Chapter 9
Transcriptomic and Proteomic Analysis of the Plant Response to Nematode Infection

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9.1 Parasitic Nematode Interaction with Plants

The rhizosphere is a niche environment exploited by a wide variety of microorganisms and plant roots are continuously subjected to a plethora of biotic stresses including being fed upon by plant-parasitic nematodes (Grunewald et al. 2009). Plant-parasitic nematodes can be ecto- or endoparasites and either sedentary or migratory. In the latter case, the nematodes feed upon and quickly destroy plant cells. Sedentary endoparasitism has evolved in the order *Tylenchida*. Nematodes that employ this feeding strategy become immobile after initiating a permanent feeding site. The most studied plant-parasitic nematodes are the sedentary endoparasitic root-knot (*Meloidogyne* spp.) and cyst (*Globodera* and *Heterodera* spp.) nematodes. These nematodes have evolved complex interactive relationships with host cells to form highly specialized nematode feeding sites (NFSs) called giant-cells (GCs) and syncytia, respectively, in infected plant roots from which they withdraw nutrients to sustain a sedentary parasitic lifestyle (Curtis 2007). Understanding the complex signal exchange that occurs during infection of plants is an important parameter for defining those processes that govern parasitic nematode interactions with plant hosts. In order to establish feeding sites, nematodes use secreted effector proteins to manipulate the endogenous molecular and physiological pathways of their hosts. To date, significant progress has been made to identify stylet-secreted effector proteins originating from the pharyngeal glands which assist nematode invasion, migration, and feeding site formation in root tissues (Curtis 2007; Abad and Williamson 2010). On the plant side, molecular studies have shown that physiological changes are accompanied by extensive alterations in plant gene expression (reviewed by Gheysen and Mitchum 2009; Caillaud et al. 2008; Li et al. 2009). However, the functional role in feeding site formation of many of the genes with altered expression remains a mystery. An approach that combines the use of proteomic technology with genet-

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ics is expected to further increase our knowledge of protein function in the plant response to nematode infection. The integration of existing functional genomic data with proteomic analyses will help elucidate the complete picture of the parasitic interaction.

9.2 A Historical View of Methods Used to Study Transcriptional Changes During Plant-Nematode Interactions

The first attempts to identify and clone differentially expressed genes during plant-nematode interactions identified the first GC-induced gene, *TobRB7,* from a tobacco root cDNA library (Opperman et al., 1994). The methodologies available at the time were restricted to differential screening of libraries which required large amounts of RNA, presenting a challenge to obtain enough infected biological material for analysis. Despite this challenge, 11 differentially expressed genes were successfully identified in a study using 1 mg of RNA isolated from tomato roots infected with *Meloidogyne incognita* (Van der Eycken et al., 1996). In a separate report, differentially expressed genes encoding catalases were identified in potato infected with *Globodera pallida* (Niebel et al., 1995). In all studies, timepoints represented late infection stages when the nematode was nearing completion of its life cycle.

The limitation of obtaining enough material enriched in NFSs and the ability to obtain enough RNA for gene expression analyses was soon overcome by the development of PCR-based techniques for transcript amplification. This step forward demanded less starting biological material and allowed for the dissection of tissues limited to the infection area at earlier infection stages than were analyzed in previous studies. From approximately 50 mg of hand-dissected mature tomato GCs, 11 µg of RNA was isolated and 297 differentially expressed transcripts were identified (Wilson et al. 1994). Other studies isolated tissues enriched for NFSs from *Medicago* and tomato roots during the early stages of infection (12–72 h post-inoculation; hpi) with *Meloidogyne* spp. or soybean infected with *Heterodera glycines* (Potenza et al. 2001; Lambert and Williamson 1993; Khan et al. 2004).

PCR-based techniques for transcript amplification, such as differential display and cDNA-AFLP, were also employed for studying plant gene expression changes in response to nematode infection. One of the first studies based on differential display with syncytia enriched material identified 15 differentially expressed transcripts from only 400 ng of RNA using 10 primer combinations in soybean infected with *Heterodera glycines* at 24 hpi (Hermsmeier et al. 1998). Several studies rendered similar numbers of identified clones when meticulous selection and enrichment of the infection structures were used to isolate starting material (Hermsmeier et al. 2000; Vercauteren et al. 2001). One of the most significant differential display analyses, yielding 27 unique sequences, was conducted on microaspirated *Meloidogyne javanica* GCs at 25 days post infection (dpi) (Wang et al. 2003). The

only technical limitation of this analysis was that high turgor pressure precluded the collection of cytoplasm from GCs younger than 15 dpi. In contrast to differential display, only one study based on cDNA-AFLP has been reported. In this study, 15 differentially expressed transcripts were identified during the incompatible interaction of sugar beet (carrying the resistance gene *HS1*$^{pro\text{-}1}$) with *Heterodera schachtii* (Samuelian et al. 2004).

While global analyses of gene expression were ongoing, other analyses focused on characterizing the activation of specific plant gene promoters or enhancers in NFS, based on promoter:gene reporter fusions or enhancer traps employing β-glucuronidase (GUS) or green fluorescent protein (GFP) (Gheysen and Fenoll 2002; Li et al. 2009). The use of luciferase (LUC) as a reporter for studying gene expression changes in NFSs has been limited to genes with transient expression patterns, such as cell cycle genes (Goverse et al. 2000). Although the identification of minimal regulatory sequences from these nematode-activated promoters is scarce, a few promoters such as *TobRB7, AtPYK10, AtPYK20*, and *CaMV35S* have been studied by deletion analysis (Bertioli et al. 1999; Nitz et al. 2001; Opperman et al. 1994; Puzio et al. 1999). Furthermore, putative *cis* elements associated with NFS expression have been proposed based only on *in vitro* analyses (Escobar et al. 1999). Attempts to identify GC activated *cis*-elements determined that *HSEs* are indispensable for the activation of *HSPs* in GCs (Escobar et al. 2003; Barcala et al. 2008).

More recently, technological advancements for single cell isolation such as laser capture microdissection (LCM) and microaspiration, coupled with DNA microarray technology and linear amplification of RNA, have constituted a substantial step forward in the understanding of global transcriptional changes occurring in developing NFSs during plant-nematode interactions.

9.3 Microarray Analysis of Nematode-Infected Root Tissues

DNA microarrays allow the simultaneous analysis of expression changes from a large number of genes, and in some cases, such as in *Arabidopsis*, they are representative of the whole genome. Although this holistic approach constituted considerable progress in the understanding of the transcriptional changes occurring during plant-nematode interactions, microarrays of other plant species such as tomato and soybean have provided a partial picture of transcriptional regulation after nematode infection. This poses a limitation for cross-species comparison of gene expression changes within galls, GCs and/or syncytia. In the coming years, this is expected to change as the genome sequences of several plant species, including tomato and soybean (O'Rourke et al. 2009), have been completed and full genome microarrays become available. Meta-analyses of the microarray data will likely identify commonalities of transcriptional regulation among genes and pathways in galls and syncytia formed in different plant species.

9.3.1 Cyst-Nematodes

The first microarray study to identify plant gene expression changes during cyst nematode parasitism utilized an *Arabidopsis* Affymetrix GeneChip containing probesets for 8,200 genes, representative of approximately one-third of the total genome (Table 9.1; Puthoff et al. 2003). In this study, a comparative analysis between *Heterodera glycines*-infected *Arabidopsis* roots (incompatible interaction) and *H. schachtii*-infected *Arabidopsis* roots (compatible interaction) at 3 dpi was conducted. Infected whole roots were used as the starting material for RNA isolation and 116 genes were identified to be differentially regulated in response to *H. schachtii*. Only 12 genes were found to be differentially regulated by *H. glycines*, but these were also differentially regulated in response to *H. schachtii*. Interestingly, genes uniquely regulated by *H. glycines* were not identified. In fact, the lack of an active defense response at the transcriptional level suggested that the inability of *H. glycines* to parasitize the non-host plant *Arabidopsis* was more likely due to an inability of the two species to communicate (Puthoff et al. 2003). The microarray analysis confirmed previous studies demonstrating that during the compatible interaction the nematode alters the regulation of genes involved in hormone responses, cell wall modification, and cell cycle for syncytium development. Moreover, the downregulation of genes by the nematode is likely to be just as important as upregulaton of specific plant responses for successful infection.

The next phase of microarray studies were conducted on the *H. glycines*-soybean interaction using infected whole root pieces to first probe partial cDNA microarrays (Khan et al. 2004; Alkharouf et al. 2006) and later the Soybean Affymetrix GeneChip during both compatible (Ithal et al. 2007a; Klink et al. 2007a) and incompatible (Klink et al. 2007a; Klink et al. 2010) interactions. These studies (Table 9.1) identified a number of genes involved in primary metabolism, biosynthesis of phenolics, cell wall modification, cell signaling, and transcriptional regulation. A clear trend was the general activation of plant defense genes in response to *H. glycines* during a compatible interaction. In addition, it was found that soybean responds differently, both qualitatively and quantitatively, to avirulent and virulent populations of the *H. glycines* prior to feeding site establishment (Klink et al. 2007a).

9.3.2 Root-Knot Nematodes

The number of microarray based studies for root-knot nematode interactions are limited compared to that of cyst nematode-plant interactions (Table 9.1; reviewed in Li et al. 2009). The first studies were performed on either infected whole roots or hand-dissected galls at early-middle and late infection stages using different oligonucleotide array platforms including Affymetrix, CATMA, and 50-mer MWG Biotech (Hammes et al. 2005; Jammes et al. 2005; Fuller et al. 2007) or cDNA arrays (Bar-Or et al. 2005). Only two plant species, *Arabidopsis* and tomato, have been analyzed in response to root-knot nematodes during a compatible interaction.

Table 9.1 Summary of plant gene expression analyses in response to cyst and root-knot nematodes based on microarrays

Nematode	Population	Plant species	Infection timepoints and isolation methods			Analysis	Reference	Database
			GCs	Syncytia	Biological material			
Heterodera schachtii, H. glycines	OP50, HG-type 1.2.3.5.6.7; *H. schachtii* on cabbage	*Arabidopsis thaliana* (Col-0)			Excised whole root pieces 3 dpi	Affymetrix Arabidopsis GeneChip ~8,200 genes	Puthoff et al. (2003)	Data presented in paper
Heterodera glycines	NL1-RHg, HG-type 7	*Glycine max* Kent			Excised whole root pieces 2 dpi	Soybean cDNA microarray ~1,300 genes	Khan et al. (2004)	http://bioinformatics.towson.edu/SGMD/MicroarrayExps/2D_KentPaper.htm
Heterodera glycines	NL1-RHg, HG-type 7	*Glycine max* Kent			Excised infected whole root pieces 6, 12, 24 hpi; 2, 4, 6, 8 dpi	Soybean cDNA microarrays ~6,000 genes	Alkharouf et al. (2006)	http://bioinformatics.towson.edu/SGMD/Publications/KentTmeline/index.htm
Heterodera glycines	PA3, HG-type 0	*Glycine max* Williams 82			Excised infected whole root pieces 2, 5, 10 dpi	Affymetrix Soybean GeneChip	Ithal et al. (2007a)	ArrayExpress Accession #E-MEXP-808
Heterodera glycines	NL1-RHg, HG-type 7; TN8, HG-type 1.3.6.7	*Glycine max* Peking			Excised whole root pieces 12 hpi, 3 and 8 dpi	Affymetrix Soybean GeneChip	Klink et al. (2007a)	Soybean Genomics and Microarray Database; http://www.towson.edu/nalkharo/SGMD/SupplementalSites/GmWholeRootIC/
Heterodera glycines	PA3, HG-type 0	*Glycine max* Williams 82		LCM 2, 5, 10 dpi		Affymetrix Soybean GeneChip	Ithal et al. (2007b)	ArrayExpress Accession #E-MEXP-876
Heterodera glycines	NL1-RHg, HG-type 7; TN8, HG-type 1.3.6.7	*Glycine max* Peking		LCM 3, 8 dpi		Affymetrix Soybean GeneChip	Klink et al. (2007b)	Soybean Genomics and Microarray Database; http://bioinformatics.towson.edu/SGMD3/
Heterodera schachtii	*H. schachtii* on mustard	*Arabidopsis thaliana* (Col-0)		Micro-aspiration, 5, 15 dpi		Affymetrix Arabidopsis (ATH1) GeneChip	Szakasits et al. (2009)	http://bioinf.boku.ac.at/pub/Szakasits2008/

Table 9.1 (continued)

Nematode	Population	Plant species	Infection timepoints and isolation methods			Analysis	Reference	Database
			GCs	Syncytia	Biological material			
Heterodera glycines	NL1-RHg, HG-type 7	*Glycine max* PI88788		LCM 3, 6, 9 dpi		Affymetrix Soybean GeneChip	Klink et al. (2010)	Soybean Genomics and Microarray Database; http://bioinformatics.towson.edu/SGMD3/
Meloidogyne incognita		*Arabidopsis thaliana* (Wassilewskija)			Hand-dissected galls 7, 14, 21 dpi	CATMA array	Jammes et al. (2005)	Array Express (accession number E-MEXP-233; http://www.ebi.ac.uk/arrayexpress)
Meloidogyne incognita		*Arabidopsis thaliana* (Col-0)			Hand-dissected galls 21 dpi	Arrays printed with the MWG 25,000 Arabidopsis 50mer oligonucleotide set.	Fuller et al. (2007)	TAIR (http://www.arabidopsis.org/tools/bulk/go/index.jsp)
Meloidogyne incognita		*Arabidopsis thaliana* (Col-0)			Whole infested roots 7, 15, 30 dpi	Affymetrix Arabidopsis ATH1	Hammes et al. (2005)	MIAME: www.mged.org/Workgroups/MIAME/miame.html
Meloidogyne javanica		*Arabidopsis thaliana* (Col-0)	LCM, 3 dpi		Hand-dissected galls 3 dpi	*Arabidopsis* synthetic 70-mer oligonucleotides set, version 3 from Qiagen-Operon obtained from Dr. David Galbraith (University of Arizona).	Barcala et al. (2010)	Array express http://www.ebi.ac.uk/microarray-as/aer/entry
Meloidogyne javanica		*Solanum lycopersicum* Mill cv. Moneymaker	LCM, 3, 7 dpi		Hand-dissected galls 3, 7, 14 dpi	TOM1 microarray slides from the Center for Gene Expression Profiling (CGEP) at the Boyce Thompson Institute	Portillo et al. (2009; unpublished)	Data presented in paper

These analyses (Table 9.1) have revealed interesting information regarding different aspects of transcript regulation across the infection stages. For example, only 11.4% of the genes differentially expressed at 5 dpi in tomato galls were common to 10 dpi galls, suggesting qualitative differences in gene expression throughout infection. In addition, the amplitude of variation among the common genes was higher at 10 dpi than at 5 dpi, which indicates a more vigorous response at the later stage (Bar-Or et al. 2005). Certain groups of genes, such as those with functions in cell wall and cytoskeleton remodeling and hormone–associated genes are similarly regulated in galls in different plant species and experiments. In contrast, the group of genes related to transcriptional regulation and defense, show more heterogeneity in their responses. For example, in tomato galls most pathogenesis-related (PR) genes were induced, including a defensin and a harpin-induced gene (*hin-1*) (Bar-Or et al. 2005). Similarly, *Arabidopsis* microarray studies of *M. javanica* galls at 3 dpi and *M. incognita* galls at 21 dpi found that most of the biotic stress genes were up-regulated in hand-dissected galls (Fuller et al. 2007; Barcala et al. 2010). In contrast, genes encoding PR proteins and several WRKY coding transcription factors, mainly involved in plant defense against pathogens, were found to be repressed in an *Arabidopsis* microarray study of *M. incognita* galls at either 7, 14, or 21 days post-infection (Jammes et al. 2005). The discrepancies among studies may be attributed to differences in the infection stages and the reference tissues used in each study, or perhaps subtle differences in the plant responses to the nematode populations used.

To date, there have been few attempts to compare gene expression of hand-dissected galls or whole root pieces containing syncytia with that of isolated GCs or syncytia at the same early infection stage in the same plant system; however, these studies have revealed important differences (Ithal et al. 2007b; Barcala et al. 2010). Normalization of expression data obtained from different microarray platforms and further modifications in the interpretation of data that could account for the lack of experimental uniformity will be necessary in order to exploit the available microarray datasets for a better understanding of plant-nematode interactions. This is an endeavor that will no doubt require cross-disciplinary expertise in bioinformatics.

9.3.3 Analysis of Isolated Feeding Cells

Giant-cells are embedded in a voluminous root structure called a gall that forms as a result of hyperplasia of surrounding cells (Gheysen and Fenoll 2002). The volume contribution of five to eight GCs to the total volume of a gall is quite small at least at early developmental stages. Similarly, a syncytium, which can form a complex of up to 200 cells, is still only a small fraction of the total root cell population. Therefore, for detailed information of the molecular changes occurring within these specialized cells (GCs and syncytia), their specific isolation is crucial. The first attempts to isolate GCs was by hand-dissection from tomato galls from late stage infection (1–2 months) (Wilson et al. 1994). Since then, different methodologies

have been employed or developed for the isolation of individual plant cells or cell types for microanalysis of plant-microbe interactions (He et al. 2005; Ramsay et al. 2006). Some methods, such as microfabricated nanocutting devices of high precision (Chang et al. 2006) have only been used in animal systems. However, for GC and syncytium isolation, laser-capture microdissection (LCM) and microaspiration with a modified pressure probe attached to an oil-filled microcapillary (Fig. 9.1a, b, respectively; Ramsay et al. 2006; Portillo et al. 2009) have rendered interesting results (Table 9.1).

One of the first demonstrations of the strong dilution effect of GC-specific transcripts in whole galls by qPCR was performed from microcapillary-aspirated tomato GCs at 25 dpi (Wang et al. 2003). Again, cytosolic material was extracted only from late infection stages, possibly because a high turgor pressure precluded the aspiration of younger cells. LCM established a clear improvement, as GCs were isolated as early as 48 to 92 hpi, when their first morphological features are clearly distinguishable in sections, either in paraffin-embedded or in optimal cutting tem-

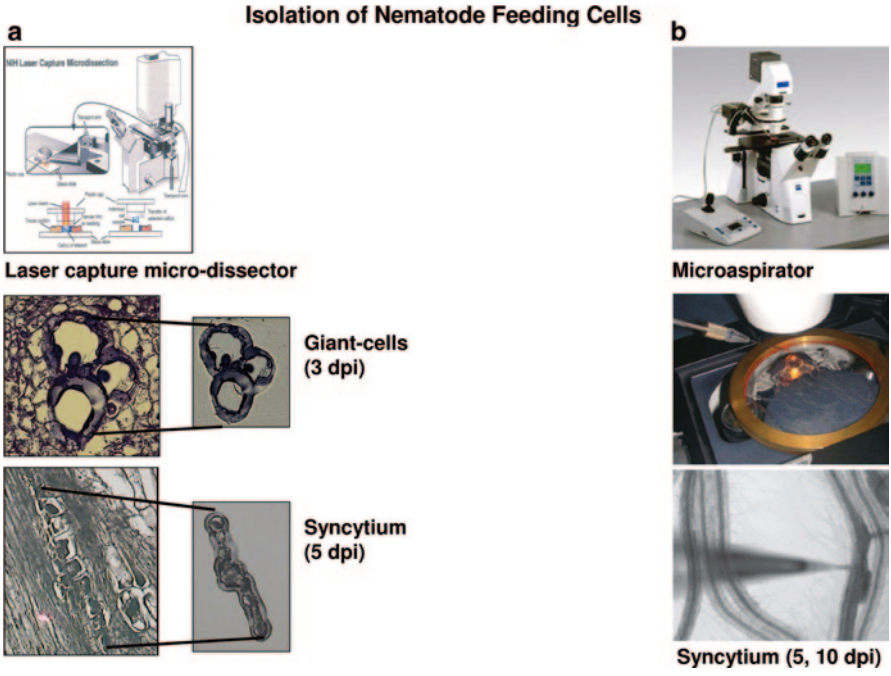

Fig. 9.1 Schematic representation of two methods used for isolation of nematode-feeding cells. **a** upper panel, laser capture microdissector device; second and third panels represent the capture of giant cells (GCs) and syncytia respectively. **b** upper panel, microaspirator device; second panel, metal ring fixed under an inverse microscope (Zeiss, http://www.zeiss.com) holds a thin glass plate covered with medium enclosing the roots; third panel, a microcapillary is guided towards the roots by a micromanipulator (Eppendorf, http://www.eppendorf.com) for piercing a single syncytium. (Szakasits et al. 2009)

perature media (OCT) from cryosections (Ramsay et al. 2004; Fosu-Nyarko et al. 2009; Portillo et al. 2009). As little as two collection caps containing 100 LCM GCs, was sufficient for successful PCR amplification of 4 out of 7 genes tested, including a loading control. Transcripts from a *MAPK* gene and the *LeCycD3;3* and *LeCycD3;2* genes were clearly detected (Ramsay et al. 2004). However, it is important to note that the mRNA recovery and its amplification, as well as an adequate RT-PCR product of a particular gene does not in itself guarantee the structural integrity of RNA after LCM (Fig. 9.2; Portillo et al. 2009). Thus, sensitive techniques, such as electropherograms, are recommended to test the quality and integrity of the amplified RNA (aRNA) before using it for transcriptomic analysis. One parameter crucial for achieving good integrity RNA from cryosections is the quantity of starting material. For *Arabidopsis* and tomato GCs, 200–300 GCs isolated at 3 and/or 7 dpi have been shown to yield high quality RNA for subsequent microarray analysis. Whether RNA integrity also depends on the fixation and embedding protocols used for galls is something that remains to be determined as there are currently only two

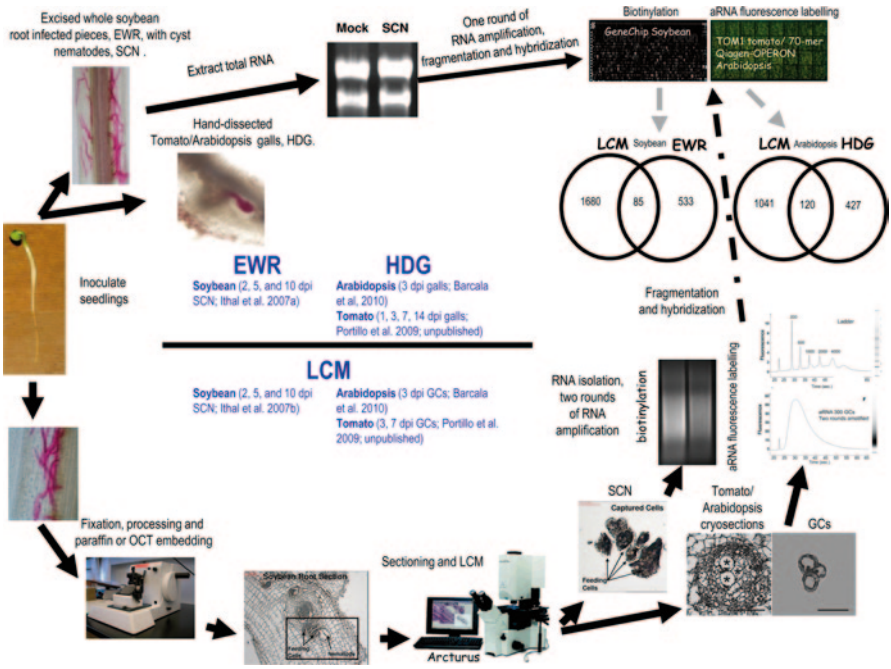

Fig. 9.2 Diagram representing the steps required for the RNA extraction of excised whole soybean root pieces (EWR), infected with cyst nematodes (SCN) and hand dissected galls (HDG) formed by root-knot nematodes for subsequent microarray hybridization, upper part. Steps required for laser micro-dissection of giant cells (GCs), and soybean syncytia (SCN), RNA amplification and quality checking for microarrays hybridisation, lower part. A Venn diagram representing the common genes in the intersection of EWR, *versus* LCM-SCN, as well as HDG *versus* LCM-GCs. OCT, optimal cutting temperature media; LCM, laser capture micro-dissection

LCM GC studies published, both from cryosectioned galls (Portillo et al. 2009; Barcala et al. 2010).

The use of giant-cell cytoplasm as starting biological material for further transcriptomic analysis provided more accurate information on the relative levels of gene expression in GCs, either by classical PCR, qPCR or microarray analysis (Ramsay et al. 2004; Barcala et al. 2010), and has also allowed the identification and isolation of particular genes by differential display and library construction (Table 9.1; Wang et al. 2003; Fosu-Nyarko et al. 2009). Additional confirmation of a strong dilution of the GC transcripts in galls was provided from microarray data of LCM GCs isolated at 3 dpi compared to their corresponding hand-dissected galls (Barcala et al. 2010) (Fig. 9.2). This effect was clearly enhanced for genes with lower expression changes (fold change values of −1 to −3 and 1 to 3) in GCs as compared to uninfected cells from vascular tissue. In these fold change ranges, most of the differentially expressed GC transcripts were not detected in the whole gall transcriptome (Barcala et al. 2010). Similarly, this trend was confirmed in tomato at the same and even later infection stages (3, 7 dpi; Portillo et al. (2009); unpublished). Only 120 genes out of 1,161 differentially expressed in GCs were shared with those of the gall transcriptome in *Arabidopsis* (Fig. 9.2) and the tendency was similar in tomato. Reliability of the comparison was high as both analyses were performed using the same microarray platform with exactly the same experimental design, hybridization steps and data processing. One of the most striking differences between galls and GCs was the identification of a high number of down-regulated genes in GCs that were not detected as being differentially expressed in galls. Furthermore, the categories of secondary metabolism and biotic stress included a high proportion of 'gall and GC distinctive genes', but with opposite expression patterns (repressed in GCs, but up-regulated in galls). In addition, only eight out of the more than 100 genes encoding transcription factors differentially expressed in GCs were co-regulated between GCs and galls. In contrast, genes related to cell wall modification, such as expansins (EXPA6, EXPA1 and EXPA2), were mostly up-regulated co-regulated genes in GCs and galls. These data suggest that genes typically involved in defense mechanisms against pathogen attack (Dixon et al. 2002), such as those involved in the phenylpropanoid pathway were probably shut-off by the nematode exclusively in GCs, but not in the rest of the gall tissues (Barcala et al. 2010). This interpretation is supported by the identification of nematode pathogenicity factors such as secreted chorismate mutase and calreticulin which may be directly involved in plant defense suppression (Doyle and Lambert 2003; Jaubert et al. 2005).

Sensitivity in detecting gene expression changes specific to feeding cells induced by cyst nematodes was also increased when LCM and microaspiration approaches were employed to isolate the contents of syncytia (Fig. 9.2; Klink et al. 2005; Ithal et al. 2007b; Klink et al. 2007b; Szakasits et al. 2009). Microarray analyses using RNA isolated from laser-microdissected syncytia resulted in a substantial increase in the number of differentially expressed genes that were identified compared to microarray analyses using total RNA isolated from nematode-infected whole root pieces (Table 9.1; Ithal et al. 2007b; Klink et al. 2007b). Although both approaches

identified genes in common (Ithal et al. 2007b), the fold-change in expression of these genes in the LCM study was on average 26-fold higher and an additional 1,680 genes were identified. A similar increase in sensitivity was demonstrated when microaspirated syncytium cytoplasm was used for microarray analyses (Szakasits et al. 2009). In contrast to microaspiration studies at 5 dpi, which identified 18.4% of the total number of genes represented on the GeneChip as upregulated and 15.8% as downregulated (Szakasits et al. 2009), an analysis of infected whole roots at 3 dpi only identified 1% (upregulated) and 0.6% (downregulated) of the total genes represented as being differentially regulated (Puthoff et al. 2003).

A direct comparison between soybean gene expression changes identified using infected whole root pieces with those from syncytia after LCM, revealed only a small percentage of genes in common (Fig. 9.2; Ithal et al. 2007a, b; Klink et al. 2007b). Many of the co-upregulated genes included those belonging to the multibranched phenylpropanoid pathway which leads to the production of a diverse number of secondary metabolites in plants, including flavonoids, anthocyanins, and secondary cell wall components, genes involved in cell-wall related processes such as those coding for expansins and extensins, and genes that code for proteins involved in general stress responses including peroxidases, glutathione S-transferases, harpin-induced gene family members, and disease resistance-responsive family proteins (Ithal et al. 2007a, b; Klink et al. 2007b). In the LCM study reported by Ithal et al. (2007b), JA biosynthesis genes and other genes associated with abiotic and biotic stress responses including senescence-associated proteins, wound and osmotic stress responsive genes, and pathogen responsive receptor-like kinases were downregulated in syncytia. Consequently, the upregulation of genes involved in general plant defense identified from studies of infected whole root pieces may include a response of the plant to the intracellular migration and early establishment of feeding sites by the nematodes; components of which may be later suppressed by the pathogen as syncytia develop, similarly to GCs.

Although LCM has proven to be an effective tool to study gene expression in nematode feeding cells, GCs and syncytia are not clearly distinguished in sections at very early differentiation stages (12–48 hpi). This is partly due to the intrinsic characteristics of the developing feeding cells, and partly due to the tissue processing steps prior to LCM. In general, before microdissection, GCs and syncytia can be recognized in sections by their dense cytoplasm, sometimes slightly plasmolysed due to the fixation and dehydration treatments (Barcala et al. 2010). In addition, LCM requires mild fixation treatments to preserve macromolecules, but it produces a poorer preservation of the histological structures (Ramsay et al. 2004, 2006; Portillo et al. 2009). It is recommended to omit histochemical staining during sample processing to minimize RNA degradation (Ramsay et al. 2004). In addition, GCs and syncytia at very early developmental stages do not show unambiguous morphological features. Thus, the isolation of GCs and syncytia during the early stages of differentiation will require the development of new strategies, such as the combination of reporter lines activated at early infection stages during GC and syncytia differentiation to aid in the identification of the developing cells before LCM is

applied. The combination of LCM coupled to epifluorescence microscopes could allow for the isolation of emergent GCs and syncytia from their precursor cells that still do not show unequivocal morphological characters.

9.4 Next Generation Sequencing Technology to Study Plant Responses to Nematode Infection

Next generation rapid sequencing technology has been used only once for the study of the plant response to nematode infection (Hewezi et al. 2008). In this study, small RNA molecules were purified from total RNA isolated from cyst nematode-infected *Arabidopsis* roots by size fractionation and 100,000 sequence reads were obtained using 454 sequencing technology. Of 16 miRNAs checked after *H. schachtii* infection, 14 were altered at 4 dpi and 7 were altered at 7 dpi (Hewezi et al. 2008). Such approaches open the possibility of using different plant species from which scarce sequence data are available. Cross-species comparisons of data could perhaps also reveal physiological responses in galls and syncytia conserved among species or identify responses unique to each plant species-nematode interaction.

9.5 Proteomic Analysis of the Plant Response to Nematode Infection

9.5.1 Application of Proteomics to Investigate Plant-Microbe Interactions

The term proteome refers to the complete set of proteins present in a cell, organ or organism at a given time (Wilkins et al. 1995). Advances in proteomics have been made possible due to improvements in protein separation by two dimensional-gel electrophoresis (2-DE) (Görg et al. 2000), multidimensional liquid chromatography (MudPIT) (Washburn et al. 2001), peptide sequencing by mass spectrometry (MS) (Steen and Mann 2004; Venable et al. 2004), and bioinformatics (Apweiler et al. 2004). However, progress in defining proteomes is expected to proceed at a slower pace than genome sequencing (Jorrin et al. 2006). The application of proteomics in plant pathology is being used to characterize pathogen virulence factors, as well as to identify changes in protein levels in plant hosts upon infection (Kav et al. 2007). It is well known that nematode feeding site ontogeny is a reflection of extensive gene expression modification in infected root cells (Caillaud et al. 2008; Li et al. 2009). In contrast, strategies aimed at studying the proteomic plant response to nematodes are still in their infancy. Technical advances in the quality and reproducibility of 2-DE gels, software packages to process digitized images of gels, the development of non-gel based high-throughput protein separation techniques, and

analytical mass spectrometry should facilitate an increase in proteomic approaches to study plant-nematode interactions (Chen and Harmon 2006; Domon and Aebersold 2006). In order to establish a reliable, specific proteomic study, cell-specific analysis is a prerequisite as tissues are usually composed of heterogeneous cell populations and molecular analysis of biological samples as a whole may be of limited value. LCM and microaspiration provide powerful new tools to extract proteins from feeding sites for molecular analysis of the plant-nematode interactions. Although transcriptomic analyses have already been performed (Sect. 9.3.3), proteomics studies utilizing LCM to analyze the plant responses underlying GC and syncytium formation are still at a very early stage.

9.5.2 *Understanding Plant-Nematode Interactions in Light of Proteomic Studies*

The current knowledge on plant-nematode interactions is primarily based on genome and transcriptome analysis with few studies focused on the proteomic profiles of plants infected by nematodes. One of the first studies to examine changes in protein expression in response to nematode parasitism compared *Globodera rostochiensis* infected and uninfected roots of potato carrying the *H1* resistance gene. The presence of the nematodes in the root system did not cause any changes at 6 and 21 hpi, or at 3 and 6 dpi. Surprisingly, variations were observed in young leaves of infected plants (Hammond-Kosack et al. 1990). In contrast, Callahan et al. (1997) reported different results on one- and 2-DE analysis of resistant and susceptible cotton (*Gossypium hirsutum* L.) root protein extracts. Several polypeptides were differentially expressed in response to root-knot nematode infection. A novel 14 k Da polypeptide was more abundantly expressed in young galls of the resistant isoline at 8 dpi (Callahan et al. 1997). The profile of genes or proteins induced by the nematodes during feeding site formation can generate potential targets for reverse genetics. For example, the tomato expansin gene *LeEXPA5* found to be expressed during *Meloidogyne javanica* parasitism, facilitates cell expansion *in vivo* and was presumed to be important for the expansion of GCs. Consistently, the ability of nematodes to complete their life cycle on *LeEXPA5*-antisense transgenic roots was reduced (Gal et al. 2006). Thus, proteomic approaches coupled with functional genomics tools presents a powerful approach for the identification of targets for the development of transgenic crops resistant to nematodes.

Root proteomics aimed at discovering plant defense-related proteins in roots have been studied in nematode-resistant cotton and coffee cultivars infected with *Meloidogyne paranaensis* and *M. incognita*, respectively. A 2-DE analysis comparing infected versus non-infected roots identified a class III chitinase of *C. arabica*, known to be involved in defence responses to pathogens (Jaubert et al. 2002). Another differentially expressed protein in cotton was a quinone reductase 2 (QR2) that catalyzes the divalent reduction of quinones to hydroquinones to protect plant cells from oxidative damage (Sparla et al. 1999). These findings emphasize the importance of root

proteomics in the isolation of resistance and defense-related proteins against nematodes. Ultimately, transcriptomic, proteomic and metabolomic analyses will need to be integrated to elucidate the complex nature of the plant response to nematodes.

9.6 Conclusions

A vast list of differentially expressed genes identified from comparisons of either infected roots *versus* uninfected roots or from hand-dissected root nematode induced structures, is available in different databases and publications. Furthermore, the combination of precise cell-specific isolation techniques, such as LCM and microaspiration, together with holistic approaches for gene expression analysis based on microarrays, differential display, and EST sequencing have identified a diverse catalogue of genes differentially expressed in GCs and syncytia. Nevertheless, information pertaining to which genes have restricted expression in NFS is limited. Similarly, there is still fragmented information on comparative analyses among different experiments and plant-nematode interactions. Although in their infancy, the application of next generation sequencing technologies and proteomics analysis promises to provide more functional information on the sophisticated interactions between nematodes and their host plants.

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