**Title**: Nematode feeding sites: unique organs in plant roots

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**Abstract**

Although generally unnoticed, nearly all crop plants have one or more species of nematodes that feed on their roots, frequently causing tremendous yield losses. The group of sedentary nematodes, which are among the most damaging plant-parasitic nematodes, cause the formation of special organs called nematode feeding sites (NFS) in the root tissue. In this review we discuss key metabolic and cellular changes correlated with NFS development, and similarities and discrepancies between different types of NFS are highlighted.

**Keywords**: sedentary nematode, giant-cell, syncytium, phytohormones

Plant-parasitic nematodes are generally subdivided in groups according to their infection style. Migratory endoparasitic nematodes cause mechanical damage to the root system, by moving through and destroying the cells from which they feed. On the other hand, sedentary endoparasitic nematodes only move within the root during migration as the second stage juvenile. Once they reach a suitable site in the host root they manipulate the normal root physiology to induce a specialized nematode feeding site (NFS) called syncytium (cyst nematodes, CN; *Heterodera* and *Globodera* spp.) or giant-cells (root-knot nematodes, RKN; *Meloidogyne* spp.). For both types of nematodes, the second stage infective juveniles hatch from the eggs and invade the plant root generally in the elongation zone. CN penetrate the host root tissue by breaking and puncturing cells during migration. Conversely, RKN move intercellularly downwards the root towards the root apex, where they make a U-turn and move upwards in the vascular cylinder. RKN transform four to eight root cells into giant-cells from which the nematodes feed (Fig. 1A). The first sign of giant-cell induction is the formation of binucleate cells (de Almeida Engler et al. 2011). Repeated rounds of nuclear division and cell growth in the absence of cytokinesis lead to the formation of multinucleate, hypertrophied giant-cells up to 100-times the size of normal root vascular parenchyma cells. Hyperplasy of surrounding cells causes the formation of typical root-knots or galls. CN select only one cell, often of the vascular parenchyma or cortex, to pierce and induce the formation of a syncytium (Fig. 1B). This syncytium becomes a large multinucleate cell formed by the breakdown of plant cell walls, and subsequent fusion of adjacent protoplasts (Grundler et al. 1998). At their feeding site, the RKN and CN become sedentary and extract nutrients from the enlarged plant cells for their growth and reproduction. Through a further 3 moults they develop into females or eventually males. Female CN mature into cysts that contain several hundred eggs, whereas female RKN exude egg masses directly into the rhizosphere or within the gall tissue.

Despite their different ontogeny, many of the functions and cellular features of the two types of NFS are similar. Both have a dense cytoplasm, multiple enlarged nuclei, small vacuoles and show proliferation of smooth endoplasmic reticulum, ribosomes, mitochondria, and plastids. Both types of feeding sites involve a major developmental reprogramming (Gheysen and Fenoll 2002), correlated with a differential activity of genes related to metabolism, stress responses, protein synthesis, cell division, transport and signal transduction, and hence they should be considered to be a unique plant organ.

**The role of NFS as a source of nutrients for the nematode**

Nematode feeding sites (NFS) are the sole source of nutrients for the developing sedentary nematodes within the root tissue. All transcriptome analyses report the induction of the primary metabolism in both types of NFS (e.g. Jammes et al. 2005; Ithal et al. 2007a & b; Kyndt et al. 2012a). Metabolic analyses on syncytia induced by *Heterodera schachtii* in *Arabidopsis thaliana* showed that this high activity leads to elevated sucrose levels, and the accumulation of starch in the plastids (Hofmann et al. 2007, [2008](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2718214/#bib18)), which probably acts as a carbohydrate buffer to compensate for changing solute uptake by the nematode (Hofmann et al. 2008). A further in-depth metabolic profiling also revealed increased levels of many amino acids and phosphorylated metabolites in syncytia, as well as a high accumulation of specific sugars, such as 1-kestose (Hofmann et al. 2010).

In addition to the modified metabolism inside the NFS there are also many reports exemplifying massive intra- and intercellular solute allocations. Investigations on phloem transport revealed that labeled solutes were able to enter CN-induced syncytia or RKN-induced giant-cells ([Dorhout et al. 1993](http://www.plantphysiology.org/content/138/1/383.full#ref-7); [Bockenhoff et al. 1996](http://www.plantphysiology.org/content/138/1/383.full#ref-4)). These observations suggested that these probes might be symplastically unloaded from the phloem into NFS via connecting plasmodesmata. Nevertheless, reports about symplastic isolation of NFS have later been made, making this a point of discussion.

Plasmodesmata connecting the syncytium with neighboring cells were detected in young syncytia (Hoth et al. 2005; Hoth et al. 2008), but their limited number, and temporal callose deposition along these plasmodesmata indicated impaired symplasmic exchange (Grundler et al. 1998; Hofmann et al. 2010). Indeed, experiments with carboxyfluorescein diacetate confirmed the symplasmic isolation of young syncytia (Hofmann et al. 2007), indicating that transporters are required for nutrient import in almost all young syncytia. Nevertheless, at later developmental stages, syncytial cells are no longer symplastically isolated, and functional plasmodesmata between syncytia and the phloem have been detected (starting around 10 dpi; Hofmann and Grundler 2006; Hofmann et al. 2007).

The symplastic isolation of giant-cells is also under discussion. Just like in syncytia, the connection between giant-cells and neighbouring cells might differ depending on their developmental stage. Huang and Maggenti (1969) concluded that giant-cells were symplastically isolated based on the lack of plasmodesmata on their cell wall ingrowths. However, Hoth et al. (2008) showed that the giant-cells are connected by plasmodesmata between-them, but isolated from the surrounding tissue. And later, Hofmann et al. (2010) reported the presence of not only numerous plasmodesmata along the cell wall between giant-cells but also in cell walls towards neighbouring cells.

Whether or not NFS are symplastically isolated, transporter proteins also take part in an active nutrient uptake into syncytia and giant-cells. Distinct sets of transporters, among which aquaporins, Ca2+-ATPase genes, sucrose and amino acid transporters, seem to fulfill this role at different developmental stages in syncytia or giant-cells (Opperman et al. 1994; Hammes et al. 2005; Jammes et al. 2005; Barcala et al. 2010).

**Cell expansion and cell wall modifications in NFS**

Both CN and RKN secrete an arsenal of plant cell-wall degrading and modifying enzymes into the plant tissue (Haegeman et al. 2012). However, cell growth and expansion of the NFS are hypothesized to be mediated mainly by up-regulation of plant genes encoding proteins that promote wall loosening such as endoglucanases, pectinases, and expansins (Wieczorek et al. 2006, 2008; reviewed by Gheysen and Mitchum 2009).

Cell expansion is generally associated with structural changes in cell wall xyloglucans, the major component of hemicellulose, which play an important role in cell wall elasticity and rigidity ([Scheller and Ulvskov 2010](#_ENREF_55)). The xyloglucan endo-transglycosylase/hydrolase (XTH) genes, belonging to the GHF16 family, encode proteins that can potentially have two distinct catalytic activities: xyloglucan endo-transglycosylase activity results in the nonhydrolytic cleavage and ligation of xyloglucan chains, whereas xyloglucan endo-hydrolase activity yields irreversible chain shortening (Eklöf and Brumer 2010). In plant cells, XTHs likely contribute towards cell wall reinforcement once expansion is complete (Campbell and Braam 1999). The role of XTHs during NFS formation is elusive, as this gene family seems to be up or downregulated depending on the developmental time point of the NFS, the type of NFS and the gene family member (reviewed by Gheysen and Mitchum 2009). Due to re-allocation of metabolites into the NFS, turgor pressure rises ([Böckenhoff 199](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2718214/#bib3)5). To withstand the rising pressure, both giant-cells and outer walls of syncytia induce thickening of the cell wall, probably linked to cell-wall reinforcing mechanisms like induction of XTHs, callose or lignin deposition (Grundler et al. 1998), and peroxidase activity (Ithal et al. 2007b).

Although many similarities in cell wall composition have been observed, giant-cells and syncytia encompass a different activity of cellulose synthase genes. The activity of these genes, whose primary responsibility is cellulose anabolism, is reduced in syncytia (Ithal et al. 2007b; Ibrahim et al. 2011), which is not surprising since cell fusion is needed to form this type of NFS. On the other hand, in RKN infection sites many members of the Arabidopsis cellulose synthase A (*CesA*) gene family, responsible for both primary and secondary cell wall synthesis, were induced. *CesA* genes showed maximum activity in RKN infection sites at five days post-inoculation, where secondary cell wall *CesA* genes were primarily expressed within the giant-cells and expression of primary cell wall *CesA* genes was mainly localized to neighboring hyperplastic cells (Hudson 2008).

**Nematodes trigger and sustain the ongoing plant host cell cycle machinery**

As a consequence of a cross talk between nematode secreted proteins and various host molecular pathways (de Almeida Engler and Gheysen 2013), the development of both giant-cells and syncytia involves coordinated cell cycle programs ensuing the formation of these multinucleate feeding cells (Fig. 1A-B). Formation of a NFS comprises an acytokinetic mitotic phase for giant-cells and a period of DNA synthesis linked to endoreduplication for both giant-cells and syncytia. This results in the enlargement of nuclei in these feeding sites.

The control of the cell cycle among eukaryotes is quite conserved, with both mitotic or endoreduplication cycles driven by an analogous cell cycle machinery (Inzé and De Veylder 2006). Progression through the cell cycle depends on the timely activation of the *bona fide* cyclin-dependent kinases (CDKs), which are monitored by the concerted action of multiple transcriptional and post-translational mechanisms that control their activity via the core cell cycle machinery. This includes their association with cyclins (CYC), CDK inhibitors, kinases and phosphatases, ubiquitin-dependent proteolysis and intracellular trafficking (Inzé and De Veylder 2006).

Early investigations revealed that the induction of NFS involves not only the induced transcription of both *CDKA;1* and *CYCB1;1*, and several D-type cyclins, but also the activation of G2-to-M phase transition genes like *CDKB1;1* and *CYCA2;1* (Niebel et al. 1996; de Almeida Engler et al. 1999). Besides, *in situ* hybridization analyses of 61 core cell cycle genes revealed their transcriptional activity in both types of feeding sites (de Almeida Engler et al. unpublished data), suggesting that all these components will orchestrate cell cycle progression during the different stages of NFS development (de Almeida Engler and Gheysen 2013). The mitotic phase will be acytokinetic in giant-cells, and by cytokinesis in neighboring cells of galls and syncytia. Recurring DNA replication cycles generate ample numbers of enlarged nuclei (Fig. 1C-D) to sustain the vital metabolic activity phase (Wiggers et al. 1990; Starr 1993; de Almeida Engler and Gheysen 2013). In plant cells the switch from the mitotic to the endoreduplication cycle can be linked to the increased expression of cyclin-dependent kinase inhibitory proteins like KRPs (Verkest et al. 2005) and SIMs (Churchman et al. 2006), or by up-regulation of the APC/C components like CCS52A (Vinardell et al. 2003). As cells enter the endocycle program, they lose CDKB activity leading to a stabilization of KRPs and resulting in a subsequent inhibition of CDKA activity (Verkest et al. 2005). It is likely that a balance of the mitotic cycle followed by an increase in ploidy levels during nematode infection will ultimately affect giant-cell’s fate and maintenance. The decreased expression of *CDKB1;1*, concerted with other mitotic cyclins in developing giant-cells suggests an exit of the G2-to-M phase during giant-cell expansion and maturation (de Almeida Engler et al. 1999). Components known to be involved in this switch have recently been examined in developing galls and syncytia. Overexpression of CDK inhibitors like *KRP1*, *KRP2* (Fig. 1E-G) and *KRP4* has shown to significantly reduce gall size and to affect progression of nuclear division in giant-cells (Vieira et al. 2012a, 2013). The involvement of particular endocycle genes with feeding sites development brought us a step closer to understand how the plant cell cycle is usurped by these endoparasitic nematodes. Important components of the endocycle machinery in NFS are: anaphase-promoting complex (APC) genes (*CCS52A* and *CCS52B*), the endocycle repressor DP-E2F-like (*E2F/DEL1*) gene and the ROOT HAIRLESS 1 PROTEIN (RHL1), which is part of a multiprotein complex of the toposiomerase VI (Koltai et al. 2001; Favery et al. 2002; de Almeida Engler et al. 2012). On the whole, manipulation of cell cycle gene expression in Arabidopsis plants by down- or over-expression strategies strongly influences the extent of both mitotic and endoreduplication cycles in galls as well as in syncytia leading to a disturbance of the nematode’s life cycle and reproduction (Fig. 1H-I).

**Rapidly expanding nematode-induced feeding sites require cytoskeleton reshuffling**

Studies on cytoskeleton organization in nematode feeding cells illustrated that partial depolymerisation of the microtubule as well as the actin cytoskeleton occur in giant-cells induced by RKN as well as in syncytia produced by CN in host plant root cells.

Expression analyses have shown that tubulin genes, as well as actin-2 and -7 are highly transcribed in galls and syncytia (de Almeida Engler et al. 2004; Banora et al. 2011). *In situ* immunocytochemical analyses of actins and tubulins and *in vivo* examination of microtubules in nematode infected root cells revealed that cytoplasmic microtubules (Fig. 2A), as well as actin filaments (Fig. 2B), are disorganized and appear partially depolymerized. Interestingly, the cortical cytoskeleton in giant-cells remains dense and filamentous (de Almeida Engler and Favery 2011).

In multinucleate giant-cells multiple diverse shaped phragmoplasts develop in late anaphase. Misaligned phragmoplasts may cause a wrong positioning of vesicles and guidance of the fusion process, therefore disturbing proper cell wall formation (de Almeida Engler and Favery 2011). Alternatively, double ring-like phragmoplasts of antiparallel microtubules and actin filaments gradually expand outwards along with the formation of new cell plate fragments of different sizes. These will eventually touch one side of the giant-cell resulting in a wall stub. Other giant-cell nuclei do not form a phragmoplast. The end result is aborted cytokinesis and the formation of giant-cells where the cell plate is either affected or absent. Mitotic figures or mitotic cytoskeleton structures such as phragmoplasts were to date not spotted in syncytia, suggesting the absence of nuclear division within the feeding site. This mitotic activity seems to be restricted to cells neighboring the feeding site before fusion (de Almeida Engler and Favery 2011).

Recent studies have reported that γ-tubulin is most likely a component of microtubule organizing centers (MTOCs) in NFS. γ-tubulins are highly expressed in syncytia (Fig. 2C) and galls (Fig. 2D,E) and are possibly responsible for *de novo* synthesis of microtubules (Banora et al. 2011). γ-tubulins also co-localise with α-tubulins (Youssef-Banora et al. 2011) supporting the hypothesis of its involvement in the MTOCs. Concentration of γ-tubulin in galls is comprehensible, considering the amplified mitotic activity displayed in giant-cells and intense division of neighboring cells. Curiously, the antibody against Arabidopsis γ-tubulins recognized a microtubule binding protein potentially secreted by the RKN, *Meloidogyne incognita*, during parasitism (Fig. 2E; Vieira et al. 2012b).

Studies employing cytoskeleton-disrupting drugs suggested that a depolymerized cytoskeleton might help giant-cell expansion, and nematode feeding by decreasing the density of the giant-cell cytoplasm (de Almeida Engler et al. 2004). Some of these data have been validated by functional studies of a cytoskeleton binding protein, actin-depolymerizing factor 2 (ADF2). Decreased ADF2 levels stabilize the actin cytoskeleton in plant cells and block giant-cell development resulting in decreased nematode reproduction (Clément et al. 2009).

Microtubule binding proteins such as MAP65;1 (Fig. 2F; de Almeida Engler et al. unpublished data) and MAP65;3 are highly expressed in giant-cells. A functional analysis of MAP65;3 suggests its involvement in cytokinesis occurring in normal plant cells, while aborted in giant-cells (Calliaud et al. 2008).

**The role of plant hormones in NFS: at the interface between cell development and plant defence**

Nematode feeding sites are characterized by an induction of specific developmental pathways, whereas plant defense responses need to be suppressed to allow the biotrophic pathogen to survive inside the root tissue. Plant hormones are controlling this trade-off between cellular development and plant defense, and hence many of the hormone pathways are influenced during NFS formation. The major hormonal changes inside young (initiation, 2-3 dai) and older (maturation, 7-14 dai) NFS and the surrounding infected tissues are summarized in Figure 3, and will be discussed in the following paragraphs. Since no actual hormone measurements have been executed on NFS, this overview is based on reports about the expression of hormone biosynthesis genes, hormone responsive genes and reporter genes in NFS and neighboring root tissue.

During migration, nematodes will cause damage to host cells, leading to activation of defense responses. For instance, the wound-responsive jasmonate and ethylene pathways are strongly induced upon migratory nematode infection in rice ([Kyndt et al. 2012a, 2012b](#_ENREF_41)). Tomato cells have also been shown to respond to RKN attack by producing superoxide (O2.–) and hydrogen peroxide (H2O2), both of which are toxic to the parasite (Melillo et al. 2006). Nevertheless, once the nematode has become sedentary, a strong suppression of several genes involved in plant defense is needed to accommodate the biotrophic pathogen to survive inside this hostile environment.

In the case of RKN, a strong attenuation of defence-related hormone pathways, mainly of the salicylate (SA) and ethylene (ET) pathways, has been observed in giant-cells as well as the surrounding root tissues and developing galls at early time points (Jammes et al. 2005; Barcala et al. 2010; Kyndt et al. 2012a & b; Hamamouch et al. 2011; Ji et al. unpublished data; Portillo et al. 2013). Remarkably, even above-ground tissues of RKN-infected plants show a reduced expression of the salicylic acid (SA)-biosynthesis gene *OsICS1* in rice (Kyndt et al. 2012b) and SA-responsive *PR*-genes in Arabidopsis (Hamamouch et al. 2011). This defence suppression is however temporarily, and once the feeding site is at the maturation phase, SA and ET-defence responses are activated in the gall tissue, probably due to the growing NFS and nematode (Kyndt et al. 2012a; Glazer et al. 1983; Ibrahim et al. 2011). Nevertheless, most defence pathways stay repressed inside the giant-cells, even at later time points (Ji et al. unpublished data).

In the case of CN infection, SA and JA-related plant defense genes are strongly induced in infected roots, mainly at early time points (Ithal et al. 2007a; Hamamouch et al. 2011). This might be attributed to the fact that CN migrate intracellularly, while RKN intercellularly, and hence elicit a stronger plant defense response along their migration path. The fact that this response is instantly attenuated in RKN infected tissue might be due to their subtleness when moving in-between cells, and by the power to quickly suppress potentially provoked plant defense responses. Nevertheless, CN also suppress defense pathways, albeit more locally. In studies on isolated NFS a strong and consistent repression of the jasmonate pathway was observed in syncytia ([Ithal et al. 2007b](#_ENREF_31)) just like in 7 dai giant-cells (Ji et al. unpublished data). In addition, although less striking than upon RKN infection, also CN parasitism probably involves some level of suppression of the SA signaling pathway (Wubben et al. 2008). For instance, the CN effector 10A06 (Hewezi et al. 2010) was recently shown to interact with Arabidopsis spermidine synthase, hence most likely mediating down-regulation of SA-responsive genes.

Genes involved in Brassinosteroids (BR) biosynthesis, a class of hormones involved in many developmental and defence-related processes have recently been found to be consistently induced in RKN-induced galls (Kyndt et al. 2012a). Nahar et al. (2013) revealed that in rice, BR suppress defense against RKN through their antagonism with the defense-inducing jasmonate pathway. An activation of the BR-pathway might be important for the nematode to overcome root defense but might at the same time activate developmental changes in the galls. BR promote cell expansion and elongation, and also regulate general root development and cell wall modification through their effect on the expression of XTHs and cellulose synthase, in a concentration-dependent manner (reviewed by Wolf et al. 2012).

A more recently detected class of peptide hormones, called the CLAVATA3 (CLV3)/ESR (CLE) hormones are noteworthy to mention here as well, since peptides that mimic CLE hormones are being secreted by cyst nematodes (Lu et al. 2009; Wang et al. 2010, 2011). These CLE-like effector proteins act as ligand mimics of plant CLE peptides, which are regulating differentiation of meristematic cells (Kratsir et al. 2011). CLAVATA-like signaling components are supposed to control cell division and differentiation and thus maintain integrity of cyst nematode-induced syncytia (Replogle et al. 2011).

Data about the role of ethylene (ET) in feeding site formation is complex and seems contradictory, probably due to its pleiotropic role in development and defense, two mechanisms that might be of importance at different stages of NFS formation. Early reports by Glazer et al. (1983, 1985) showed that chemical blocking of ethylene production inhibited gall development in tomato and that treatment of the tomato plants with an ET-precursor lead to better giant-cell enlargement. They were contradicted by later reports showing an important role for ET-responses in activation of JA-dependent defense against RKN (Nahar et al. 2011; Fudali et al. 2013). Fudali et al. (2013) showed that ET-overproducing Arabidopsis plants are less attractive to RKN. These results obtained with RKN are in marked contrast to each other and to the data from the sugar beet CN, *H. schachtii*, where root exudates from ET-overproducing mutants were found to be more attractive to CN (Wubben et al. 2001), and the plants are hyper-susceptible ([Goverse et al. 2000](#_ENREF_24); Wubben et al. 2001). Because ethylene is known to induce cell expansion and to inhibit lignification, it was suggested that this plant growth regulator plays a major role in the development of syncytia (Goverse et al. 2000). However, data from ET-responsive gene expression are contradictory to this hypothesis, and rather support a role for ET in defence against cyst nematodes. For instance, an ET-responsive mRNA encoding the RAP2.3 protein is accumulating in surrounding root tissues of CN infected *Arabidopsis* *thaliana* plants, but is locally down-regulated inside the syncytium (Hermsmeier et al. 2000). Another Ethylene-Responsive Element-Binding Protein gene, *GmEREBP1*, was downregulated in CN infected whole root tissue of susceptible soybean (Mazarei et al. 2002), while it was highly expressed in infected resistant roots. The recent data of Ali et al. (2013) confirms the role of ET-responses in defence against cyst nematodes, which might be related to JA-activation. The ET-reponsive *RAP2.6* was shown to be strongly downregulated in 5, 10 and 15 dpi syncytia (Ali et al. 2013), and overexpression of this gene lead to activation of the JA-pathway and callose deposition.

Further investigations are clearly needed to elucidate the exact role of ET in NFS development and plant defence against nematodes. The observed discrepancies for this hormone might be explained by differences in experimental design (e.g. concentrations of chemicals, time of application, time of infection evaluation), but may also be related to the role of ET in root cell development. Interestingly, Swarup et al. (2007) demonstrated that ET induces auxin biosynthesis, and this interaction leads to a strong inhibition of root cell elongation. However, auxin facilitates radial expansion in the root elongation zone (Strader et al. 2010). ET probably plays different roles at different stages of the nematode infection process: (1) having a restraining role by activating nematode repellents and JA-biosynthesis or (2) having an activating role, potentially through its positive effect on auxin biosynthesis, facilitating radial expansion of the giant-cells. Auxin (AUX) manipulation is well-known to be an important process during initiation and early development of NFS of sedentary plant-parasitic nematodes ([Grunewald et al. 2009b](#_ENREF_26)), since induced AUX levels have been found inside young NFS ([Karczmarek et al. 2004](#_ENREF_36); [Grunewald et al. 2009a](#_ENREF_25)). During root development, AUX is mainly responsible for cell division, and establishing and maintaining root primordia ([De Smet et al. 2010](#_ENREF_13)). In Arabidopsis, CN have been shown to hijack the AUX distribution route in order to facilitate infection (Grunewald et al. 2009). This might be accomplished by enhanced expression of the auxin-importer AUX1 in the primary syncytial cell (Mazarei et al. 2003) and by the down-regulation of the efflux transporter PIN1. Seeing that AUX-mutants are less susceptible, these responses are important for nematode infections (Hutangura et al. 1999; Goverse et al. 2000; Karczmarek et al. 2004). The Hs19C07 effector of *H. schachtii,* specifically targets LAX3, one of the AUX influx transporters in Arabidopsis roots (Lee et al. 2011). In this way, the nematode affects the AUX distribution upon infection, potentially leading to improved cell division and cell wall loosening, but probably also playing a part in plant defense suppression through its antagonism with the SA-pathway [(Robert-Seilaniantz et al.](#_ENREF_15) 2011).

Auxins and cytokinins (CK) have contrasting roles in cell division in plant roots (Dello Ioio et al. 2007). It is known that CKs, in contrast to AUX, play an inhibitory role in root meristem size, cell division and lateral root formation in Arabidopsis, while they promote root cell differentiation ([Chapman and Estelle, 2009](#_ENREF_7)). Aux and CKs regulate the expression and/or the activity of the CDKs and the mitotic cyclins, and by doing so they control cell cycle progression (Trehin et al. 1998), an important process during NFS development (see above). Auxin accumulation in young syncytia and giant-cells is transient and the AUX response shifts to neighboring cells at 2-5 days after infection (Hutangura et al. 1999; Karczmarek et al. 2004) probably aiding in its radial expansion. In experiments with the CK-responsive promoter *ARR5* in RKN-infected *Lotus japonica*, a high level of expression was induced when the RKN juveniles reached the differentiating vascular bundle and during early stages of the nematode-plant interaction. At later stages (7 dai- 3 weeks after infection) ARR5 activity was absent inside the mature giant-cells, although neighboring cells continued to express the reporter gene (Lohar et al. 2004). CKX (cytokinin oxidase) overexpressing *L*. japonica hairy roots had reduced gall formation indicating a requirement for cytokinins in the establishment of giant-cells. However, as established giant-cells did not exhibit ARR5 expression, it must be the initial stages of giant-cell induction that require cytokinins and consequently some form of cell differentiation. Taken together, these data indicate that AUX and CK are necessary for NFS initiation and early development, while at later stages they accumulate in the neighboring cells. The role of CK and its crosstalk with AUX inside the developing NFS remains to be further investigated. The fact that chlorophyll is accumulating in dark-grown syncytia (Szakasits et al. 2009) and giant-cells (Ji et al. unpublished data), could for instance be related to the interference with AUX-CK homeostasis, since Kobayashi et al. (2012) demonstrated a positive effect of cytokinin signaling on chlorophyll biosynthesis, while auxin action repressed chlorophyll accumulation in Arabidopsis roots.

Also gibberellins play an important role in stimulating both cell division and expansion ([Richards et al. 2001](#_ENREF_52)). In galls, giant-cells and syncytia, gibberellin biosynthesis genes are strongly and constantly expressed (Klink et al. 2007; Kyndt et al. 2012). These observations suggest that gibberellins are important players throughout the initiation, development, maintenance and maturation of giant-cells, although detailed functional studies, and the potential crosstalk with other hormones like auxin are currently lacking.

**Epigenetic processes controlling NFS development**

Recent studies have revealed a role for epigenetic mechanisms during NFS formation. Sequence data of small RNA libraries isolated from syncytia in Arabidopsis (Hewezi et al. 2008) and cyst-nematode infected soybean roots (Li et al. 2012) suggest that small RNAs are mediating gene regulation processes during the plant-nematode interaction. One of these small RNAs, miR396, was functionally studied in Arabidopsis and shown to target a set of Growth-regulation Factor (GRF) genes, *GRF1* and *GRF3*. Micro-array analysis showed that the miR396-GRF regulatory system can alter the expression of 44% of the more than 7,000 genes reported to change expression in the Arabidopsis syncytium, demonstrating its key regulatory function and hence an ideal molecular target for the nematode to manipulate. miR396 is strongly suppressed in the early developing syncytium, when nematodes are at the J2 and early J3 stage. The authors suggested that the inactivation of miR396 increases GRF1 and GRF3 mRNA abundance to a threshold that enables these transcription factors to regulate gene expression reprogramming events that direct the early differentiation and formation of the syncytium (Hewezi et al. 2012). At later stages of syncytium development (when the nematode is at the J3/J4 stage), this miRNA is induced in comparison with the neighbouring cells. This will lead to reduced expression of GRF1 and GRF3, thereby ending the induction/formation phase of the syncytium and starting its maintenance phase (Hewezi et al. 2012).

Epigenetic processes are probably involved in giant-cell development as well. Genes involved in chromating remodeling, DNA methylation, small RNA formation, and histone modifications are all highly expressed inside these cells, starting at 3 dai (Portillo et al. 2013), and increasing at 7 dai (Portillo et al. 2013; Ji et al. unpublished data). Further detailed investigations on their target loci and functional relevance are currently lacking.

**Concluding remarks**

NFS development interferes with basic developmental processes in plant roots, among which the cellular metabolism, cell wall architecture, cell cycle, cytoskeleton, hormone homeostasis and epigenetic mechanisms.

Further studies of mutants with cytoskeletal defects and of proteins implicated in signaling pathways that support microtubule, actin reordering and cell wall modifications, will confer more understanding on how these structures are critical for NFS expansion.

Also more work is needed to elucidate the complicated hormonal cross-talk in these highly specialized organs. Data about cross-talk between pathways in other plant organs cannot be simply translated, because of the unique physiology and cellular architecture of NFS. Measuring actual hormonal levels inside these organs could bring us a step closer to unraveling the hormonal network underlying NFS initiation and development.

Further elucidating the role of epigenetic reprogramming to maintain transcriptional changes inside NFS will undoubtedly reveal remarkable molecular mechanisms targeted by the sedentary nematodes to control development of these unique plant organs.

**Figure legends**

**Fig. 1 Functional analyses of core cell cycle genes in galls induced by *Meloidogyne incognita* or syncytia induced by *Heterodera schachtii* in Arabidopsisroots.**

**A-B.** Longitudinalsections of gall (A) and syncytium (B) stained with toluidine blue. **C-D.** 3D confocal projections of serial optical sections of whole-mount root samples cleared and stained with propidium iodide. **C.** Young gall displaying the nuclei within four giant-cells, delimited by white lines. Arrow indicates a single giant-cell with synchronized nuclear mitotic divisions. **D.** Distribution of nuclei within a maturing giant-cell (14 dai). **E.** A gall section at 14 dai showing promoter activity of *ICK2/KRP2* (red color). **F-G.** Longitudinalsections of a gall at 30 dai (stained with toluidine blue) of an overexpressing *ICK2/KRP2* line (**F**), containing giant-cells showing a significant reduction in nuclei number and decreased neighboring cell division in comparison to wild-type gall (**A**). This results in the arrest of gall and nematode development (stained with acid fuchsin) (**G**). **H-I.** Sections of a gall (**H**) and syncytium (**I**) of a *CCS52A* overexpressing infected root. Both present reduced size and a disturbed phenotype compared to control feeding sites in **(A)** and **(B)**. Asterisk, giant-cell; n, nematode. S, syncytium. Bars = 20 µm (except C-D = 10 µm). More details about the experimental conditions can be found in de Almeida Engler et al. 2012 (A-B); Vieira et al. 2012a (C-D), Vieira et al. 2013 (E-G), and de Almeida Engler et al. 2012 (H-I)

**Fig. 2 The microtubule and actin cytoskeleton are remodeled during a susceptible interaction in nematode feeding sites.**

The microtubule (**A**) and the actin (**B**) cytoskeleton are partially depolymerized (arrows) in root-knot nematode induced giant-cells. High γ-tubulin expression is observed in syncytia (**C**) as well as in galls by immunolocalization (**D**). Root-knot nematodes secrete a cytoskeleton binding protein during parasitism (**E**). MAP65;1 is highly expressed in root-knot nematode induced galls (**F**). G: Gall; GC: Giant-cell; n: nematode; s: syncytium. More details about the experimental conditions can be found in de Almeida Engler et al. 2004 (A-B-F), Banora et al. 2011 (C-D-F), and Vieira et al. 2012b (E)

**Fig. 3 Schematical representation of the transcriptional changes in plant hormone pathways upon nematode infection in a compatible interaction with plant roots and in isolated nematode feeding sites.** This scheme is based on transcriptional activation or repression of genes involved in hormone biosynthesis pathways or reporter genes responsive to hormone accumulation. Activation or repression is shown in comparison with the corresponding uninfected root tissue, and is illustrated as big or small lettering respectively. For hormones not indicated on the graph the data is contradictory, or no data is available

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