Plant Gene Expression in Actinorhizal Nodules of *Alnus glutinosa*

Changhui Guan

Promotor:	dr. A. van Kammen, hoogleraar in de moleculaire biologie
Co-promotors:	dr. T. Bisseling, universitair hoofddocent moleculaire biologie
	dr. K. Pawlowski

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Statements

1. The uninfected cortical cells in *Alnus* nodules do not seem to be involved in nodule functioning.

2. The expression of the glutamine synthetase gene in the pericycle of the nodule vascular bundle in *Alnus* nodules implies that free ammonium exists in this tissue.

This thesis.

3. Ag13, a glutamic acid- and proline-rich protein represents a member of a new family of acidic extracellular proteins in plants.

This thesis.

4. Ag135, a homologue of jojoba fatty acid reductase, must play an important role in nodule metabolism though its function is not clear yet.

This thesis.

5. When Suharjo and Tjepkema suggested that in *Alnus* nodules, hemoglobin is localized within the *Frankia* vesicles, they overlooked that to get there, the protein has to pass the invaginated plasma membrane of the host, the vesicles envelope and the vesicle membrane.

Suharjo, U. K. J. and Tjepkema, J. D., 1995. Physiol. Plant. 95: 247-252.

6. When testing the function of different domains of the auxin-binding protein (ABP) from *Zea mays* (ABPzm1), Thiel *et al.* overlooked the ER retention signal (KDEL) of the active C-terminal peptide (Pz151-163).

Thiel, G., *et al.*, 1993. Proc. Natl. Acad. Sci. USA 90: 11493-11497.

7. Crespi et al. did not provide any evidence that *ENOD40* functions at the RNA level in plant development.

Crespi, M. D., *et al.*, 1994. EMBO J. 13: 5099-5112.

8. The assumption of a decline in lipid triggered gene expression in the model of transcriptional control of the malate synthase gene in cucumber, proposed by Graham *et al.*, is not supported by experimental evidence.

Graham, I. A., *et al.*, 1992. Plant Cell 4: 349-357.
(see Mclaughlin, J. C., and Smith, S. M., 1994.
Planta 195: 22-28.)

9. Though Chinese economy has been speeding up, China is still a developing country.

10. The disclosures in books about China should be taken with a grain of salt. Some memorialists write books to earn money rather than to tell the truth.

11. Failure carries within itself the success. Failure brings up an expert.

CONTENTS

Outline

Plants that can be nodulated by actinomycetes of the genus *Frankia* are collectively called actinorhizal plants and comprise mostly woody plant species. Compared to *Rhizobium*-legume interactions, actinorhizal symbioses are poorly understood, especially in their molecular aspects. The goal of the research described in this thesis is to study plant gene expression during the development and function of actinorhizal nodules of *Alnus glutinosa*, by characterizing cDNA clones isolated from a nodule cDNA library. Chapter 1 gives an overview about the development and functioning of actinorhizal nodules, in comparison with legume-*Rhizobium* interactions.

By differential screening, several *A. glutinosa* cDNA clones were isolated, representing genes expressed at markedly elevated levels in actinorhizal nodules compared to roots. These cDNAs were found to encode products involved in nitrogen metabolism (chapter 2), a hitherto unknown metabolic pathway (chapter 3), and senescence (chapters 4 and 5).

Like in legume nodules, ammonium assimilation in actinorhizal nodules is performed by the common glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway. The exported form of fixed nitrogen in *Alnus* nodules is citrulline. Two cDNA clones isolated were found to encode products related to nitrogen metabolism. pAg11 encoded a glutamine synthetase (GS), the key enzyme responsible for ammonium assimilation; pAg118 encoded an acetylornithine transaminase (AOTA) which is involved in the biosynthesis of citrulline (chapter 2). By determining their sites of expression new insight was gained in reassimilation of ammonium in actinorhizal nodules.

One nodule-specific clone, pAg135, was found to encode a polypeptide homologous to a fatty acid reductase, but since fatty alcohols are not found in *A. glutinosa* nodules it remains to be examined in which metabolic pathway Ag135 is active (chapter 3).

A cDNA (pAg13) encoding a proline-rich polypeptide was also isolated. Apart from proline, the potential mature peptide was also rich in glutamic acid. *In situ* hybridization showed that this gene was expressed in infected cells during endosymbiont degradation and in the nodule pericycle (chapter 4). Ineffective root nodules that cannot fix nitrogen because the *Frankia* bacteria do not form vesicles, can be induced by certain *Frankia* strains on *A. glutinosa*. They represent compact structures and contain higher amounts of polyphenols than the effective nodules. A comparison of *ag13* expression between effective and ineffective nodules of *A. glutinosa* is presented, implying that ag13 expression is indeed correlated with senescence (chapter 5).

So far, about 15 nodule-specific/enhanced cDNA clones have been isolated and identified in *Alnus glutinosa* nodules. In chapter 6, the results currently achieved in plant molecular studies on *Alnus glutinosa* nodules are summarized and discussed.

Chapter 1 Introduction

Nodulation in legumes and actinorhizal plants

Changhui Guan, Katharina Pawlowski and Ton Bisseling. In: Nitrogen Fixation: Fundamentals and Applications. Proceedings of the 10th International Congress on Nitrogen Fixation (I. A. Tikhonovich, N. A. Provorov and W. E. Newton, eds). 1995, pp. 49-59. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Nodulation in legumes and actinorhizal plants

Changhui Guan, Katharina Pawlowski and Ton Bisseling

Department of Molecular Biology, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

I Introduction

All nitrogen in living organisms is ultimately derived from atmospheric dinitrogen which gets incorporated into organic compounds by biological or chemical nitrogen fixation. Since biospheric nitrogen is subjected to a rapid turnover by denitrification, maintenance of the biosphere has to be achieved by nitrogen fixation. Biological nitrogen fixation is an energy-consuming process performed by the enzyme nitrogenase which is irreversibly denatured by oxygen. Nitrogenase is formed only by prokaryotes who in some cases fix nitrogen in symbiosis with higher plants. In these symbioses, bacteria are hosted inside plant cells in special organs, the so-called root nodules. The product of nitrogen fixation, ammonium, is exported to the plant, while the plant in turn is providing its symbiont with energy sources. Of the symbiotic nitrogen fixers, two distinct phylogenetic groups are (*Azo-*, *Brady-*) *Rhizobium* and *Frankia* who fix atmospheric nitrogen in association with higher plants, leading to the *Rhizobium*-legume symbioses and *Frankia*-actinorhizal symbioses (Young, 1992). *Rhizobium* enters symbioses only with leguminous plants (with the exception of *Parasponia*; Trinick, 1979), while *Frankia* is able to nodulate a taxonomically diverse group of plants which recently have been found to be closely related amongst each other and with legumes (Soltis et al., 1995). These plants are collectively referred to as actinorhizal plants.

Leguminous root nodules represent stem-like structures with peripheral vascular bundles and infected cells in the central tissue. Two types of leguminous root nodules have been defined, the determinate and the indeterminate nodules. Indeterminate nodules are characterized by a persistent distal meristem (Newcomb, 1976). Due to its activity, a developmental gradient from the meristem to the proximal senescence zone is present in the central tissue of the nodule, by which the central tissue can be divided into specific zones (Vasse et al., 1990; Fig. 1A). The meristem (zone I) is followed by the prefixation zone (zone II), where infection of the cortical cells takes place. In the so-called interzone II-III, bacterial nitrogen fixation is induced (Yang et al., 1991) and proceeds throughout the nitrogen fixation zone (zone III). In the senescence zone (zone IV), bacteroids are degraded by the plant. Determinate nodules do not have a persistent meristem (Newcomb et al., 1979). The nodule meristem ceases to divide at an early stage of development. As a result,

all the cells of the central tissue are at a similar stage of development at any given time point.

The presence of nodular structures (actinorhizae) on the roots of actinorhizal plants was first reported for alder in 1829 (Meyen, 1829). Since then, actinorhizae have been found among over 25 genera of dicotyledonous plants belonging to eight different families (Benson, Silvester, 1993). Actinorhizal root nodules display an indeterminate growth pattern (Fig. 1B). In contrast to legume nodules, they represent coralloid structures composed of several modified lateral roots without root caps (lobes). Actinorhizal nodule lobes contain a central vascular bundle, and infected cells in the cortex (reviewed by Silvester et al., 1990). In some cases, the nodule lobe meristems differentiate agraviotropically growing roots instead of root caps. These "nodule roots" contain large air spaces and serve to aerate the nodule tissue.

The interaction between host plants and microsymbionts starts with signal exchange and recognition of the symbiotic partners. From this step until nodule formation and functioning, many genes from both partners participate in the process. Some of them are nodule-specific plant genes, called nodulin genes (Van Kammen, 1984) in *Rhizobium*-legume symbioses, and actinorhizin genes in actinorhizal symbioses (Tremblay et al., 1986). Nodulins are grouped into early and late nodulins based on the time course of their expressions (Nap, Bisseling, 1990).

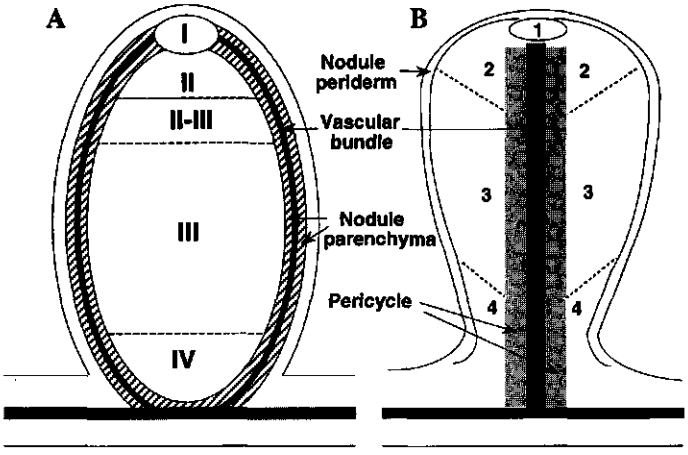

Figure 1. Comparison of an indeterminate legume nodule and an actinorhizal nodule.

(A) Legume nodules contain a peripheral vascular system and infected cells in the central tissue. An O_2 diffusion barrier is present in the nodule parenchyma surrounding the nodule vascular bundles. Since the nodule parenchyma is interrupted by the meristem (zone I), an O_2 gradient forms that extends from the distal to the proximal end of the nodule. Due to the activity of the meristem, the cells in the central tissue are arranged in a developmental gradient (see text). II, prefixation zone; II-III, interzone; III, fixation zone; IV, senescence zone.

(B) Actinorhizal nodule lobes contain a central vascular bundle and are surrounded by a periderm. Due to the activity of the distal nodule meristem (1), a developmental gradient is formed in the cortex. In the infection zone (2) cortical cells enlarge and some of them are infected and gradually filled by *Frankia* hyphae. In the fixation zone (3) *Frankia* vesicles have formed and nitrogen fixation (*nif*) genes are expressed, resulting in nitrogen fixation. In the senescence zone (4), plant and bacterial material is degraded in the infected cells.

II Nodule induction and Nod factors

1. Nodule development

In most cases, infection of legume plants by rhizobia starts by root hair deformation (Bauer, 1981). While rhizobia attach to the root hair, the root hair deforms as a response to rhizobial Nod factors (Lerouge et al., 1990; Heidstra et al., 1994), and the bacteria get trapped in the root hair curl. By hydrolyzing the local root hair cell wall (Callaham, Torry, 1981), the bacteria enter the epidermal cell in an infection thread which proceeds to grow through the cortical cells. Simultaneously, cortical cells are mitotically activated, giving rise to the nodule primordium (Dart, 1977). Infection threads grow toward the primordium and then the bacteria are released into the cytoplasm of the host cells, surrounded by a plant-derived peribacteroid membrane (PBM). The nodule primordium thereupon develops into a mature nodule, while the bacteria differentiate into their endosymbiotic form, bacteroids (Fig. 2A). At this stage, bacteroids synthesize nitrogenase which catalyzes the reduction of nitrogen. In a few cases, however, infection follows a so-called crack-entry mechanism without formation of infection threads (Chandler, 1978).

Frankia can infect its host plants in two different ways, root hair infection or epidermal intercellular penetration. Which of these ways is realized depends on the plant species (Miller, Baker, 1985; Racette, Torry, 1989). In root hair infections, *Frankia* hyphae are entrapped in the deformed root hair. The trapped *Frankia* hyphae branch and initiate the digestion of the primary cell wall of the root hair, and the host plant in turn begins to build an additional cell wall around the site of digestion (Callaham et al., 1979; Berry et al., 1983). Continued digestion by the bacteria and triggered wall building by the host plant create a tubular ingrowth which is termed encapsulation (Lalonde, Knowles, 1975; Newcomb, Wood, 1987). This structure is analogous to the infection thread in *Rhizobium*-legume symboses. Mitotic activity in the root cortex and cell expansion result in the formation of the prenodule whose cells are infected by encapsulated *Frankia* hyphae. Subsequently, one or several nodule lobe primordia are initiated in the root pericycle. *Frankia* hyphae grow from the root cortex into the developing nodule lobe primordia through cortical cells. Upon entering the primordia, once again, *Frankia* hyphae branch and invade numerous cells of the young nodule cortex, being surrounded by encapsulating material derived from the host cell wall as well as by the host plasma membrane (Fig. 2B). Infected cells are filled with hyphae from the center outward; then *Frankia* begins to produce differentiated vesicles where nitrogenase is induced and nitrogen fixation starts (reviewed by Berry, Sunell, 1990). Vesicles are terminal swellings or differentiate from short branch hyphae. In contrast to *Rhizobium*, *Frankia* can fix nitrogen also in the free-living state under atmospheric oxygen tension by forming these specialized vesicles (Benson, Silvester, 1993). In the vesicles, nitrogenase is protected from oxygen by the multilayered lipid

envelope (Berry et al., 1993).

2. *Rhizobium* Nod factors and *Frankia* factor

The so-called Nod factors are *Rhizobium*-derived lipo-chitooligosaccharides, that play a key role in the induction of the initial stages of nodulation. The bacterial genes involved in Nod factor synthesis are the *nod* (nodulation) genes. These genes are not expressed in free-living bacteria, with the exception of *nodD,* which is expressed constitutively. Its product NodD is able to bind to specific flavonoids secreted by the roots of the host plant (Goethals et al., 1992); upon binding to flavonoids, it acts as a transcriptional activator of the other *nod* genes (Fisher, Long, 1992), which encode enzymes involved in the synthesis of Nod factors. The structure of a Nod factor from *R. meliloti* was first determined in 1990 (Lerouge et al., 1990) and since then Nod factor structures have been determined from other rhizobia (Van Rhijn, Vanderleyden, 1995). In general, Nod factors consist of a backbone of three to five β-1,4-linked *N*-acetylglucosamines bearing a fatty acid on the non-reducing sugar residue. Furthermore, the factors can have various substitutions on the reducing and the non-reducing terminal sugar residues.

Due to the striking similarities of the initial infection steps between *Rhizobium*-legume symbioses and the *Frankia*-actinorhizal symbioses, similar signaling mechanisms between the microsymbionts and the hosts are expected. However, investigations to detect any sequences homologous to the *nod* genes in the *Frankia* genome have failed so far (Simonet et al., 1990; Chen et al., 1992). This might mean that the *Frankia nod* genes are not conserved at the DNA level, but proteins with the corresponding functions may exist in *Frankia*. However, no functional complementation has been observed yet (Chen et al., 1992; Reddy et al., 1992). In most recent studies, it was found that (a) *Frankia* factor(s) eliciting root hair deformation on host plants is released into the growth medium, and is produced constitutively by *Frankia* strain ArI3 (Van Ghelue, 1994). Culture filtrate of *Frankia* strain ArI3 only led to deformation of the root hairs of *Alnus glutinosa* and not of non-host plant species tested, indicating a degree of specificity. So far no root hair deformation factors from *Frankia* have been purified and their chemical properties remain unknown.

3. Gene induction by Nod factors

Rhizobium Nod factors can induce the expression of several plant genes in the epidermis of legume roots (Vijn et al., 1995). The early nodulins *ENOD5* (Scheres et al., 1990b) and *ENOD12* (Scheres et al., 1990a), which encode proline-rich proteins, and *Mtrip1* (Cook et al., 1995), which encodes a peroxidase, represent such genes. The latter gene is expressed in the root pericycle of uninoculated roots; all three genes are induced in the epidermis

within a few hours after application of Nod factors (Horvath et al., 1993; Journet et al., 1994; Cook et al., 1995). The induction of *ENOD12* and *Mtrip1* expression occurs in a relatively broad zone of the root, starting just above the root tip, where root hairs have not yet emerged, and extending to the region containing mature root hairs (Pichon et al., 1992; Cook et al., 1995). Cytological studies have shown that Nod factors elicit the expression of these genes in all epidermal cells (Journet et al., 1994; Cook et al., 1995), and that a direct contact between Nod factors and epidermal cells is required (Journet et al., 1994).

Moreover, Nod factors can induce nodulin gene expressions in the cortical cells which are mitotically reactivated to form the nodule primordium. *ENOD12* and *ENOD40* represent such genes (Vijn et al., 1993). Furthermore, *ENOD40* is also induced by Nod factors in the root pericycle (Kouchi, Hata, 1993; Yang et al., 1993; Asad et al., 1994). This gene has a phytohormone effect when expressed in the non-legume tobacco (K. Pawlowski, R. Walden, personal communication).

III Onset of nitrogen fixation

1. Symbiosis-specific differentiation

In *Rhizobium*-legume interactions, the intracellular bacteria differentiate into their symbiosis-specific form, the bacteroids, after release from the infection thread into nodule primordium cells (Fig. 2A). Because both plant (Häser et al., 1992) and bacterial (Glazebrook et al., 1993) mutants have been identified that are specifically defective in bacteroid differentiation, this process may be independent of internalization of bacteria by the infected cells. Bacterial mutants specifically defective in the release of bacteria from the infection thread are known as well (De Maagd et al., 1989). Bacterial *nod* genes are expressed in the distal part of the prefixation zone II (Schlaman et al., 1991), indicating that Nod factors may play a role in signal exchange within the nodule. However, since bacterial release and bacteroid development can be impaired in bacterial strains with functional *nod* genes, other bacterial and/or plant signals must also play a role in these steps of development.

In all the actinorhizal genera except *Casuarina* and *Allocasuarina*, the onset of nitrogen fixaton is associated with the appearance of *Frankia* vesicles in the infected host cortical cells (Berry, Sunell, 1990). Vesicles are also formed in free living *Frankia* cultures under aerobic conditions when substrate nitrogen is limiting (Tjepkema et al., 1980). Within the cytoplasm of the vesicle, septations may occur which divide the cell into compartments (Fig. 2B). The function of these septa is unknown. Within nodule tissue the extent of vesicle formation, the shape and the spatial organization of the vesicles are controlled by the host plant. Symbiotic

vesicles may be spherical, club-shaped, elliptical, or filamentous (Newcomb, Wood, 1987; Racette, Torrey, 1989). Thus, like *Rhizobium*, *Frankia* shows some symbiosis-specific differentiation.

2. Establishment of the interface between the partners

Root nodules provide a proper environment to allow efficient nitrogen fixation by the microsymbiont and regulated nutrient exchange between both symbionts. The nutrient exchange is regulated by occurrence of plant-derived membranes that in all cases surround the "intracellular" microsymbiont. In legume nodules, bacteroids are enclosed in peribacteroid membranes (PBMs; Fig. 2A). They form the interface between the symbiotic partners across which signals and metabolites are exchanged and prevent a defense response by the plant against the "intracellular" bacteria (Nap, Bisseling, 1990; Verma, 1992; Werner, 1992). This process of endosymbiont internalization and propagation requires massive membrane synthesis, and in the case of soybean nodules equals 30 times the amount of plasma membrane synthesis (Verma, 1992).

The PBM of legume nodules has a phospholipid (Perotto et al., 1995) and protein composition that is different from that of the plasma membrane (Verma, 1992) and that (presumably) endows it with specialized functions. The PBM contains several plant proteins and may even contain a rhizobial protein (Fortin et al., 1985; Miao et al., 1992). Within the peribacteroid space (PBS) between the bacteroids and the PBM, several proteins are present that are also found in vacuoles, e.g. α-mannosidase II (Kinnback et al., 1987; Mellor, Werner, 1987), proteases (Mellor et al., 1984), and protease inhibitor (Garbers et al., 1988; Manen et al., 1991). Thus, the PBM may have adapted some properties of the tonoplast membrane (Mellor, Werner, 1987). Indeed, it has been proposed that the symbiosome (the PBM with enclosed bacteroids) has properties of a lytic compartment that is continuously being neutralized by ammonia exported by the bacteroids (Kannenberg, Brewin, 1989). According to this hypothesis, one would expect that the lack of bacterial nitrogen fixation would lead to bacteroid degradation. In fact, there is evidence for premature bacteroid degradation of non-fixing *Rhizobium* mutants (e.g. Hirsch, Smith, 1987). Because the PBM constitutes the interface between bacteroids and host plants, it plays an important role in controlling the exchange of metabolites. These metabolites include ammonium, the product of nitrogen fixation, and heme, the prosthetic group of the oxygen transport protein leghemoglobin, which are exported by the bacteroids to the host cytoplasm (Nadler, Avissar, 1977; O'Gara, Shanmugan, 1976), and carbon sources and probably also assimilated ammonium, which are supplied by the host to the bacteroids (De Bruijn et al., 1989; Werner, 1992). Which proteins are involved in the transport of these compounds is largely unclear. Nodulins Ngm-26 and Ngm-23 have been localized in the peribacteroid membrane and have been supposed to play a role in PBM structure or function (Jacobs et

al., 1987; Sandal et al., 1987; Ouyang et al., 1991).

In actinorhizal nodules, the interface between the microsymbiont and the host plant is the invaginated plasma membrane formed around the growing hyphae. Within this membrane, *Frankia* is still surrounded by the pectinaceous cell wall-like encapsulation. It is not clear yet if nodule-specific plant proteins are incorporated into the invaginated plasma membrane. However, it has been postulated that the product of a nodule-specific cDNA in *Alnus glutinosa*, *ag12*, which encodes a subtilisin-like protease with a putative signal peptide, might be involved in the processing of a protein that is part of the encapsulation material surrounding the bacteria or of another protein with an undeterminated function (Ribeiro et al., 1995; Fig. 3-C, 3-D). The *ag12* transcript was found mainly in the newly infected cells of zone 2 in the nodule where nitrogen fixation has not started yet.

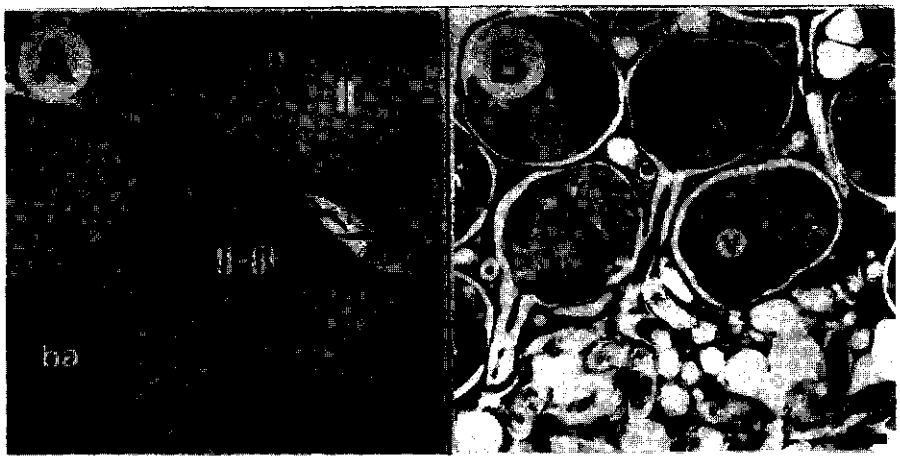

Figure 2. Intracellular nitrogen-fixing bacteria.

(A) Intracellular rhizobia in a nodule formed on clover by *R. trifolii*. A magnification of the transition of the prefixation zone II to the interzone II-III is shown. In the cell of zone II, intracellular bacteria (b) have not yet differentiated into their nitrogen-fixing form. In the cell of interzone II-III, which contains amyloplasts (a), nitrogen-fixing bacteroids (ba) have differentiated. Intracellular bacteria are surrounded by the plant-derived peribacteroid membrane (PBM). The photograph was kindly provided by U. Bialek and A. van Lammeren.
(B) Intracellular *Frankia* in a nodule of *Alnus serrulata*. Vegetative hyphae (h) and nitrogen-fixing septate vesicles (v) can be seen. Vesicles are surrounded by a lipid envelope (arrow) that provides O_2 protection of the nitrogen fixation process. Both hyphae and vesicles are surrounded by encapsulating cell wall-like material and the invaginated plasma membrane of the host cell. The photograph was kindly provided by R.H. Berg.

3. Nodule metabolism

1) Assimilation and transport of ammonium

The form in which nitrogen is transported depends on the plant: temperate legumes, which generally form indeterminate nodules, export amides, whereas tropical legumes, which form

determinate nodules, export ureides. Actinorhizal plants mostly export amides, with the exception of *Alnus* sp. and *Casuarina equisetifolia,* which are citrulline exporters (Schubert, 1986; Sellstedt, Atkins, 1991). In all cases, ammonium is exported by the microsymbiont as the first product of nitrogen fixation and is assimilated in the cytoplasm of nodule cells via the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway (Schubert, 1986). Subsequently, glutamate will be metabolized into nitrogen transport forms.

In legume nodules, the products of several late nodulin genes play a role in this metabolism. In ureide producing determinate legume nodules, the assimilation of ammonium by GS and the biosynthesis of ureides are spatially separated to some extent: whereas GS is expressed in both infected and uninfected cells of soybean nodules (Miao et al., 1991), uricase (nodulin-35), one of the key enzymes in purine oxidation which catalyzes the oxidation of uric acid to allantoin, has been found in peroxisomes of uninfected cells only (Hanks et al., 1981; Nguyen et al., 1985). Allantoinase, which is catalyzing the next step in purine oxidation, has also been localized in the uninfected cells (Hanks et al., 1981). The uninfected cells of determinate nodules also seem to be involved in transport of fixed nitrogen. The presence of GS in the infected cells of actinorhizal nodules of *Alnus glutinosa* has been confirmed by both enzymatic activity measurements and immunological studies (Hirel et al., 1982), and by *in situ* hybridization (C. Guan, A. Ribeiro, T. Bisseling and K. Pawlowski, unpublished results).

2) Carbon metabolism

The carbon source transported from the leaves to the nodules is sucrose (Hawker, 1985), which is introduced into nodule metabolism through degradation by sucrose synthase. This enzyme is present at high levels in both legume and actinorhizal nodules (Thummler, Verma, 1987; M. Van Ghelue, A. Ribeiro, A. Akkermans, B. Solheim, A. Van Kammen, T. Bisseling and K. Pawlowski, unpublished observations). In case of *Rhizobium*-induced nodules, bacteroids express a dicarboxylic acid uptake system. Isolated bacteroids take up dicarboxylic acids, and mutants in this uptake are symbiotically ineffective (Ronson et al., 1987; Werner, 1992), indicating that dicarboxylic acids are likely to be the carbon source supplied by the plant to the intracellular bacteria. It has been suggested that nodulin Ngm-26 transports the dicarboxylic acids to the bacteroids (Ouyang et al., 1991). However, its low substrate specificity *in vitro* indicates that it is more likely to form a pore responsible for the uptake of ions or small metabolites in general (Weaver et al., 1994).

The form of carbon that is supplied to symbiotic *Frankia* in actinorhizal nodules is not clear, yet a malate/aspartate shuttle between host and microsymbiont has been suggested (Akkermans et al., 1981). Based on the hypothesis, like in legume nodules, sucrose has to be metabolized to phosphoenolpyruvate (PEP) to provide the carbon source for the microsymbiont. Sucrose synthase or invertase and glycolytic enzymes would be involved.

Subsequent carbon dioxide fixation by PEP carboxylase would lead to the formation of dicarboxylic acids which can be reduced to malate. High activity of PEP carboxylase have been found in legume (King et al., 1986) as well as actinorhizal nodules (McClure et al., 1983).

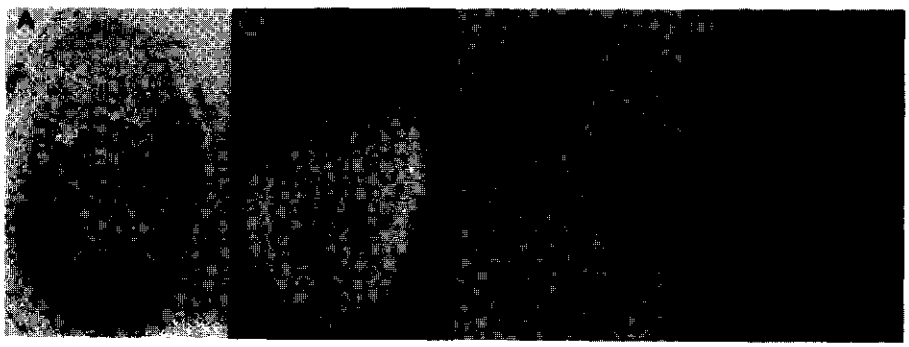

Figure 3. Nodule-specific gene expression.

(A,B) Leghemoglobin (*lb*) is expressed in zones II, II-III and III in a pea nodule. Thus, *lb* expression is preceding nitrogen fixation which starts in the interzone II-III. The photographs were kindly provided by W. C. Yang.
(C,D) Expression of *ag12* in a nodule of *A. glutinosa* is confined to the infected cells of zones 2 and 3. Highest expression levels are found in the infected cells of zone 2 where nitrogen fixation does not take place. *ag12* encodes a subtilisin-like protease.
(A,C) represent bright field micrographs where silver grains denoting hybridization are visible as black dots. The nodule vascular bundle (V) and periderm (P) are labeled (see also Fig. 1). In the dark field micrographs **(B,D)**, silver grains are visible as white dots.

IV Oxygen control

The enzyme nitrogenase is highly oxygen sensitive, because one of its components, the iron molybdenum cofactor, is irreversibly denatured by oxygen (Shaw, Brill, 1977). On the other hand, the large amount of energy required for this reaction has to be generated by oxidative processes; thus, there is a high demand for oxygen in nodules. Different strategies are used in different symbiotic interactions to cope with this paradox.

In legume nodules, a low oxygen tension in the central part of the nodule is achieved by a combination of a high metabolic activity of the microsymbiont and an oxygen diffusion barrier in the periphery of the nodule, that is, in the nodule parenchyma (Witty et al., 1986; Fig. 1A). Oxygen is supposed to diffuse via the intercellular spaces. The nodule parenchyma contains very few and small intercellular spaces where nodulin genes such as *ENOD2* are expressed whose protein products might contribute to the formation of the oxygen barrier (Van de Wiel et al., 1990). In the infected cells of the central part of the nodule, high levels of the oxygen carrier protein leghemoglobin facilitate oxygen diffusion to the sites of respiration (Fig. 3-A, 3-B). In this way, the microsymbiont is provided with

sufficient oxygen to generate energy within a low overall oxygen concentration (Appleby, 1984). In contrast to *Rhizobium*, *Frankia* bacteria can form specialized vesicles in which nitrogenase is protected from oxygen (Berry et al., 1993; Benson, Silvester, 1993). However, vesicle formation during symbiosis does not take place in all *Frankia*-actinorhizal interactions (Benson, Silvester, 1993) and does not always seem to provide full oxygen protection of nitrogenase in others (Tjepkema, 1983; Kleemann et al., 1994). In *Casuarina* symbioses, an oxygen diffusion barrier is established around groups of infected cells by lignification of the walls of both infected and adjacent uninfected cells (Berg, McDowell, 1988; Zeng et al., 1989). In addition, the oxygen transport protein hemoglobin, the equivalent of leghemoglobin, is expressed in the infected cells of *Casuarina* nodules (Fleming et al., 1987; Tjepkema, Asa, 1987; Jacobsen-Lyon et al., 1995). Also in *Myrica* nodules, an oxygen diffusion barrier is present (Zeng, Tjepkema, 1994) and high amounts of hemoglobin have been found, although *Frankia* forms vesicles in this symbiosis (Tjepkema, Asa, 1987).

The leghemoglobin genes have been extensively studied in *Rhizobium*-induced symbioses. Promoter analysis of these genes has led to the identification of a so-called organ specific *cis*-acting element (OSE; Ramlov et al., 1993) which was also found in the promoter of the nodule-specific hemoglobin gene of the actinorhizal plant *Casuarina glauca* (Jacobsen-Lyon et al., 1995). A *C. glauca* hemoglobin promoter-GUS fusion was expressed in the infected cells of *Rhizobium*-induced nodules on *Lotus corniculatus* (Jacobsen-Lyon et al., 1995), implying that similar regulatory factors are involved in both legume and actinorhizal systems. This, in addition to the newly found phylogenetic relationship between different actinorhizal plant genera on the one hand and between actinorhizal plants and legumes on the other hand (Soltis et al., 1995), leads to the hypothesis that the ability for plants to enter symbioses is a trait which developed only once in evolution. Thus, actinorhizal and *Rhizobium*-legume symbioses seem to be closely related.

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Chapter 2

Nitrogen metabolism in actinorhizal nodules of *Alnus glutinosa*: Expression of glutamine synthetase and acetylornithine transaminase

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Nitrogen metabolism in actinorhizal nodules of *Alnus glutinosa*: Expression of glutamine synthetase and acetylornithine transaminase

Changhui Guan[1], Ana Ribeiro[1], Antoon D.L. Akkermans[2], Yuxiang Jing[3], Ab van Kammen[1], Ton Bisseling[1] and Katharina Pawlowski[1*]

[1]Department of Molecular Biology, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands (*author for correspondence)
[2]Department of Microbiology, Agricultural University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands
[3]Institute of Botany, Academia Sinica, 20 Nanxincun, Xiangshan, Beijing 100093, PR China

Key words: actinorhizal nodules, *Alnus glutinosa*, nitrogen metabolism, glutamine synthetase, acetylornithine transaminase, *in situ* hybridization

Abstract

Differential screening of an actinorhizal root nodule cDNA library of *Alnus glutinosa* yielded two clones related to nitrogen metabolism. The insert of pAg11 encoded a glutamine synthetase (GS), the key enzyme responsible for the assimilation of ammonium, while the insert of pAg118 encoded an acetylornithine transaminase (AOTA) involved in the biosynthesis of citrulline, the exported form of fixed nitrogen in *Alnus* nodules. Southern hybridization results suggested that both cDNAs were encoded by small gene families. The expression patterns of the corresponding genes were analyzed by Northern hybridization with RNA isolated from roots, nodules, leaves, shoot tips, flowers and developing fruits of *A. glutinosa*, respectively. GS mRNA was found in all tissues tested, with the highest level of expression in nodules. AOTA transcripts were found at very low levels in roots, and at high levels in nodules. *In situ* hybridization showed that GS was specifically expressed in the infected cells as well as in the pericycle of the vascular bundle of the nodule lobes, while AOTA was expressed in the infected cells. These data suggest that citrulline biosynthesis takes place mainly in the infected cells in root nodules of *A. glutinosa*.

Introduction

The major enzyme responsible for the first step in ammonium assimilation is glutamine synthetase (GS; EC 6.3.1.2) which catalyzes the ATP-dependent condensation of ammonium with glutamate to yield glutamine [29, 33]. GS isoenzymes are located in the cytosol or the chloroplast in plants [33]. Different GS isoforms are encoded by small multigene families whose members have been shown to be differentially expressed in an organ-specific manner [3, 41, 49]. GS expression has been extensively investigated in legumes, especially in root nodules in which GS plays a crucial role in the assimilation of ammonium, the product of rhizobial nitrogen fixation. At least one nodule-specific form of GS has been found in soybean [48] and alfalfa [17], while pea and *Medicago truncatula* do not seem to contain a nodule-specific GS [49, 51].

Actinomycetes of the genus *Frankia* can induce actinorhizal nodules (actinorhizae) on the roots of several woody plants from eight different families. These plants are collectively called actinorhizal plants. Actinorhizal nodules structurally resemble modified lateral roots without root caps, with a central vascular tissue and infected cells in the expanded cortex [4]. Like in legume nodules, ammonium assimilation in actinorhizal nodules is performed by the common GS/GOGAT pathway [45]. GS activity has been found in root nodules of *A. glutinosa* and associated with the cytosol of the large inner cortical cells [8, 24].

The product of ammonium assimilation, glutamate, is further metabolized in root nodules to yield nitrogen transport form which is brought into the xylem. The nitrogen transport form depends on the host plant, temperate legumes which generally form indeterminate nodules, export amides, whereas tropical legumes which form determinate nodules, export ureides [2]. Actinorhizal plants mostly export amides, with the exception of *Alnus* sp. and *Casuarina equisetifolia,* which are citrulline exporters [45, 47]. Citrulline is synthesized via the arginine biosynthetic pathways in microorganisms and plants. The enzyme acetylornithine transaminase (AOTA; also known as acetylornithine aminotransferase, ACOAT; EC 2.6.1.11) catalyzes the fourth step in this pathway, the conversion of N-acetyl-γ-glutamate semialdehyde to N-acetylornithine [13, 14, 32]. AOTA genes from *Escherichia coli, Anabaena* sp. and *Saccharomyces cerevisiae* have been cloned and sequenced [19, 22].

To analyze nitrogen metabolism in actinorhizal nodules, a cDNA library constructed from *A. glutinosa* nodule RNA was screened differentially for nodule-specific or -enhanced cDNA clones. cDNAs encoding enzymes involved in nitrogen metabolism were selected for further analysis.

Materials and Methods

Plant and bacterial growth conditions

A. glutinosa seeds were collected from a local source (Weerribben, The Netherlands). The plants were grown in a greenhouse at 25°C under 16 hrs light, 8 hrs dark. Seeds were germinated in sterile gravel and wetted with sterile tap water for three weeks. Then they were transferred to sterile gravel wetted with 1/4 strength Hoagland solution [25] and each plantlet was infected with 1 ml of a 1:5 diluted dispersed culture of *Frankia* HFPArI3 [5] grown in P medium [34]. Nodules were harvested 5-8 weeks after infection depending on the growth state of the plants. For isolation of root RNA, seedling roots were collected from uninfected plantlets 2-3 weeks after germination. For isolation of shoot tip RNA or genomic DNA, shoot tips including two youngest unfolded leaves were collected from plants 5-13 weeks after infection. Male and female flowers as well as developing fruits were collected from a local stand (Wageningen, The Netherlands). Flowers were collected in March 1994, developing fruits in April, June, and September 1994, respectively.

Pisum sativum ssp. *sativum* L. cv. Rondo was grown in gravel trays and inoculated with *Rhizobium leguminosarum* biovar *viciae* 248 as described [7]. *Phaseolus vulgaris* L. cv. negro Jamapa was inoculated with *R. leguminosarum* biovar *phaseoli* strain CE330 [16] grown in YEM medium [6]. Legume nodules were harvested 2-3 weeks after infection.

Isolation of DNA and RNA

Nucleic acids were isolated from *A. glutinosa* as described [42]. Total RNA was isolated from legumes as described [40]. Poly(A) RNA was isolated from total RNA using Dynabeads (Dynal, Oslo, Norway) according to the protocol provided by the manufacturer.

Construction and screening of a cDNA library

A cDNA library from poly(A) RNA of *A. glutinosa* nodules was custom-made by Stratagene (La Jolla, CA, USA) in λZapII. Differential screening of the library has been described elsewhere [42].

Sequencing procedures and data analysis

DNA manipulations were carried out as described by Sambrook et al. [43]. The nucleotide sequences were determined using the dideoxy chain termination method [44]. Sequence data were analyzed using the programs of the Wisconsin Genetics Computer Group (GCG) [15]. Database searches were performed using the BLAST algorithm [1] in the nucleotide sequence databases of the National Center of Biotechnology Information (NCBI), National Library of Medicine, NIH, in Bethesda, MD.

Southern and Northern hybridization conditions

Southern and Northern hybridizations were carried out as described [42]. The complete cDNA inserts were used as probes. Hybridization signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In situ *hybridization*

The preparation of sections of fixed plant material has been described elsewhere [42]. Pretreatment, hybridization and washing were performed essentially as described by Cox and Goldberg [12] and adapted by Van de Wiel et al. [52].

For the preparation of *A. glutinosa* GS sense and antisense RNA probes, a 650 bp *Eco*RI fragment containing the 5' half of the cDNA was subcloned in pBluescript KS^+ (Stratagene). The resulting clone was linearized with *Xba*I and antisense RNA was transcribed using T3 RNA polymerase, or it was linearized with *Sal*I and sense RNA was transcribed using T7 RNA polymerase. For AOTA, a *Sst*I deletion derivative of pAg118, containing the 5' 400 bp of the cDNA was linearized with *Eco*RI and antisense RNA was transcribed using T3 RNA polymerase, or it was linearized with *Sst*I and sense RNA was transcribed using T7 RNA polymerase. For *P. vulgaris* γGS, a 360 bp *Eco*RI/*Bam*HI fragment of the coding region was subcloned in pBluescript KS^+. The resulting plasmid was linearized with *Eco*RI and antisense RNA was transcribed with T7 RNA polymerase, or it was linearized with *Bam*HI and sense RNA was transcribed using T3 RNA polymerase. The production of *Frankia nifH* antisense RNA has been described elsewhere[42].

Results

pAg11 and pAg118 from an A. glutinosa *nodule cDNA library encode a glutamine synthetase and an acetylornithine transaminase, respectively*

An *A. glutinosa* nodule cDNA library was screened differentially with nodule and root cDNA, respectively. 24 clones hybridizing with nodule cDNA but not or only weakly with root cDNA were purified. Their plant origin was confirmed by Southern hybridization with total DNA from the host plant *A. glutinosa* and the microsymbiont *Frankia* HFPArI3, and their nodule-enhanced expression was confirmed by Northern hybridization with total RNA isolated from roots, nodules and shoot tips of *A. glutinosa*, respectively (data not shown). The ends of the cDNA clones were sequenced and the deduced amino acid sequences were used for homology searches in the NCBI databases. Two clones whose products are involved in nitrogen metabolism were selected for further characterization.

The insert of the cDNA clone pAg11 was found to encode a 356 amino acid polypeptide, showing high amino acid sequence homology with all plant GS enzymes. Highest homologies were found to a cytosolic GS (GS20) from soybean (95% amino acid similarity) [35] and a nodule-enhanced GS (GS13) from alfalfa (93%; Fig. 1A) [50]. Thus, Ag11 was termed AgGS1. Because AgGS1 is highly homologous to cytosolic GS enzymes from other plants and does not contain a signal peptide sequence at the 5'-end, it probably represents a cytosolic GS isoform. This is consistent with the result of previous physiological studies [24]. Two GS isoenzymes have been reported in *Alnus glutinosa* nodules, a major isoform and minor isoform. The major one showed the same chromatographic behavior as root GS and probably they were the same enzyme [24]. Up to now, the data are not sufficient to conclude which isoform AgGS1 represents.

The insert of the cDNA clone pAg118 encoded a polypeptide (Ag118) of 451 amino acids, homologous to an acetylornithine transaminase (AOTA) encoded by the *argD* gene of *Anabaena* (43%) [19] and an AOTA encoded by the *ARG8* gene from *Saccharomyces cerevisiae* (42%) [22]. The amino acid sequence comparison is shown in Fig. 1B. The similarity between the three polypeptides extended over most of their sequence. Large discrepancies were only seen in the N-terminal regions which are probably representing signal peptides for specific subcellular localization. These data strongly suggested that pAg118 represents an AOTA. Ag118 also showed homology to ornithine aminotransferases (OATs) of *Drosophila ananassae* (GenBank accession number dbjlD50331), rat [36] and human [26] (data not shown), indicating an evolutionary relationship of both groups of enzymes catalyzing analogous reactions in different metabolic pathways [22].

A

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AgGS1     1 MSLLSDLINLNLSDATDKVIAEYIWIGGSGTDLRSKARTLTGPVNHPSKLPKWNYDGSST 60
GmGS20    1 ..............T.E.............M.........P...SD..E........... 60

AgGS1    61 GQAPGEDSEVIYILRQFFKDPFRRGNNILVICDTYTPAGEPIPTNKRHGAAKIFSHPEVV 120
GmGS20   61 .........A..-THKP.Q..............A..............A...V....D.. 119

AgGS1   121 AEVPWYGIEQEYTLLQKDVKWPLGWPVGGYPGPQGPYYCGIGADKAWGRDIVDAHYKACL 180
GmGS20  120 ...................IQ.........F..........V.....F............I 179

AgGS1   181 YAGINISGINGEVMPGQWEFQVGPSVGISAGDEVWAARYILERITEIAGVVLSLDPKPIQ 240
GmGS20  180 ................................I................V.F.....P 239

AgGS1   241 GDWNGAGAHTNYSTKSMRNNGGYEIIKKAIEKLGLRHKEHIAAYGEGNERRLTGRHETAD 300
GmGS20  240 ...................ED....V.....D...KK........................ 299

AgGS1   301 INTFKWGVANRGASIRVGRDTEKEGKGYFEDRRPASNMDPYVVTSMIAETTLLWKP 356
GmGS20  300 ....L.........V.........A.......................D....... 355
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B

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Ag118     1 MTSLQYFSLNRPVFPATHLHRPGIRHLQVSACANVEVQAPSSVKKQGVSKEVMEAAGRVL  60
ArgD      1                                MSLQTLIEQATNPPESGSAASSPFSTDSFDAS...V  33
ARG8      1                                      MFKRYLSSTSSRRFTSILEEKAFQV  25

Ag118    61 VGTYAR.VPVVLSRGKGCKLY.DPEGREYLDLSAGIAVNVLGHADSDWLRAVTEQAATLT 118
ArgD     34 MSTYGR.FPLALERGAGCRVW.DTQGKEYLDFVAGIATCTLGHAHPAMVEAVTRQIQKLH  91
ARG8     26 .TTYSRPEDLCITRGKNAKLYDDVNGKEYIDFTAGIAVTALGHANPKVAEILHHQANKLV  84

Ag118   119 HVSNVFY.......SIPQVELAKRLVASSFADRVFFSNSGTEANEAAIKFARKFQRFTRP 171
ArgD     92 HVSNLYYIPEQGELAQWIIQ.......HSCADRVFFCNSGAEANEAAIKLARKYAHTVLD 144
ARG8     85 HSSNLYFTKECLDLSEKIVEKTKQFGGQHDASRVFLCNSGTEANEAALKFAKKHG.IMKN 143

Ag118   172 DEKQPATEFVSFSNSFHGRTMGSLALTSKENYRSPFEPVMPGVTFLEYGNIEAATQLI.. 229
ArgD    145 IEK...PIILTANASFHGRTLATITATGQAKYQKYFDPLVPGFHYVNYNDISAVEAAISE 201
ARG8    144 PSKQ...GIVAFENSFHGRTMGALSVTWNSKYRTPFGDLVPHVSFLNLNDEMTKLQSYIE 200

Ag118   230 ...QRRKIAAVFVEPIQGEGGVYSATKEFLYALRKACDDSGTLLVFDEVQCGLGRTGYLW 286
ArgD    202 LDEGDYRVAAILIEPLQGEGGVRPGDVEYFQKLRQICDDTGILLMFDEVQVGMGRSGKLW 261
ARG8    201 TKKDE..IAGLIVEPIQGEGGVFPVEVEKLTGLKKICQDNDVIVIHDEIQCGLGRSGKLW 258

Ag118   287 AHEIY..DVFPDIMTLAKPLAGGLPIGAVLVTERVASAITYGDHGTTFAGGPLVCKAALT 344
ArgD    262 GYEYLGVE..PDIFTSAKGLGGGIPIGA.MMSKKFCDVFQPGEHASTFGGNPFACGVALA 318
ARG8    259 AHAYLPSEAHPDIFTSAKALGNGFPIAATIVNEKVNNALRVGDHGTTYGGNPLACSVSNY 318

Ag118   345 VLDKILRPGFLASVSKKGHYFKEMLINKLGG.NSHVREVRGVGLIVGIELD....VSASP 399
ARGD    319 VCQTLERENILQNVQDRGEQLRSGLRAIAAKYPHHLTEVRGWGLINGLELAADIPLTAAD 378
ARG8    319 VLDTIADEAPLKQVSKKSDILQKRLREIQAKYPNQIKTIRGKGLM....LGAEFVEPPTE 374

Ag118   400 LVNACLNSGLLVLTAGKGNVVRIVPPLIITEQELEKAAEILLQCLPALDRHG 451
ArgD    379 VVKAAINEGLLLVPAGPK.VVRFVPPLIVTEAEINTALKLLEKALATVTA   427
ARG8    375 VIKKARELGLLIITAGKS.TVRFVPALTIEDELIEEGMDAFEKAIEAVYA   423
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Figure 1. Amino acid sequence comparisons. **(A)** Comparison between the amino acid sequence of Ag11 (AgGS1) and the nodule-specific soybean GS20 [35]. The amino acid sequence of AgGS1 is shown in full. The amino acid sequence of GS20 is given only where it differs from the AgGS1 sequence while the consensus amino acids are shown as dots (.). The only gap is denoted by a hyphen at position 73 in GS20. **(B)** Comparison between the amino acid sequence of Ag118 and the sequences of *Anabaena* AOTA (ArgD) [19] and yeast AOTA (ARG8) [22]. Gaps were introduced to optimize the alignment. Identical amino acids are given in bold print.

Southern hybridization analysis of ag11 *and* ag118

To analyse the organization and complexity of GS and AOTA genes in *A. glutinosa*, Southern blots containing total DNA of *A. glutinosa* digested with *Eco*RI, *Bam*HI and *Hin*dIII were hybridized with the complete inserts of pAg11 and pAg118, respectively. The results are shown in Fig. 2. Both GS and AOTA seem to be encoded by small gene families in *A. glutinosa*. The GS Southern analysis (Fig. 2A) indicates that there are at least two members in the GS gene family of *A. glutinosa*. The hybridization with *ag118* shows a more complicated pattern, containing several weaker hybridizing bands (Fig. 2B). These bands may represent genes encoding other aminotransferases.

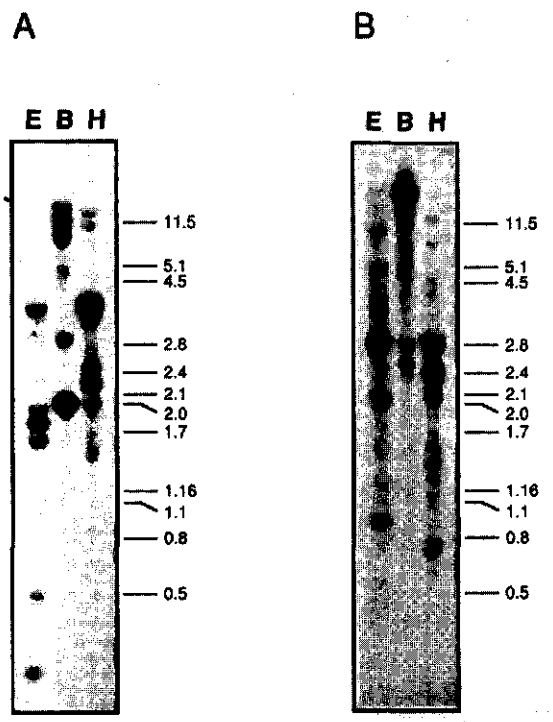


Figure 2. Southern hybridization analysis. Southern blots containing total DNA of *Alnus glutinosa* digested with *Eco*RI (E), *Bam*H I (B) and *Hin*d III (H) were hybridized with ^{32}P-labeled inserts of pAg11 (A) and pAg118 (B), respectively. The *ag11* cDNA contains an *Eco*RI-, a *Hin*dIII- and two *Bam*HI-sites. The *ag118* cDNA contains a *Hin*dIII-site.

GS and AOTA exhibit an nodule-enhanced expression pattern

Northern hybridization was performed to check the organ-specific expression of GS and AOTA genes using RNA isolated from roots, leaves, stems, shoot tips, flowers, and developing fruits of *A. glutinosa*. As shown in Fig. 3, GS transcripts were found in all the tissues tested, with highest levels in the nodule (Fig. 3A). AOTA mRNA was found to be

present at high levels in nodules and at very low levels in roots (Fig. 3B). Regarding the low expression levels found in roots, it should be noted that the roots for RNA isolation were taken from seedlings germinated in the absence of nitrogen sources.

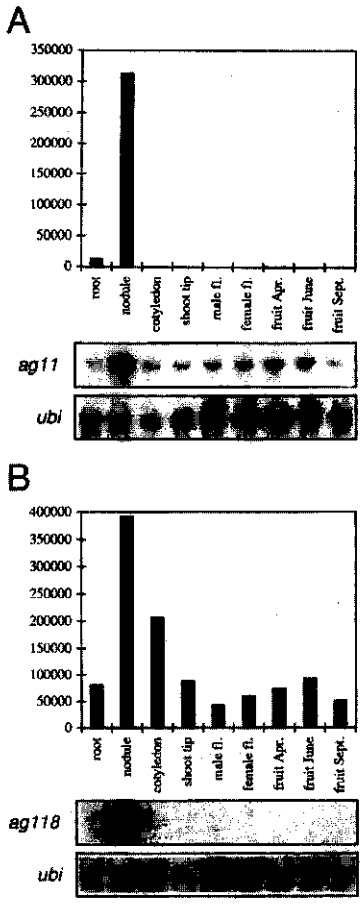
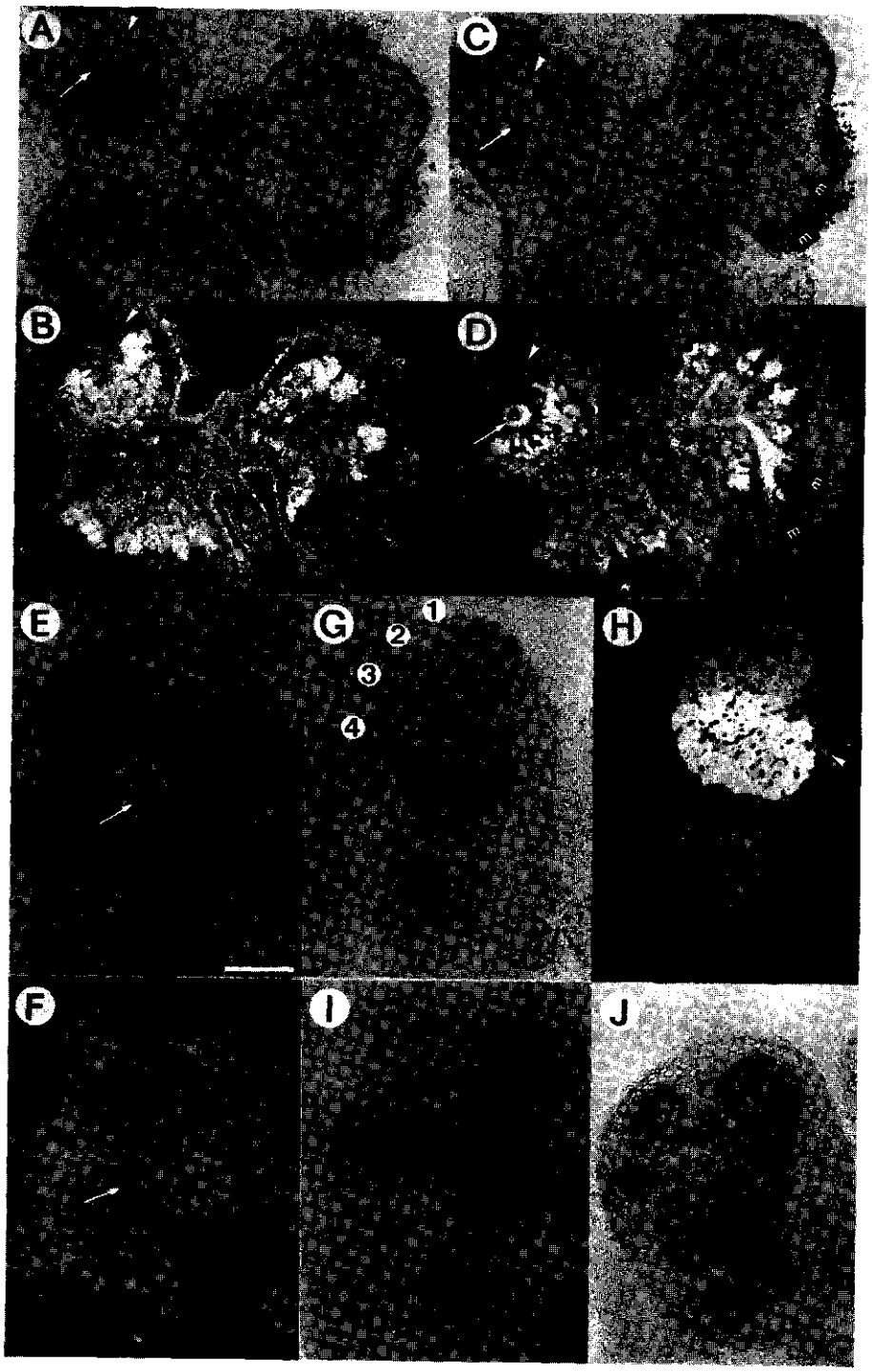


Figure 3. Northern hybridization analysis. Northern blots con-taining about 10mg total RNA per slot were hybridized with *ag11* **(A)** and *ag118* **(B)**, respectively. The amount of mRNA on the filters was determined by hybridization with a soybean ubiquitin probe [27]. Signal was measured by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) in all cases. Expression levels are shown as relative area units calculated from comparison with ubiquitin expression.

1, roots; 2, nodules; 3, cotyledons; 4, shoot tips; 5, male flowers; 6, female flowers; 7, immature fruits collected in April; 8, immature fruits collected in June; 9, immature fruits collected in September.

Localization of GS and AOTA transcripts in nodules of A. glutinosa

The expression patterns of *ag11* and *ag118* in the nodule were determined by *in situ* hybridization of longitudinal sections of *A. glutinosa* nodules with ^{35}S-labeled antisense and sense RNA probes, respectively. For a marker of the developmental gradient of the infected cells, *in situ* hybridizations with antisense RNA of the nitrogenase structural gene *nifH* from


A
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Frankia HFPArI3 were performed on adjacent sections [39, 42]. The results are shown in Fig. 4. GS mRNA was found in the infected cortical cells and in the cells of the multilayered pericycle of the central vascular bundle of the nodule lobe. The highest amounts of GS transcripts were found in the infected cells showing the highest level of *Frankia nifH* expression. GS mRNA could not be detected in infected cells before the onset of bacterial nitrogen fixation (Figs. 4A/B, C/D). The AOTA gene showed a different expression pattern in *A. glutinosa* nodules. It was highly expressed in the infected cells of the nodule, but no AOTA mRNA could be found in the pericycle of the nodule vascular bundle (Figs. 4E/F), indicating that expression in this tissue was below the detection limit. For both GS and AOTA, no signal was detected in hybridizations with sense RNA probes (data not shown).

←

Figure 4: *In situ* localization of GS and AOTA mRNA.

In bright field micrographs (**A, C, E, G, I, J**) silver grains denoting hybridization are visible as black dots. In dark field micrographs (**B, D, H**), silver grains appear as white dots. In dark field micrographs taken under epipolarized light (**F**), silver grains appear yellow. (**A/B**) Section of a 8-week-old *A. glutinosa* nodule hybridized with *Frankia nifH* antisense RNA. *nifH* expression is confined to the infected cells filled with *Frankia* material (black arrowhead), while no expression can be detected in young infected cells not yet completely filled with hyphae (white arrowheads). A white arrow points at the pericycle in the cross-section of a nodule vascular bundle. A short black arrow points at the root pericycle. (**C/D**) An adjacent section of the same nodule hybridized with *A. glutinosa GS* antisense RNA. In infected cortical cells not yet completely filled with hyphae, i.e. not expressing *Frankia nifH* and not fixing nitrogen, *GS* expression cannot be detected (white arrowheads), while high levels of *GS* expression are visible in the nitrogen fixing infected cells (black arrowheads). High levels of *GS* expression are also found in the pericycle of the nodule vascular bundle (white arrow), but expression in the pericycle of the root is below the detection level (short black arrow). No *GS* expression is detectable in the meristems of nodule lobes (thick black arrows; m). (**E/F**) Cross section of a 12-week-old nodule lobe hybridized with *A. glutinosa AOTA* antisense RNA. While infected cortical cells are showing hybridization (black arrowhead), no hybridization is found in the pericycle of the nodule vascular bundle (white arrow). (**G/H**) Longitudinal section of a 18-day-old pea nodule hybridized with *P. vulgaris* GS_γ antisense RNA. The zonation of the nodule according to Vasse et al. [54] is indicated. *GS* expression is detected in the meristem (1), in the prefixation zone (2), in the interzone (3) and in the fixation zone (4). A senescent zone has not yet developed. Highest expression levels are found in the fixation zone. *GS* is also expressed in the nodule vascular system (arrowhead). (**I**) Section of a 14-day-old white *P. vulgaris* nodule, not yet fixing nitrogen, hybridized with *P. vulgaris* GS_γ antisense RNA. Expression is confined to the infected cells of the central tissue (arrow). (**J**) Section of a 21-day-old pink nitrogen-fixing *P. vulgaris* nodule, hybridized with *P. vulgaris* GS_γ antisense RNA. GS_γ expression is still confined to the infected cells of the inner tissue (arrow).

The bars denote 500 µm. The bar in (**A**) is valid for **A-D**, the bar in (**E**) is valid for **E-J**.

GS expression in legume nodules

To compare the expression patterns of GS between actinorhizal and legume nodules, *in situ* hybridization of legume GS was performed. Longitudinal sections of mature indeterminate nodules of pea and determinate nodules of *Phaseolus vulgaris* were hybridized with *Phaseolus vulgaris* nodule-specific GS (*gln-γ*) [3] antisense RNA, respectively. Results are shown in Fig. 4. In pea nodules, GS was found to be expressed in the nodule meristem, at decreased levels in the prefixation zone and interzone, and at highest levels in the fixation zone, in both infected and uninfected cells. Its transcripts were detected also in the nodule vascular bundles (Figs. 4G/H). These results are consistent with those of promoter analysis of pea *GS3A* in transgenic alfalfa nodules [9]. In *Phaseolus vulgaris* nodules, GS_γ transcripts were confined to the infected cells (Figs. 4I/J).

Discussion

Localization of ammonium assimilation and citrulline biosynthesis in A. glutinosa *nodules*

In *A. glutinosa* nodules, GS and AOTA genes were both found to be expressed in the infected cortical cells, while GS expression was also found in the pericycle of the nodule vascular system. In the infected cells, GS and AOTA expression was confined to the nitrogen fixation zone, i.e. to those infected cells where *Frankia* is fixing nitrogen and exporting ammonium to the plant cytoplasm. Since GS catalyzes the first step of ammonium assimilation, while AOTA catalyzes the penultimate step of citrulline biosynthesis, the synthesis of acetylornithine [30], it is very probable the assimilation of ammonium exported by symbiotic *Frankia*, and the biosynthesis of the nitrogen transport form, citrulline, occurs in the infected cells of *A. glutinosa* nodules. Citrulline would then have to be exported from the infected cells to the xylem elements.

It is known that yeast AOTA (*ARG8*), human OAT and rat OAT are all located in the mitochondrial matrix [22, 36]. This would suggest that Ag118 is also located in the mitochondria. Despite the fact that there is no consensus for mitochondrial signal peptides, it has been found that they are enriched for arginine, leucine, serine and contain less asparagine, glutamate, valine and isoleucine, and that the first 10-15 residues of mature, imported mitochondrial proteins are rich in proline and serine [55]. The N-terminus of Ag118 fulfills these criteria (Fig. 1B). The mitochondrial localization of AOTA also agrees with cytochemical data on the localization of carbamoyl transferase, another enzyme involved in citrulline biosynthesis which was found in the mitochondria of host cells in *Alnus* root

nodules [46], indicating that citrulline is mainly synthesized in the mitochondria of the infected cortical cells.

Role of the A. glutinosa *nodule pericycle in nitrogen transport*

The special multilayered pericycle of the central vascular system of *Alnus* nodule lobes consists of small cells with a dense cytoplasm and high metabolic activity [10, 11, 53]. The fact that GS transcripts were also found in the pericycle of the nodule vascular system (Fig. 4C/D) indicates that free ammonium is present in this tissue. Thus, either ammonium is diffusing from the infected cells to the pericycle, or assimilation products are degraded in the pericycle, yielding ammonium for reassimilation. The latter hypothesis is supported by the fact that the composition of nitrogenous solutes in the stem xylem and the nodules is different, arguing for a degradation and reassimilation process of nitrogenous solutes during transport to the plant vascular system. In comparison to the stem xylem, *A. glutinosa* nodules contain relatively high amounts of serine, while glutamate is enriched in the stem xylem compared to nodules [8]. Serine might be degraded in the pericycle, and ammonium reassimilated by the GS/GOGAT cycle to yield glutamate. Such degradation/reassimilation processes have been postulated by Lea and Miflin [28], who have estimated that a nitrogenous solute can be catabolized and reassimilated five or more times before ending up in the seed, and GS is involved in the reassimilation of ammonium released in a variety of metabolic pathways [29, 31]. Thus, we postulate that nitrogenous solutes are degraded and ammonium is reassimilated in the vascular pericycle of *Alnus* nodules in course of transport to the xylem.

Comparison with legume nodules

Also in legume nodules, GS expression is found in the nodule vascular system, arguing for reassimilation of ammonium in course of transport to the xylem [20, 50] (Fig. 4G/H; Fig. 4I/J). Which nitrogen transport forms are synthesized depends on the plant species [45], as do sites of ammonium assimilation and nitrogen transport form biosynthesis [20, 35]. For instance, in determinate legume nodules exporting ureides, the biosynthesis of the nitrogen transport form takes place in the uninfected cells [21, 37], and both infected and uninfected cells of the central tissue express GS [35]. However, all legume nodules examined thus far have in common that GS gene expression is controlled developmentally as well as metabolically [23]. Metabolic control, i.e. induction by ammonium, has been confirmed by the fact that GS expression is much lower in ineffective than in effective nodules [18, 56]. The fact that GS expression is always induced before the onset of nitrogen fixation and the export of ammonium by bacteroids [20, 38, 50] (Fig. 4G/H; Fig. 4I/J), as well as the induction in Fix^- nodules, argue for developmental control.

This is different from the situation in actinorhizal nodules of *A. glutinosa*, where GS induction does not precede the onset of bacterial nitrogen fixation. Thus, developmental control of GS expression seems to be lacking in *A. glutinosa* nodules, and only metabolic control is taking place. This is in agreement with the fact that actinorhizal symbioses are more primitive than *Rhizobium*-legume symbioses in general.

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Chapter 3

Expression of a homologue of fatty acid reductase in actinorhizal nodules of *Alnus glutinosa* and in *Arabidopsis thaliana*

Guan, C., Akkermans, A.D.L., van Kammen, A., Bisseling, T. and Pawlowski, K.
In preparation.

Expression of a homologue of fatty acid reductase in actinorhizal nodules of *Alnus glutinosa* and in *Arabidopsis thaliana*

Changhui Guan[1], Antoon D.L. Akkermans[2], Ab van Kammen[1], Ton Bisseling[1] and Katharina Pawlowski[1,*]

[1]Department of Molecular Biology, Agricultural University, Dreijenlaan 3, 6703HA Wageningen, The Netherlands
[2]Department of Microbiology, Agricultural University, Hesselink van Suchtelenweg 4, 6703CT Wageningen, The Netherlands

*Author for correspondence
Department of Molecular Biology
Dreijenlaan 3
Agricultural University
6703HA Wageningen
The Netherlands
fax: +31-317-483584
phone: +31-317-482640
E-mail: Katharina.Pawlowski@mac.mb.wau.nl

running title: Fatty acid reduction in *Arabidopsis* and *Alnus*

Keywords: *Alnus glutinos*a, actinorhiza, *Arabidopsis,* wax, fatty acyl reductase, jojoba

Summary

A cDNA library made from poly(A) RNA from actinorhizal nodules of *Alnus glutinosa* was screened differentially with nodule and root cDNA, respectively, to isolate nodule-specific cDNAs. The gene corresponding to one of these cDNAs, *ag135*, was found to be expressed at high levels in nodules, at lower levels in male flowers and at very low levels in shoot tips, female flowers and during the first stages of fruit development. The expression in nodules was confined to the infected cortical cells and to the pericycle of the nodule vascular bundles. A homologue of this gene was identified in *Arabidopsis thaliana*, *ara135*, mapping close to, but not at the *ms1* locus. *ara135* was found to be expressed in the epidermis of some floral organs, i.e. of sepals, petals and filaments, and in the epidermis of ovules. The encoded proteins show high homology to Ms2 from *A. thaliana* (Aarts *et al.*, 1993). The possible function of Ag135 in actinorhizal nodules of *A. glutinosa* is discussed.

Introduction

Research on the model plant *Arabidopsis thaliana* is providing information on many areas of plant biology (Höfte *et al.*, 1993; Somerville, 1993; Newman *et al.*, 1994). However, there are some processes in plant development which cannot be addressed using *A. thaliana*, e.g. secondary stem growth or plant-bacterial symbioses resulting in the formation of nitrogen fixing nodules. Two systems of these nitrogen fixing symbioses are known: legume/*Rhizobium* symbioses (Hirsch, 1992) and actinorhizal symbioses between actinomycetous bacteria of the genus *Frankia* and dicotyledonous plants of different families, mostly trees and woody shrubs (Benson and Silvester, 1993). Actinorhizal nodules are perennial, coralloid structures consisting of multiple nodule lobes which represent modified lateral roots with infected cortical cells (Berry and Sunell, 1990). While legume nodules are rather uniform, the only variability being their either determinate or indeterminate growth pattern (Goodchild, 1977), the organization of *Frankia* in infected cells and the arrangement of the infected cells themselves in actinorhizal nodule lobes is more variable as can be understood from the fact that actinorhizal symbioses include different plant families (reviewed by Silvester *et al.*, 1990; Baker and Mullin, 1992). Due to their simple structure and variability, actinorhizal symbioses seem well suited for analysis of the general principles of nodule formation. However, both plant and bacterial partners of actinorhizal symbioses represent recalcitrant species not well suited to genetic and molecular biological analysis. There is no genetics of the microsymbiont, since *Frankia* cannot be transformed (Benson and Silvester, 1993), and the woody nature of actinorhizal plants has hampered the molecular genetic analysis of the host (Séguin and Lalonde, 1991). The question arises whether the achievements of studies on model systems like *A. thaliana* and rice (Uchimiya *et al.*, 1992; Sasaki *et al.*, 1994), particularly the accumulating sequence data (Höfte *et al.*, 1993; Newman *et al.*, 1994) can facilitate the investigation of special developmental processes like the formation of actinorhizal nodules.

Studies on legume nodules have shown that nodule-specific genes either represent duplications from preexisting genes active in non-symbiotic development or have been adopted from other developmental pathways (Nap and Bisseling, 1991). Thus, nodule-specific genes can be expected to have homologues in non-symbiotic plants which should be found eventually amongst the randomly sequenced cDNAs of *A. thaliana* (Höfte *et al.*, 1993; Newman *et al.*, 1994) or rice (Sasaki *et al.*, 1994). Assuming that also the nodule-specific host genes expressed in actinorhizal nodules have their non-symbiotic counterparts, we set about to functionally analyze the products of actinorhiza-specific genes using *A. thaliana* as a dicotyledonous model plant, thereby circumventing the problems of working with woody plants.

Results

Isolation and characterization of nodule-specific cDNAs

To isolate nodule-specific cDNA clones, an *A. glutinosa* nodule cDNA library was screened differentially with nodule versus root cDNA. cDNA clones hybridizing with nodule but not or only weakly with root cDNA were purified. All these cDNAs clones represented *A. glutinosa* genes as was shown by hybridization against Southern blots containing DNA from *A. glutinosa* as well as *Frankia*. Nodule-specific or -enhanced expression was confirmed by Northern blot analysis using RNA from roots and nodules. The characterization of one of these nodule-specific cDNA clones, pAg135, whose insert hybridized to a 1.7 kb mRNA present in nodules, but not in roots will be described in this paper.

The expression of the *ag135* gene was not confined to nodules, as shown in Figure 1A. *ag135* is expressed at high levels in nodules and at lower levels in male flowers, while very low levels of *ag135* transcripts are found in shoot tips and in female flowers and during the first stages of fruit development. In roots and cotyledons, *ag135* mRNA was not detectable. Southern hybridization analysis showed that *ag135* is hybridizing with five or more fragments in each digest (Figure 2). Thus, *ag135* seems to be encoded by a small gene family in the *A. glutinosa* genome.

Localization of ag135 *mRNA in* A. glutinosa *nodules*

The expression pattern of *ag135* in root nodules was determined by *in situ* hybridization of longitudinal sections and cross sections of nodules with antisense and sense RNAs, respectively. As a marker of the developmental stage of the infected cells, *in situ* hybridizations with antisense RNA of the nitrogenase structural gene *nifH* from *Frankia* HFPArI3 were performed on adjacent sections (Ribeiro *et al.*, 1995). The results are shown in Figures 3A to 3D. *A. glutinosa* nodules display a developmental gradient in which four zones can be distinguished (Ribeiro *et al.*, 1995; Figure 3A): first, the meristematic zone (zone 1) at the tip of the nodule, consists of small dividing cells which do not contain bacteria. Second, the prefixation zone (zone 2), contains enlarging cortical cells some of which being infected and getting gradually filled with hyphae from the center outwards (Lalonde, 1979; Schwintzer *et al.*, 1982; Berry and Sunell, 1990). Third, in the fixation zone (zone 3), bacterial *nif* gene expression is switched on and nitrogenase is produced (Huss-Danell and Bergman, 1990). The induction of *Frankia nifH* expression is used as marker for the transition of zone 2 to zone 3 (Pawlowski *et al.*, 1995). In the senescence zone (zone 4), host cytoplasm as well as microsymbiont material is degraded due to the senescence of

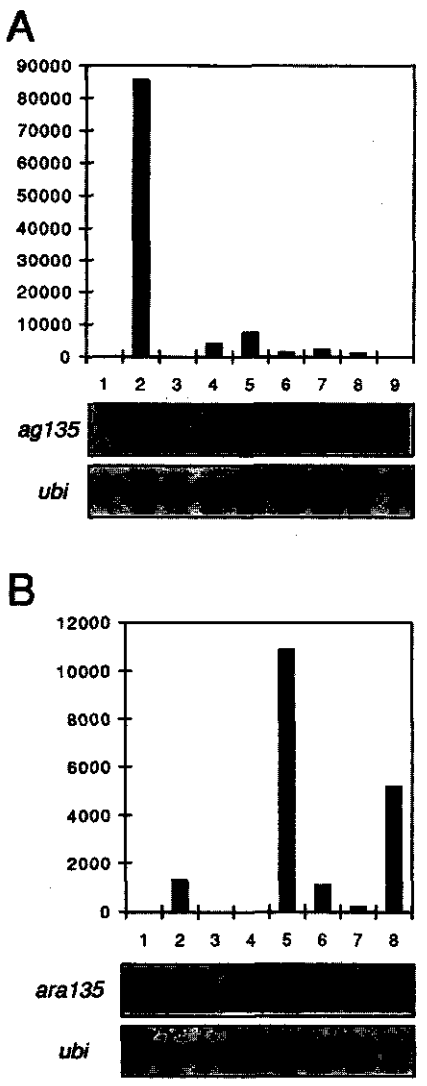


Figure 1: Northern hybridization analysis. **(A)** expression of *ag135* in different organs of *A. glutinosa:* 1, roots; 2, nodules; 3, cotyledons; 4, shoot tips (including the three or four youngest leaves); 5, male flowers; 6, female flowers; 7, immature fruits collected in April; 8, immature fruits collected in June; 9, immature fruits collected in September. *A. glutinosa* is flowering in March and fruit development is finished late in November.

(B) expression of *ara135* in different organs of *A. thaliana:* 1, roots; 2, rosette leaves; 3, stem leaves; 4, stems; 5, flowers; 6, siliques I; 7, siliques II; 8, siliques III. Stages of silique development: I, wilted parts of the flower still attached to silique base, less than 6 mm in length; II, silique has not yet reached mature length and diameter; III, silique has reached mature length and diameter but desiccation has not started yet.

Northern blots containing about 10 µg of total RNA per slot were hybridized against *ag135* **(A)** or *ara135* **(B)**, respectively. Afterwards, the amount of mRNA on the filters was determined by a hybridization with a soybean ubiquitin (*ubi*) probe (Kouchi and Hata, 1993). Signal was determined by using a PhosphoImager (Molecular Dynamics, ImageQuant^{TM}). The diagrams show the expression levels of *ag135* and *ara135* as relative area units calculated from comparison with ubiquitin expression.

cortical cells (data not shown). *ag135* mRNA was found in the infected cortical cells and in the cells of the multilayered pericycle of the nodule vascular bundles (Figures 3C and 3D) which are located in the center of the nodule lobes. *ag135* was expressed in infected cells of zone 2, thus preceeding the onset of *nifH* expression, but the highest levels of *ag135* mRNA were found in cells of the first layers of zone 3 which are showing the highest level of *Frankia nifH* expression as well. *ag135* expression could not be detected in zone 4 (data not shown). No signal was obtained using sense RNA (data not shown).

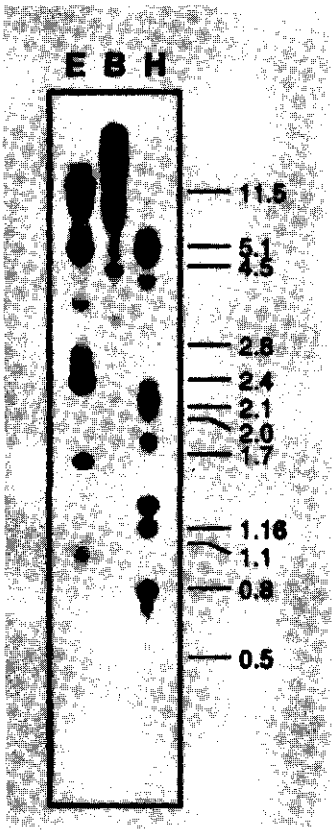


Figure 2: ***Ag135*** **is encoded by a small gene family.**

A Southern blot with 10 µg/slot genomic DNA of *A. glutinosa* digested with *Eco*RI (E), *Bam*HI (B) and *Hin*dIII (H) was hybridized against *ag135* (full size cDNA). The cDNA does not contain any *Eco*RI-, *Bam*HI- or *Hin*dIII sites.

Sequence of ag135 *and identification of homologues in* Arabidopsis thaliana

The DNA sequence of the full size cDNA clone pAg135 was determined and the encoded amino acid sequence was used for homology searches in the NCBI databases in order to identify homologues in *A. thaliana*. Ag135 showed homology to the protein product of the male sterility locus *ms2* in *A. thaliana* (Aarts *et al.*, 1993). Furthermore, a homologue of *ag135* was identified among the randomly sequenced *A. thaliana* cDNA clones (genbank accession T04771) which was designated *ara135*. The sequence of *ara135* was determined. Since nuclear male sterility can be the effect of several physiological disturbances (Chaudhury, 1993; Vedel *et al.*, 1994), the homology with Ms2 did not yield information on the possible biochemical function of the Ag135 protein. Therefore, analysis of the other *ag135*-homologue, *ara135*, was conducted. First, *ara135* was mapped on the *A. thaliana* genome. This should indicate whether it was transcribed from a locus characterized by a mutation which might help in determining its physiological function. Furthermore, the possible function of *ara135* was assessed by determining its *in situ* expression pattern.

RFLP mapping: ara135 *maps close to, but not at the* ms1 *locus*

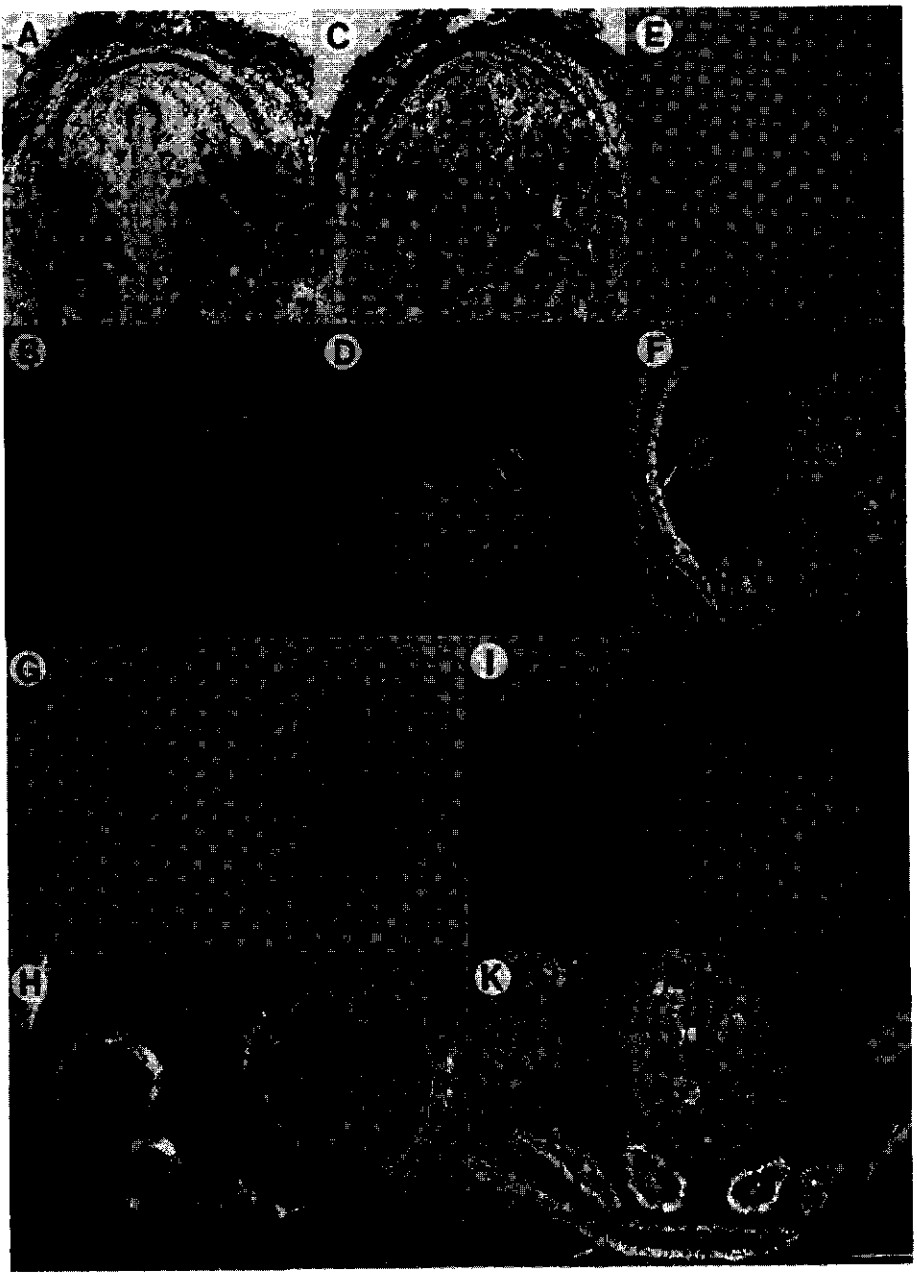
RFLP mapping was performed using the mapping population of recombinant inbred lines of Lister and Dean (1993). Digestion of *A. thaliana* ecotypes Landsberg *erecta* and Columbia DNA with *Cla*I yielded an RFLP marker for *ara135*. *ara135* was mapped on chromosome 5 between the markers g4560 and m291 (Lister and Dean, 1993). In this region, the mutations *pistillata* (Bowman *et al.*, 1991) and *ms1* (male sterility 1; Van der Veen and Wirtz, 1968;

Dawson *et al.*, 1993) are located in the integrated map of *A. thaliana* (Hauge *et al.*, 1993). Since Ara135 shows homology to the product of another male sterility locus, Ms2, it was essential to determine whether it is encoded by *ms1*. To analyze whether the *ms1* mutation represents a mutation in the promoter of ara135, expression levels of *ara135* were compared in flowers of *A. thaliana* ecotype Landsberg *erecta* and in *ms1* mutant flowers. No significant differences in expression levels could be found by Northern analysis (data not shown). To check for possible mutations in the coding region, *ara135* cDNA was amplified by RT-PCR from RNA isolated from wildtype and *ms1* mutant flowers, respectively (see Experimental Procedures). Sequencing of one subcloned PCR product each from mutant and wildtype revealed some point mutations, only one of which led to an amino acid change, namely of the conserved asparagine$_{119}$ (see Figure 4) to serine. A ca. 400 bp region including this point mutation was sequenced in different subcloned PCR products from wildtype (seven) and mutant (nine) plants (see Experimental Procedures). Since the mutation could not be refound, it had to be ascribed to a PCR error. Thus, since the expression level of *ara135* is not altered and its coding region is not mutated in the *ms1* mutant, it is very unlikely that *ara135* represents *ms1*. Thus, the RFLP mapping yielded no information on the function of *ara135*.

Expression of ara135 *in* A. thaliana

In the next attempt to get a clue to the function of *ara135* in *A. thaliana*, its expression pattern was analyzed. To find out whether *ara135* has a similar function in *A. thaliana* as *ag135* has in *A. glutinosa*, expression patterns in non-symbiotic development were compared. *ara135* expression was analyzed by Northern hybridization and found to take place in flowering inflorescences and, to a lower level, in rosette leaves (but not in caulinary leaves or stems) and immature siliques of *A. thaliana* (Figure 1B). During silique development, *ara135* expression decreases and then increases again. Like for *ag135*, expression of *ara135* in roots was not detectable (Figure 1B). Thus, expression patterns of *ara135* and *ag135* are similar: both genes seem to be expressed in flowers and to some extent in developing fruits and young leaves, but not in roots.

The expression pattern of *ara135* in *A. thaliana* flowers was analyzed by *in situ* hybridization of sections of inflorescences against ^{35}S labeled *ara135* antisense RNA (Figures 3E to 3K). *ara135* mRNA could be localized in the epidermis of petal primordia beginning from phase3 of flower development as defined by Smyth *et al.* (1990; Figures 3G and 3H). In later stages of development, *ara135* mRNA was present in several parts of the flower. In the epidermis of the sepals *ara135* expression was gradually reduced during development from phase 6 to 13 (Figures 3G and 3H; Smyth *et al.*, 1990). *ara135* mRNA was also found at and near the flower base, as well as in the epidermis of the petals (Figures 3E and 3F) and in the epidermis of the filaments (Figures 3I and 3K). Furthermore, *ara135* expression was found


A
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in the epidermis of the ovules (Figures 3E and 3F, 3I and 3K). Thus, *ara135* expression was specific to the epidermis of some, but not all floral organs, and to the epidermis of ovules.

Ag135/Ara135 show homology with fatty acyl reductase from jojoba seeds

Ag135, Ara135 and Ms2 show high homology to a NADPH dependent seed fatty acyl reductase (FAR) from jojoba (*Simmondsia chinensis*) which catalyzes the reduction of long chain fatty acyl-CoA substrates to fatty alcohols (Metz *et al.*, 1994; Figure 4). The amino acid homologies between Ag135 and the fatty acyl reductase (74.4% amino acid similarity) on the one hand and between Ag135 and Ara135 (73.4%) on the other hand are in the same range, significantly higher than the homologies between Ms2 versus Ag135, Ara135 and the jojoba enzyme (63.5 - 65.8% amino acid similarity), respectively.

←

Figure 3: Localization of *ag135* and *ara135* mRNA

(A,C,E,G,I) represent bright field micrographs, i.e. silver grains denoting hybridization are visible as black dots, **(B,D)** represent dark field/epipolarization micrographs in which silver grains are visible as yellow dots. **(F,H,K)** represent dark field micrographs in which silver grains are visible as white dots. The bar denotes 500 µm.

(A/B) Expression of *Frankia nifH* in a longitudinal section of a root nodule lobe of *A. glutinosa*.

(C/D) Expression of *ag135* in an adjacent section of the same nodule lobe. The pericycle (P) of the central vascular bundle is indicated. Arrowheads point at infected cells not yet completely filled with hyphae, i.e. not containing vesicles and not yet fixing nitrogen in **(A-D)**.

(E/F) Expression of *ara135* in a flower bud (cross section) of *A. thaliana* ecotype Landsberg *erecta*. *Ara135* mRNA is localized in the epidermis of the petal (thin arrow) and in the epidermis of the ovules (thick arrow).

(G/H) Expression of *ara135* in an inflorescence (longitudinal section). Expression becomes confined to the epidermis of the sepals from stage 3 (long arrows) to stage 13 (short arrows; Smyth *et al.*, 1990).

(I/J) Cross section of a flower bud near the base: *ara135* expression is found in the epidermis of sepals (thin arrow) and filaments (arrowheads) and in the epidermis of the ovules (thick arrows).

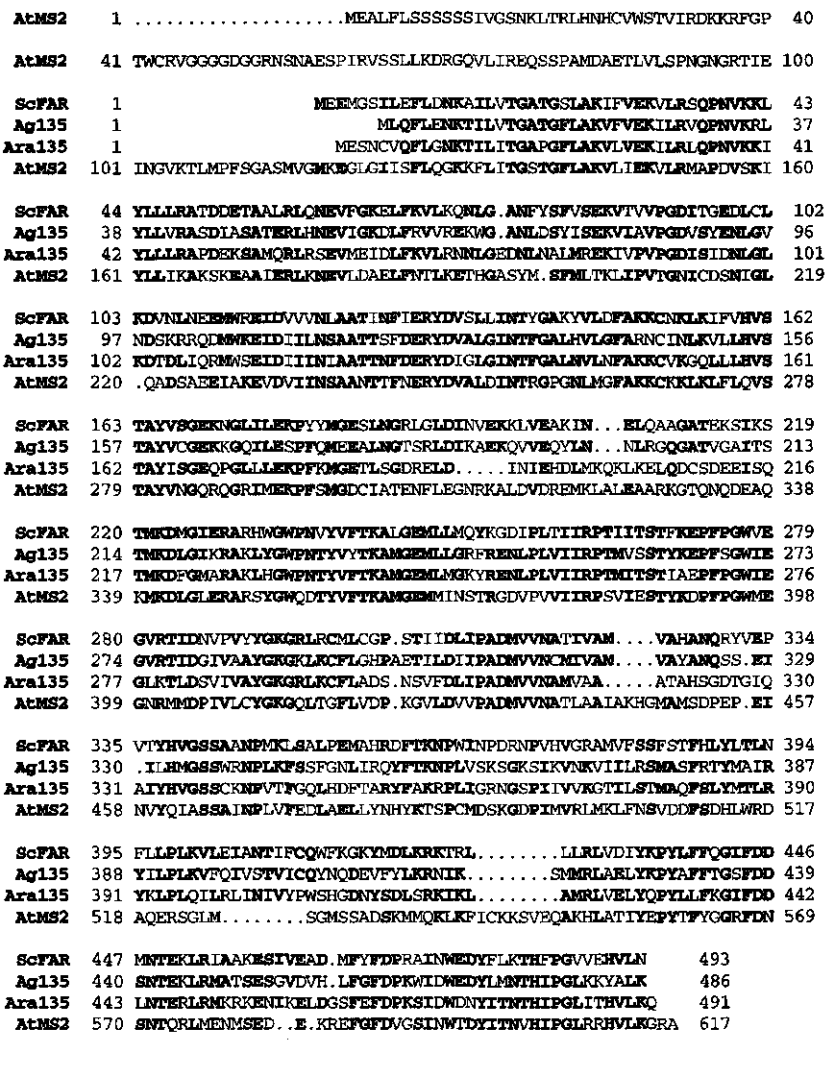

Figure 4: Amino acid sequence comparison between jojoba fatty acyl reductase (ScFAR), Ag135, Ara135 and Ms2.

Residues conserved in at least two sequences are given in bold print. The amino acid sequence of AtMs2 is based on the DNA sequence of the genomic clone. Based on the homology, we propose that translation actually starts at M_{118}.

Discussion

Ag135/Ara135: cuticular wax biosynthesis via the reductive pathway from jojoba seeds

We showed that the *A. glutinosa* gene *ag135* was expressed specifically in the infected cortical cells and in the pericycle of vascular bundles of actinorhizal nodules. Ag135 displays high homology to the products of two *Arabidopsis* genes, the male sterily gene *ms2* (Aarts *et al.*, 1993) and the gene corresponding to a randomly sequenced cDNA termed *ara135* which maps close to, but not at the *ms1* locus. Ag135, Ara135 and Ms2 are homologues of the fatty acyl reductase involved in wax biosynthesis in jojoba seeds. In jojoba, the fatty alcohols in turn are esterified with fatty acids to produce waxes which form the seed energy reserve ("liquid wax"; Pollard *et al.*, 1979). In *Arabidopsis* flowers, *ara135* is expressed specifically in the epidermis of some floral organs and of ovules. Since these cells produce cuticular waxes, this expression pattern is consistent with a role of Ara135 in the synthesis of such waxes or their precursors. Up to now it was generally assumed that cuticular wax esters are exclusively produced by the reduction of fatty acyl-CoA to the corresponding aldehyde which in turn is reduced to a primary alcohol to be esterified with a fatty acid (Kolattukudy, 1971; Kolattukudy, 1980; Von Wettstein-Knowles, 1995). This is to our knowledge the first report suggesting that the jojoba pathway of single-step fatty acyl reduction (Pollard *et al.*, 1979) also is involved in cuticular wax biosynthesis.

The plant cuticle consists of cutin, an insoluble hydroxy fatty acid polymer (Holloway, 1982), associated with so-called intracuticular waxes which include long chain fatty acids esterified with alcohols, and free alcohols. The cuticle is covered by the epicuticular waxes, comprising very long chain non-polar lipids. Since we have no evidence on whether the fatty acyl transferase from the jojoba liquid wax biosynthetic pathway is also present in cells expressing *ara135*, it is not clear whether the fatty alcohols produced by Ara135 are in turn esterified to form wax esters, or are forming a part of the intracuticular lipids as free alcohols.

The expression of *ara135* is confined to some floral organs, ovules, and to young rosette leaves, while expression in caulinary leaves could not be detected (Figure 1B). These data suggest that the expression of the jojoba pathway of fatty acyl reduction is under strict developmental control, and even very related organs might have cuticles with a different composition. This is consistent with the observation that in maize, marked differences in wax composition of young versus mature leaves have been found (Bianchi *et al.*, 1984). Homologues of *ara135* have also been found amongst randomly sequenced maize cDNAs from a library made from etiolated seedlings ten days after germination (genbank accessions T18413 and T15324; data not shown), implying that the jojoba pathway also might take place in cuticular wax biosynthesis in monocotyledonous plants.

Mutation of another *A. thaliana* gene encoding homologue of the jojoba fatty acyl reductase, *ms2* leads to a surprising phenotype, male sterility by pollen abortion shortly after release from the tetrads (Aarts *et al.*, 1993). It is probable that the loss of fatty acyl reductase function can cause alterations of the pollen wall, leading to pollen abortion. This would imply that *ms2* is expressed in the pollen or in the tapetum. However, at present it is not known in which cells *ms2* expression is taking place.

Wax biosynthesis in actinorhizal nodules?

Ag135 and Ara135 have a similar degree of homology with the jojoba fatty acyl reductase (see Results). Therefore we expect that also Ag135 is a fatty acyl reductase. *ag135* is expressed in nodules, flowers and shoot tips. In the latter organs, cuticular wax biosynthesis has to take place. However, it is remarkable that *ag135* is expressed in infected cells and the pericyle of the nodule vascular bundles (Figures 3C and 3D). Since it is very unlikely that such cells produce cuticular waxes or long chain fatty alcohols, it is possible that waxes form part of secondary cell wall modifications also in non-surface cells. This is indicated by the occurrence of LTPs implicated to be involved specifically in the synthesis of the extracellular cuticular matrix also in for example phloem parenchymatic cells (Pyee *et al.*, 1994) or stem cortical cells (Thoma *et al.*, 1994). Furthermore, secondary cell wall modifications of non-surface cells can include suberization, and suberin contains long chain fatty alcohols (Kolattukudy, 1980; Von Wettstein-Knowles, 1995). Detailed studies of secondary cell wall modifications in *A. glutinosa* nodules are needed to understand the function of fatty acyl reductase in non-surface cells.

Up to now, several genes whose products are involved in nodule carbon and nitrogen metabolism have been found to be expressed in the infected cells as well as in the specialized pericycle of the nodule vascular bundles of *A. glutinosa*, e.g. glutamine synthetase (chapter 5) and sucrose synthase (Van Ghelue *et al.*, 1996). The fact that *ag135* is also expressed in these two tissues and moreover, at a relatively high level, seems to suggest a function of Ag135 in nodule metabolism, maybe not having the same substrate specificity as the jojoba enzyme which was shown to have a preference for C24:1-CoA as a substrate *in vitro* (Metz *et al.*, 1994).

Does Arabidopsis *facilitate studies on actinorhizal nodules?*

The accumulated information from the random cDNA sequencing project on *Arabidopsis* (Höfte *et al.*, 1993; Newman *et al.*, 1994) has enabled us to easily obtain an *Arabidopsis*

homologue of our *A. glutinosa* cDNA *ag135* and to study its expression pattern in a system whose development is well known. Furthermore, several other cDNAs from *A. glutinosa* representing genes expressed specifically or at elevated levels in nodules have turned out to have homologues amongst the random cDNA sequences *from A. thaliana* (chapter 6). This confirms our working hypothesis that most or all of those genes have their counterparts in non-symbiotic plant development (Nap and Bisseling, 1991). Moreover, it shows that at the present stage of the random *Arabidopsis* cDNA sequencing project, there is a high probability for finding *Arabidopsis* homologues for any plant cDNAs, at least for those representing genes expressed at high levels. The availability of quick mapping procedures and an integrated genetic map for *Arabidopsis* (Hauge *et al.*, 1993; Lister and Dean, 1993) allowed to determine the position of *ara135* on the *Arabidopsis* genome by which we could check whether this gene had already been mutated. For *ara135*, this was not the case. However, it can be expected that when the map has become more saturated with mutant loci in the near future, it will be very easy to assign mutant phenotypes to cDNAs. Thus, the comparative analysis of homologous cDNAs from the non-actinorhizal plant *Arabidopsis* can contribute to the understanding of the function of actinorhiza-specific cDNAs.

Experimental Procedures

Plant and bacterial growth conditions

Alnus glutinosa seeds were collected from a local source (Weerribben, The Netherlands). The plants were grown in a greenhouse at 25°C under 16 hrs light, 8 hrs dark. Seeds were germinated in trays containing sterile gravel wetted with sterile tap water. After three weeks, the seedlings were transferred to sterile gravel wetted with 1/4 strength Hoagland solution (Hoagland and Arnon, 1938) and each plantlet was infected with 1 ml of a 1:5 diluted dispersed culture of *Frankia* HFPArI3 (Berry and Torrey, 1979) grown in P medium without nitrogen (Meesters *et al.*, 1985). Nodules were harvested 5-13 weeks after infection. Nodules for *in situ* hybridization were grown in a hydroponic tank with 1/4 strength Hoagland solution. Male and female flowers as well as developing fruits were collected from a local stand (Wageningen, The Netherlands); flowers were collected in March 1994, fruits in April, June and September 1994, respectively. Roots and cotyledons were harvested from non-inoculated seedlings two to three weeks after germination, i.e. before development of primary leaves. *Arabidopsis thaliana* ecotype Landsberg *erecta* was grown in a greenhouse in pot soil. Seeds were germinated on filter paper wetted with tap water for one day at 4°C and for two days at 21°C in the light before transfer to pot soil. *A. thaliana* roots were obtained

by germinating seeds and proceeding cultivation in liquid LS medium (Linsmaier and Skoog, 1965) with 2% sucrose on a shaking incubator at 21°C in the light.

Isolation of DNA and RNA

DNA was isolated from *A. glutinosa* leaves according to Dellaporta *et al.* (1983) with some modifications. DNA from *Frankia* HFPArI3 was isolated as described in Ribeiro *et al.* (1995). DNA was isolated from *A. thaliana* according to Tai and Tanksley (1990). Total RNA was isolated from *A. glutinosa* as described by Pawlowski *et al.* (1994). Poly(A) RNA was isolated from total RNA using dynabeads (Dynal, Oslo, Norway) according to the protocol provided by the manufacturer. Total RNA was isolated from *A. thaliana* as described by Pawlowski *et al.* (1994).

Construction and screening of a cDNA library

A cDNA library from poly(A) RNA of *A. glutinosa* nodules harvested 5-8 weeks after infection was custom-made by Stratagene (La Jolla, CA, USA) in λZapII. After *in vivo* excision, DNA was isolated from 224 randomly picked plasmid clones, digested with *Eco*RI and subjected to agarose gel electrophoresis and Southern blotting. Parallel Southern blots were hybridized against radiolabeled cDNA from roots and nodules of *A. glutinosa*, respectively. Complementary DNA was produced from 10 mg total RNA using reverse transcriptase (Amersham, Little Chalfont, UK) and amplified using a random primed DNA labeling protocol (Feinberg and Vogelstein, 1983) in the presence of α-^{32}P-dATP. After hybridization (Sambrook *et al.*, 1989), filters were washed at 65°C with decreasing salt concentrations down to 0.5xSSC, 0.1% SDS.

Cloning and sequencing procedures

DNA manipulations were carried out as described by Sambrook *et al.* (1989). Deletion clones were prepared using the kit from Promega (Madison, WI, USA). The nucleotide sequences were determined using the dideoxy chain termination method (Sanger *et al.*, 1977) using an automatic sequencer (Applied Biosystems, Foster City, CA, USA; model 373A). Sequence data were analyzed using the programs of the Wisconsin Genetics Computer Group (Devereux *et al.*, 1984). Database searches were performed using the BLAST algorithm (Altschul *et al.*, 1990) in the nucleotide sequence databases of the National Center of Biotechnology Information (NCBI), National Library of Medicine, NIH, in Bethesda, MD, USA.

Southern and Northern hybridization conditions

Total RNA was denatured in DMSO/glyoxal and electrophoresed on 1.2% agarose gels (Sambrook *et al.*, 1989). DNA was separated on 0.8% agarose gels (Sambrook *et al.*, 1989). Nucleic acids were transferred to GeneScreen^{TM} (New England Nuclear, Boston, MA, USA) filters (RNA) or to Amersham Hybond N$^+$ (Amersham, Little Chalfont, UK) filters (DNA). Northern hybridizations were performed in buffer containing 50% formamide at 42°C (Sambrook *et al.*, 1989). Southern hybridizations were performed according to the protocol provided by the manufacturer for Amersham Hybond N$^+$. Filters were washed at 65°C with decreasing salt concentrations down to 0.5xSSC, 0.1% SDS.

PCR

RT-PCR was performed as described by Horvath *et al.* (1993). For amplification of *ara135* cDNA from wildtype *A. thaliana* ecotype L. *erecta* and *ms1* mutant plants, oligonucleotides 5'-GGAATTCTTGTGTTTCTCTTAGGTT-3' and 5'-GGAATTCTTTTGATATTACA CGCCA-3' were used. PCR products were subcloned using the kit from Promega (Madison, WI, USA). For sequencing of the potentially mutated region of the PCR products, the synthetic primers 5'-AGAGAAAATACTAAGGTTGCA-3' and 5'-TCTCTAGTAACA ATCCAGGT-3' were used.

In situ *hybridization*

Tissues were fixed in 100 mM sodium phosphate pH 7.2 containing 4% paraformaldehyde and 0.25% glutaraldehyde for 4 hrs under vacuum, dehydrated via a graded ethanol series and embedded in para clean (Klinipath, Duiven, The Netherlands). Sections (7 µm thick) were dried on polylysine-coated slides at 42°C overnight, deparaffinized with xylene and rehydrated via a graded ethanol series. Hybridization pretretment, hybridization and washing were performed essentially as described by Cox and Goldberg (1988) and adapted by Van de Wiel *et al.* (1990).

For the *Frankia nifH* probe, pFnifH1 (Ribeiro *et al.*, 1995) was linearized with *Eco*RI and antisense RNA was transcribed using T7 RNA polymerase. For *A. glutinosa ag135* sense and antisense probes, the *Eco*RI/*Eco*RV fragment containing the first 440 bp of the original cDNA clone (position 143 to 565 in the full size sequence) was subcloned in pBluescript KS$^+$ yielding pAg135EE which was linearized with *Hin*dIII and transcribed with T7 RNA

polymerase (sense) or linearized with *Eco*RI and transcribed with T3 RNA polymerase (antisense), respectively. For *A. thaliana ara135* sense and antisense probes, a 495 bp *Hin*dIII fragment of the coding region of *ara135* was subcloned in pBluescript KS$^+$ yielding pAra135H which was linearized with *Sal*I and transcribed with T7 RNA polymerase (sense) or linearized with *Eco*RI and transcribed with T3 RNA polymerase (antisense), respectively.

After washing, the slides were coated with microautoradioography emulsion LM-1 (Amersham, Little Chalfont, UK) and exposed for 4 weeks at 4°C. They were developed in Kodak D19 developer for 5 min. and fixed in Kodak fix. Sections were counterstained with 0.02% ruthenium red and 0.025% toluidine blue 0 for 5 min. each and mounted with DPX (BDH Laboratory Supplies, Poole, UK).

RFLP mapping

The *A. thaliana* mapping population from Lister and Dean (1993) was used. The Southern hybridization data were analyzed using the JoinMap program (Stam, 1993).

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Chapter 4

ag13 is expressed in *Alnus glutinosa* nodules in infected cells during endosymbiont degradation and in the nodule pericycle

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ag13 is expressed in *Alnus glutinosa* nodules in infected cells during endosymbiont degradation and in the nodule pericycle

Changhui Guan, Antoon D. L. Akkermans, Ab van Kammen, Ton Bisseling and Katharina Pawlowski

C. Guan, A. van Kammen, T. Bisseling, and K. Pawlowski (corresponding author), Dept. of Molecular Biology, Agricultural Univ., Dreijenlaan 3, 6703 HA Wageningen, The Netherlands; A.D.L. Akkermans, Dept. of Microbiology, Agricultural Univ., Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands.

Key words - actinorhiza, *Alnus glutinosa*, *Frankia*, glutamic acid-rich protein, proline-rich protein, endosymbiont degradation.

We isolated and characterized an *Alnus glutinosa* cDNA clone, pAg13, which corresponds to a gene expressed at elevated levels in nodules induced by *Frankia* compared to roots. The deduced polypeptide sequence is rich in glutamic acid and proline and contains a putative signal peptide indicating an extracellular or vacuolar location of the mature protein. *In situ* hybridization showed that *ag13* is expressed in the pericycle of the nodule vascular bundle and in infected cells that exhibited degradation of the endosymbiont.

INTRODUCTION

Actinomycetous bacteria of the genus *Frankia* induce nitrogen-fixing root nodules on actinorhizal plants which comprise members of eight different dicotyledonous plant families, mostly trees or perennial woody shrubs (for reviews, see Simonet et al. 1990; Baker and Mullin 1992; Benson and Silvester 1993). Actinorhizal root nodules are coralloid structures consisting of multiple lobes. The nodule lobes represent modified lateral roots without root caps, with a superficial periderm, a central vascular cylinder and infected cells in the cortex (Benson and Silvester 1993). In all the actinorhizal symbioses except for those with *Casuarina* and *Allocasuarina*, bacterial nitrogen fixation takes place in *Frankia* vesicles. These are formed as terminal swellings on *Frankia* hyphae or short side branches in the infected cortical cells (Fontaine et al. 1984).

Due to the activity of the apical meristem, actinorhizal nodule lobes have an indeterminate growth pattern, and thus the nodule cells are aligned in a developmental gradient. Starting from the apical meristem, four different zones have been defined in a mature actinorhizal nodule lobe (Ribeiro et al. 1995). The meristematic zone (zone 1) consists of small dividing cells. The infection zone (zone 2) contains enlarging cortical cells, some of which are infected by *Frankia* hyphae and in turn become larger than uninfected cells (Lalonde 1979; Schwintzer et al. 1982; Berry and Sunell 1990). In the fixation zone (zone 3), vesicles have been formed, where the bacterial nitrogen fixation *(nif)* genes are expressed and nitrogenase is produced (Huss-Danell and Bergman 1990; Pawlowski et al. 1995). In the senescence zone (zone 4), bacterial *nif* gene expression is switched off, vesicles disappear and *Frankia* material is degraded in the infected cells, and eventually, infected cells become senescent (Schwintzer et al. 1982; Berry and Sunell 1990).

In nitrogen-fixing nodules induced by *Rhizobium* on legume roots, several nodule-specific genes such as *ENOD2, ENOD5, ENOD12,* and *MtPRP4* have been found to encode proline-rich proteins (Franssen et al. 1987; Scheres et al. 1990; Wilson et al. 1994). Their transcripts are found in special tissues of the nodule (Scheres et al. 1990; Van de Wiel et al. 1990; Wilson et al. 1994). Using polyclonal antibodies, proline-rich proteins have been localized in the infection thread matrix, in the nodule endodermis and in tissues of the vascular cylinder (Sherrier and VandenBosch 1994). Thus, expression of proline-rich cell wall protein genes during nodule development plays a prominent role in the morphogenesis and the infection process of *Rhizobium*-induced nodules. Expression of proline-rich protein genes can be modulated in response to elicitors (Sheng et al. 1991), and their products can become insolubilized in the cell wall as part of the defense response against fungal pathogens (Bradley et al. 1992; Brisson et al. 1994).

At present, no data are available on the function of proline-rich cell wall proteins in actinorhizal nodule development. Here we report the characterization of an *Alnus glutinosa* cDNA clone, pAg13, encoding a glutamic acid- and proline-rich polypeptide present at high levels in actinorhizal root nodules.

MATERIALS AND METHODS

Plant and bacterial growth conditions

Alnus glutinosa seeds were collected from a local source (Weerribben, The Netherlands). Growth conditions and inoculation with *Frankia* strain HFPArI3 (Berry and Torrey 1979) have been described elsewhere (Ribeiro et al. 1995). Nodules were harvested about 10 weeks after infection. For isolation of root RNA, seedling roots were collected from uninfected plantlets 2-3 weeks after germination. For isolation of shoot tip RNA or genomic DNA, shoot tips including the two youngest unfolded leaves were collected from plants 5-13 weeks after infection. Male and female flowers as well as developing fruits were collected from a local stand (Wageningen, The Netherlands). Flowers were collected in March 1994, fruits in April, June, and September 1994, respectively.

Arabidopsis thaliana ecotype Landsberg *erecta* plants were grown in a greenhouse. Seeds were germinated on filter paper wetted with sterile distilled water for one day at 4°C and subsequently for two days at 25°C in the light before being transfered to pot soil.

Isolation of DNA and RNA

Nucleic acids were isolated from *A. glutinosa* and *A. thaliana* as described by Ribeiro et al. (1995).

Construction and screening of a cDNA library

The construction and screening of the cDNA library have been described elsewhere (Ribeiro et al. 1995). For isolation of full size cDNA clones of *ag13*, $4x10^4$ plaques from the amplified library were plated and probed with a 350bp *Eco*RI/*Sty*I fragment from pAg13.

Sequencing procedures and data analysis

DNA manipulations were carried out as described by Sambrook et al. (1989). The nucleotide sequences were determined using the dideoxy chain termination method (Sanger et al. 1977). Sequence data were analyzed using the programs of the Wisconsin Genetics Computer Group (GCG) (Devereux et al. 1984). Database searches were performed using the BLAST algorithm (Altschul et al. 1990) in the nucleotide sequence databases of the National Center of Biotechnology Information (NCBI), National Library of Medicine, NIH, in Bethesda, MD.

Southern and Northern hybridization conditions

Southern and Norhtern hybridizations were carried out as described by Ribeiro et al. (1995). Hybridization signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In situ hybridization

Fixation of plant materials and preparation of sections has been described elsewhere (Ribeiro et al. 1995). *In situ* hybridization was performed essentially as described by Cox and Goldberg (1988) and adapted by van de Wiel et al. (1990). For *in vitro* transcription of *Frankia nifH*, pnifH1 (Ribeiro et al. 1995) was linearized with *Eco*RI, and antisense RNA was transcribed using T7 RNA polymerase. For ag13, a subclone of a 350 bp *Eco*RI/*Sty*I fragment of 5' part of the cDNA in the *Sma*I site of Bluescript KS^+ was linearized with *Bam*HI and transcribed with T3 RNA polymerase to obtain antisense RNA, or linearized with *Hin*dIII and transcribed with T7 RNA polymerase to obtain sense RNA, respectively. The preparation of sucrose synthase sense and antisense RNA has been described in Van Ghelue et al. (1996).

RESULTS

Isolation and characterization of *ag13* cDNAs

Previously we isolated 12 cDNA clones from an *Alnus glutinosa* nodule cDNA library, representing genes expressed at elevated levels in actinorhizal nodules (Ribeiro et al. 1995). Sequencing of the ends of the selected clones revealed that one cDNA clone, pAg13, encoded a proline-rich polypeptide. This clone was selected for further analysis. Using a subfragment of the coding region of pAg13, the cDNA library was screened for full-length clones, yielding eight different clones. Their ends were sequenced and the longest cDNA was selected for further analysis.

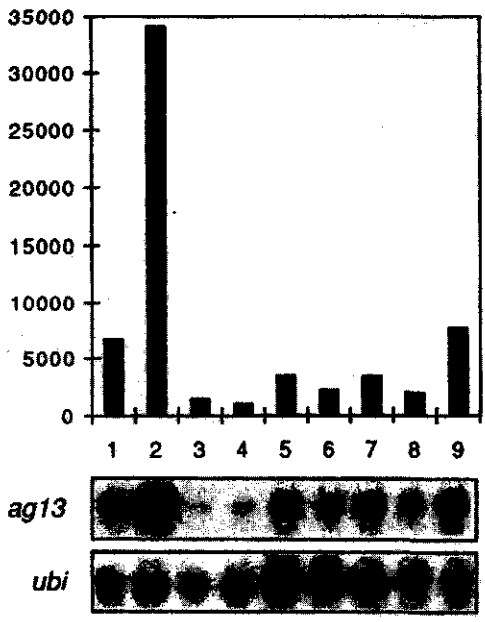

Figure 1: Expression of *ag13* in different organs of *Alnus glutinosa*

Bar 1, roots; bar 2, nodules; bar 3, cotyledons; bar 4, shoot tips; bar 5, male flowers; bar 6, female flowers; bar 7, developing fruits collected in April; bar 8, developing fruits collected in June; bar 9, developing fruits collected in September.

Northern blots containing about 10 µg total RNA per slot were hybridized with *ag13*. Afterwards, the amount of mRNA on the filters was determined by a hybridization with a soybean ubiquitin probe (Kouchi and Hata 1993). Signal was determined by using a Phosphoimager (Molecular Dynamics, ImageQuant™). Expression levels of *ag13* are shown as relative area units calculated from comparison with ubiquitin expression.

Northern hybridizations with RNA from different organs of *A. glutinosa* showed that the insert of pAg13 hybridized with an mRNA of about 1.0 kb present at high levels in nodules. At markedly lower levels, *ag13* was also expressed in roots, shoot tips, cotyledons, flowers and developing fruits (Fig. 1). Southern hybridization results suggested that Ag13 was encoded by a small gene family of at least two genes (Fig. 2), which was confirmed by comparing the sequences of 3' regions of the eight different full-length cDNAs (data not shown).

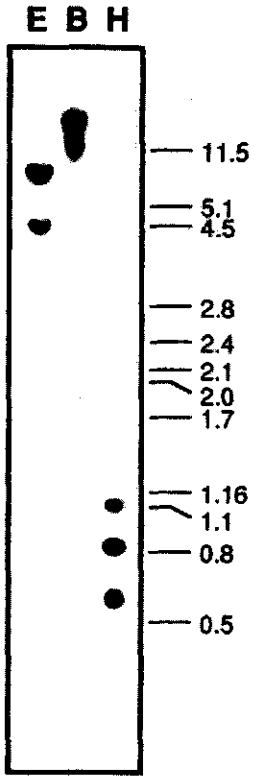

Figure 2: Ag13 seems to be encoded by a small gene family.

A Southern blot containing total DNA of *A. glutinosa* digested with *Eco*RI (E) and *Hin*dIII (H), respectively, was hybridized with the insert of pAg13. The sizes of the marker fragments are given. *Eco*RI and *Hin*dIII do not cut the cDNA.

Search for homologues of Ag13

The insert of pAg13 encoded a polypeptide consisting of 185 amino acids which contained a putative N-terminal signal peptide suggesting that Ag13 enters the endomembrane system (Von Heijne 1986; Fig. 3). The putative mature polypeptide was rich in glutamic acid (30%), proline (15%), valine (14%), and alanine (13%), and had an isoelectric point of 3.97. Three homologues were found in the NCBI databases (Fig. 3), encoded by a cDNA representing a gene expressed during fruit development of kiwifruit (Ledger and Gardner 1994; Kiwi501), by a partial cDNA clone from immature castor (*Ricinus communis*) fruits (Van de Loo et al. 1995; polypeptide termed Rc13), and by a partial *A. thaliana* cDNA clone from the random sequencing program (NCBI accession number 21371; polypeptide termed Ara21371). These polypeptides showed a striking similarity in amino acid composition (Tab. 1). The homology between Ag13 and Kiwi501 also covered the putative signal peptide sequences (Fig. 3). No typical repetitive motifs identified in other proline-rich polypeptides were found in Ag13 or its homologues, yet Ag13 contains a few repetitive sequences, e.g., two continuous repeats of nine amino acids (EEEAPKETP; see Fig. 3).

The *A. thaliana* homologue Ara21371 was further analysed by Northern hybridization with RNA from different tissues of *A. thaliana* and was found to be expressed in stems, flowers and siliques, but not in roots or leaves (data not shown).

Amino acid	Ag13	Kiwi501	Rc13*	Ara21371*
Ala	13	23	11	15
Glu	30	28	35	36
Lys	11	6	11	17
Pro	15	16	11	9
Thr	7	7	12	4
Val	14	9	11	15
Others	11	11	8	4

Table 1. Amino acid composition of plant glutamic acid-rich proteins. The putative mature amino acid sequences from Ag13 and Kiwi501 were used for evaluation. The partial sequences of Rc13 and Ara21371 were marked by stars. Amino acid frequencies were given in %

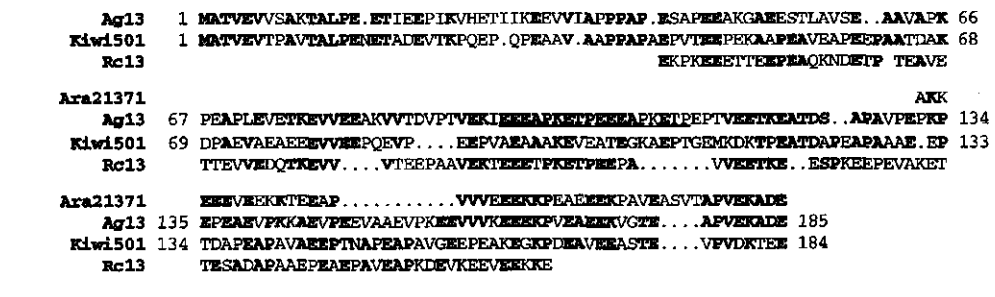

Figure 3: Amino acid sequence comparison between the Ag13, Kiwi501, Rc13 and Ara21371.
Identical amino acids are marked by bold print. Underlined parts in Ag13 stand for repetitive sequences (see text). The putative signal peptide cleavage site is marked by an arrow. In case of different conservations in the same column among the four sequences, identity is taken preferentially according to the Ag13 sequence.

Localization of *ag13* mRNA in root nodules of *A. glutinosa*

To localize the expression of *ag13* in *A. glutinosa* nodules, *in situ* hybridizations of longitudinal and cross sections of nodules with both sense and antisense RNA probes of *ag13* were carried out. The results are shown in Fig. 4. *ag13* mRNA was detected in the pericycle of the nodule vascular bundle in the younger part of the nodule and in older infected cells (Figs. 4A/4B). In higher magnification, it was visible that in cells expressing *ag13*, *Frankia* vesicles start to disappear (Fig. 4C). This implied that *ag13* was expressed in infected cells of zone 4, the senescence zone. A comparison with *Frankia nifH* expression was performed on adjacent sections to visualize the transitions from zone 2 to zone 3 and from zone 3 to zone 4. *ag13* expression was detected in infected cells that did not express *Frankia nifH* anymore (Figs. 4D/E). A comparison of the expression patterns of *ag13* and *A. glutinosa* sucrose synthase (Van Ghelue et al. 1996) on adjacent sections confirmed these results, showing that *ag13* expression took place in cells which had stopped to express sucrose synthase (Figs. 4F/G). Thus, *ag13* was expressed in infected cells of zone 4 and in the pericycle of the nodule vascular bundle in zone 2 and zone 3.

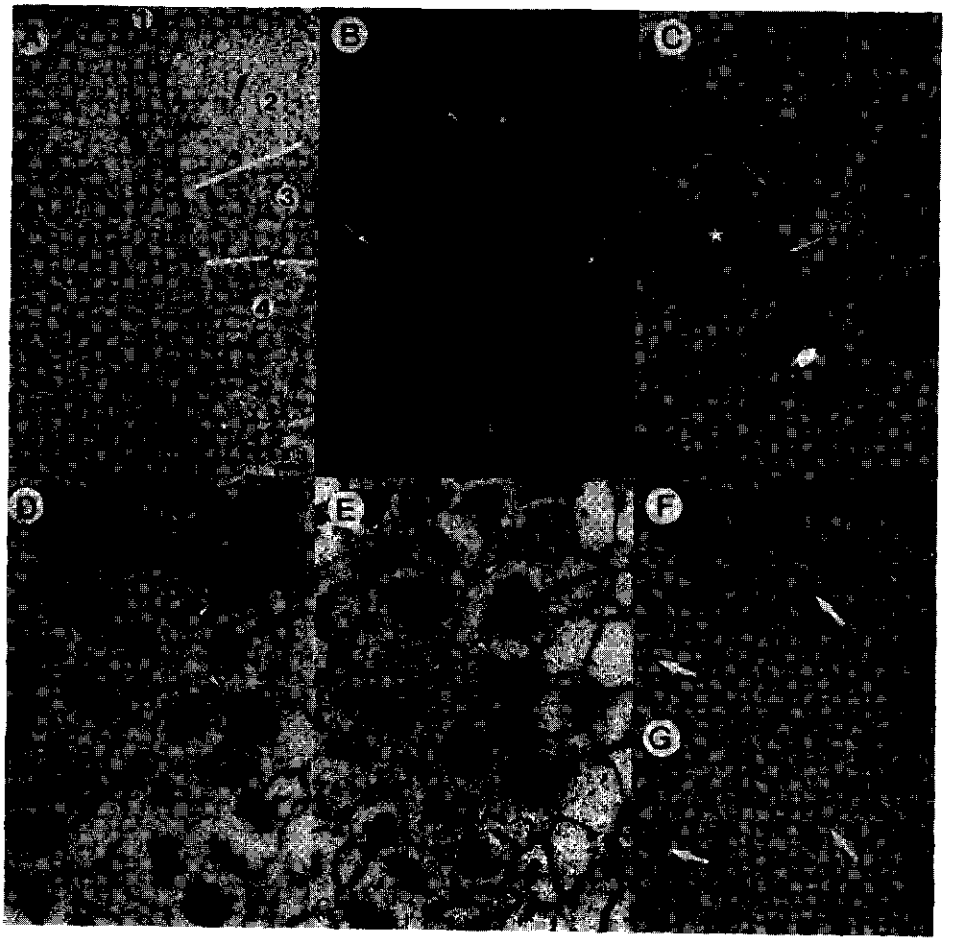

Figure 4: ***In situ*** **localization of** ***A. glutinosa ag13*** **mRNA in root nodules.**
In bright field micrographs **(A, C, D, E, F, G)** silver grains denoting hybridization are visible as black dots. In the dark field micrograph **(B)** photographed under epipolarized light, silver grains are visible as yellow-green dots. **(A/B)** Expression of *ag13* in a longitudinal section of a root nodule lobe of *A. glutinosa*. Hybridization is found in the younger part pericycle of the nodule vascular bundle (arrowheads) and in infected cells of the older part of the nodule lobe (arrows). The zonation of the nodule lobe is indicated. 1, meristematic zone; 2, infection zone; 3, fixation zone; 4, senescence zone. Bar = 250 µm. **(C)** Detail of infected cells: cells containing clearly visible *Frankia* vesicles do not express *ag13* (star), while those where vesicles are not visible in the whole cell anymore show *ag13* expression (thin arrows). In some cells, ag13 expression can be detected although some vesicles are still present (thick arrow). Bar = 100 µm. **(D/E)** Expression of *Frankia nifH* **(D)** in a longitudinal section of a nodule lobe, and of *A. glutinosa ag13* **(E)** in an adjacent section of the same nodule lobe. Arrowheads point at cells showing hybridization with *Frankia nifH* antisense RNA, but not with *ag13* antisense RNA. Arrows point at cells hybridizing with *ag13*, but not with *Frankia nifH* antisense RNA. Bar see **(C)**. **(F/G)** Expression of *A. glutinosa* sucrose synthase **(F)** in a longitudinal section of a nodule lobe, and of *ag13* **(G)** in an adjacent section of the same nodule lobe. Arrowheads point at cells showing hybridization with sucrose synthase antisense RNA, but not with *ag13* antisense RNA. Arrows point at cells hybridizing with *ag13*, but not with sucrose synthase antisense RNA. Bar = 250 µm.

DISCUSSION

We have shown that *ag13* encodes a glutamic acid- and proline-rich acidic polypeptide whose expression in the nodule is confined to the pericycle of the vascular cylinder and the infected cells of the senescence zone. While Ag13 contains a signal peptide for cotranslational insertion into the ER (von Heijne 1986), it does not contain a KDEL signal for retention in the ER (Andreas et al. 1990). Thus, the mature protein should be either localized in the vacuole or be secreted. While the determinants of vacuolar localization are diverse (Nakamura and Matsuoka 1993) and thus do not allow a decision for or against vacuolar targeting, it should be noted that up to now, no proline-rich protein has been localized in the plant vacuole. Thus, the fact that the putative mature protein is rich in proline (15%) and very hydrophilic, suggests that Ag13 is a structural protein forming part of the cell wall or the extracellular matrix. However, Ag13 does not show homology to any of group of well-defined plant cell wall proline-rich glycoproteins like e.g. extensins, gum arabic glycoproteins, and repetetive proline-rich proteins, since it does not contain any of the characteristic repetitive peptide motifs which characterize these protein classes (Showalter 1993; Kieliszewski and Lamport 1994). Although Ag13 resembles arabinogalactan proteins (AGPs) in being rich in proline and alanine and being acidic, in contrast to AGPs it is not rich in serine/threonine (Fincher et al. 1983). *ag13* shows quite a different expression pattern both temporally and spatially compared to proline-rich nodulin genes in legumes whose expression patterns are correlated with early events in nodule development (*ENOD2*, Franssen et al. 1987; *ENOD5* and *ENOD12*, Scheres et al. 1990; *MtPRP4*, Wilson et al. 1994). Furthermore, no proline-rich protein has been found to be expressed specifically in the pericycle thus far.

ag13 expression in infected cells is confined to the senescence zone where the expression of *Frankia nifH* has been turned off and *Frankia* vesicles are subjected to degradation. As has been shown before, *Frankia nifH* expression and plant sucrose synthase expression are switched off before *Frankia* vesicles start to disappear in the infected cells (Pawlowski et al. 1995; Van Ghelue et al. 1996). In legume as well as actinorhizal nodules, the loss of nitrogenase activity has been found to be the first symptom of senescence (Swaraj et al. 1993; Vikman et al. 1990). The loss of *Frankia nifH* expression has been used to mark the transition from the nitrogen fixation zone to the senescence zone (Pawlowski et al. 1995; Ribeiro et al. 1995). These data indicate that *ag13* expression might serve as a marker for the senescence zone and thus, for endosymbiont degradation in the nodule. In this context, the expression of *ag13* might be part of a defense response of *Alnus* against *Frankia*, as other proline-rich proteins are expressed during defense against pathogens (Corbin et al. 1987).

Electron microscopical studies have shown that during legume as well as actinorhizal nodule senescence, endosymbiont and plant cytoplasm seem to be degraded simultaneously (Vance et al. 1980; Schwintzer et al. 1982; Vikman et al. 1990; Bosabalidis and Tsaftaris 1993). Thus,

ag13 expression might actually be linked to plant cell senescence and not to endosymbiont degradation. This hypothesis would agree with the fact that *ag13* homologs are expressed during fruit development (Ledger and Garner 1994; Van de Loo et al. 1995), since senescence and fruit development show strong similarities (Vercher and Carbonell 1991; Smart 1994). However, this does not explain the expression of *ag13* in the younger part of the pericycle of the nodule vascular bundle, a tissue which has been implied to mediate the transport of carbon and nitrogen compounds between xylem/phloem and the cortical cells (Burgess and Peterson 1987a, b). Further studies will be needed to understand the function of acidic cell wall proteins in general and Ag13 in particular in relation to cell differentiation and function.

Ag13 and its homologues are characterized by their high content of glutamic acid, followed by proline and alanine (Tab. 1). In animals, several kinds of structural proteins have been found to be glutamic acid- and proline-rich proteins, for example a family of neurofilament proteins (Julien et al. 1986; Myers et al. 1987). In higher plants, to our knowledge there have been only two reports about glutamic acid-rich proteins, one of them about a wheat protein rich in proline, lysine, glutamic acid and methionine, not showing any similarity with Ag13 (Raines et al. 1991). The other reported plant glutamic acid-rich protein, Kiwi501 (Ledger and Garner 1994) together with Ag13, Rc13 and Ara21371 might represent a new family of acidic plant extracellular proteins, characterized by sequence similarity and amino acid composition with glutamic acid as the most frequent amino acid.

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Chapter 5

Gene expression in ineffective actinorhizal nodules of *Alnus glutinosa*

Changhui Guan, Diederick J. Wolters, Cornelis van Dijk, Antoon D. L. Akkermans, Ab van Kammen, Ton Bisseling and Katharina Pawlowski. Acta Botanica Gallica, in press.

Gene expression in ineffective actinorhizal nodules of *Alnus glutinosa*

Changhui Guan[1], Diederick J. Wolters[2], Cornelis van Dijk[2], Antoon D. L. Akkermans[3], Ab van Kammen[1], Ton Bisseling[1] and Katharina Pawlowski[1]

[1]Department of Molecular Biology, Agricultural University, 6703 HA Wageningen, The Netherlands
[2]Department of Plant-Microorganism Interactions, Netherlands Institute of Ecology, 6666 GA Heteren, The Netherlands
[3]Department of Microbiology, Agricultural University, 6703CT Wageningen, The Netherlands

Corresponding author:
Katharina Pawlowski
Department of Molecular Biology
Dreijenlaan 3
Agricultural University
6703 HA Wageningen
The Netherlands
Phone: +31-317-483278
Fax: +31-317-483584
E-mail: katharina.pawlowski@mac.mb.wau.nl

Summary. -

Several *Frankia* strains have been shown to induce ineffective, i.e. non-nitrogen fixing nodules, sometimes in a host-plant dependent manner. Previous studies have demonstrated that the resistance to nodulation of *Alnus glutinosa* by ineffective *Frankia* strains is genetically determined. In this study, ineffective nodules induced on susceptible *Alnus glutinosa* clones by soil suspensions from a local swamp were analysed cytologically. Comparisons with effective nodules showed that ineffective nodules contain higher amounts of polyphenols than effective nodules, indicating a plant defense reaction. Polyphenols were found even in the infected cortical cells. *In situ* hybridization with a *Frankia* antisense 16S rRNA probe showed that *Frankia* is degraded at an early stage of development of infected cells. The mRNAs of two plant genes, *ag12* and *ag13*, which had been found to be expressed in the infected cells of effective nodules, were localized in ineffective nodules. Their expression patterns seemed to be analogous in both types of nodules.

INTRODUCTION

Ineffective actinorhizal root nodules lack the ability to fix nitrogen. They have been observed on seedlings of different host species, induced by differenct sources of inoculum (Hahn *et al.*, 1988; Van Dijk *et al.*, 1988). In most cases, the induction of ineffective nodules depends on the host plant species (Van Dijk *et al.*, 1988; Bosco *et al.*, 1992). However, also presumably host-independently ineffective *Frankia* strains were isolated (Hahn *et al.*, 1988; van Dijk and Sluimer-Stolk, 1990) which seem to be phylogenetically related (Hahn *et al.*, 1989; D. Wolters, A.D.L. Akkermans and J. Woldendorp, unpublished observations).

Effective actinorhizal nodules consist of multiple lobes representing modified lateral roots without root caps, with a superficial periderm and infected cells in the expanded cortex. Due to the activity of the apical meristem, a developmental gradient of infected cells is formed in the cortex which can be divided into four zones. The meristematic zone (zone 1) consists of small dividing cells that are not infected by *Frankia*. The infection zone (zone 2) contains enlarging cortical cells some of which are infected by *Frankia* and are gradually filled with *Frankia* hyphae from the center outward (Lalonde, 1979; Schwintzer *et al.*, 1982; Berry and Sunell, 1990). In cells completely filled with hyphae, provesicles are formed as terminal swellings on hyphae (Fontaine *et al.*, 1984). In the course of the differentiation of provesicles into vesicles, bacterial nitrogen fixation (*nif*) gene expression is induced and nitrogenase is produced (Huss-Danell and Bergman, 1990; Pawlowski *et al.*, 1995). The onset of *nif* gene expression marks the transition from the infection zone to the fixation zone (zone 3). In the senescent zone (zone 4), *nif* gene expression is switched off and degradation of endosymbiont material and host cytoplasm is taking place (Berry and Sunell, 1990). The expression of plant genes along this developmental gradient has been analysed. *ag12*, encoding a nodule-specific serine protease, was found to be expressed at highest levels in infected cells of the infection zone (zone 2; Ribeiro *et al.*, 1995). *ag13*, encoding a nodule-enhanced putative cell wall protein, was expressed in the pericycle of the central vascular bundle of nodule lobes and in infected cells of the senescence zone (zone 4; Guan *et al.*, 1996).

Since in ineffective nodules, no nitrogen fixation takes place, the plant does not have to provide the structures for efficient nitrogen assimilation as well as nitrogen and carbon transport. In order to find out whether the expression patterns of genes whose products are not directly involved in actinorhizal nodule metabolism, are influenced by the absence of nitrogen fixation, we studied the expression of *ag12* and *ag13* in ineffective nodules of *A. glutinosa*. The viability of the endosymbiont in infected cells of these nodules was examined by *in situ* hybridization with a *Frankia* 16S rRNA antisense probe.

MATERIALS AND METHODS

Plant growth conditions

Stem cuttings of *A. glutinosa* were used to obtain ineffective nodules because they were found to be susceptible to nodulation by ineffective *Frankia*. Stem cuttings of 2-4 cm were surface sterilized by wiping with a tissue with 70% ethanol, and put on perlite with demineralized water. The perlite containers were covered with perspex plates and placed in climate-chamber at 23°C, with an illumination of 16 hrs and a relative humidity of 70%. After roots emerged, plants were transfered to half strength modified Hoagland solution (Quispel, 1954). Trace elements were added according to Allen and Arnon (1955), and Fe-citrate (0.02 mM) was used as an iron source. The soil sample used for inoculation was collected at a the swamp-area Boezem van Brakel (Dutch State Survey Grid 0132.6 424.0) where high amounts of ineffective *Frankia* and low effective/ineffective nodulation ratio had been found (Wolters *et al.*, 1996). Plants with a sufficiently large root system (at least 10 cm of roots) were transfered to full strength Hoagland solution with reduced N-content, with addition of 80 ppm of Previcur N (Schering Aagrunol B.V., The Netherlands), according to Van Dijk and Sluimer (1994). This fungicide treatment was continued after the inoculation of the plants until the end of the experiment. Inoculation was carried out after one week, with freshly prepared soil-inocula. These were added to the plant root system at the equivalent of 20 g of soil per liter of Hoagland solution. Nodules were harvested about eight weeks after inoculation.

Cytology and *in situ* hybridization

Fixation and embedding of nodules have been described by Ribeiro *et al.* (1995). *In situ* hybridization was performed as described by Cox and Goldberg (1988) and adapted by Van de Wiel *et al.* (1990). For photography, sections were stained with 0.02% Ruthenium Red for 5 min. and with 0.025% Toluidine Blue for 2 min., dehydrated via a graded ethanol series and mounted with DePeX (BDH Laboratories, Poole, UK).

The preparation of sense and antisense probes of *ag12* and *ag13* has been described by Ribeiro *et al.* (1995) and Guan *et al.* (1996), respectively. For the *Frankia* 16S rRNA antisense probe, rDNA was amplified from ineffective *A. glutinosa* nodules using primer 5'-CACGGATCCAGAGTTTGAT(C/T)(A/C)TGGCTCAG-3' for the conserved eubacterial sequence, and primer 1115*Hin*dIII for the sequence specific for ineffective strains (Embley *et al.*, 1988; D. Wolters, A.D.L. Akkermans and J. Woldendorp, unpublished observations). Polymerase chain reaction (PCR) conditions were 3 min at 95°C, then 35 cycles of 1 min at 93°C, 2 min at 48°C, 3 min at 72°C, 5 min at 72°C, then 5 min at 72°C. An *Bam*HI-*Eco*RI subfragment (corresponding to *E. coli* rRNA positions 7 to 674) of the PCR product was subcloned into Bluescript KS^+ (Stratagene, La Jolla, CA, USA). For *in*

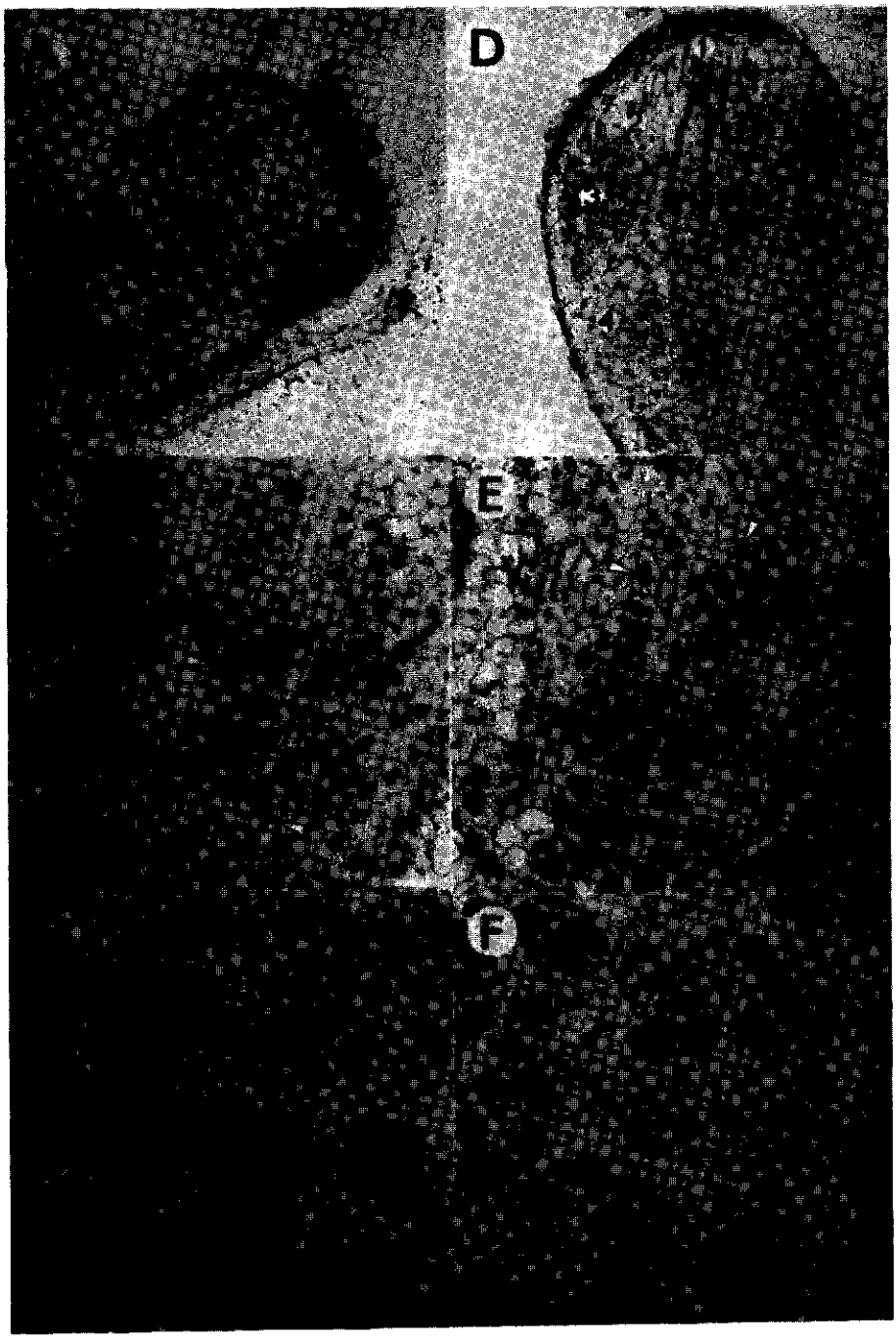
vitro transcription, the resulting plasmid was linearized with *Bam*HI, and antisense RNA was produced using T3 RNA polymerase.

RESULTS AND DISCUSSION

Ineffective nodules of *Alnus glutinosa* are characterized by slow growth and an almost complete deficiency of *Frankia* vesicle development, i.e. the absence of mature vesicles (Van Dijk and Sluimer-Stolk, 1990). We have analysed ineffective actinorhizal nodules induced by soil suspensions from a local source on susceptible *A. glutinosa* plants (see Materials and Methods). The soil suspensions led to the induction of ineffective as well as effective nodules, which were harvested eight weeks after inoculation and embedded in paraffin. The symbiotic efficiency of the nodules was investigated by microscopical examination for the presence of nitrogen-fixing vesicles in infected cortical cells in nodule sections. The structures of effective and ineffective nodules were compared (Figures 1A and 1D). In ineffective nodules, development of lobes stops very soon, leading to a very compact structure (Figure 1A). Ineffective nodules contain higher amounts of polyphenols than effective nodules (Figures 1A and 1D), and often, infected cells of ineffective nodules contain polyphenols (Figures 1B and 1C), a phenomenon rarely observed in infected cells of effective nodules.

The zonation of the cortex in the lobes of effective nodules is based on nitrogen fixation by *Frankia* in infected cells of the fixation zone (Ribeiro et al. 1995) and therefore not applicable to ineffective nodules. However, infected cells are filled with *Frankia* hyphae from the center outwards in ineffective as well as in effective nodules (Figures 1B and 1E). Thus, it is possible to distinguish between a zone 2 (infection zone) equivalent in ineffective nodules, containing infected cells partially filled with *Frankia* hyphae, and a zone 3 containing infected cells filled completely with hyphae. A difficulty arises when the senescence zone (zone 4) equivalent has to be defined. Using paraffin sections and light microscopy, it is not possible to distinguish between cells containing viable *Frankia* hyphae (Figure 1B) and cells displaying senescence (Figure 1F). Therefore, sections of ineffective nodules were hybridized with a *Frankia* 16S rRNA probe to distinguish between cortical cells with rRNA containing viable *Frankia* hyphae and those with collapsed hyphae. The results are shown in Figure 2A. Only at the tip of the nodule lobe, all infected cells showed hybridization with the *Frankia* rRNA probe. Below that area, non-hybridizing and hybridizing cells were found. Thus, endosymbiont senescence in ineffective nodules seems to start shortly after the cortical cells have been filled with hyphae material, but not in an uniform manner. This variability of reaction of the plant against non-nitrogen fixing *Frankia* is also visible in the differences in polyphenol content of infected cells (see above).

Expression of *ag12* and *ag13* was localized in ineffective *A. glutinosa* nodules by *in situ* hybridization with antisense RNA probes. *ag12* expression was found to be restricted to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a few infected cells in an early developmental stage, some of them incompletely and others completely filled with *Frankia* hyphae (Figures 2D and 2E). This pattern could be equivalent to the high levels of *ag12* expression in the infection zone of effective nodules. In effective nodules, *ag13* has been found to be expressed in the pericycle of the central vascular bundles of the nodule lobes, and in the infected cells of zone 4. In the pericycle of the nodule vascular bundles of the ineffective nodules, *ag13* expression was below the detection level (Figure 2F and 2G). However, *ag13* expression could be detected in several infected cells of ineffective nodules (Figures 2F and 2G). Developmentally, these cells were older than those expressing *ag12*, but a clear correlation between endosymbiont degradation and *ag13* expression could not be established (Figures 2A, 2B and 2C). Although in cells expressing *ag13*, no *Frankia* rRNA hybridization could be detected, a considerable amount of infected cells did not hybridize with either probe. No signal was found in hybridizations with sense RNA probes (data not shown). Altogether, the results of localization of *ag12* and *ag13* expression imply that both genes have analogous expression patterns in effective and ineffective *Alnus* nodules, namely in each case depending on the developmental stage of the infected cortical cells. Both Ag12 and Ag13 have been hypothesized to be localized in the cell wall-like matrix surrounding *Frankia* in the infected cells, Ag13 as part of the matrix and Ag12 being involved in the processing of a matrix component (Ribeiro *et al.*, 1995; Guan *et al.*, 1996). Thus, the structure of the material surrounding intracellular *Frankia* seems to be

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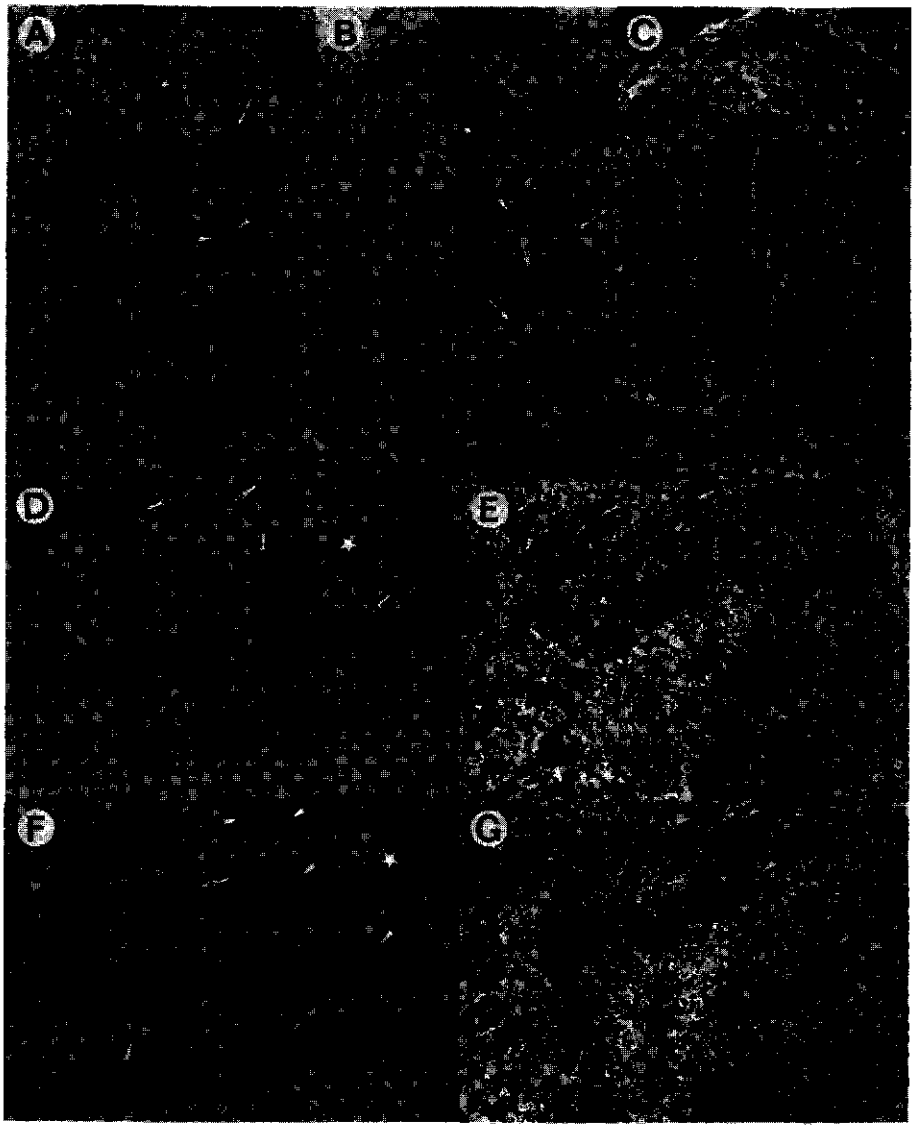
Figure 1. Cytological comparison between ineffective and effective nodules of *A. glutinosa*.
Nodules were harvested eight weeks after inoculation, fixed and embedded in paraffin. 7 mm thick sections were put on slides, deparaffinized and stained with Ruthenium Red and Toluidine Blue. Magnifications are the same in **A,D** and in **B,C,E,F**, respectively. The shift from zone 2 to zone 3 and from zone 3 to zone 4 in effective nodules has been defined by *Frankia nifH* expression in the infected cells, therefore it can here only be approximated based on the presence of *Frankia* vesicles. *Frankia nifH* expression starts some time after vesicles are visible and is switched off some time before vesicles disappear (Pawlowski *et al.*, 1995).
(A) Section of an ineffective nodule. The area magnified in **(B)** is indicated. V, vascular bundle; P, periderm. Bar = 500 µm.
(B) Detail of **(A)**: no vesicles are visible in the infected cells (arrows). Several infected cells contain dark-staining polyphenols (arrowheads). Bar = 125 µm.
(C) Detail of another ineffective nodule: high amounts of polyphenols are present. Bar see **(B)**.
(D) Longitudinal section of a lobe of an effective nodule. Based on the developmental stage of the infected cortical cells, four zones can be distinguished: 1, the meristem at the tip of the nodule lobe; 2, the infection zone containing cells which are being filled with *Frankia* hyphae from the center outward; 3, the fixation zone with nitrogen-fixing cells containing fully differentiated *Frankia* vesicles; and 4, the senescence zone where *Frankia* material and plant cytoplasm are degraded. The areas magnified in **(E)** and **(F)** are indicated by white boxes. Bar see **(A)**.
(E) Detail of **(D)**: vesicles can be seen in the infected cells of zone 3 (arrows), but not yet in the infected cells of zone 2 (arrowheads). Bar see **(B)**.
(F) Detail of **(D)**: in the infected cortical cells of zone 4 (arrows), *Frankia* vesicles have disappeared. Bar see **(B)**.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similar in effective and ineffective symbioses. However, further studies are needed to analyse the structure of this interface and its function in the symbiosis.

A comparison of *ag12* and *ag13* expression levels in effective versus ineffective nodules was not attempted since it cannot be conclusive, regarding the differences in growth and metabolic activity between both types of nodules. However, the relation of expression levels of both genes seems to differ in both types of nodules. In effective nodules, using both RNA gel blot and *in situ* hybridization techniques, *ag12* expression levels have always been found to be higher than *ag13* expression levels (data not shown), while in two independent *in situ* hybridizations of ineffective nodules, *ag13* expression levels were significantly higher than those of *ag12*. In view of the fact that in effective nodules, *ag12* expression is related to early and *ag13* expression to late developmental stages of infected cells (Ribeiro *et al.*, 1995; Guan *et al.*, 1996), this is consistent with the early degradation of the endosymbiont in infected cells in ineffective nodules, as indicated by the *Frankia* rRNA hybridization results.

While ineffective nodules have been described for several actinorhizal symbioses, it is not clear which bacterial and plant functions are determining symbiotic efficiency. Since in some cases, the same *Frankia* strain has been found to form effective or ineffective symbioses depending on the host plant (Van Dijk *et al.*, 1988; Bosco *et al.*, 1992),

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Figure 2: Gene expression in ineffective nodules of *A. glutinosa*.

A,B,D,F represent bright field micrographs; silver grains denoting hybridization are visible as black dots. **C,E,G** represent dark field photographs; silver grains denoting hybridization are visible as white dots. Polyphenols show white fluorescence. Bar in **(A)** = 500 µm.

(A) Hybridization of the section of an ineffective nodule with a *Frankia* antisense 16S rRNA probe. The nodule lobe meristem is marked by a little white star. At the tip of the nodule lobe, all infected cells show hybridization (arrows). Below, infected cells containing viable bacteria, i.e. showing hybridization are intermingled with non-hybridizing cells. Arrowheads point at two adjacent cells, one hybridizing with the *Frankia* probe, the other one not.

(B/C) An adjacent section of the same nodule lobe as in **(A)** was hybridized with an *A. glutinosa ag13* antisense RNA probe. The nodule lobe meristem is marked by a little white star in **(B)**. Arrows point at infected cells showing hybridization. White-and-black arrowheads point at infected cells containing polyphenols, but not hybridizing with *ag13*; the white fluorescence of the polyphenols can be mistaken for hybridization but the view under epipolarized light shows that no silver grains have accumulated in these cells (data not shown). A white arrowhead points at a cluster of infected cells hybridizing with *Frankia* 16S rRNA in **(A)**, but showing no hybridization with *ag13*.

(D/E) Oblique section of an ineffective nodule hybridized with an *A. glutinosa ag12* antisense RNA probe. A white star marks the approximate position of the nodule lobe meristem in **(D)**. Arrows point at infected cells showing hybridization with *ag12*.

(F/G) An adjacent section from the same nodule as in **(D/E)** was hybrized with an *A. glutinosa ag13* antisense RNA probe. The approximate position of the nodule lobe meristem in **(F)** is marked by a white star. Arrowheads point at infected cells hybridizing with *ag12* in **(D/E)**, but showing no hybridization with *ag13*. Arrows point at infected cells hybridizing with *ag13*.

symbiotic efficiency cannot be solely dependent on the ability of the endosymbiont to fix nitrogen, but must have something to do with the signal exchange between both symbiotic partners. The increased polyphenol content of ineffective nodules (Figure 1) points at a plant defense reaction against *Frankia,* but it is not clear whether this is the reason for or a consequence of symbiotic inefficiency.

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Chapter 6 General discussion

Current achievements in plant molecular studies of actinorhizal nodules of *Alnus glutinosa*

Changhui Guan, Katharina Pawlowski and Ton Bisseling. In preparation.

Current achievements in plant molecular studies of actinorhizal nodules of *Alnus glutinosa*

Changhui Guan, Katharina Pawlowski and Ton Bisseling

Department of Molecular Biology, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

For a long time, studies on the molecular aspects of actinorhizal nodule formation were hampered by the lack of methods to isolate sufficiently pure DNA and RNA from actinorhizal plants. The host plants mostly are woody shrubs or trees, and only recently methods have been developed to isolate their nucleic acids. The development of these techniques paved the way to initiate studies on actinorhizal nodule development. Meanwhile, several plant genes/cDNAs have been cloned that are expressed at elevated levels during actinorhizal nodule development. Such cDNAs have been used to study different aspects of actinorhizal nodule functioning and development.

Comparing actinorhizal nodules and lateral roots, we can expect that genes showing a nodule-specific or -enhanced expression pattern can fall into four different groups. First, there are structural differences between actinorhizal nodule lobes and lateral roots, like the lack of a root cap in nodule lobes. Genes whose products are involved in determining these differences can be expected to be expressed in the nodule meristem. Second, nodules contain cells infected by *Frankia* bacteria, i.e. an interaction between the plant cells and the bacterial symbiont is taking place. Genes whose products are involved in this interaction can be expected to be expressed in the infected cells. Third, nodules are metabolically highly specialized organs: The product of bacterial nitrogen fixation, ammonium, which is exported by the bacteria, has to be assimilated via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle and to be metabolized to nitrogen transport forms (Schubert, 1986). Acceptor molecules and energy for nitrogen assimilation and carbon sources for bacterial nitrogen fixation have to be provided. Also the supply of the intracellular bacteria with other than carbon or nitrogen sources, for instance with iron and molybdenum ions for nitrogenase function, has to occur via the plant. Fourth, genes whose products are involved in nodule-specific functions not directly related to infection, but for establishing the physiological conditions to facilitate N_2 fixation and ammonium assimilation, can be expected to be expressed at various sites.

I Nomenclature of actinorhiza-specific genes

Previously the term "actinorhizin gene" was used for actinorhizal nodule-specific genes (Tremblay et al., 1986) and was applied to plant as well as bacterial genes. In contrast, in the legume-*Rhizobium* symbioses, the term "nodulin" is exclusively used for nodule-specific plant proteins (van Kammen, 1984). We will use the term "actinorhizal nodulin" to refer to actinorhizal nodule-specific plant proteins. Nodule-specificity will be defined by comparison of the accumulation of transcripts in nodules and roots. When a gene is expressed in nodules but not in roots, it will be called nodule-specific, irrespective of whether it is expressed in other organs. In legume nodules, nodulins have been divided into early nodulins and late nodulins according to the timing of their gene expression (Nap and Bisseling, 1990). The growth pattern of *Alnus* nodules is not suitable for time course studies of gene expression, because they represent coralloid structures consisting of multiple lobes, and new nodule lobes are formed continuously. But we can base the distinction of early and late actinorhizal nodulins on gene expression patterns along the developmental gradient of nodule cortical cells as detected by *in situ* hybridizations.

Actinorhizal nodule lobes have an indeterminate growth pattern. Therefore, their tissues are of graded age. The youngest tissues of the nodule lobe are at the distal part, the apex of the nodule lobe, where the meristem is located. The meristem differentiates continuously, adding new cells to the different nodule lobe tissues. The tissues near the root attachment site are the oldest. The presence of different developmental stages of cortical cells in actinorhizal nodule lobes provides good opportunities to determine via *in situ* hybridization at which stage of development a gene is induced. Starting from the apical meristem, four different zones have been defined in a mature actinorhiza (Ribeiro et al., 1995). The meristematic zone (zone 1) consists of small dividing cells. The infection zone (zone 2) contains enlarging cortical cells. Some of them are infected and in turn become larger than uninfected cells (Lalonde, 1980; Schwintzer et al., 1982; Berry and Sunell, 1990). The fixation zone (zone 3) is characterized by formation of vesicles by the bacteria and the induction of bacterial nitrogen fixation (*nif*) genes the products of which are localized in these vesicles (Huss-Danell and Bergman, 1990; Ribeiro et al., 1995). In the senescence zone (zone 4), the infected cells degenerate, and degradation of the host cytoplasm and *Frankia* hyphae is occurring (Schwintzer et al., 1982).

Actinorhizal nodulin genes expressed at highest levels in the meristematic zone (zone 1) and/or in the infection zone (zone 2) before the onset of nitrogen fixation will be called early actinorhizal nodulin genes. Those expressed at highest levels in the fixation zone (zone 3) and/or senescence zone (zone 4) will be called late actinorhizal nodulin genes.

II Gene expression in *A. glutinosa* nodules

The *Alnus-Frankia* symbiosis was taken by a few groups (Séguin and Lalonde, 1991; Mullin et al., 1993; Pawlowski et al., 1994b,c; Van Ghelue et al., 1996) as a system to study molecular aspects of actinorhiza development and functioning because of the availability of basic knowledge about nodule physiology (reviewed by Huss-Danell, 1990) and of cell and tissue culture systems (Séguin and Lalonde, 1990). Furthermore, several *Frankia* strains that induce actinorhizae on *Alnus* have been cultured (Benson and Silvester, 1993).

Before suitable RNA isolation methods were available, actinorhizal gene expression was studied at the protein level. For example, polypeptide patterns of uninfected roots and nodules of *Alnus* were compared (Séguin and Lalonde, 1993). By this approach, five nodule-specific polypeptides were observed, but it was unclear whether they were encoded by the host or the microsymbiont. Improvements in poly(A) RNA isolation from *Alnus* made it possible to construct nodule cDNA libraries (Twigg and Mullin, 1990; Pawlowski et al., 1994a). By differential screening of the libraries with root and nodule cDNA, respectively, a dozen of nodule-specific/enhanced cDNA clones have been isolated. The results obtained with these clones are summarized and discussed in the following sections of this chapter.

1. cDNA clones isolated from nodule cDNA libraries of *A. glutinosa*

By differential screening of nodule cDNA libraries, 14 nodule-specific or nodule-enhanced cDNA clones have been isolated so far. The plant origin of these 14 clones has been confirmed by Southern hybridization which also showed that all these cDNAs are encoded by small gene families. Nodule-specific and nodule-enhanced cDNA clones of *A. glutinosa* are listed in Table 1.

2. Sequential gene induction in *Alnus* nodules

As mentioned before, genes whose products are involved in the interaction with the endosymbiont can be expected to be expressed in the infected cells. Since in mature, nitrogen-fixing infected cells, the interaction has already been established, we expect these genes to be expressed at highest levels in the infected cells of zone 2, where infection is still in progress. In contrast, genes whose products are involved in the functioning of mature infected cells, i.e. in the metabolic specialization of the nodule, are expected to be induced at a later stage of

development. Therefore, the induction pattern of a nodule-specific or -enhanced gene may yield information on the function of its product. Genes expressed during different stages of development of infected cortical cells will be discussed in the following chapters.

Table 1. *Nodule-specific or -enhanced cDNA clones isolated from A. glutinosa* nodules

clone name	organs in which expressed	expression site in the nodule	function based on sequence	references
pAg3-1	all organs tested	infected cells and pericycle	sucrose synthase	van Ghelue et al., 1996
pAg6	all organs tested	infected cells of zone 2, 3 and pericycle	enzyme involved in biosynthesis of thiazole	Ribeiro et al., 1996
pAg11	all organs tested	infected cells of zone 3 and pericycle	glutamine synthetase	chapter 2
pAg12	nodule and shoot tip	infected cells of zone 2, weaker in zone 3	subtilisin-like protease	Ribeiro et al., 1995
pAg13	all organs tested	infected cells of zone 4 and pericycle	rich in glutamic acid and proline	chapter 4
pAg24	root, nodule, shoot tip, cotyledon; other organs n. t.	n. d.	carbamoyl phosphate synthetase	unpublished
pAg67	nodule and developing fruit	n. d.	unknown	unpublished
pAg118	root and nodule	infected cells of zone 3	acetylornithine transaminase	chapter 2
pAg135	nodule, shoot tip and flower	infected cells and pericycle	fatty acid reduc-tase homologue	chapter 3
pAg144	all organs tested	infected cells of zone 2, 3 and pericycle	enolase	van Ghelue et al., 1996
pAg164 (pAgNS84)	nodule	infected cells of zone 2	rich in glycine and histidine	unpublished
pAg172	nodule	n. d.	transmembrane transporter	unpublished
pAgNg203-118	nodule and flower	n. d.	cysteine protease (AgNOD-CP1)	Goetting-Minesky and Mullin, 1994; K. Pawlowski, unpubl. result

A) Gene expression at early stages of infected cell development

ag12 and *ag164* are two early actinorhizal nodulin genes identified in *A. glutinosa* nodules. Both are expressed at highest levels in the infected cells of zone 2.

ag12 is highly expressed in nodules, furthermore it is expressed in shoot tips, but at a low level (Ribeiro et al., 1995). In nodules, the expression occurs mainly in infected cells of zone 2 where bacterial *nif* genes are not yet expressed, but also in infected cells of zone 3, although at a lower level. *ag12* encodes a serine protease of the subtilisin family. The presence of a putative N-terminal signal peptide sequence indicates that Ag12 is probably active in the extracellular space. Since *ag12* is expressed in early stages of nodule development, Ag12 is not likely to be involved in senescence. Based on its expression patterns, Ag12 has been postulated to be involved in processing proteins that are part of the cell wall-like matrix material surrounding the bacteria.

ag164 and *agNS84*, two members of the same gene family, are also expressed at early stages of nodule development (K. Pawlowski, P. Twigg, C. Guan and B. C. Mullin, unpublished). Their transcripts are found in the infected cells of zone 2, an expression pattern similar to that of *ag12* (Tab. 1). Like Ag12, Ag164/AgNS84 contain a putative N-terminal signal peptide, suggesting that they are exported to the extracellular space, i.e. probably to the space between *Frankia* bacteria and the invaginated plasma membrane surrounding them. The mature Ag164 and AgNS84 polypeptides are rich in alternating glycine and histidine residues, a pattern also found in the metal-binding domains of a yeast protein (Conklin et al., 1992). Thus, Ag164/AgNS84 might represent metal-binding proteins present in the extracellular compartment where *Frankia* is localized. The presence of metal-binding proteins in the space between plant membrane (peribacteroid membrane) and the endosymbiont (peribacteroid space) has also been postulated for legume symbioses. In pea, the early nodulins ENOD3/14 and Nod6 which contain a cysteine-rich domain have been proposed to represent metal-binding proteins of the peribacteroid space (Kardailsky et al., 1993). In soybean, the nodulin-A family (Sandal et al., 1987), and their homologue in *Phaseolus vulgaris*, Npv30 (Campos et al., 1995), have been suggested to represent metal-binding proteins, some of which are localized in the peribacteroid membrane. Since the bacteria need molybdenum and iron for nitrogenase biosynthesis provided by the host plant, we can expects that there are special systems to transport metal ions to the endosymbionts. Ag164/AgNS84 and the nodulins mentioned above might be part of such systems.

Thus, the two gene families expressed early during the infected cell development encode proteins which are probably exported to the space between invaginated plasma membrane and *Frankia*. This supports the working hypothesis that the products of such genes should be involved in the interaction with the endosymbiont.

B) Gene expression during nodule functioning

During nitrogen fixation, the nodule has to provide a micro-environment to support the bacterial nitrogen-fixing process. The host plant has to supply carbohydrates for plant and bacterial respiration in nitrogen-fixing nodules (Akkermans et al., 1981; Vance and Heichel, 1991) and to assimilate the fixed nitrogen (Schubert, 1986). Physiological studies have provided insight in metabolic pathways of carbon and nitrogen metabolism in actinorhizal nodules (Huss-Danell, 1990).

Several cDNAs encoding enzymes involved in carbon and nitrogen metabolism have been cloned (Tab. 1), e. g. sucrose synthase, enolase, glutamine synthetase and acetylornithine aminotransferase. All these *A. glutinosa* enzymes are encoded by small multigene families and since in all cases, only one family member has been studied, it is not clear yet if nodule-specific forms exist.

i) C metabolism related gene expression

The main transport form of carbohydrate in plants is sucrose (Pate, 1962) which can be introduced into metabolism either by the action of intracellular sucrose synthase or extracellular invertase (Morell and Copeland, 1984, 1985). In nodules, sucrose is needed for four different processes. First, the energy for ammonium assimilation and for the biosynthesis of the nitrogen transport form, citrulline, has to be generated by glycolytic processes. Second, the plant has to provide carbon sources to the bacteria, supposedly C_4-dicarboxylic acids (Akkermans et al., 1981). Third, carbon backbones for ammonium assimilation have to be supplied. Fourth, shoot carbohydrates are used to build up starch in the uninfected nodule cortical cells (Wheeler et al., 1983). For the first three processes, which are supposed to take place in the infected cortical cells and in tissues involved in transport processes, sucrose has to be degraded to phosphoenolpyruvate (PEP) which in turn is phosphorylated by the action of PEP carboxylase. PEP carboxylase has been localized in the infected cortical cells (Perrot-Rechenmann et al., 1981).

From *A. glutinosa*, nodule-enhanced cDNAs have been cloned encoding sucrose synthase and enolase (Van Ghelue et al., 1996), the latter of which is catalyzing the synthesis of PEP. Another nodule-enhanced cDNA (*ag6*) encodes a product is involved in the biosynthesis of thiamine, a cofactor of enzymes involved in glycolysis and Calvin cycle (Ribeiro et al., 1996). The expression patterns of the corresponding genes in nodules have been analyzed by *in situ* hybridization (Van Ghelue et al., 1996; Ribeiro et al., 1996). The transcripts of these genes are found in the infected cells of zone 2 and zone 3, and in the pericycle of the nodule vascular bundle. This expression pattern fits the expectations, since glycolytic activity is required in the pericycle to provide energy for transport processes, and in the infected cells, carbon sources for the bacteria as well as carbon backbones and energy for ammonium assimilation have to be supplied. Surprisingly, sucrose synthase expression could not be detected in the starch-containing uninfected cortical cells of *A. glutinosa*. This might mean that or sucrose is metabolized by invertase in the uninfected cells, or it could be a problem of the detection limit in *Alnus* nodule *in situ* hybridization.

ii) N metabolism related gene expression

In actinorhizal and legume nodules, assimilation of ammonium exported by the bacteria takes place via the GS/GOGAT pathway. The nitrogen transport form in *Alnus* is citrulline (Schubert, 1986). Two nodule-enhanced cDNA clones have been found to encode proteins that are related to nitrogen metabolism. pAg11 encodes a glutamine synthetase (GS), the key enzyme responsible for ammonium assimilation; pAg118 encodes an acetylornithine transaminase (AOTA) which is catalyzing the penultimate step in the biosynthesis of citrulline (chapter 2). Transcripts of GS were found in the infected cells of zone 3 and the pericycle of the nodule vascular bundle, while those of AOTA were detected in infected cells of zone 3 only. These data suggest that in *A. glutinosa* nodules, citrulline synthesis mainly takes place in the infected cells. This is different from the situation in determinate legume nodules exporting ureides which are synthesized in the uninfected cells of the central tissue (chapter 2; Nguyen et al., 1985), but similar to the situation in indeterminate legume nodules where uninfected cells of the central tissue only seem to have storage functions (Bauchrowitz et al., 1995). However, while *Alnus* is exporting a ureide, citrulline is not synthesized via *de novo* purine biosynthesis as in determinate legume nodules, but via ornithine (chapter 2). It is suprising that GS is active in the nodule pericycle, indicating that there must be high levels of free ammonium in this tissue, either due to diffusion from the infected cells, or due to degradation of nitrogenous solutes (chapter 2). In view of the differences in the relative

concentrations of amino acids in nodules and in the stem xylem, it is likely that degradation of assimilation products, and reassimilation of ammonium, take place in the nodule pericycle (chapter 2).

iii) O_2 metabolism related gene expression

In legume nodules, the plant is providing O_2 protection of nitrogenase by generating an O_2 diffusion barrier, and at the same time, facilitating O_2 transport to the sites of respiration by supplying large amounts of the O_2 transport protein leghemoglobin. In actinorhizal nodules, the situation is less uniform, since *Frankia* can provide its own oxygen protection mechanism by forming vesicles. In *Casuarina* symbioses, the situation resembles that in legumes nodules, since the infected cells are surrounded by an O_2 diffusion barrier (Berg and McDowell, 1987a), and high amounts of hemoglobin, the homologue of leghemoglobin, are present (Fleming et al., 1987), while *Frankia* does not form vesicles (Berg and McDowell, 1987b). In *Alnus* nodules, however, no O_2 diffusion barrier has been found (Silvester et al., 1990), and although nodules contain increased amounts of hemoglobin compared to roots, their hemoglobin content is much lower than that of *Casuarina* nodules (Suharjo and Tjepkema, 1995). Thus, further research is required to ascertain to what extent *Alnus* nodule hemoglobin is contributing to O_2 protection of nitrogenase.

iv) Gene expression for an unknown metabolic pathway

The isolation of nodule-specific/-enhanced cDNAs from *A. glutinosa* nodules has led to the discovery of a gene whose product is involved in a novel metabolic reaction. cDNA clone pAg135 showed an *in situ* hybridization pattern corresponding to that of a late actinorhizal nodulin gene. The expression of *ag135* in nodules is confined to the infected cells and to the pericycle of the vascular bundle (chapter 3). *ag135* mRNA is already detected in early infected cells of zone 2, but the highest levels of expression are found in the infected cells of zone 3 where bacterial *nif* genes are highly expressed. Based on the analysis of its homologue Ara135 in *Arabidopsis* and the sequence comparison with the jojoba (*Simmondsia chinensis*) fatty acyl reductase (Metz et al., 1994), Ag135 was postulated to represent a fatty acyl reductase for fatty alcohol biosynthesis. However, no long chain fatty alcohols could be detected in *A. glutinosa* nodules (Penny von Wettstein-Knowles, personal communication). Thus Ag135 seems to represent a carboxyl reductase with a substrate specificity different from that of the jojoba enzyme. Since it shows the same expression pattern as other nodule-

enhanced clones whose products are involved in nodule metabolism, Ag135 might be involved in nodule primary metabolism in a novel metabolic pathway. The function of Ag135 remains to be analyzed.

C) Gene expression during nodule senescence

A. glutinosa is a perennial actinorhizal plant showing a seasonal growth pattern of root nodules (Schwintzer et al., 1982). It is common that endophyte is degraded and nitrogen-fixing infected cells die in autumn, while new infected cells are formed in spring (Akkermans and Van Dijk, 1976). The knowledge about nodule senescence is limited just as plant senescence in general is not well understood (Smart, 1994).

The nodule-enhanced cDNA clone pAg13, seems to represent a senescence-related gene (chapter 4). *ag13* transcripts were localized in infected cortical cells of zone 4 where *nif* gene expression has been switched off and host cytoplasm as well as *Frankia* bacteria are subjected to degradation. The cDNA encodes a polypeptide with a putative signal peptide suggesting an extracellular location (4). Mature Ag13 and its homologues have a peculiar amino acid composition being rich in glutamic acid, proline, alanine and valine, indicating that they might represent a new family of acidic plant extracellular proteins (chapter 4). One of the homologues of *ag13* was found to be expressed induring fruit development in kiwifruit (Ledger and Gardner, 1994). Since senescence and fruit development show strong similarities (Vercher and Carbonell, 1991; Smart, 1994), Ag13 is probably involved in nodule senescence rather than in endosymbiont degradation. But, in either case, such a function does not explain the expression of *ag13* in the pericycle of the nodule vascular bundle (chapter 4).

Another actinorhizal nodulin that might be involved in senescence is AgNOD-CP1 (Goetting-Minesky and Mullin, 1994). Northern analysis showed that the corresponding gene is expressed at high levels in nodules and at lower levels also in flowers of *A. glutinosa*, and that its expression levels in old nodules are higher than in young ones (K. Pawlowski, unpublished observations). Sequence analysis has revealed that the cDNA encodes a cysteine protease (Goetting-Minesky and Mullin, 1994). Cysteine protease activities have been found in senescing legume nodules (Pladys and Vance, 1993) and *Arabidopsis* leaves (Hensel et al., 1993). In fact, proteolysis is an important feature in old legume nodules and might play a role in recovery of nitrogenous compounds from the senescing tissues (Pfeiffer et al., 1983a,b; Pladys and Rigaud, 1985; Pladys et al., 1991; Pladys and Vance, 1993). These data suggest

that AgNOD-CP1 may represent a late actinorhizal nodulin that may function in nodule senescence.

3. Gene expression in the pericycle of the nodule vascular bundle

One of the characteristics of *Alnus* nodule lobes is that they possess a huge central vascular system surrounded by a multilayered pericycle consisting of small cells with dense cytoplasm, rich in mitochondria (Burgess and Peterson, 1987a). This multilayered pericycle of the nodule vascular system represents a unique differentiated tissue, neither found in *Alnus* roots (Burgess and Peterson, 1987a) nor in legume nodules. The pericycle might be important in nodule metabolism as interface between the xylem/phloem and the nodule cortical cells. It has been speculated to function in nutrient transport between vascular system and infected cortical cells (Burgess and Peterson, 1987b). So far, all nodule-enhanced genes examined, and *ag135* as well, have been found to be expressed in the pericycle of the nodule vascular bundle in *Alnus* (Table 1). The expression of sucrose synthase, enolase and *ag6* supports the cytological observations indicating a high metabolic activity of this tissue (Van Ghelue et al., 1996; Ribeiro et al., 1996). GS expression in the pericycle has led to the hypothesis that degradation of nitrogenous solutes and reassimilation of ammonium is taking place in this tissue.

III Perspectives

In course of the studies presented in this thesis, several cDNAs representing nodule-specific or -enhanced genes of *A. glutinosa* have been cloned, and their sites of expression in root nodules have been determined using *in situ* hybridization. Thus, in spite of their woody character, actinorhizal plants can be examined using standard molecular biological techniques. However, more detailed studies of actinorhizal symbioses are still impaired by the fact that neither for the host nor for the microsymbiont, a genetic system is available. Fortunately, most nodule-specific genes have counterparts in non-symbiotic plant development. Thus, the accumulated information from the random cDNA sequencing project on *Arabidopsis* (Höfte et al., 1993; Newman et al., 1994; Cooke et al., 1996) has made it possible to identify *Arabidopsis* homologues of nodule-specific genes/cDNAs of interest. Most cDNAs isolated from *A. glutinosa* nodules turned out to have homologues among the random cDNA sequences from *Arabidopsis* (Tab. 2). The availability of quick mapping procedures and an integrated genetic map for *Arabidopsis* (Lister and Dean, 1993; Hauge et al., 1993) can lead to

the identification of the function of the *Arabidopsis* homologues of nodulin genes by assigning mutant phenotypes to cDNAs. Complementation studies can be performed to ascertain the gene function. For such studies, yeast has also been used successfully (Ribeiro et al., 1996). Furthermore, *Arabidopsis* represents a suitable system for analysing actinorhizal

Table 2. Actinorhizal nodulin homologues in *Arabidopsis*
Nodule-enhanced clones are marked by a asterisk.

A. glutinosa cDNA clones	*Arabidopsis* homologues	function of the gene	references
Ag12	Ara12	serine protease	Ribeiro et al., 1995
Ag135	MS2, Ara135	Jojoba fatty acyl reductase homologue	Aarts et al., 1993; chapter 3
Ag172	*Atchl-1*	ion transporter	Tsay et al., 1993; C. Guan and K. Pawlowski, unpubl. results
Ag3-1*	*Asus1*	sucrose synthase	Martin et al., 1993; Van Ghelue et al., 1996
Ag6*	Ara6 (*Thi4*)	enzyme involved in thiazole synthesis	Ribeiro et al., 1996; Machado et al., 1996
Ag13*	Ara21371	glu-, pro-rich protein	chapter 4

gene function by examining the effects of antisense expression or ectopic expression of their *Arabidopsis* homologues.

Because of their lateral root like structure, actinorhizal nodules have been considered to be more "primitive" than legume nodules. This is certainly correct with regard to the developmental program leading to actinorhizal nodules, which shows few deviations from the endogenous program for lateral root development. However, the isolation of up to now five different cDNAs representing nodule-specific genes has indicated that both systems seem to be equally sophisticated with regard to the specialization of infected cells. This also seems to apply to the specialization of non-infected cells. The lateral root like structure and absence of an oxygen diffusion barrier in *Alnus* nodules may previously have led to the hypothesis that the only symbiosis-related specialization of *Alnus* nodule cells was that of infected cells. Now, we know that the nodule pericycle is similarly specialized, involving the expression of several nodule-enhanced and at least one nodule-specific gene (Van Ghelue et al., 1996; chapters 2, 3 and 4). There may be just one aspect of legume symbioses not present in actinorhizal nodules, which is requiring nodule-specific functions. Legume nodule cells can

contain two infection-related compartments, the infection thread matrix and the peribacteroid space, for which there is no equivalent in actinorhizal nodules. However, the function of peribacteroid space proteins in legume nodules may be fulfilled by proteins of the encapsulation in actinorhiza. Thus, altogether both types of symbiotic organs, actinorhiza and legume nodules, seem to involve a similar degree of specialization.

Since decades, one of the final aims of symbiotic nitrogen-fixation research has been to transfer the ability to enter nitrogen-fixing symbioses to major crops like rice and wheat. The discussion has mostly been centered on extending the host range of rhizobia. However, the actinorhizal microsymbiont *Frankia* is less restricted than rhizobia regarding host specificity. Host plants nodulated by *Frankia* comprise a diverse group of plant species from eight different families (Benson and Silvester, 1993), although recently actinorhizal plants have been found to be closely related with each other and with leguminous plants (Soltis et al., 1995). In contrast to rhizobia, *Frankia* is capable of fixing nitrogen in free living state under aerobic conditions, while nitrogenase is protected from oxygen by the multilayered lipid envelope of the vesicle (Berry et al., 1993). Also during symbioses, *Frankia* can form vesicles which contribute to oxygen protection of nitrogenase, while in legume nodules oxygen protection has to be provided solely by the plant (Silvester et al., 1990). Actinorhizal plants from different families show a high degree of variability in structure, e.g. in the arrangement of infected cells and in nodule aeration/oxygen protection mechanisms (Silvester et al., 1990) as well as in the plant-dependent shape and localization of *Frankia* vesicles in infected cells (Baker and Mullin, 1992), demonstrating that in different plant families, divers ways can be taken to solve the oxygen dilemma imposed by bacterial nitrogen fixation. Thus, the analysis of actinorhizal symbioses can show us how different ways are open for plants to build up an ecological niche for nitrogen-fixing symbionts.

The most important part in any endophytic nitrogen-fixing symbiosis is the interaction in the infection process. Here, *Rhizobium*-legume and actinorhizal symbioses show strong similarities (chapter 1). Most of the nodule-specific genes identified in both systems are expressed in the infected cells and thus, probably encode proteins involved in the interaction with the microsymbiont. Thus, for analysing the infection process, a comparison of homologous nodule-specific genes in legumes and actinorhizal plants, respectively, will be useful. Unfortunately, homologous nodulin genes have not been identified in both systems thus far. However, the accumulating *Arabidopsis* sequence information will make it possible to compare the sequence of any *A. glutinosa* actinorhizal nodulin and its *Arabidopsis* homolog and to identify conserved amino acid sequences. This would then allow the cloning of homologues of legume nodulins from actinorhizal plants, and from actinorhizal nodulin genes

from legumes, by PCR using degenerate primers. In that way it will become possible to study the expression of the homologues of nodule-specific/-enhanced actinorhizal genes in a system with different morphology, e.g. legumes or *Arabidopsis* itself, which may open more ways to a functional analysis.

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Summary

Samenvatting

Alnus glutinosa kan geïnfecteerd worden door de actinomyceet *Frankia*. Dit leidt tot de vorming van stikstofbindende wortelknollen. Om de funktie van plant gecodeerde genen, die in de knol tot expressie komen te karakteriseren, is een cDNA bank van *A. glutinosa* wortelknollen gemaakt. Deze is differentieel gescreend met knol- en wortel cDNA. Verschillende cDNA klonen van genen die knolspecifiek tot expressie komen of in de knol sterk geïnduceerd worden zijn geïsoleerd. De sequenties van deze klonen zijn bepaald en vergeleken met sequenties in databanken. De plaats van de genexpressie in de knol is bepaald met behulp van *in situ* hybridisatie.

cDNA's van genen die coderen voor de stikstofmetabolisme gerelateerde enzymen glutamine synthetase (GS) en acetylornithine transaminase (AOTA) zijn geïsoleerd. GS is het sleutelenzym verantwoordelijk voor de assimilatie van het door *Frankia* bacteriën gesynthetiseerde ammonium en AOTA is betrokken bij de biosynthese van citrulline, de transport vorm van gefixeerd stikstof in *Alnus* knollen. Het genexpressie patroon van deze genen is geanalyseerd op Northern blots. GS word in alle geteste organen gevonden, met het hoogste expressie niveau in knollen. Geringe hoeveelheden AOTA transcripten zijn gevonden in wortels, en grote hoeveelheden in knollen. *In situ* hybridisatie laat zien dat GS specifiek tot expressie komt in geïnfecteerde cellen en in de pericykel van de vaatbundels van de knollobben, terwijl AOTA alleen in geïnfecteerde cellen tot expressie komt. De gegevens suggereren dat ammonium assimilatie plaatsvindt in de geïnfecteerde cellen en in de pericykel van de vaatbundel van de knol, en dat citrulline voornamelijk in de geïnfecteerde cellen gemaakt wordt.

Een cDNA is geïsoleerd van een gen dat kodeert voor een eiwit dat homoloog is aan een vetzuurreductase van jojoba zaden (ScFAR). Het hiermee overeenkomende gen, *ag135*, komt sterk tot expressie in knollen, en zwak in andere organen. *ag135* transcripten worden gevonden in geïnfecteerde cortex cellen en in de pericykel van de vaatbundel van de knol. In *Arabidopsis thaliana* is een homoloog gen geïdentificeerd, *ara135*. Dit mapt dichtbij, maar niet op het *ms1* locus. *ara135* komt tot expressie in de epidermis van een aantal bloemorganen, namelijk van kelkbladeren, kroonbladeren, helmdraden en van zaadknoppen. Eiwitten gekodeerd door *ag135* en *ara135* vertonen grote homologie met Ms2 van *A. thaliana*, dat verantwoordelijk is voor mannelijke steriliteit.

De cDNA kloon pAg13 komt met een gen overeen dat sterker tot expressie komt in *Frankia* geïnduceerde knollen dan in wortels. De afgeleide eiwitsequentie is rijk aan glutaminezuur en proline, en bevat mogelijk een signaalpeptide wat zou kunnen duiden op een extracellulaire lokatie van Ag13. *In situ* hybridisatie laat zien dat *ag13* tot expressie komt in de pericykel van de vaatbundel van de knol en in geinfecteerde cellen tijdens de afbraak van de endosymbiont. Van een gen homoloog met *ag13* is gevonden dat het tijdens de vruchtontwikkeling van kiwi tot expressie komt, en homologe cDNA klonen zijn ook

gevonden in *A. thaliana* en *Ricinus communis*, wat suggereert dat Ag13 behoort tot een nieuwe familie van zure extracellulaire eiwitten.

Verschillende *Frankia* stammen induceren ineffectieve, dwz. niet stikstof fixerende knollen, wat soms afhankelijk is van de gastheer plant. Ineffektieve knollen geïnduceerd door bodem suspensies op vatbare *A. glutinosa* klonen zijn cytologisch geanalyseerd. Vergeleken met effectieve knollen, bevatten ineffektieve knollen grotere hoeveelheden polyfenolen. Deze worden zelfs in de geïnfecteerde cortexcellen gevonden, hetgeen op een verdedigingsreaktie van de plant wijst. *In situ* hybridisatie met een *Frankia* antisense 16S rRNA probe laat zien dat *Frankia* inderdaad in een vroeg ontwikkelingsstadium van de geïnfecteerde cellen in ineffectieve knollen word gedegradeerd. De mRNAs van twee plantegenen, *ag13 en ag12*, zijn gelocaliseerd in ineffectieve knollen. In effectieve knollen komt *ag12* tot expressie in jonge geïnfecteerde cellen. Het expressiepatroon van *ag12* en *ag13* lijkt vergelijkbaar te zijn in effectieve en ineffectieve knollen.

Summary

Alnus glutinosa can be infected by actinomycetous *Frankia*, which leads to the formation of nitrogen-fixing root nodules. To characterize the function of host-encoded genes expressed in the nodule, a cDNA library of *A. glutinosa* root nodules was constructed and screened differentially with nodule and root cDNA, respectively. Several nodule-specific and -enhanced cDNA clones were isolated. These clones were sequenced and the deduced polypeptide sequences were used for homology searches in the databases. The localization of gene expression in the nodule was determined by *in situ* hybridization.

cDNAs encoding nitrogen metabolism related enzymes glutamine synthetase (GS) and acetylornithine transaminase (AOTA) have been isolated. GS is the key enzyme responsible for the assimilation of ammonium synthesized by *Frankia* bacteria, and AOTA is involved in the biosynthesis of citrulline, the exported form of fixed nitrogen in *Alnus* nodules. Their gene expression patterns were analyzed by Northern hybridization. GS mRNA was found in all organs tested, with the highest level of expression in nodules. AOTA transcripts were found at very low levels in roots, and at high levels in nodules. *In situ* hybridization showed that GS was specifically expressed in the infected cells as well as in the pericycle of the vascular bundle of the nodule lobes, while AOTA was expressed only in the infected cells. The data suggested that the ammonium assimilation most likely takes place in both the infected cells and the pericycle of the nodule, and citrulline seems to be synthesized mainly in the infected cells.

A cDNA encoding a homologue of a fatty acyl reductase from jojoba seeds (ScFAR) was isolated. The corresponding gene, *ag135*, was found to be expressed at high levels in nodules and at lower levels also in other tissues. *ag135* transcripts were confined to the infected cortical cells and to the pericycle of the nodule vascular bundle. A homologue of this gene, *ara135*, was identified in *Arabidopsis thaliana*, mapping close to, but not at the *ms1* locus. *ara135* was found to be expressed in the epidermis of some floral organs, i.e. of sepals, petals and filaments, and of ovules. Proteins encoded by both *ag135* and *ara135* showed high homology to Ms2 from *A. thaliana*.

The nodule-enhanced cDNA clone pAg13 corresponds to a gene expressed at elevated levels in nodules induced by *Frankia* compared to roots. The deduced polypeptide sequence is rich in glutamic acid and proline, and contains a putative signal peptide indicating an extracellular location of Ag13. *In situ* hybridization showed that *ag13* is expressed in the pericycle of the nodule vascular bundle and in infected cells that exhibited degradation of the endosymbiont. An *ag13* homologue has been found to be expressed during kiwifruit development, and homologues have also been found in *Arabidopsis* and castor bean, suggesting that Ag13 might be the member of a novel family of acidic extracellular proteins.

Several *Frankia* strains have been shown to induce ineffective, i.e. non-nitrogen fixing nodules, sometimes in a host-plant dependent manner. Ineffective nodules induced

on susceptible *A. glutinosa* clones by soil suspensions were analysed cytologically. Comparisons with effective nodules showed that ineffective nodules contain higher amounts of polyphenols, indicating a plant defense reaction. Polyphenols were found even in the infected cortical cells. *In situ* hybridization with a *Frankia* antisense 16S rRNA probe showed that *Frankia* is degraded at an early stage of development of infected cells in ineffective nodules. The mRNAs of two plant genes, *ag12* and *ag13*, which had previously found to be expressed in the infected cells of effective nodules, were localized in ineffective nodules. Their expression patterns seemed to be analogous in both types of nodules.

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Curriculum Vitae

I was born in Shenyang, Liaoning Province, China on April 26, 1964. I enjoyed my primary and secondary school time from 1972 to 1980. After that, I spent two years for my high school education at the 83th High School of Shenyang in Xinchengzi District. From 1982 to 1987, I studied in the Department of Biology of Fudan University in Shanghai and obtained my bachelor degree in plant physiology. In September 1987, I began my MSc studies in the Nitrogen Fixation Laboratory, Institute of Botany, Chinese Academy of Sciences (CAS), under the supervision of Prof. Yuxiang Jing and Prof. Bin Wang (Institute of Genetics, CAS). In July 1990, I obtained my M.S. degree in plant molecular biology and started research as a probationer in the same lab working mainly on rice nodulation. In March 1993, I was sent by CAS as a visiting scholar to Dr. Ton Bisseling's lab at the Department of Molecular Biology, Wageningen Agricultural University, The Netherlands, to work with Dr. Katharina Pawlowski on the identification and characterization of nodule-specific and -enhanced cDNA clones of *Alnus glutinosa*. Eight months later, I was accepted as a PhD candidate with Prof. Ab van Kammen as supervisor. I finished my PhD research in February 1996. In March 1996, I started my postdoctoral research in Dr. Klaus Palme's Laboratory at the Max-Planck-Institut für Züchtungsforschung, Cologne, Germany.