Plant genes involved in giant cells formation induced by root-knot nematodes in Arabidopsis and Medicago

Bruno Favery, Fabien Jammes, Liudmila L. Chelysheva, Philippe Lecomte, Arnaud Complainville, Peter Mergaert, Martin Crespi, Pierre Abad

11-th International Congress on Molecular Plant-Microbe Interactions (MPMI) 18-26 July St Petersburg (Russia).
In **Biology of Plant-Microbe Interactions** (Tikhonovich I., Lugtenberg, B., Provorov, N. ,eds) Vol. 4, pp 301-304.

Plant genes involved in giant cell formation induced by root-knot nematodes in *Arabidopsis* and *Medicago*

Bruno FAVERY [1], Fabien JAMMES [1], Liudmilla. CHELYSHEVA, Philippe LECOMTE [1], Arnaud COMPLAINVILLE [2], Peter MERGAERT [2], Martin CRESPI [2], Pierre ABAD [1].

[1] Unité Interactions Plantes-Microorganismes et Santé Végétale, INRA, 123, Bld F. Meilland, BP 2078, 06606 Antibes Cedex, France ; favery@antibes.inra.fr
[2] Institut des Sciences Végétales, CNRS, Avenue de la Terrasse, 91198 Gif sur Yvette, France.

Abstract

Root-knot nematodes *Meloidogyne* spp. are obligate plant endoparasites. They induce the redifferentiation of root cells into multinucleate hypertrophied feeding cells. To understand the biotrophic plant nematode interaction, two strategies were developed. We first compared the nitrogen-fixing nodules and giant cell formation for overlapping regulatory pathways in *Medicago*. We studied expression pattern of nodule-expressed genes after infection with *M. incognita* using promoter-GUS fusions and macroarray analysis. A promoter trapping strategy was also developed in *Arabidopsis* to identify plant genes involved in giant cell formation. Analysis of T-DNA tagged lines showing an activation of the GUS reporter gene in galls allowed the characterization of plant genes involved in different cellular processes. Results obtained on the functional analysis of an early upregulated gene in giant cell, *NEC*, encoding a calcium-binding protein are described.

Introduction

The root-knot nematodes *Meloidogyne* spp. are obligate sedentary endoparasites. Nematode growth and reproduction depend on the establishment of specialized feeding sites (NFS) within the root. Root-knot nematodes induce the redifferentiation of root cells into multinucleated and hypertrophied feeding cells. These “giant cells” result from repeated nuclear divisions without cytokinesis and cycles of endoreduplication [14]. These cells are metabolically active and present modifications of their cell wall and cytoplasm content. The identification of nematode responsive plant genes represents a major challenge in understanding how nematodes dramatically alter root development to produce and maintain these giant cells. These complex morphological and physiological changes during the establishment of the giant cells are reflected by altered gene expression in affected root cells [10]. Because *Meloidogyne* species can induce similar giant cells in several thousand host species, they probably interact with some fundamental key steps of the plant cell cycle [4, 12]. To understand the biotrophic plant nematode interaction, two strategies were developed. The first one is a comparison between root knot nematode parasitism and other microbe interaction especially with the nodule induced by symbiotic rhizobia in *Medicago*. To identify plant genes involved in giant cell formation, a promoter trap strategy with a promoterless GUS gene was developed in *Arabidopsis.*

Results and Discussion

Comparison between root knot nematode parasitism and symbiotic rhizobia in *Medicago*.

The first strategy discussed was a comparison between root knot nematode parasitism and other microbe interaction. Root-knot nematodes establish and maintain an intimate relation with the plant cell and therefore are considered as biotroph organisms. Because plants have engaged

associations with a wide range of mutualistic and parasitic biotrophic organisms, it is quite conceivable that these biotrophic interactions might have evolved certain common core components affecting cellular functions such as cell-wall reorganisation, membrane synthesis, metabolite fluxes or cytoskeleton rearrangements [13]. In spite of the fact that the development and physiology of biotrophic interactions are specific and significantly different from each other (depending on the type of microorganism), those ones occurring between plants and endoparasitic nematodes, and the legume-*Rhizobium* symbioses are among the most elaborate interactions. As for giant cell formation, differentiation of the nodule primordium starts by division arrest, and these cells undergo multiple rounds of endocycles and a gradual increase in the cell volume. However in contrast to root-knot nematode, these endoreduplicating and growing cells can only be invaded by rhizobia and develop to symbiotic nitrogen-fixing cells. Nod factors secreted by rhizobia play a central role as external mitogenic signals that induce cell division in the root cortex [9]. Among the genes shown to be involved in nodulation, *ENOD40* has been studied both as a marker for the early rhizobial Nod factor-induced responses in plants as well as a regulator of primordium formation [2, 3, 6, 15]. In nodule formation, *ENOD40* induction has been proposed as a limiting step in primordium formation, sensitising the cortical cells for division. The CCS52 protein is an APC activator involved in mitotic cyclin degradation and in regulation of endoreduplication in root nodule organogenesis [1]. Two *CCS52* genes have been identified in the model legume *M. truncatula*. However, *ENOD40* and *CCS52* are not exclusively associated with the nodulation process [9].

To address the open question of common regulatory pathways between root symbioses and biotrophic pathogenic interactions, we studied the expression of genes, previously shown to be important for the nodulation process, during gall formation [8]. In a first step, the expression patterns of two key genes for the nodulation process in *Medicago truncatula* were analysed after infection with *M. incognita.* Analysis of transgenic plants carrying promoter-GUS fusions showed that *ENOD40* and *CCS52a* were expressed at 7 days post-infection (dpi) in galls. In uninfected root *ENOD40* is weakly expressed in the vascular cylinder whereas *CCS52* is expressed in the root apical meristem and lateral root initiation sites. Time-course experiments showed that both genes were induced in young galls 3 dpi, and persisted in 15 dpi galls. However, cross sections of galls revealed different patterns of *ENOD40* and *CCS52a* expression: *ENOD40* was not induced in the giant cells but only in cells surrounding the giant cells and the nematode. On the contrary, a high induction of the *CCS52a* gene was observed in giant cells and also in surrounding cells, consistently with its function in endoreduplication and differentiation.

The involvement of *ENOD40* and *CCS52* in both nodule and gall formation suggested that there are other common elements in the development of these two neoformed organs. To compare changes in gene expression profiles during nodule and gall development, the expression patterns of 192 cDNAs selected from a nodule cDNA library (including cell cycle genes and putative elements of signal transduction pathways) were analysed using macroarray after infection with *M.incognita* and *S. meliloti* [11]. Filters were hybridised with ^{33}P-labelled cDNAs synthesized from control uninfected roots, dissected nematode galls (7 dpi and 14 dpi), and root nodules (20 dpi). Our data revealed that only a few genes are significantly upregulated in both interactions. In nodules 21 dpi, 38 genes were found to be upregulated. These genes correspond to different gene classes such as known nodulin genes or metabolic genes (e.g. *ENOD 40* or nodulin 25 precursor with a 500-fold increase, leghemoglobins..), putative regulatory genes and new nodulin genes with no homology in databases. In galls, only few changes are observed and no more than five genes were upregulated at 7 and 14 dpi. Among them nodulin 26 (*NOD26*) and cyclin D3 (*CYCD3*) were the only genes activated at both time points. *NOD26*, encoding an aquaporin, showed a 44-fold induction in nodules and a 4 to 5.4-fold induction in galls. The *CYCD3* was shown to be activated 3-fold only in galls in our conditions. No induction of *ENOD40* and *CCS52* genes was detectable in galls. The *NOD26* and *CYCD3* induced expressions in galls were confirmed using semi-quantitative RT-PCR and the induction levels were comparable to those obtained with the macroarrays. *NOD26* encodes an aquaporin channel able to transport water and glycerol [5]. As in *Rhizobium* symbiosis we could anticipate that NOD26 plays a role in the giant cells since osmoregulation has been shown to be

modified. The upregulation of *CYCD3,* encoding a D-type cyclin, induced in the G1 phase of the cell cycle, suggests that these cyclins play also a key role in the response of root cells to nematodes to proceed into the cell cycle and/or to initiate endoreduplication. During gall development, expression patterns of other key regulatory genes such as the mitotic cyclin *CYCB1;1, CDC2bAt* and *CYCA2;1* have been reported in *Arabidopsis* [4].
In conclusion these first data suggest that specific signals from biotrophic organisms trigger signalling pathways in the plant host. However, our data on *ENOD40, CCS52a, NOD26, CYCD3* and other regulatory proteins required for the establishment of meristems expression (Koltai et al., 2001), suggest that certain cellular processes dealing with endoreduplication, cell cycle regulation, cell to cell communication and water transport are shared by the complex developmental processes of nodule organogenesis and gall formation [8].

Promoter trapping in *Arabidopsis*

To identify new genes and obtain a more comprehensive view of the molecular mechanisms underlying the induction and maintenance of giant cells, a promoter trapping strategy was developed with a promoterless GUS gene construct being introduced randomly into the *Arabidopsis* genome via *Agrobacterium* T-DNA transformation. A large scale screening of more than 20 000 T-DNA tagged *Arabidopsis* lines for GUS expression after *Meloidogyne* infection allowed the identification of about 200 lines with an increased GUS expression in galls. These lines presented also GUS expression in the healthy plant in different cell types or at different developmental times [7]. These results support the hypothesis that 'normal' biochemical functions have been recruited to play key roles in allowing pathogen growth and confirm the complex morphological and physiological changes in cells during their modification into nematode feeding sites. The first induced gene characterized encodes a key enzyme of the pentose phosphate pathway, the D-ribulose-5-phosphate 3-epimerase (RPE). We showed that this gene is essential for the early steps of giant cell formation induced by *M. incognita* [7]. The characterization of the RPE gene validated the promoter trap strategy for the study of the plant-nematode interaction.
The second interesting line, present a GUS activation less than two days after infection with *Meloidogyne*, that is to say less in the first 24 hours after giant cell induction. The precise localisation of GUS expression in galls from this line showed a GUS staining restricted to giant cells and absent in the surrounding cells. No GUS expression was detected after *Heterodera schachtii* infection. Thus, this gene represents the earliest induced gene specifically expressed during giant cell formation. During plant development, the GUS staining is observed in other parts of the plant but the expression is limited to tissues with meristematic activity such as the root meristem, lateral root primordia and the apical shoot meristem.
Molecular and genetic analyses indicate that only one T-DNA was inserted. We showed that the insertion of the T-DNA took place into the last exon of the *NEC* gene, and its resulting integration placed the ATG initiation codon of the *GUS* gene in frame with the *NEC* ORF allowing a functional gene fusion. This gene exists as a single copy in *Arabidopsis.* Blast researches do not reveal homologies with known protein. However an unique “calcium-binding” like motif is present. These EF-Hands motifs are present in calcium binding proteins like calmodulins. Blast researches revealed only one homologue with 60% identities in *Arabidopsis*. However, this homologous protein did not present a mitochondrial signal peptide.
We confirmed by RT-PCR that the gene is expressed in galls and also in meristems. In order to prove that this protein is able to bind calcium, we have expressed the protein in bacteria, and perform a Ca-binding test with radioactive calcium. After Ca^{45} hybridisation, signals were observed only with NEC and calmodulin proteins indicating that NEC is a calcium-binding protein.
To test the implication of NEC in giant cell formation and plant development, we have isolated homozygous *nec* plants and looked for phenotype. However no mutant phenotype was observed during plant development and also no modification in nematode infection or development. This lack of phenotype could be due to the fact that the T-DNA is inserted in the 3’ end of the gene and induce only a small deletion allowing the fusion protein still to be functional. Therefore, the effect

of NEC suppression was studied using plants expressing anti-sense gene. Interestingly, primary transformants presents a phenotypic defect with a reduced root and aerial part development. The T2 plants are currently analysed for level of expression of NEC and also for response to nematode infection. Finally, to localize the protein, the *NEC* cDNA was fused to GFP (NEC:eGFP) under the control of a 35S promoter. Confocal observations of tobacco BY-2 cells transformed with this construct showed dots of fluorescence in the cytoplasm reminding mitochondria. We confirmed that these dots were mitochondria using a specific Mitotracker staining. Thus, we can conclude that NEC is indeed a mitochondrial protein.

In conclusion, the promoter trap is a powerful strategy to identify new genes involved in giant cell formation. This strategy allowed the identification of the *RPE* gene encoding a ribulose-5-phosphate-3-epimerase, which has been shown to be essential for the NFS formation during infection with the *M. incognita* [7]. Moreover, this strategy allowed the identification of genes with unknown function that are expressed in restricted parts of the plant. This is the case for *NEC*, which is induced in the early steps of feeding cell formation but also in meristematic tissues. The *NEC* protein binds calcium and could be involved in signal transduction. Therefore, the root-knot nematode combined to this strategy represents an efficient tool to understand plant development and may serve in identifying genes that regulate aspects of cell division and differentiation.

Acknowledgements

This work was supported by the French National Institute for Agriculture Research (INRA), the National Centre for Scientific Research (CNRS) and GENOPLANTE. This work was partly supported by AFIRST (Association Franco-Israélien pour la Recherche Scientifique et Technologique) and by a grant from “Action Puces à ADN – CNRS”. A.C. and F. J. are supported by a fellowship of the Ministère de la Recherche et de l'Enseignement Supérieure.

References

1. Cebolla A. et al. (1999) EMBO J. 18: 4476-4484.
2. Charon C. et al. (1997) Proc. Nat. Acad. Sci. USA 94: 8901-8906.
3. Charon C. et al. (1999) Plant Cell 11: 1953-1965.
4. de Almeida Engler J. et al. (1999) Plant Cell 11: 793-807.
5. Dean R. M. et al. (1999) Biochemistry 38: 347-353.
6. Fang Y. and Hirsh, A. M. (1998) Plant Physiol. 116: 53-68.
7. Favery B. et al (1998) EMBO J. 17: 6799-6811.
8. Favery B. et al. (2002) Mol. Plant Microbe Interact 15: 1008-1013.
9. Foucher F. and Kondorosi E. (2000) Plant Mol. Biol. 43: 773-786.
10. Gheysen G. and Fenoll C. (2002) Annu. Rev. Phytopathol. 40: 191-219.
11. Györgyey J. et al. (2000) Mol. Plant-Microbe Interact. 13: 62-71.
12. Niebel et al. (1996) Plant J. 10: 1037-1043.
13. Parniske M. (2000) Curr. Opin. Plant Biol. 3: 320-328.
14. Wiggers R. J. et al. (1990) Phytopathology, 80: 1391-1395.
15. Yang, W.C. et al. (1993) Plant J. 3: 573-585.