathogen during the compatible interaction. Conceivably, components necessary for the defense reaction (e.g., a functional Mi protein) are absent from susceptible plants, in which the hypersensitive response is, therefore, not elicited. Another possibility is that induction of PR proteins is a consequence of a general stress of a metabolic nature, generated by nematode parasitism. Whether the expression of these genes reflects a localized induction or a systemic induction, similar to that involved in the process of systemic acquired resistance (SAR), remains to be determined.

Expression of genes encoding two WRKY transcription factors was significantly up-regulated during the progress of nematode infection (established by both chip and qPCR results). WRKY was hypothesized to be a common repressor of *PR-1* regulon genes (i.e., genes with common regulation patterns), including peroxidases (Maleck et al., 2000; Chen et al., 2003). Several PR genes, including a peroxidase, were down-regulated in our microarray data during nematode infection (cluster A); the down-regulation of peroxidase was verified in our qPCR datasets. These may have been repressed, during nematode infection by the up-regulated WRKY transcription factors. A similar pattern of up-regulation of WRKY transcription factor was also reported for *Arabidopsis* response to cyst nematodes (Puthoff et al., 2003), and this may be a reflection of a commonality in the host genetic pathways that are activated in response to root knot and cyst nematodes.

*Hormone-associated genes*

The transcription of a gene putatively encoding a gibberellin (GA) 2-oxidase-like protein was up-regulated during the parasitic process (established by both chip and qPCR results). GA 2-oxidases are enzymes involved in GA deactivation, a com-

plex and tightly regulated process, which controls plant elongation (Lester et al., 1999; Thomas et al., 1999), and their up-regulation could reflect reduced levels of active GA during RKN infection. Interestingly, a similar pattern of induction was reported for GA 2-oxidase in the cyst nematode–*Arabidopsis* interaction (Puthoff et al., 2003). Thus, GA-regulated signaling may be common to the plant responses to both root knot and cyst nematodes.

The induction of the expression of two ethylene-responsive elements, an ethylene-responsive element binding protein (EREBP) and an ethylene-responsive transcriptional coactivator, was detected especially at 10 days (through integration of chip and qPCR data). The expression pattern of the ethylene-responsive elements may reflect ethylene involvement in RKN parasitism. This notion was previously suggested by Glazer et al. (1983, 1984), who detected a coincidence of high ethanol production with RKN feeding site development, especially during the second week after inoculation. EREBP expression and involvement of ethylene in the *Arabidopsis* and soybean responses to cyst nematodes were reported previously (Hermsmeier et al., 2000; Wubben et al., 2001; Mazarei et al., 2002; Puthoff et al., 2003). Thus, ethylene-associated genetic pathways may be induced in hosts during parasitism by both cyst and root knot nematodes.

*Development-associated transcription factors*

The kinetics of the down-regulation of transcripts that follows nematode infection, initially detected at 5 days and again at 10 days, was demonstrated for development-associated transcription factors that clustered in cluster A. Of special interest may be the repression of expression of a homeobox transcription factor (a tomato *VAHOX1*) during nematode infection. This repression was demonstrated by both chip and qPCR analysis. Tornero et al. (1996) found that *VAHOX1* was specifically expressed in the phloem during phases of secondary growth, and that it participated in the regulation of the identity and/or activity of tomato phloem tissues during secondary phases of vascular development. In the present study, repression of *VAHOX1* expression during the maintenance of nematode feeding sites (confirmed by both chip and qPCR analysis at 5 and 10 d.p.i) may be indicative of a need for constant and active repression of the acquisition of a fate of vascular plant cells that are the progenitors of giant cells (Williamson and Hussey, 1996), in order to maintain nematode feeding sites.

The broad spectrum and sensitivity of the microarray approach for large-scale mRNA expression profile suggested involvement of putative regulators, such as transcription- and hormone-regulated factors, and putative downstream elements of genetic pathways, such as PR proteins and antimicrobial proteins, in the maintenance of the parasitic association at two examined time points. Examination of additional early and late events of nematode parasitism, and future functional studies of the roles of these genes and genetic pathways, will provide further insights into the genetic web of the compatible response of plant hosts to parasitic RKN.

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