



Conference Review

Differentiation of plant cells during symbiotic nitrogen fixation

Ben Trevaskis¹, Gillian Colebatch¹, Guilhem Desbrosses¹, Maren Wandrey¹, Stefanie Wienkoop², Gerhard Saalbach² and Michael Udvardi^{1*}

¹Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany

²Risoe National Laboratory, Plant biology and Biogeochemistry Department, DK-4000 Roskilde, Denmark

*Correspondence to:

Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany.

E-mail:

Udvardi@mpimp-golm.mpg.de

Abstract

Nitrogen-fixing symbioses between legumes and bacteria of the family Rhizobiaceae involve differentiation of both plant and bacterial cells. Differentiation of plant root cells is required to build an organ, the nodule, which can feed and accommodate a large population of bacteria under conditions conducive to nitrogen fixation. An efficient vascular system is built to connect the nodule to the root, which delivers sugars and other nutrients to the nodule and removes the products of nitrogen fixation for use in the rest of the plant. Cells in the outer cortex differentiate to form a barrier to oxygen diffusion into nodules, which helps to produce the micro-aerobic environment necessary for bacterial nitrogenase activity. Cells of the central, infected zone of nodules undergo multiple rounds of endoreduplication, which may be necessary for colonisation by rhizobia and may enable enlargement and greater metabolic activity of these cells. Infected cells of the nodule contain rhizobia within a unique plant membrane called the peribacteroid or symbiosome membrane, which separates the bacteria from the host cell cytoplasm and mediates nutrient and signal exchanges between the partners. Rhizobia also undergo differentiation during nodule development. Not surprisingly, perhaps, differentiation of each partner is dependent upon interactions with the other. High-throughput methods to assay gene transcripts, proteins, and metabolites are now being used to explore further the different aspects of plant and bacterial differentiation. In this review, we highlight recent advances in our understanding of plant cell differentiation during nodulation that have been made, at least in part, using high-throughput methods. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: symbiosis; nitrogen-fixation; differentiation; transcriptomics; metabolomics; proteomics

Received: 11 February 2002

Accepted: 12 February 2002

Introduction

Nitrogen is an essential element for plant growth and reproduction. Lack of mineral nitrogen in the soil often limits plant growth and, as a result, modern agriculture uses approximately 80 million tons of nitrogen fertilizer annually to maintain the world's food supply. However, some plants are able to utilize the enormous pool of gaseous N₂ present in the atmosphere by forming symbioses with nitrogen-fixing bacteria, which produce ammonium for the plant. Such plants thrive in nitrogen-poor soils and contribute significantly to the global nitrogen cycle and to agriculture. The best-known

nitrogen-fixing symbioses occur between legumes (*Fabaceae*) and bacteria of the *Rhizobiaceae* family.

The legume-rhizobia symbiosis is initiated by the exchange of signal molecules between the plant and microbe. Flavonoid compounds released by the plant attract rhizobia and trigger the production and release of nodulation (Nod) factors by the bacteria [42]. Nod factors are specific lipochito-oligosacharrides [32] that affect the host plant in a number of ways. The first visible effect of Nod factors on the plant root is the curling of root hairs. Nod factors also trigger cell division in cortical cells, which leads to the formation of the nodule meristem [9,13,46].

Rhizobia bound to curled root hairs cause localised cell wall degradation and enter the plant root via a specialised extracellular structure known as the infection thread, which grows through the root towards dividing cells, the nodule primordia, within the root cortex [4]. When the rhizobia reach the nodule primordia they infect root cells and are internalised within the peribacteroid or symbiosome membrane [4,11]. Within these organelle-like compartments the bacteria divide and differentiate into nitrogen-fixing bacteroids [55]. Cell division continues in the root tissue also, eventually forming an outgrowth containing the infected plant cells. The infected cells are surrounded by uninfected tissue that contains vascular tissue for the delivery and export of metabolites to the nodule. Another uninfected cell layer forms an oxygen barrier that helps to protect the oxygen sensitive nitrogenase enzyme [29,40]. Within the infected cells of a mature nodule the bacteroids fix nitrogen in return for nutrients from the plant [52].

There are differences between the nodule developmental programmes of different legumes. In the case of indeterminate nodules, such as the nodules formed by pea or *Medicago truncatula*, the nodule meristem differentiates from inner-cortical cells and is persistent, with new cells being constantly produced and infected. In determinate nodules, such as those of soybean or *Lotus japonicus*, the nodule meristem differentiates from cells in the outer cortex and is only transiently active. The infected region of a determinate nodule contains infected host cells that are all at a similar developmental stage [3,23].

Nodule meristems produce two major types of tissue: the central tissue containing infected and uninfected cells; and the peripheral tissue consisting of cortical, endodermal, and parenchymal cells, which contain the vascular bundles [37]. The central tissue of indeterminate nodules can be divided into several zones, differing in their degree of cell differentiation: the meristematic zone; the pre-fixation or invasion zone, where cell division is arrested and cells differentiate and are invaded; the nitrogen fixation zone, where plant cells and bacteria are terminally differentiated for nitrogen fixation; and the senescent zone, closest to the root, which contains degenerated plant and bacterial cells [55]. Cells of the meristem contain 2C-4C DNA, depending upon whether they are in G1 or G2, whilst cells in the other zones are endoreduplicated [6]. Increased ploidy levels may promote infection or allow increased cell size and metabolic activity in infected

cells [6]. In contrast to the situation in indeterminate nodules, cells of the infected zone of determinate nodules differentiate in unison. The infected zone of determinate nodules also contains polyploid cells [3].

The study of rhizobia has yielded a wealth of information. The entire genomes of *Sinorhizobium meliloti*, the symbiotic partner of Medicago, and of *Mezorhizobium loti*, the symbiotic partner of Lotus, have been sequenced [19,28]. The roles and regulation of many genes required for effective symbiosis are well understood. Genes required for nodulation (*nod* genes) [34,12], metabolism of dicarboxylic acids (*dct* genes) [57,58], and synthesis or activity of the nitrogenase complex (*nif* and *fix* genes) [26], are examples of rhizobial genes that have been found to be essential for symbiosis. Many of these genes are induced in the differentiating rhizobia during symbiosis. Progress in understanding plant-controlled aspects of nodule development has been slower, hindered by longer generation times and by the added complexity of the eukaryotic system. It is clear that the plant host controls many aspects of nodule development and in some legumes nodule-like structures can spontaneously develop in the complete absence of rhizobia [51]. Recent advances in plant genomics are starting to accelerate progress in understanding plant-controlled aspects of the legume-rhizobia nitrogen fixing symbiosis. In this review we highlight past work in this area and describe more recent results obtained with high-throughput molecular methods that provide new insights into plant cell differentiation during symbiotic nitrogen fixation.

Nodulins

The term nodulin was originally used to describe 'nodule-specific host polypeptides' [31]. Genes encoding nodule-specific proteins are likely to play important roles in nodule differentiation and development. Nodulin genes have been isolated using a number of methods of molecular biology, including cold-plaque screening [16,27], mRNA differential display [48], subtractive hybridisation [20,30] and differential screening of nodule cDNA [18,41]. Nodulin genes have been classified into two groups: those that are induced soon after contact with rhizobia, the early nodulins; and those expressed late during the nodulation process or in mature nodules, the late nodulins. Early nodulins are likely to be involved in

the early stages of nodule development, such as cortical cell division and infection thread synthesis, and include several proline-rich structural proteins of the cell wall [33,45]. The classic late nodulin is leghemoglobin, which is found in high concentrations in the cytoplasm of infected cells of mature nodules where it binds oxygen and buffers free oxygen levels to prevent inactivation of the oxygen sensitive nitrogenase enzyme [1]. Other late nodulins appear to be involved in primary nitrogen and carbon metabolism, as well as transport of nutrients between the bacteria and plant [54,25,49]. It is now clear that only a few of the genes that are up-regulated during nodule development are expressed exclusively in nodules. Many previously described nodulins have been shown to be expressed in other plant tissues when assayed with more sensitive expression analysis techniques such as RT-PCR (e.g. [50]). In this respect the original nodulin concept is over-simplistic; in reality there are probably few genes that are expressed exclusively in nodules. Regardless of whether these genes are nodule specific or up-regulated in nodules, they are likely to be important for symbiotic nitrogen fixation. Nodulin genes have also been useful as markers for examining various aspects of the early nodulation process, such as the potential involvement of plant hormones [35] and for determining which processes are affected in mutants that are defective in the nodule differentiation process, the analysis of the DMI mutants for example [5].

Transcriptome analysis

The genes that have previously been identified as nodulins represent only a subset of all of the genes that are induced during nodule differentiation and development. High-throughput methods for expression analysis are now being used to compare gene expression between symbiotic and non-symbiotic plant tissues, and thus to identify large numbers of genes that are up-regulated during nodule development. One such method relies on DNA arrays, on which thousands of genes or gene fragments are placed and used as 'probes' to detect the amount of specific transcripts in a given RNA sample. For example, we have produced nylon membranes containing 2300 *Lotus japonicus* nodule cDNAs, and have used them to identify 83 genes that were up-regulated in mature nodules compared to roots [10]. These included several nodulin genes of

unknown function. Also included were genes involved in sucrose breakdown and glycolysis, CO₂ recycling, and amino acid synthesis, which are all processes that are accelerated in nodules compared to roots. Genes involved in membrane transport, hormone metabolism, cell wall and protein synthesis, signal transduction, and regulation of transcription were also induced in nodules. Genes that may subvert normal plant defence responses, including two encoding enzymes involved in detoxification of active oxygen intermediates, and one that may prohibit phytoalexin synthesis were also identified. More than half of the genes identified in our work have never before been identified as induced in nodules. Lotus transcript profiling also uncovered genes that were repressed during nodule development and differentiation. In fact, many of the genes examined were expressed at higher levels in roots than nodules (unpublished). This may reflect the specialised function of nodules in nitrogen acquisition. Thus, DNA array analysis is providing multiple new leads for symbiosis research.

Proteomics and the symbiotic interface

A key difference between infected cells of legume root nodules and all other plant cell types is the presence in the former of nitrogen-fixing 'organelles' called symbiosomes, which consist of one or more bacteroids surrounded by the plant-derived symbiosome membrane [11]. The symbiosome membrane is the interface between plant and bacteroids, and is likely to contain many proteins essential for nutrient and signal exchange between the plant and bacteroids [52]. Several previously identified nodulin proteins are located in and on the symbiosome membrane, including Nodulin 26, an aquaglyceroporin protein whose exact function is unclear [15,21]. There are likely to be many more as yet unidentified proteins on this membrane that fulfill functions critical to symbiotic nitrogen fixation.

Systematic approaches are now available to identify proteins in biological samples. The most effective of these, in terms of throughput and sensitivity, are based on mass spectrometry (MS), which can determine the masses of protein fragments produced by protease digestion or the sequence of small peptides, both of which can serve as a 'fingerprint' to identify the protein of interest [38,59]. In many instances the identification of

proteins has been aided greatly by access to genome sequence of the organism being studied. For organisms such as yeast, whose genomes have been completely sequenced, identification of thousands of different proteins can be done quickly and routinely [56] and quantification of proteins is also possible [22]. This new field of work has been dubbed 'proteomics'.

We are using proteomics to study proteins on the symbiosome membrane. Utilizing methods to purify symbiosome membranes from the legumes soybean and *Lotus japonicus* [53,8], together with proteomics tools, we have identified numerous proteins associated with the symbiosome membrane. Some of these are peripheral membrane proteins [39], while others are integral membrane proteins, including Nodulin 26 as well as a putative cation channel regulator from soybean and a sulfate transporter homologue from Lotus (unpublished). Interestingly, the putative sulfate transporter matched precisely the protein encoded by one of the genes that we identified as nodule-induced using Lotus cDNA arrays. Real-time RT-PCR analysis showed that transcripts from this gene were 500-fold higher in mature nodules than in uninfected roots [10]. This type of result may turn out to be common for genes encoding symbiosome membrane proteins because of the highly specialised role of the membrane and the proteins it contains. Our results provide a nice example of the way in which transcriptome and proteome analysis can complement one another in the quest to document and understand cellular differentiation. Another complementary analytical tool is provided by the discipline of metabolomics, described briefly below.

Metabolomics and nodule cell differentiation

Unlike roots, which acquire a range of essential mineral nutrients from the soil, nodules are specialised in nitrogen acquisition and are produced only when mineral nitrogen is limiting. Such specialisation might be expected to be reflected by a more streamlined metabolism, carried out by fewer proteins and orchestrated by fewer genes. Over the past few years, the tools of analytical chemistry have been developed to the point that high-throughput analysis of hundreds to thousands of metabolites is now possible [14]. Such analyses typically employ either liquid or gas chromatography in series with

mass spectrometry [14,43]. We have used GC-MS to profile metabolites present in nodules and other organs of Lotus. Each organ exhibits a different metabolite profile, although, of course, the profile changes with organ development and physiological status. Detailed analysis of nodule and root metabolite profiles may help us to identify novel aspects of nodule metabolism that underpin symbiotic nitrogen fixation. For example, our comparison of root and nodule transcript levels in Lotus has uncovered nodule-induced genes encoding enzymes that have not previously been associated with symbiotic nitrogen fixation. Quantification of the reactants and products of these enzymes may provide some insight into their importance in nodules. Metabolomics is also likely to prove useful in analysing the nodule phenotypes of symbiotic interactions formed with either mutant bacteria or mutant plants. In yeast it has been shown that seemingly silent mutations show clear phenotypes at the metabolome level [43].

Classical and reverse genetics

Classical genetic analysis of legumes has been successful in identifying loci that are either essential for nodule development or for effective nitrogen fixation. Hypernodulating mutants have also been identified [47]. By examining mutants for defects in visible features of nodule development, such as root hair curling or cortical cell division, and examining expression of early nodulin genes, it has been possible in many instances to determine where in the nodulation process such mutants are blocked. The first plant gene essential for nitrogen fixing symbiosis has now been isolated, the *Nin* gene of Lotus, which encodes a transcription factor that is essential for initiating cortical cell division [44]. The *Nin* gene was cloned using a transposon tag, and with more tagged lines being generated in Lotus it is likely that mutants defective in other loci essential for symbiotic nitrogen fixation will be identified soon. Increasingly detailed genetic maps in model legume species such as Lotus and Medicago, combined with increased genome and EST sequencing efforts are also facilitating map based cloning and it is likely that a number of interesting genes involved in nodulation will be cloned in the near future [47].

Transformation protocols have been developed for a number of legume species, including *Lotus*

japonicus and *Medicago truncatula*, which enable reverse genetics to be used to study nodule development [24,2]. Obvious targets for reverse genetics are nodulin or nodule-induced genes, several of which have now been studied. The early nodulin gene *enod40* is induced by Nod factors and expressed in nodule primordia in pea [36]. Constitutive over-expression of *enod40* induced dedifferentiation and division of cortical cells in *Medicago truncatula*, suggesting a role for the protein in nodule initiation [7]. Anti-sense suppression of the transcription factor *Mszpt2-1*, which is normally expressed in vascular bundles of developing alfalfa nodules, resulted in non-functional nodules in which differentiation of the nitrogen fixing zone and bacterial invasion were arrested [17]. The *ccs52* gene, which encodes an inhibitor of mitosis, was also implicated in cellular differentiation in legume nodules by reverse genetics. Expression of *ccs52* was enhanced in cells undergoing endoreduplication. Anti-sense suppression of the gene in *Medicago* resulted in a decreased number of endocycles and reduced volume of the largest nodule cells [6]. Thus, *ccs52* appears to be necessary for endoreduplication and differentiation of nodule cells.

Outlook

The high-throughput technologies of functional genomics are opening our eyes to the true complexity of cellular differentiation that underpins symbiotic nitrogen fixation. Integration of results of transcriptome, proteome, and metabolome analyses with those obtained from forward and reverse genetics will undoubtedly lead to a better understanding of nitrogen-fixing symbioses. Ultimately, this may lead to an extension of symbiotic fixation of nitrogen to agriculturally important non-legumes.

Acknowledgement

The Max Planck Society, Deutsche Forschungsgemeinschaft, Alexander von Humboldt Society, and European Union are thanked for their support of this work.

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