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Analysis of the plant genes involved in the nitrogen fixing symbiosis. *Sesbania rostrata* as a model

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Summary

During nitrogen fixing nodule formation on leguminous plants, several plant genes, so-called nodulin genes, are specifically expressed. In this paper the current knowledge on the expression, induction and regulation of nodulin genes in the different symbiosises studied so far is summarized, together with main results obtained in the case of *Sesbania rostrata* on nodule-specific plant gene expression.

Introduction

Each stage in the *Rhizobium*-legume symbiosis is characterized by a series of developmental events concerning both bacteria and plant, resulting in a complex, well organized and well coordinated plant organ. This has been well described from a morphological point of view (Bauer, 1981).

By classical genetic experiments, several plant genes involved in nodulation and symbiotic nitrogen fixation have been identified in pea, soybean, clover and alfalfa (Nutman 1981 ; la Rue *et al.*, 1985). Mutations in the plant genome can result in disturbed nodul development, varying from the absence of nodules to the developement of wild-type like but ineffective nodules (Vincent, 1980).

The knowledge of nodule formation has made rapid progress since the rise of molecul biological techniques. It has been found that a number of genes in both plant and bacteriu are only expressed in nodules. An effective symbiosis is accomplished by differentiati of bacteria into bacteroïds on the one hand, and differenciation of plant cells into a root nodule on the other hand. The major part of research activity has concentrated on the nitrogen fixing bacteria, which are more easily accessible to genetic manipulation in comparison with legume plants. Most of the bacterial symbiotic genes (genes for nodulation and nitrogen fixation) have been identified and are well documented. The plant is equally important, providing the right environment, the energy and using the fixed nitrogen for its growth and development. Over the last few years, interest in the role of the plant in the symbiosis has considerably intensified, with the evidence of plant genome-encoded, nodule-specific proteins, called nodulins (Legocki and Verma, 1980) differentially expressed during nodule development.

Compared with other plant differentiation processes, root nodule development is unique in the involvement of a prokaryote in the induction and control of plant development. Developmental program leading to root nodules, as an organ, is as complex as other developmental programs in plants and involves numerous genes. Nodulin gene expression is one of the most specific aspects of nodule differentiation and its study may be a useful approach to understand root nodule development.

Nodulin definition. By definition, nodulins are plant gene-encoded proteins, which are found only in root nodules and not in uninfected roots nor in other part of the host plant (Van Kammen, 1984). Nodulin genes are, by consequence, plant genes exclusively expressed during the development of the symbiosis. Around 10 to 40 different nodulins have been found in the various systems studied so far, like soybean (Legocki and Verma 1980), pea (Bisseling *et al.*, 1983), *Medicago sativa* (Lullien *et al.*, 1987), *Phaseolus vulgaris* (Campos *et al.*, 1987), *Sesbania rostrata* (De Lajudie and Huguet, 1988). Nodulin genes are differentially expressed during nodule development (Bisseling *et al.*, 1983 ; Gloudemans *et al.*, 1987). The majority of nodulin genes is expressed around the onset of nitrogen fixation, like leghemoglobin, and are called nodulin genes. They most probably function in establishing and maintaining a proper environment within the nodule that allows nitrogen fixation and ammonium assimilation to occur. Few nodulins are detectable at earlier stages of development when the nodule structure is being formed, which are called early nodulin genes.

Nodulin functions. In all systems studied so far, the use of mutated or engineered bacterial strains that arrest nodule development at different stages has enabled to establish parallels events at the levels of nodule structure and nodulin gene expression by coupling histological and molecular biological data. Very few nodulins have been identified. Moreover, nodulins must play roles at every stage of nodule developement and function : (i) for plant-microbe recognition : lectins, enzymes of host origin capable of degrading the capsular polysaccharides of the bacteria (Bauer, 1981) ; (ii) during infection process : root hair curling, infection thread formation, development of a meristem from cortex cells, release of bacteria in the meristematic cells and differentiation of host cells and bacteria into a nitrogen fixing nodule ; (iii) for the transport of metabolites including organic acids, amino acids and sugars, as well as N_2 and O_2 ; (iv) for the assimilation of fixed nitrogen produced by bacteroïds ; (v) roles in all the morphological, cytological and physiological changes that take place in the infected cell, like modification of endomembrane systems, increased abundance of free ribosomes, polyribosomes, proplastids and mitochondria ; carbon, nitrogen and oxygen metabolism changes dramatically during the differentiation of the meristem into a root nodule, as indicated by the increase in activities of several enzymes such as PEP carboxylase, malate deshydrogenase, glutamine synthetase and uricase (for a review see Verma and Long, 1983).

A few early nodulin genes have been identified. Among them only one has been well characterized : ENOD2, a nodulin of soybean (*Glycine max.*), which has been also found in other legumes like pea, vetch, alfalfa and clover (Govers *et al.*, 1987). The structural features of this nodulin, like its hydroxyproline-rich sequence, suggest that it is a cell wall protein closely related to the extensin (Nap, 1988).

Late nodulins are more documented. The most famous is leghemoglobin, which constitutes 30% of the total soluble proteins. Leghemoglobin is a hemoprotein with high oxygen affinity, which provides high yield of oxygen to bacteroïds at a low partial tension, compatible with nitrogen fixation. It is generally detectable just before nitrogenase activity can be measured. Leghemoglobin is a true symbiotic protein since the heme is a product of the bacteroïd and protein is plant-encoded. In all legumes studied so far, there are several leghemoglobins, encoded by several genes (Marcker *et al.*, 1984). Other late nodulins have been identifield. One is a soybean nodule-specific uricase, a key enzyme in the ureide biosynthetic pathway for ammonia assimilation (Bergman *et al.*, 1983). Two enzymes of glutamine synthetase, which catalyses the first reaction in the assimilation of ammonia into organic nitrogen, are present in nodules of *Phaseolus vulgaris*, one of which is nodule-specific (Cullimore *et al.*, 1983). A sucrose synthase, an enzyme which catalyzes the degradation of sucrose into fructose and glucose, has been found specific to nodules of soybean (Thummler and Verma, 1987). Other nodule-specific forms of enzymes that differ in physical, kinetic and immunochemical properties from the corresponding enzymes in roots have been found : phosphoenolpyruvate carboxylase, choline kinase, xanthine deshydrogenase, purine nucleosidase, malate deshydrogenase (Nap, 1988). But there is still no evidence that they derive from nodule-specific gene expression or from nodule-specific modifications of root enzymes.

Several other nodulins, whose functions are yet unknown, have been found associated with peribacteroïd membrane, the site of closest interaction and exchanges between the bacteroïds and the host cell.

Molecular studies on nodulin genes have progressed a lot since the last few years : gene cloning, organization in the genome, regulation, sequence analysis (for a review, see Bisseling *et al.*, 1986).

Sesbania rostrata

Sesbania rostrata is a tropical legume which forms nitrogen fixing nodules on stem and roots. This makes *Sesbania* a very efficient system for nitrogen fixation and a real potential as green manure in tropical agriculture. Two bacterial genera are capable to induce nodules on *S. rostrata* : *Azorhizobium caulinodans* and *Rhizobium sp.* (Dreyfus *et al.*, 1988). Due to its particular properties (in vitro nitrogen fixation), *A.caulinodans* has been extensively studied. Opposite to this, few data are available on the plant itself (Bogusz *et al.*, 1987 ; De Lajudie and Huguet, 1988, 1989). We focussed on *S. rostrata* to investigate plant genes activated during nodule development and function. *S. rostrata* constitutes a good model in such a study since, opposite to that of root nodules, age of stem nodules can precisely be known : stem nodules are homogenous in age, opposite to root nodules that form continuously.

Our approach consisted in measuring the level of translatable mRNA present in each tissue by comparing one-and-two dimensional polyacrylamide gel electrophoretic patterns of in vitro translation products of poly(A)$^+$ RNA from uninfected stems and stem nodules, uninfected roots and root nodules.

Plant gene expression in stem nodules appears very similar to that in root nodules, except some quantitative differences in the intensity of certain spots which could be explained by the heterogenous age of root nodules. We found 16 nodule-specific polypeptides, in addition to around 20 other present at different relative intensities in nodules and in uninfected tissues. During stem nodule development, majority of nodule-specific genes are expressed 12 days after inoculation, concommitantly with leghemoglobin and initiation of nitrogen fixation. These could be correlated with transport of substrates towards bacteroïds and assimilation of fixed nitrogen. Some other genes are expressed either during the early stages of infection, or transiently, suggesting a role in plant-microbe recognition, infection process, and nodule morphogenesis. Some others, expressed later (day 18), could have a role in maintaining nodule structure and function, or in nodule senescence. All these observations indicate that there is a sequence in the activation of nodule-specific genes during nodule life, like in other described systems.

Among the nodule-specific polypeptides, we identified six in vitro translation products that cross reacted with a serum anti-leghemoglobin of *Sesbania rostrata*. During nodule development, these leghemoglobin components are differently expressed and their relative proportions vary : some of them are first major to become minor during the late stages of nodule development.

Root nodules induced either by *Rhizobium* sp. (ORS 51) or *A. caulinodans* (ORS 571) are very similar except for the expression of one more specific gene in the case of ORS 51. One of the great differences pointed out between stem and root nodules is the presence of chloroplasts and photosynthesis in stem nodules. We found interesting to compare plant gene expression in stem nodules developed either in the dark or under light : they show only minor differences ; moreover, stem nodules in the dark and root nodules show some striking similarities. This, together with other arguments like the same seven leghemoglobin isomeres (Bogusz *et al.*, 1987), the same infection process in stem and root nodules (Ndoye and Truchet, in preparation), or the fact that root nodules developed under light are photosynthetic, suggests that stem and root nodules are not of completely different nature.

We used different nitrogen fixation deficient mutant strains of *A. caulinodans* ORS 571 and observed that induction of nodule-specific gene expression is not dependant on nitrogen fixation. However the level of expression of leghemoglobin and several other nodulin genes is lowered when there is no nitrogen fixing activity in nodule. This is in agreement with all systems described so far.

Conclusion

Besides the agronomic aspect, nitrogen fixing symbiosises are a tools towards elucidating several fundamental biological problems. They constitute a model for the study of plant microbe interactions, which are diverse and numerous in nature, especially of parasitism and pathogenicity.

Opposite to *Agrobacterium tumefaciens*, the other genus of the *Rhizobiaceae* family, which induces tumor formation, a set of undifferentiated cells, *Rhizobium* is able to bypass the plant defense mechanisms and to induce a de-differentiation of plant cells and the development of a nodule, a true organ specialized for nitrogen fixation. Therefore, *Rhizobium*-Legume symbiosis with *Frankia* actinorhizal plants are an interesting and rare example for plant molecular biology of the induction by a prokaryote of an organogenesis in a eukaryote.

In less than a decade, our knowledge on plant gene expression during symbiosis has progressed a lot, and we begin to know more about the complex mechanisms of symbiosis, bacterial genes implied in the induction of nodulin genes, which is a first step in identifying bacterial signals to the plant that induces expression of the successive classes of nodulin genes. A better understanding of all the mechanisms implied in symbiosis will help in a better domestication for a practical use in the field.

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