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An insight into critical endocycle genes for plant-parasitic nematode feeding sites establishment

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Root-knot and cyst nematodes are biotrophic parasites that invade the root apex of host plants and migrate toward the vascular cylinder where they cause the differentiation of root cells into galls (or root-knots) containing hypertrophied multinucleated giant-feeding cells, or syncytia, respectively. The precise molecular mechanisms that drive the formation of such unique nematode feeding sites are still far-off from being completely understood. The diverse gene expression changes occurring within the host cells suggest that both types of plant-parasitic nematodes modulate a variety of plant processes. Induction and repression of genes belonging to the host cell cycle control machinery have shown to be essential to drive the formation of such specialized nematode feeding cells. We demonstrate that nematodes usurp key components regulating the endocycle in their favor. This is illustrated by the involvement of 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, in the proper formation of nematode feeding sites. Altering the expression of these genes in Arabidopsis plants by down or overexpressing strategies strongly influences the extent of endoreduplication in both types of nematode feeding site leading to a disturbance of the nematode's life cycle and reproduction.

Cell Cycle Activation is Crucial for Nematode Feeding Site Generation

Stimulation of the host cell cycle machinery by the action of molecular triggers seems to be an important process applied by some biotrophic plant pathogens (bacteria, virus, fungus), and plant symbionts (bacteria) to promote the formation of infection sites.[1,2] Also, plant-parasitic nematodes are able to control the host cell cycle machinery by promoting the formation of multinucleated giant cells and syncytia induced respectively by root-knot (*Meloidogyne* spp) and cyst nematodes (*Heterodera* spp).[3,4] These specialized feeding cells provide the food source required for completion of the nematode life cycle and offspring production. During the development and maturation of nematode feeding sites, both giant cells and syncytia undergo a differentiation program involving multiple rounds of DNA replication escorted by nuclear and cell expansion, ending up in large multinucleated and polyploid feeding cells.[4] Nevertheless, while giant cells become multinucleated due to acytokinetic mitoses, syncytia accumulate multiple nuclei through cell fusion. Based on the observed cellular and nuclear changes in giant cells and syncytia, it has long been assumed that the host endocycle machinery plays an essential role to generate high-ploidy nuclei associated with feeding site expansion and maturation. Until now, the molecular basis of this correlation was poorly understood.

Are Key Components of the Plant Endocycle Usurped by Nematodes to Induce their Feeding Cells?

A first question we addressed was to find out if nuclei amplification, as observed in feeding cells, implicated the classical endocycle machinery of plant cells. Therefore, a functional analysis of a set of genes currently known to be involved in the plant endoreduplication cycle was undertaken in feeding cells.[5] We focused on four candidate genes: *CCS52A, CCS52B, DEL1* and *RHL1*. CELL CYCLE SWITCH 52 (CCS52) proteins (CCS52A and CCS52B) are part of the Anaphase-Promoting Complex/Cyclosome (APC/C) and play a vital role in cell cycle progression by targeting mitotic cyclins for degradation, stimulating the conversion of mitotic cycles to endocycles.[6,7] A third candidate investigated was a transcription factor of the DP-E2F-like family

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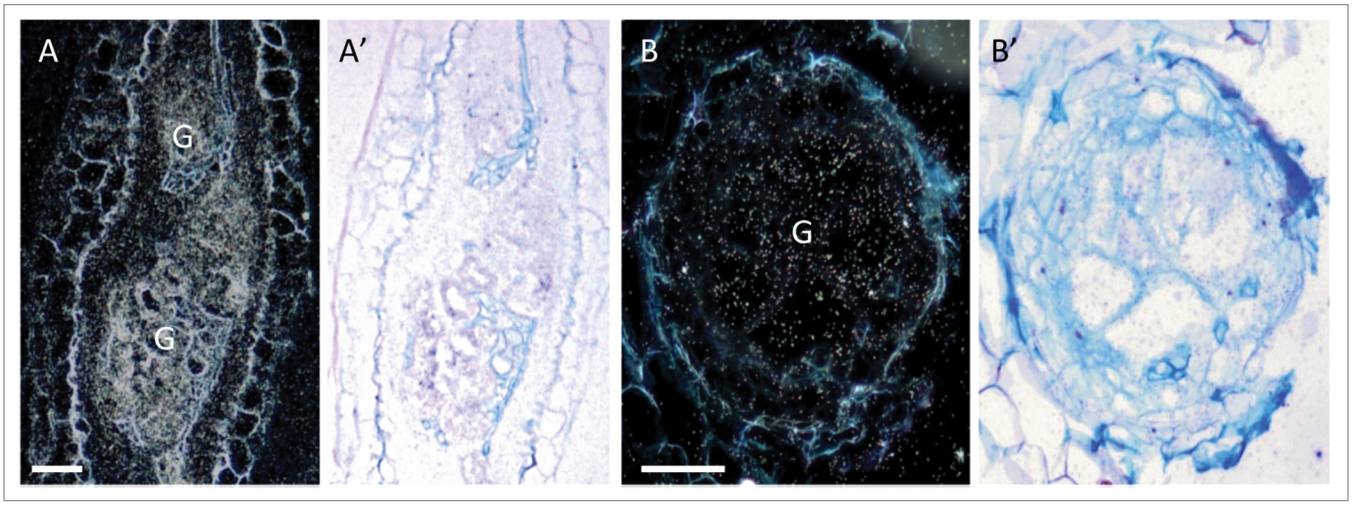

Figure 1. In situ transcript localization of genes involved in the endocycle control in galls. The different expression levels, high for *CCS52A* (**A and A′**) and low for *DEL1* (**B and B′**), in nematode-induced galls suggest that root-knot nematodes make use of the cell cycle machinery of the plant host. Hybridization signals are visible as white dots under dark-field optics and black dots under bright-field optics. Bars = 50 μm.

(*E2Fe/DEL1*), which preserves the mitotic state of proliferating cells by suppressing transcription of genes required for cells to enter the DNA endoreduplication cycle. One of its targets is the CCS52A protein, which levels are controlled up to a certain threshold in the cell, consequently preventing endoreduplication.[8,9] Finally, a fourth endocycle regulator RHL1, the plant homolog of the archaeal DNA topoisomerase VI (*RHL1*) plays an essential role in the multiprotein complex of the plant topo VI binding to DNA, therefore this protein is most likely involved in decatenation reaction of topo VI.[10]

Functional analysis performed in roots infected by root-knot and cyst nematodes revealed that *CCS52* genes, as well as *DEL1* and *RHL1*, play an active role during the development of both types of feeding sites.[11] Both *CCS52* genes were shown to be highly expressed in galls (**Fig. 1A**) and syncytia. Comparable analysis of *DEL1* illustrated low expression levels (**Fig. 1B**) as this gene acts as a specific repressor of the endocycle.[8,9]

Arabidopsis transgenic plants designed to up- (*35Spro:CCS52* lines) or down- (RNAi knockdown lines) regulate the *CCS52* genes were generated and appropriate lines were selected based on flow cytometry data. Root tissues with increased *CCS52* levels revealed nuclei with DNA content up to 32C and 64C, greatly more than normally observed in wild-type roots of *Arabidopsis* (up to 16C). Consistently, feeding sites expressing low levels of *CCS52* genes showed decreased ploidy levels in plant tissues. Previous work has shown the relevance of CCS52 for endoreduplication and ploidy-dependent cell enlargement during symbiotic cell differentiation (Rhizobiaceae).[12,13] By manipulating *CCS52A* homologs in different plant species, a reduction or total knockout of this gene decreased the ploidy levels of the nitrogen-fixing cells and arrested nodule development.[12] Similarly, our data support the hypothesis that inhibiting the endocycle through *CCS52* knockdown or *DEL1* overexpression severely inhibited feeding cell expansion resulting in a reduced food source and thereby affecting nematode development (**Fig. 2**). *CCS52* knockdown or *DEL1* overexpression lines continuously showed a delay in nematode development with significant decrease in reproduction.[11] This implies that pre-parasitic juveniles penetrated both transgenic lines, developed into parasitic second-stage juveniles but often did not mature to subsequent stages (J3/J4 and females) to attain the fertile female phase. This inhibition of nematode maturation can be linked to the status of gall or syncytium development, which was consistently inhibited in these lines.

Feeding cells expressing high levels of *DEL1* show increased mitotic activity, but the consequent inhibition of the endocycle disturbed the formation of a fully functional gall or a syncytium (**Fig. 2A and C**). Giant cells formed in the DEL1-OE line contain multiple nuclei with smaller sized nucleoli and an increased amount of cell wall stubs starting from the mother wall illustrating attempts to undergo cytokinesis (**Fig. 2C**). Some giant cells accomplish mitotic division.[11] Mitotic activity in galls (**Fig. 2B**) and syncytia were confirmed by the high expression of *CDKB1;1*.[3] These observations reinforced our past investigations that highlighted that mitosis alone is not enough to drive nematode feeding site development.[3] A phase of mitotic activity followed by endoreduplication seems to be essential for proper feeding site development. Nevertheless, the molecular mechanisms behind the peculiar nuclear enlargement observed during the first nuclear division in giant cells remains to be elucidated.[3,14]

We observed that stimulation of endoreduplication by ectopic *CCS52* expression resulted in the rapid enlargement of feeding cell nuclei and an increased ratio of gall and syncytium induction in infected roots compared with wild-type. Early stages of giant-feeding cell development (7 days after inoculation) are predominately characterized by a high mitotic activity.[3,15] For galls, acytokinetic mitoses occur inside giant cells and normal mitoses in neighboring cells, whereas in syncytia, mitotic division seems only to occur in neighboring cells.[4] The premature activation of the endocycle observed by ectopic *CCS52* expression resulted in

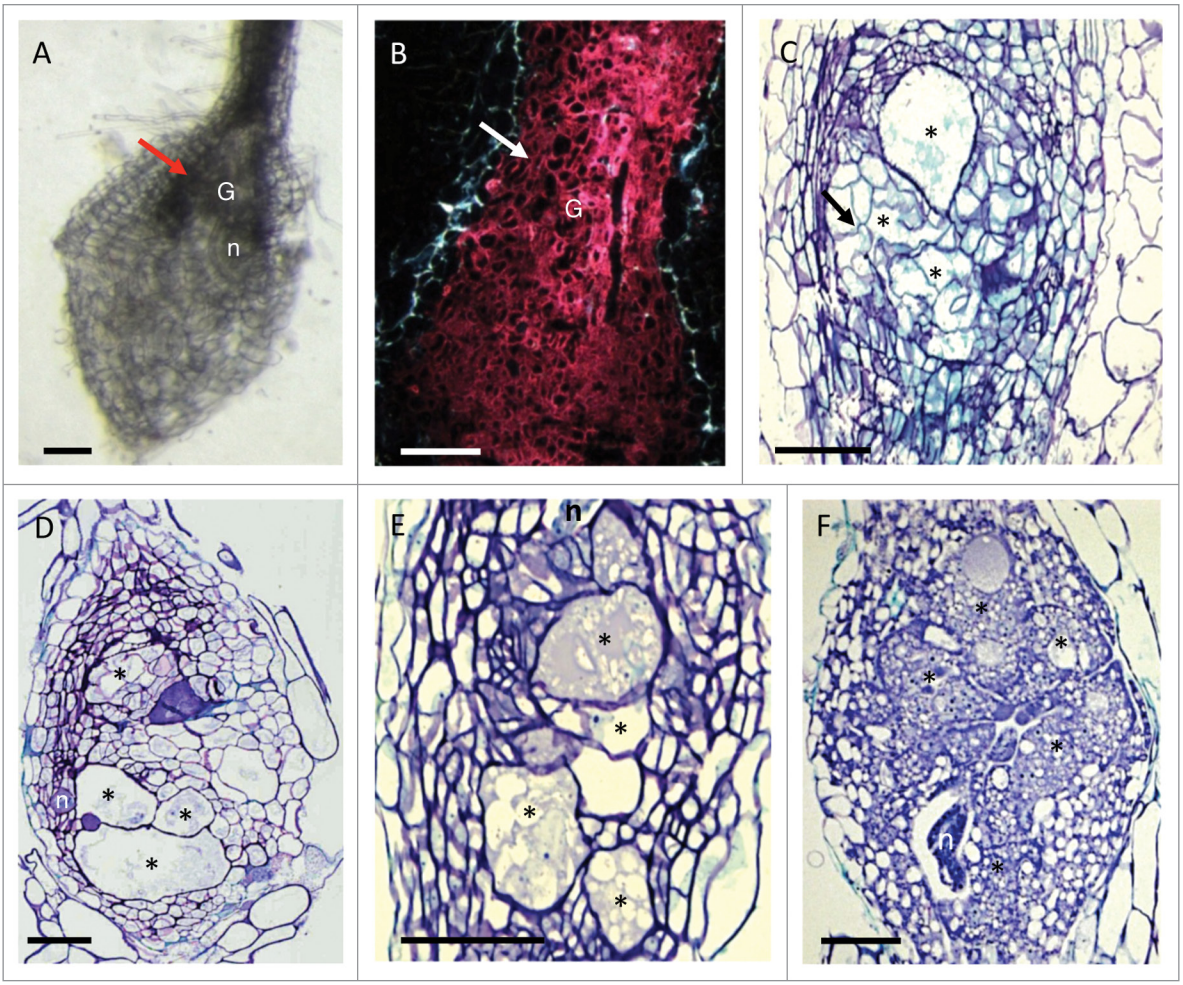

Figure 2. Ectopic expression or knock-down of *DEL1* and *CCS52* respectively affects the mitotic and endocycle progression in galls. (**A–C**) *DEL1* overexpression leads to increased mitotic activity: illustrated by increased root swelling (**A**), high *CDKB1;1* expression (**B**) and attempted giant cell division (**C**, black arrow). Knock-down (**D**) and overexpression (**E**) of *CCS52B* interfere with the endocycle, consequently affecting gall development. Image (**F**) shows a wild-type gall with the typical dense cytoplasm and highly multinucleate giant cells. G, gall; n, nematode; asterisk, giant cell. Bars = 50 μm.

small giant cells and syncytia containing fewer enlarged nuclei compared with wild-type feeding sites. The fewer mitotic cells neighboring syncytia visibly interfered with feeding site expansion. The excessive accumulation of CCS52 will promote nuclear division arrest in both giant cells and syncytia, most likely through degradation of mitotic cyclins as observed in nodules of *Medicago truncatula*.[16,17]

Examination of an *rhl1* knockout line revealed that root-knot nematodes could penetrate, induce the typical initial swelling of the infected root and succeeded to form minimal giant cells (**Fig. 3**), often binucleated. These galls were associated with young second stage juveniles, which were unable to develop further. Strangely, although cyst nematodes penetrated they could not induce a syncytium. The RHL1 protein has been linked to defects in the third round of the endocycle.[10] Therefore, repeated endocycles occurring in developing giant cells and syncytia will most likely rely on the effect of RHL1 mediated DNA decatenation.

Based on the data described above, inaccurate cell cycle responses caused by altered expression levels of the different endocycle components always lead to defects in feeding site development triggered by both root-knot and cyst nematodes.

Accurate Giant Cell and Syncytium Formation Relies on the Precise Balance between Mitosis and Endoreduplication

Orchestrating mitotic and endocycle activities in feeding cell initials seems to be essential for the optimal development of feeding cells. The temporal and regulatory transcription of cell cycle genes constitutes a core mechanism underneath the mitotic-to-endocycle transition in uninfected plant tissues,[18] and most likely also in nematode feeding sites. Temporal expression of key cell cycle regulators such as the cyclin-dependent kinases (*CDKA;1* and *CDKB1;1*) and cyclins has been reported for both giant cells and syncytia.[3,19,20,25] These data together with a recently performed broad transcript localization analysis of a large set of core cell cycle genes (de Almeida Engler et al., unpublished data) validate that plant-parasitic nematodes are able to benefit of the plant cell cycle machinery in root cells.

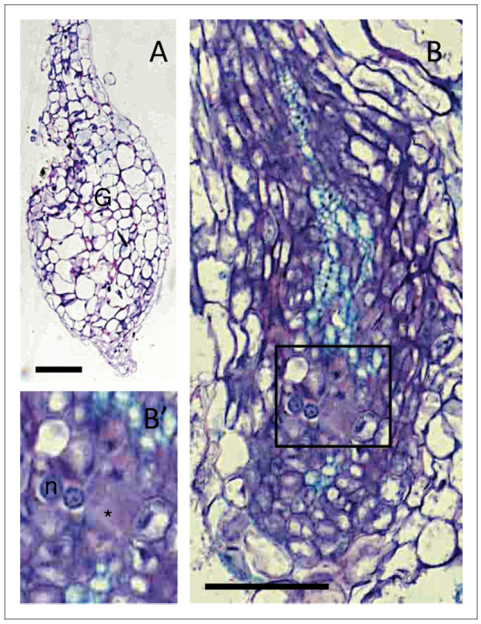

Figure 3. Knock-down of *RHL1* affects the endocycle in root-knot nematode induced galls. Nematodes penetrate the *rhl1* mutant, induce galls (**A**) containing minute giant cells (**B**) with small nuclei (**B'**). G, gall; n, nematode; asterix, giant cell. Bars = 100 μm in (**A**); 50 μm in (**B**).

In this fashion, the regulatory mechanisms governed by root-knot and cyst nematodes seem to stick to the highly conserved plant host cell cycle machinery. On-going repetitive endoreduplication cycles are preceded by a highly mitotic phase to generate a sufficient number of nuclei to sustain an appropriate level of physiological activity during the phase when nematode feeding sites accumulate nutrients essential for developing nematodes.[14] Presumably, the ability of plant-parasitic nematodes to properly drive the host cell cycle machinery will depend on other yet unidentified components. Although it is generally accepted that endoreduplication in plants is an irreversible process occurring in a post-mitotic developmental phase,[5] exceptions have been reported.[21] The artificial modulation of the plant cell cycle through gain- or -loss-of-function of key endoreduplication related genes, suggests that plant-parasitic nematodes are unable to entirely maneuver or counteract the classical cell cycle routes. Nematodes mainly usurp the normal cell cycle pathways to end up in a fully functional nematode feeding site. At this point we illustrate that DNA replication associated with a low metabolic status of feeding cells induces a noteworthy arrest on nematode development.

Conclusions

Functional analysis of endoreduplication related genes such as *CCS52A, CCS52B*, *DEL1* and *RHL1* demonstrated their strong implication in endoreduplication processes taking place in galls induced by root-knot nematodes and syncytia induced by cyst nematodes. A reduction in feeding-cell size, as a consequence of decreased DNA ploidy levels, severely impacted nematode maturation and reproduction. Repeated endoreduplication cycles occurring in giant cells and syncytia might not only facilitate cellular expansion, but also allow the induction of essential metabolic genes,[22] potentially involved in nurturing these plant-parasitic nematodes. The significant reduction of nematode feeding cells size correlated with lower reproduction suggesting that a minimal cell size is mandatory to fulfil the high nutritional demands for nematode growth and egg production. Because feeding sites are crucial for nematode survival and reproduction, modulating the cell cycle activity by interfering with genes of the host endocycle machinery might present an alternative way to control these plant pathogens. Although a concerted characterization of different Arabidopsis mutant lines upon root-knot and cyst nematodes infection has taken place in the past years,[23,24] few studies focused on cell cycle components.[3,19,25] Here we illustrated that particular endocycle related genes are crucial for feeding site development. Further characterization of the plant host cell cycle machinery, and a search for other genes potentially involved in feeding site development, might ultimately provide clues for targeting this economically important group of plant pathogens. Hence, biotechnological approaches that specifically address the cell cycle in feeding cells, could be promising to engineer resistance to plant-parasitic nematodes, and why not to a broad range of plant-pathogen species.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Doonan JH, Sablowski R. Walls around tumours - why plants do not develop cancer. Nat Rev Cancer 2010; 10:794-802; PMID:20966923; http://dx.doi.org/10.1038/nrc2942; http://dx.doi.org/10.1038/nrc2942; http://dx.doi.org/10.1038/nrc2942; http://dx.doi.org/10.1038/nrc2942
2. Wildermuth MC. Modulation of host nuclear ploidy: a common plant biotroph mechanism. Curr Opin Plant Biol 2010; 13:449-58; PMID:20542725; http://dx.doi.org/10.1016/j.pbi.2010.05.005
3. de Almeida Engler J, De Vleesschauwer V, Burssens S, Celenza JLJ Jr., Inzé D, Van Montagu MCE, et al. Molecular markers and cell cycle inhibitors show the importance of cell cycle progression in nematode-induced galls and syncytia. Plant Cell 1999; 11:793-808; PMID:10330466
4. de Almeida Engler J, Gheysen G. Nematode-induced endoreduplication in plant host cells: why and how? Mol Plant Microbe Interact 2013; 26:17-24; PMID:23194340; http://dx.doi.org/10.1094/MPMI-05-12-0128-CR
5. De Veylder L, Larkin JC, Schnittger A. Molecular control and function of endoreplication in development and physiology. Trends Plant Sci 2011; 16:624-34; PMID:21889902; http://dx.doi.org/10.1016/j.tplants.2011.07.001
6. Tarayre S, Vinardell JM, Cebolla A, Kondorosi A, Kondorosi E. Two classes of the CDh1-type activators of the anaphase-promoting complex in plants: novel functional domains and distinct regulation. Plant Cell 2004; 16:422-34; PMID:14742878; http://dx.doi.org/10.1105/tpc.018952
7. Fülöp K, Tarayre S, Kelemen Z, Horváth G, Kevei Z, Nikovics K, et al. Arabidopsis anaphase-promoting complexes: multiple activators and wide range of substrates might keep APC perpetually busy. Cell Cycle 2005; 4:1084-92; PMID:15970679; http://dx.doi.org/10.4161/cc.4.8.1856
8. Vlieghe K, Boudolf V, Beemster GTS, Maes S, Magyar Z, Atanassova A, et al. The DP-E2F-like gene DEL1 controls the endocycle in *Arabidopsis thaliana*. Curr Biol 2005; 15:59-63; PMID:15649366; http://dx.doi.org/10.1016/j.cub.2004.12.038

9. Lammens T, Boudolf V, Kheibarshekan L, Zalmas LP, Gaamouche T, Maes S, et al. Atypical E2F activity restrains APC/CCCS52A2 function obligatory for endocycle onset. Proc Natl Acad Sci U S A 2008; 105:14721-6; PMID:18787127; http://dx.doi.org/10.1073/pnas.0806510105
10. Sugimoto-Shirasu K, Stacey NJ, Corsar J, Roberts K, McCann MC. DNA topoisomerase VI is essential for endoreduplication in Arabidopsis. Curr Biol 2002; 12:1782-6; PMID:12401175; http://dx.doi.org/10.1016/S0960-9822(02)01198-3
11. de Almeida Engler J, Kyndt T, Vieira P, Van Cappelle E, Boudolf V, Sanchez V, et al. *CCS52* and *DEL1* genes are key components of the endocycle in nematode-induced feeding sites. Plant J 2012; 72:185-98; PMID:22640471; http://dx.doi.org/10.1111/j.1365-313X.2012.05054.x
12. Vinardell JM, Fedorova E, Cebolla A, Kevei Z, Horvath G, Kelemen Z, et al. Endoreduplication mediated by the anaphase-promoting complex activator CCS52A is required for symbiotic cell differentiation in *Medicago truncatula* nodules. Plant Cell 2003; 15:2093-105; PMID:12953113; http://dx.doi.org/10.1105/tpc.014373
13. González-Sama A, de la Peña TC, Kevei Z, Mergaert P, Lucas MM, de Felipe MR, et al. Nuclear DNA endoreduplication and expression of the mitotic inhibitor Ccs52 associated to determinate and lupinoid nodule organogenesis. Mol Plant Microbe Interact 2006; 19:173-80; PMID:16529379; http://dx.doi.org/10.1094/MPMI-19-0173
14. de Almeida Engler J, Engler G, Gheysen G. Unravelling the plant cell cycle in nematode induced feeding sites. In Genomics and molecular genetics of plant-nematode interactions. Genomics and molecular genetics of plant-nematode interactions 2011; 349-368; http://dx.doi.org/10.1007/978-94-007-0434-3_17
15. Wiggers RJ, Starr JL, Price HJ. DNA content and variation in chromosome number in plant cells affected by *Meloidogyne incognita* and *M. arenaria*. Phytopathology 1990; 80:1391-5; http://dx.doi.org/10.1094/Phyto-80-1391
16. Larson-Rabin Z, Li Z, Masson PH, Day CD. FZR2/CCS52A1 expression is a determinant of endoreduplication and cell expansion in Arabidopsis. Plant Physiol 2009; 149:874-84; PMID:19074624; http://dx.doi.org/10.1104/pp.108.132449
17. Kasili R, Walker JD, Simmons LA, Zhou J, De Veylder L, Larkin JC. SIAMESE cooperates with the CDH1-like protein CCS52A1 to establish endoreplication in Arabidopsis thaliana trichomes. Genetics 2010; 185:257-68; PMID:20194967; http://dx.doi.org/10.1534/genetics.109.113274
18. Inzé D, De Veylder L. Cell cycle regulation in plant development. Annu Rev Genet 2006; 40:77-105; PMID:17094738; http://dx.doi.org/10.1146/annurev.genet.40.110405.090431
19. Niebel A, de Almeida Engler J, Hemerly A, Ferreira P, Inzé D, Van Montagu M, et al. Induction of cdc2a and cyc1At expression in Arabidopsis thaliana during early phases of nematode-induced feeding cell formation. Plant J 1996; 10:1037-43; PMID:9011085; http://dx.doi.org/10.1046/j.1365-313X.1996.10061037.x
20. Ibrahim HM, Hosseini P, Alkharouf NW, Hussein EH, Gamal El-Din AelK, Aly MA, et al. Analysis of gene expression in soybean (Glycine max) roots in response to the root knot nematode Meloidogyne incognita using microarrays and KEGG pathways. BMC Genomics 2011; 12:220; PMID:21569240; http://dx.doi.org/10.1186/1471-2164-12-220
21. Weinl C, Marquardt S, Kuijt SJH, Nowack MK, Jakoby MJ, Hülskamp M, et al. Novel functions of plant cyclin-dependent kinase inhibitors, ICK1/KRP1, can act non-cell-autonomously and inhibit entry into mitosis. Plant Cell 2005; 17:1704-22; PMID:15749764; http://dx.doi.org/10.1105/tpc.104.030486
22. Damiani I, Baldacci-Cresp F, Hopkins J, Andrio E, Balzergue S, Lecomte P, et al. Plant genes involved in harbouring symbiotic rhizobia or pathogenic nematodes. New Phytol 2012; 194:511-22; PMID:22360638; http://dx.doi.org/10.1111/j.1469-8137.2011.04046.x
23. Gheysen G, Fenoll C. Gene expression in nematode feeding sites. Annu Rev Phytopathol 2002; 40:191-219; PMID:12147759; http://dx.doi.org/10.1146/annurev.phyto.40.121201.093719
24. Gheysen G, Mitchum MG. Molecular insights in the susceptible plant response to nematode infection. Plant Cell Monographs 2009; 15:45-81
25. Van de Cappelle E, Plovie E, Kyndt T, Grunewald W, Cannoot B, Gheysen G. AtCDKA;1 silencing in Arabidopsis thaliana reduces reproduction of sedentary plant-parasitic nematodes. Plant Biotechnol J 2008; 6:749-57; PMID:18554267; http://dx.doi.org/10.1111/j.1467-7652.2008.00355.x