Plant genes involved in the establishment of an actinorhizal symbiosis

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Statements

1. The fact that the change of developmental program from roots to actinorhizal nodule lobes involves only subtle changes in gene expression may explain why, up to now, no nodule-specific genes involved in the formation of the actinorhizal nodule structure have been identified.

2. The subtilisin-like proteases, Ag12/Cg12, described in this thesis, are the first of this kind to be identified in plants.

3. The non-development of genetics for actinorhizal plants can be partly compensated by using model systems like *Arabidopsis* and yeast to analyse gene function.

4. In spite of the differences between actinorhizal and legume nodules, the final functioning of the two systems is principally similar.

5. The conclusion of van den Heuvel & Ingham and Alcedo et al. that Smo (smoothened) is the hedgehog (Hh) receptor is not correct, since Smo does not bind directly to Hh.

M. van den Heuvel and P.W. Ingham, 1996, Nature 382: 547-551.
J. Alcedo et al., 1996, Cell 86: 221-232.
D.M. Stone et al., 1996, Nature 384: 129-134.
V. Marigo et al., 1996, Nature 384: 176-179.

6. The fact that TAFs (= TBP-associated factors) are required for activating transcription *in vitro* and, therefore, are obligatory targets of transcriptional activator proteins, does not prove that *in vivo* the same situation occurs.

S.S. Walker et al., 1996, Nature 383: 185-188.
Z. Moqtaderi et al., 1996, Nature 383: 188-191.

7. When suggesting that the large majority of broad bean nodulins seem to play no role in established arbuscular mycorrhiza symbiosis, Frühling et al. do not take in account that the expression levels of those nodulins could be below the detection limits.

Frühling et al., 1997, Mol. Plant-Microbe Interact. 10: 124-131.

8. The only man who never makes a mistake is the man who never does anything.

Theodore Roosevelt

9. There is only one good, knowledge, and one evil, ignorance.

Socrates

10. The Dutch enjoy one of the highest standards of living and their quality of life is excellent, but they often disguise this fact by constant complaining about this.

11. Autocracy and corruption are quite common to all third world countries and make that in these countries just a few people prosper while most suffer and starve.

Statements from the thesis entitled:
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CONTENTS

Outline

The formation of actinorhizal nodules is induced by actinomycetous bacteria of the genus *Frankia* on the roots of several dicotyledenous species belonging to eight different plant families, mostly trees or woody shrubs. These nodules are coralloid structures consisting of multiple lobes, which represent modified lateral roots with infected cortical cells. Inside the infected cells, bacterial nitrogen fixation takes place.

A detailed description and comparison of the symbiosis between *Frankia* and actinorhizal plants and the more well-known *Rhizobium*/legume symbiosis, which also leads to the formation of nitrogen-fixing root nodules, is presented in chapter 1. In spite of the fact that actinorhizal and rhizobial symbioses seem fundamentally dissimilar, the two systems share common features concerning the infection mechanism and metabolic processes. This, together with molecular phylogenetical data, suggest that both types of symbioses are closely related and that common properties are determining the capacity to develop nitrogen fixing root nodule symbioses.

The goal of this project was to study plant genes involved in the establishment of an actinorhizal symbiosis. This process requires plant functions involved in the metabolic specialization, and plant functions involved in the interaction with the microsymbiont, e.g. in the infection of cortical cells. To isolate such genes, we have differentially screened an *Alnus glutinosa* nodule cDNA library with nodule- and root cDNA, respectively, and characterized several cDNAs representing genes expressed at elevated levels in nodules compared to roots.

Most of the cDNAs that we have cloned have been shown to correspond to genes encoding enzymes involved in nodule functioning (chapters 2-4). During the nitrogen fixation process, it is a task of the host plant to provide carbon sources for the bacteria, and to assimilate the fixed nitrogen which is exported to the plant cytoplasm in the form of ammonium. Thus, enzymes involved in nitrogen (chapter 2) as well as carbon (chapters 3 and 4) metabolism are expected to be highly active in nodules.

One of the isolated cDNA clones, pAg12, was shown to represent a gene encoding a serine protease that is suggested to be involved in the process of nodule cortical cell infection (chapter 5). To assess the importance of this protease in other actinorhizal symbioses, the expression of its homolog was examined in nodules of *Casuarina glauca* (chapter 6).

An evaluation of the obtained results and a comparison of actinorhizal and rhizobial symbioses is presented in chapter 7.

Chapter 1 Introduction

Nitrogen fixing root nodule symbioses: legume nodules and actinorhizal nodules

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M.R. El-Gewely, editor.

Nitrogen fixing root nodule symbioses: legume nodules and actinorhizal nodules

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Abstract. Since decades, research has been performed to answer the question whether the ability to form an endophytic symbiosis with N_2-fixing bacteria can be transferred to agriculturally important crops. Here, two root nodule symbioses between angiosperms and N_2-fixing bacteria, *Rhizobium*/legume and symbioses between the actinomycetous bacterium *Frankia* and actinorhizal plants, will be described. In contrast to *Rhizobium*, which with one exception, can only enter symbioses with plants of the legume family, *Frankia* can enter symbioses with plant species from eight different families, mostly perennial woody shrubs. While extensive research has been done on physiological, ecological molecular and genetic aspects of *Rhizobium*/legume symbioses, molecular studies on actinorhizal symbioses have been started only recently. Nodule development, structure, and metabolism will be compared between both systems, indicating that actinorhizal symbioses represents a more primitive situation with a less sophisticated pattern of signal exchange. The developmental program of actinorhizal nodules shows less differences from the one of lateral roots than it is the case for the program of legume nodules. Also in contrast to legume nodules, there is a considerable diversity in actinorhizal symbioses regarding the differentiation of the endosymbiont in symbiosis and the oxygen protection systems provided by the plant. The implications of this comparison will be discussed.

Key words: actinorhiza, Frankia, legume, nodulin, Rhizobium, root hair deformation, root nodule, symbiotic nitrogen fixation.

Symbiotic nitrogen fixation systems

Biosphere·nitrogen is subjected to a rapid turnover, and part of it is used as a terminal electron acceptor by bacteria, and thereby lost as N_2 into the atmosphere. A continuous supply with reduced nitrogen from atmospheric N_2, is therefore required to maintain the biosphere balance. This can be provided by two processes: chemical reduction in the Haber-Bosch process, or biological N_2 fixation. However, while chemical nitrogen fixation is cost intensive and about 40–50% of the nitrogen applied as fertiliser is lost via denitrification, runoff or leaching, only 10–20% of the biologically fixed nitrogen is lost that way [1,2]. Thus, there is a strong interest in a better understanding of biological N_2 fixation in order to increase agricultural productivity.

Biological N_2 fixation can only be performed by certain prokaryotes which contain genes encoding nitrogenase. This enzyme catalyzes the reaction:

$N_2 + 8H^+ + 8e^- + 16Mg\text{-}ATP \rightarrow 2NH_3 + H_2 + 16Mg\text{-}ADP + 16P_i$

Nitrogenase consists of the homodimeric Fe protein, encoded by the nitrogen fixation (*nif*) gene *nifH*, and the tetrameric MoFe protein, encoded by *nifD* and *nifK*, which contains the FeMo-cofactor [3]. Since nitrogenase is irreversibly denatured by O_2, the process of N_2 fixation is highly O_2-sensitive [4,5]. Because of this and the high amount of energy (ATP) necessary for the nitrogenase reaction, the expression of N_2 fixation systems is strictly regulated and takes place only under nitrogen starved conditions, either under low O_2 tension or when special O_2 protection systems are provided. Several nitrogen-fixing organisms can form endophytic symbioses with higher plants, where the energy for nitrogen fixation and in most cases the O_2 protection system, is provided by the plant partner. Symbiotic N_2 fixation accounts for 70% of total biological nitrogen fixation [2]. In two groups of symbioses the prokaryotic partners are soil bacteria (rhizobia in legume symbioses and *Frankia spp.* strains in actinorhizal symbioses, respectively), while in the case of the *Nostoc-Gunnera* symbiosis [6], the cyanobacterium *Nostoc* is the N_2-fixing partner. These systems share some common features: the prokaryotes fix N_2 living as endophytes inside the cells of special organs of their host, separated from the plant cytoplasm by membranes derived from the plant plasmalemma. In the case of *Gunnera*, these infected cells are located in specialized stem glands whose development does not depend on the symbiont, while in the case of legumes and actinorhizal plants, the symbionts are hosted in root nodules that are formed by the plant upon infection with the symbiont.

Most agriculturally important plant species are belonging to the monocotyledonous plants, for example rice, corn, and wheat. To date, no monocotyledonous plants were known to form endophytic symbioses with N_2-fixing bacteria, although nitrogen fixing bacteria like *Azospirillum brasilense* are associated with the roots of several grasses. However, in this association bacterial nitrogen fixation does not contribute to plant growth, i.e., it cannot substitute for nitrogen fertilizer [7,8]. In contrast, nitrogen-fixing root nodule symbioses lead to independence of nitrogen fertilizer for the plant and are hence of major importance to design strategies by which the ability to form an endophytic symbiosis with N_2-fixing bacteria can be transferred to agriculturally important crops like rice. Recently, such a project has been initiated by the International Rice Research Institute in Manila, Philippines [9]. In this chapter we will give an overview of the *Rhizobium*/legume symbiosis as well as actinorhizal symbioses. We will describe the *Rhizobium*/legume symbiosis because the system is well studied at the molecular level and forms a paradigm for plant-microbe interactions. On the other hand, far less knowledge is available on actinorhizal symbioses. However, *Frankia* bacteria can interact with several plant families while *Rhizobium* only interacts with leguminosae. Moreover, *Frankia*-induced nodules are in fact modified lateral roots while legume nodules are in general, considered to be unique new organs. Due to the more promiscuous nature of *Frankia* as well as by the more root-like nature of actinorhizal nodules, this system might provide useful clues on how to transfer nodulation ability to other plant species.

Root nodule symbioses

In this review, the two types of nitrogen-fixing root nodule symbioses, *Rhizobium*-legume and actinorhizal symbioses, will be compared. Although the structures of the respective nodules are different, the process of nodule induction involves some steps similar in both types of symbioses.

The *Rhizobium*/legume symbiosis starts with an interaction between the bacteria and the root epidermis. In general, deformation and curling of root hairs is induced (Fig. 1A,B,C). The bacteria become entrapped in the curl and there the host cell wall

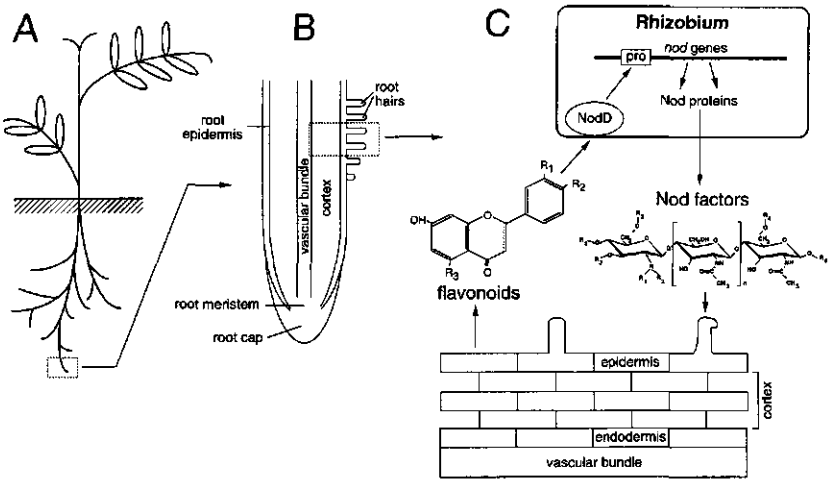

Fig. 1. Signal exchange during legume nodule induction. **A**: a schematic picture of a legume plant; **B**: a closeup of a root tip. **C**: the first steps of the interaction between legume roots and rhizobia are shown. Flavonoids present in the plant root exudate are binding the rhizobial NodD protein [236] which in turn binds the *nod* gene promoters and induces the expression of the other *nod* genes by binding to their promoters (pro) [43]. The *nod* gene products catalyze the biosynthesis of the Nod factors, that induce the deformation and curling of root hairs on the host plant.

Flavonoid structure (for review see [237]): NodD proteins from different rhizobia require specific flavonoids from their respective host plants for optimal activation. For example, hesperetin (R_1 = OH, R_2 = OCH_3, R_3 = OH) activates the NodD protein of *R. leguminosarum* biovar *viciae*, but not that of *R. leguminosarum* biovar *trifolii*, which is activated by 7-hydroxyflavone (R_1 = H, R_2 = H, R_3 = H). In addition to *nod* gene-inducing flavonoids, several flavonoids have been identified that inhibit *nod* gene activation, for example luteolin (R_1 = OH, R_2 = OH, R_3 = OH) inhibits *nod* gene induction by NodD from *R. leguminosarum* biovar *phaseoli*, but activates *nod* gene induction by NodD from *R. leguminosarum* biovars *viciae* and *trifolii*. The stippled double bound is present in luteolin and 7-hydroxyflavone, but not in hesperetin.

Nod factor structure (for review see [26,238]): The number of the N-acetylglucosamine residues can vary between three and five. The following substitutions can be found: position R_1, -H or methyl group; position R_2, acyl group (C16:1, C16:2, C16:3, C18:1 or C18:4); position R_3, -H, acetyl (O—6), or carbamyl group; position R_4, -H, sulfate, acetyl, (2-*O*-methyl)fucosyl or D-arabinosyl group; position R_5, -H or glyceryl group. A single *Rhizobium* strain can produce several Nod factors; for example *Rhizobium* NGR234 which can nodulate various tropical legumes, synthesizes 18 different Nod factors [239].

is hydrolysed and a new tubular structure, the infection thread, is formed. The bacteria invade the root hair and then the root cortex with this infection thread. Meanwhile, cells of the cortex are mitotically activated and form the nodule primordium. Infection thread grows towards this primordium and there rhizobia are released from the tips of the infection threads into the cytoplasm of the plant cells. This is an endocytotic process by which the bacteria become surrounded by a membrane derived from the host plasma membrane. In some cases, the bacteria do not enter the plant via root hairs, but between epidermal cells ("crack entry").

Frankia bacteria induce nodulation in their host plants in a slightly different way. In some interactions, root hairs are invaded by the formation of tube-like structures that resemble the infection threads in legumes. In other cases, intercellular penetration of the root and colonization of the intercellular spaces takes place. After root hair infection, like *Rhizobium*, *Frankia* induces mitotic activity in the root cortex but additionally, cell divisions are induced in the pericycle. The latter center of mitotic activity develops into a root nodule, and like in legume nodules, specialized cells become fully packed with the microsymbiont that again is surrounded by a membrane derived from the plasma membrane of the host.

Rhizobium-legume symbioses

Although leguminous plants have been used for soil enrichment by green manuring for centuries, it was first discovered in 1888, that bacteria living in symbiosis with the plant are responsible for the reduction of atmospheric N_2 to ammonium [10,11]. Gram-negative soil bacteria, members of the family *Rhizobiaceae* (including the genera *Rhizobium, Bradyrhizobium* and *Azorhizobium*), induce the formation of root nodules on their leguminous host plants. These symbioses show different degrees of host specificity, ranging from the stem-nodulating *Azorhizobium caulinodans* ORS571 that can only interact with the tropical leguminous shrub *Sesbania rostrata* [12], to the wide host range strain *Rhizobium spp* NGR234 which can induce nodules on several different tropical and temperate legume species and even on one nonlegume, *Parasponia* (Ulmaceae) [13].

In the last decades, considerable research has been devoted to the understanding of this symbiosis, not only because of the importance of biological nitrogen fixation to agriculture, but also because it provides insights in mechanisms controlling plant development.

Formation of legume nodules

Plant genes involved in nodulation. During legume nodulation, plant genes play an important role and these genes have been studied by genetic and molecular approaches. Genetic studies have revealed that certain plant genes (*sym* genes) are required during all stages of nodulation (for reviews see [14–16]). The phenotypes of the *sym* mutants show that the products of these genes are involved in, e.g., root hair deformation [17], infection [18], and bacterial release [19]. To date, none of the

sym genes have been cloned, but several researchers have started programs to isolate *sym* genes by a positional cloning strategy [20–22].

Molecular studies have concentrated on the plant genes whose expression is induced during the consecutive stages of nodulation (for review see [23–26]). Here, in most cases, the genes have been cloned, but their functions in the nodulation process are still poorly understood.

Some of these genes are not expressed in any plant organ other than nodules and are termed nodulin genes [27]. The recent use of more sensitive methods to detect gene expression, has shown that several genes which were thought to be nodule specific, are actually expressed in other organs also. For convenience, they are still being called nodulin genes in this review. Nodulin genes are thought to be derived, either from the duplication of genes involved in nonsymbiotic processes (nodulin genes sensu strictu) like in the case of leghemoglobins [28] and nodulin-26 [29], or to be genes recruited from other developmental programs, like the early nodulin genes *ENOD12* [30] and *ENOD40* [31,32]. Nodulin genes expressed before the onset of nitrogen fixation are called early nodulin genes (*ENOD*) and are probably involved in building up the nodule structure and in the infection process. Nodulin genes expressed at or after the onset of nitrogen fixation, are termed late nodulin genes (*NOD*) [33]. In general, late nodulins are involved in the metabolic specialization of the nodule. For example, leghemoglobin is the most abundant late nodulin in legume nodules. It works as an O_2 carrier in the central tissue of nodules, transporting O_2 to the sites of respiration [34].

Bacterial Nod factors. The rhizobial signal molecules that induce the early steps of legume nodulation, are the so-called Nod factors which are lipochito-oligosaccharides containing a backbone of 4-5 *N*-acetylglucosamine residues and a fatty acid at the nonreducing terminal sugar residue (Fig. 1C) (for reviews see [35–37]). All rhizobia secrete similar lipochito-oligosaccharides, but their host specificity is determined by substitutions at the terminal sugar residues. For example, in the case of *R. meliloti*, the major host determinant is a sulfate group at the reducing sugar residue [38,39]. The biosynthesis of Nod factors is mediated by enzymes encoded by the rhizobial *nod* genes (for reviews see [40,41]), whose expression is induced by flavonoids excreted by the plant roots (Fig. 1C) [42]. One of the *nod* genes, *nodD*, is expressed constitutively, and upon binding of host flavonoids, the NodD protein activates the transcription of the other *nod* genes (Fig. 1C) [43]. Nod factors can induce several responses in the host plant [39,44–48], as will be described in the following parts of this review. In some cases, as in *Medicago sativa* and *Glycine soja*, purified Nod factors are even sufficient to induce the formation of bacteria-free nodules [35,37,49].

Root hair deformation. Basically, two different ways of rhizobial infection are known: infection through deformed root hairs, and infection via so-called crack entry, where the bacteria enter the plant root through gaps in the epidermis [50,51] or enter between intact epidermal cells [52]. The latter mode of infection only occurs in some tropical legumes. The infection through deformed root hairs is the most frequently

used way, and will be described in detail in this chapter.

During root hair infection, the first microscopically visible response of the host plant on rhizobial infection, is the deformation and curling of root hairs (Figs. 1C and 2A) [53]. Microscopical studies have shown that root hair deformation is due to a new induction of root hair tip growth by the Nod factors [54]. This process is accompanied by the induction of several host genes. Examples are *Mtripl* [55], encoding a peroxidase and the early nodulin genes *ENOD5* and *ENOD12* that encode proline rich polypeptides, which probably represent cell wall components [30,56]. Purified Nod factors can induce root hair deformation, and also the expression of the above mentioned plant genes [39,47,57]. Also nodulation by crack entry depends on Nod factors [58]. Nod factors act in concentrations as low as 10^{-12} M, suggesting that they are recognized by a receptor in the root epidermis [54]. Studies on gene induction by Nod factors, have shown that the length of the N-acetylglucosamine backbone as well as the modifications at the terminal sugar residues, are crucial for

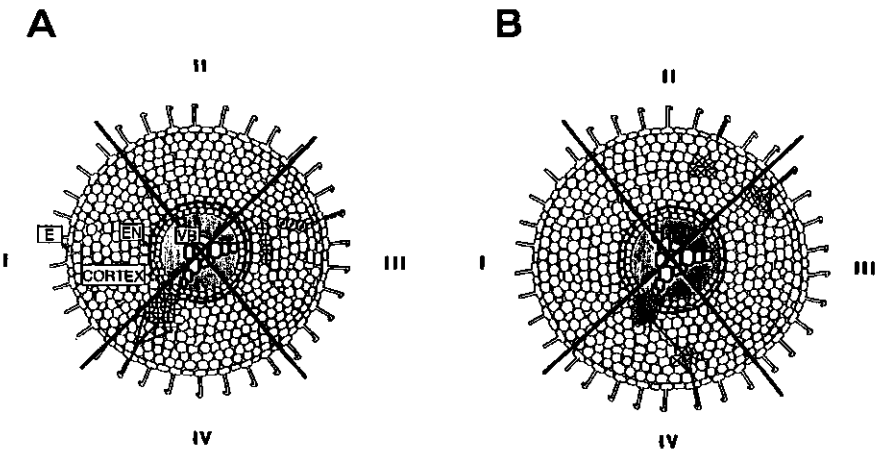

Fig. 2. Induction of the nodule primordium via root hair deformation in legumes and actinorhizal plants.

The different steps in nodule primordium induction are indicated in root cross sections. A: Induction of an indeterminate legume nodule. Stage **I** shows an uninfected root. Stage **II**, Nod factors secreted by the bacteria induce deformation and curling of root hairs. Stage **III**, after root hair deformation, an infection thread is formed in the curled root hair by which the bacteria enter the plant. At the same time, cell divisions are induced in the inner cortical layers. The outer cortical cells form preinfection thread structures preparing the passage of the infection thread. Stage **IV**, the infection thread has reached the nodule primordium in the inner cortex and cells of the primordium become infected by *Rhizobium*. (Abbreviations: E = epidermis consisting of atrichoblasts and trichoblasts (forming root hairs); EN = endodermis; VB = vascular bundle consisting of pericycle (outer layer), phloem, cambium, and xylem.) The protoxylem cells are indicated. **B**: Induction of an actinorhizal nodule. Stage **I** shows an uninfected root. Stage **II**, after root hair deformation, an infection thread-like structure is formed by which bacterial hyphae enter the plant, encapsulated in plant cell wall material. Concomitant with formation of the infection thread-like structure, cell divisions are induced in the outer cortical layers. Stage **III**, the encapsulated hyphae have grown towards the dividing cortical cells and infected them, resulting in the formation of a prenodule. Cell divisions are induced in the pericycle of the nodule vascular bundle. Stage **IV**, the encapsulated hyphae grow from the prenodule to the nodule primordium and infect cells of the primordium.

the induction of responses in the root epidermis [39,47]. However, since the presence of a fatty acyl moiety is essential but its structure is not important, probably the receptor does not recognize this part of the molecule.

Cytological studies have shown that the expression of *ENOD12* and *Mtrip1* is induced in all epidermal cells of a zone of the root, starting above the root tip even before root hairs have emerged, and extending to the region containing mature root hairs [55,59]. A direct contact between Nod factors and epidermal cells is required for the induction of such genes [39]. Thus, it is likely that within the zone of the root able to respond to Nod factors, they are recognized by all epidermal cells, not only by those containing root hairs. The function of the Nod factor induced plant genes is unclear. However, it is unlikely that all of these genes are essential for the infection process, since alfalfa plants lacking *ENOD12* can form effective nodules when inoculated with *Rhizobium* [60].

Infection thread formation. When rhizobia induce root hair curling, they become entrapped in the curls. There they induce the formation of infection threads in the crooks of curled root hairs, beginning with a local hydrolysis of the plant cell wall (Fig. 2A) [61,62]. At the site of hydrolysis the plasma membrane grows inward and new cell wall material is deposited along the invaginating plasma membrane ([62,63]; for reviews see [64,65]). This way, a tubular structure, the so-called infection thread, is formed by which the bacteria enter the plant [66]. The mechanism by which this local hydrolysis of the plant cell wall is achieved is unclear, but it seems unlikely that hydrolytic enzymes secreted by the bacteria can establish such a localized effect. Hence, it has been suggested that the bacteria induce the local secretion of hydrolytic enzymes by the plant which also happens when a trichoblast forms a root hair (for review see [65]) Bacteria inside the infection thread are surrounded by a matrix, which contains (glyco-) proteins and other compounds of the plant as well as compounds secreted by the bacteria [67]. The infection thread wall is most likely of plant origin, and has an ultrastructure similar to that of the plant cell wall [68]. The products of the early nodulin genes *ENOD5* and *ENOD12*, have been suggested to be involved in infection thread formation, since *ENOD5* and *ENOD12* are transcribed in cells containing an infection thread tip, and *ENOD12* expression is also induced in the dividing cortical cells in front of the infection thread [30,56]. It has been proposed that infection thread growth resembles the development of root hairs, but the direction of growth is inverted [46]. Thus, the mechanisms controlling initiation as well as growth of the infection thread, might be derived from root hair development.

During infection thread formation, root cortical cells are mitotically activated and form nodule primordia (see below). The infection thread grows toward the base of the root hair and subsequently toward the nodule primordium. If the primordium is formed by inner cortical cells (see below), the infection thread has to cross the outer cortex to reach the nodule primordium. Prior to infection thread penetration, the cortical cells between the infected root hair and the nodule primordium are activated and form radial tracks of cytoplasm (Fig. 2A) [62]. Such cytoplasmic structures are

called "preinfection threads" and resemble phragmosomes [46]. Therefore, it was postulated that the cells forming a preinfection thread enter the cell cycle, although they do not divide [46]. Studies on expression of cell cycle specific genes have proven that the cortical cells forming a preinfection thread indeed enter the cell cycle and become arrested in the G_2 phase [69]. Thus, preinfection thread formation is derived from the cell cycle machinery.

The infection thread penetrates root cortical cells by local hydrolysis of the cell wall and grows through the preinfection thread structures to the nodule primordium, where bacteria are endocytotically released into the plant cells and differentiate into their symbiotic form, the bacteroids [70,71].

When the plants are not infected through deformed root hairs, but by crack entry, the situation is less uniform with regard to infection thread formation. In case of *Neptunia* and during stem nodule induction on *Sesbania rostrata*, infection threads are formed when the bacteria have entered the plant [72–74], while in roots of *Arachis hypogaea*, intercellular infection centers develop and bacteria enter the cells of the nodule primordium directly via invagination of the plant plasma membrane, without infection thread formation [75]. In *Stylosanthes* and *Aeschynomene*, a similar process takes place without the formation of infection centers [50,76].

Cortical cell divisions and nodule meristem formation. Concomitant with infection of root hairs, root cortical cells, mostly opposite a protoxylem pole of the root stele [77], are activated and start dividing (Fig. 2A). Several nodulin genes are expressed in the dividing cortical cells. Examples are *ENOD12* [30], *ENOD40* [31,32,78,79] and *Gm*N93 [31]. Which of the root cortical cells divide, is determined by the plant [80,81]. In temperate legumes such as pea, vetch and alfalfa, inner cortical cells divide and form the nodule primordium [71,82]. When the infection threads reach the primordia, they ramify, and cells at the base of the primordium are infected. At the same time, a meristem is formed at the distal part of the primordium, consisting of small cells with dense cytoplasm [82]. The nodule meristem differentiates during the complete nodule life time into the different cell types that build up the nodule. Consequently, these nodules have an indeterminate development like lateral roots.

Nod factors have the ability to induce the formation of nodule primordia [48]. For this action, they have to be present in higher concentrations, and to fulfill more stringent structural requirements than for the induction of responses, in the root epidermis [83]. Thus, it has been postulated that at least two different Nod factor receptors are present in the root epidermis: a "signaling receptor" involved in the induction of reactions in the epidermis, and an "uptake receptor" that initiates the infection process and is activated only by a very specific structure [83]. However, the mechanism by which they induce mitotic activity in the cortex, is not completely understood. Cytokinin and compounds that block the polar transport of auxin, phenocopy the Nod factors, since they can cause the formation of nodule-like structures [84,85]. Therefore it is assumed that Nod factors cause a change in the cytokinin/auxin balance which subsequently results in the mitotic reactivation of cortical cells. Before cell division occurs, expression of the early nodulin *ENOD40*

is induced in the regions of the root pericycle opposite to a protoxylem pole (W.-C. Yang and T. Bisseling, unpublished results). Thus, a tissue even deeper inside the root responds faster to Nod factors than the cortex. It is possible that *ENOD40* expression in the pericycle is involved in a process which leads to a change in hormone balance or perception in the cortex, which finally causes cortical cells to divide. This hypothesis is based on the observation that *ENOD40* is affecting the response to auxin when expressed in tobacco protoplasts (K. Pawlowski, K. van de Sande, R. Walden and T. Bisseling, unpublished results).

In tropical legumes such as soybean, outer cortical cells of the roots divide to form the nodule primordium, while the inner cortical cells between the primordium and the stele are activated to divide and will in turn form the connection with the vascular bundle of the root. The growing infection threads directly invade primordium cells after penetrating the root hair (for review see [81]). Cells at the periphery of the primordium remain mitotically active and form a spherical meristem which loses its activity at an early stage of development. Thus, these nodules have a determinate growth pattern and are called determinate nodules.

Infection of cells by Rhizobium. When the infection thread has reached the nodule primordium, bacteria are released from the infection thread into the cytoplasm. During the release, the bacteria become surrounded by a plant-derived membrane, the peribacteroid membrane (PBM; Fig. 3A,B). The bacteria, together with the space within the PBM (peribacteroid space, PBS), and the PBM, form a functional structure called symbiosome [86]. The PBM works as an interface between both symbiotic partners, controlling the metabolite exchange. In accordance with its specialized function, it is different from the plasma membrane, from which it is derived, in phospholipid and protein composition [24,87]. It has been suggested that the PBM has obtained some properties of the membrane of the vacuole [24,88,89] since within the PBS, hydrolytic enzymes have been found which are also present in vacuoles [90,91]. An integral PBM protein, nodulin-26, is targeted to the vacuolar membrane when expressed in tobacco, supporting the hypothesis of the similarity between PBM and vacuolar membrane [92]. In the same line of argument, it was also proposed that the symbiosome resembles a lytic compartment, similar to the vacuole, that the bacteroides continuously have to neutralize by exporting ammonia, be a product of nitrogen fixation, in order to avoid being degraded by the plant [93]. Thus, rather than a symbiosis, the interaction between rhizobia and legumes would constitute a case of parasitism of the plant on the bacteria. This hypothesis is supported by the fact that for *Rhizobium* mutants unable to fix nitrogen, premature degradation of bacteroides can be detected in the infected cells of the nodules [94].

Legume nodule structure

Determinate and indeterminate legume nodules have a similar tissue organization, a central tissue where bacteria are hosted, surrounded by several peripheral tissues (Fig. 4A) (for review see [25,64,71]). The peripheral tissues comprise the nodule cortex, the endodermis and the nodule parenchyma [95]. The latter tissue harbors the

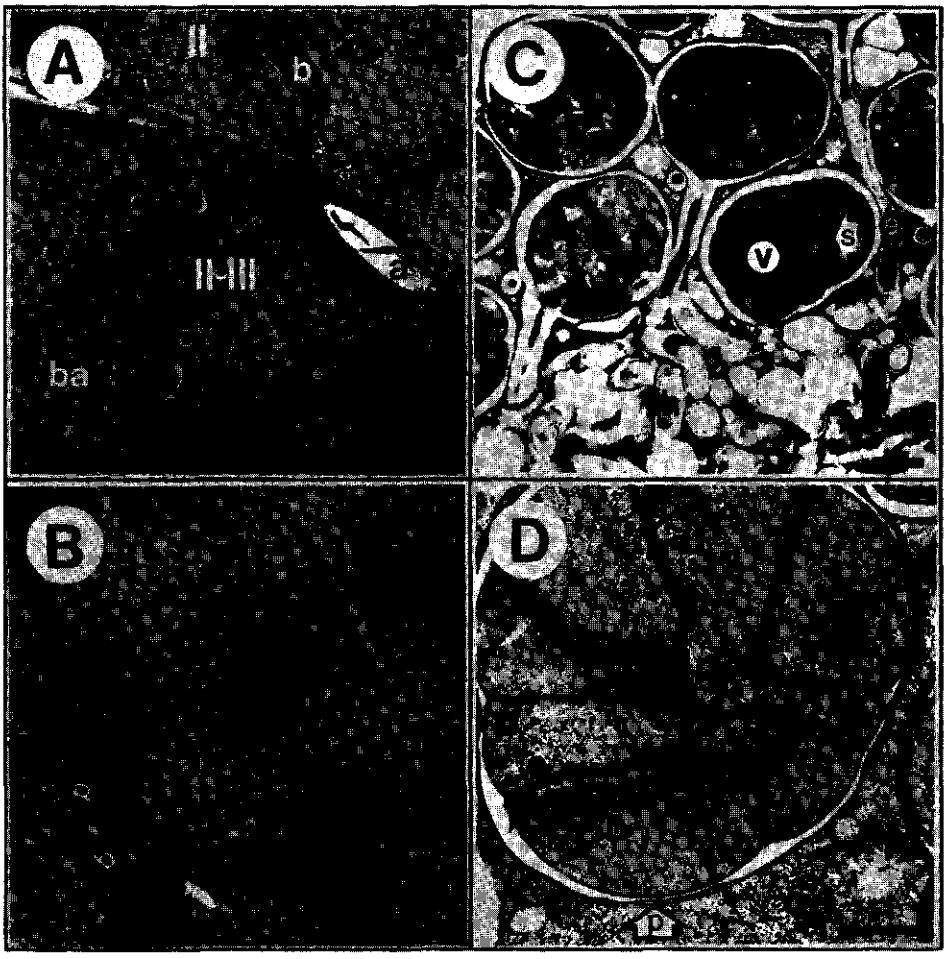

Fig. 3. Nitrogen-fixing endosymbionts in *Rhizobium*/legume and actinorhizal symbioses.

A: Intracellular rhizobia in a nodule formed on clover by *R. trifolii*. This region of the indeterminate clover nodule shows the transition of the prefixation zone II to the interzone II-III. In the upper cell (II, prefixation zone), intracellular bacteria (b) have not yet differentiated into their nitrogen-fixing form. The bottom cell (II-III, interzone) contains amyloplasts (a), and nitrogen-fixing bacteroides (ba) have differentiated. In both cells, intracellular bacteria are surrounded by a peribacteroid membrane. Bar = 1 µm. **B**: Detail – the bacterial membrane (b) and the peribacteroid membrane (p) which separate the bacteroides (ba) from the cytoplasm (cy) can be clearly distinguished. Bar = 500 nm. The photographs were kindly provided by U. Bialek and A. van Lammeren, Department of Cytology, Agricultural University Wageningen, The Netherlands. **C**: Intracellular *Frankia* in a nodule formed on *Alnus serrulata*. Vegetative hyphae (h) and nitrogen fixing vesicles (v) can be seen. Arrows point at the lipid envelope of a vesicle (e) and at a sept in a vesicle (s). Bar = 1 µm. **D**: Detail – a vesicle is separated from the plant cytoplasm (cy) by its own membrane (b), the lipid envelope (e) and the invaginated plant plasmamembrane (p). Arrowheads point at the plant cell wall-like encapsulation material between plant plasmamembrane and vesicle lipid envelope. Bar = 500 nm. Photographs were kindly provided by H.M. Berg, Biology Department, University of Memphis, Tennessee, USA.

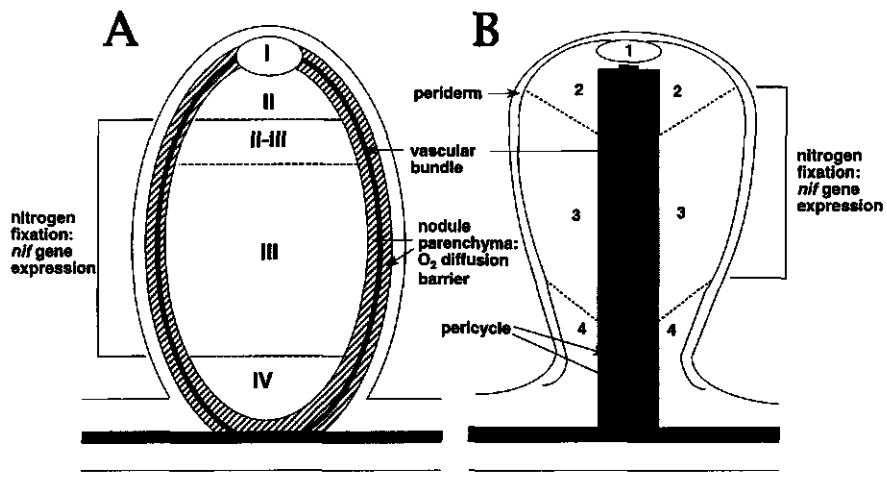

Fig. 4. Structure of indeterminate root nodules. **A**: Scheme of an indeterminate legume nodule. Zonation: I, nodule meristem; II, prefixation zone; II–III, interzone; III, nitrogen fixation zone; IV, senescence zone ([93]; see text). **B**: Scheme of an actinorhizal nodule from *Alnus glutinosa*. Zonation: 1, nodule meristem; 2, infection zone; 3, nitrogen fixation zone; 4, senescence zone ([144]; see text). The central vascular bundle contains a multilayered pericycle [136,219]. Due to the activity of the apical nodule meristem, a developmental gradient of infected cells forms in the central tissue (**A**) or in the cortex (**B**), respectively. The zones in which bacterial nitrogen fixation takes place are indicated for both types of nodules.

nodule vascular bundles. The central tissue consists of two cell types, infected and uninfected cells. The infected cells are fully packed with bacteria. A few cell layers of uninfected cells, the boundary layers, separate the central tissue from the nodule parenchyma [25,80].

Meristems of indeterminate nodules go on differentiating into the different nodule tissues. The effect is that the central tissue can be divided into several zones representing successive stages of development (Fig. 4A). A nomenclature has been developed for the successive zones of indeterminate nodules [96]. The meristem at the apex is designated as zone I. It consists of small cells with dense cytoplasm that are not infected by rhizobia. This zone is immediately followed by the prefixation zone II. In the distal part of this zone II, infection threads penetrate meristematic cells and bacteria are released into the plant cytoplasm. In the proximal part of the prefixation zone II, plant cells elongate and symbiosomes proliferate.

The interzone II–III is characterized by the start of starch accumulation in infected cells, and the presence of differentiated bacteroides (Fig. 3A,B) [96]. It is also marked by dramatic changes in gene expression by both plant and bacteria. For example, the bacteria induce expression of the N_2-fixation genes (Fig. 5A,B) [97], while the expression of bacterial *ropA* encoding an outer membrane protein is switched off [98]. The expression of the plant nodulin genes *ENOD5* and *ENOD40* [30,79] is strongly reduced at this transition, whereas several other nodulin genes like *NOD6*

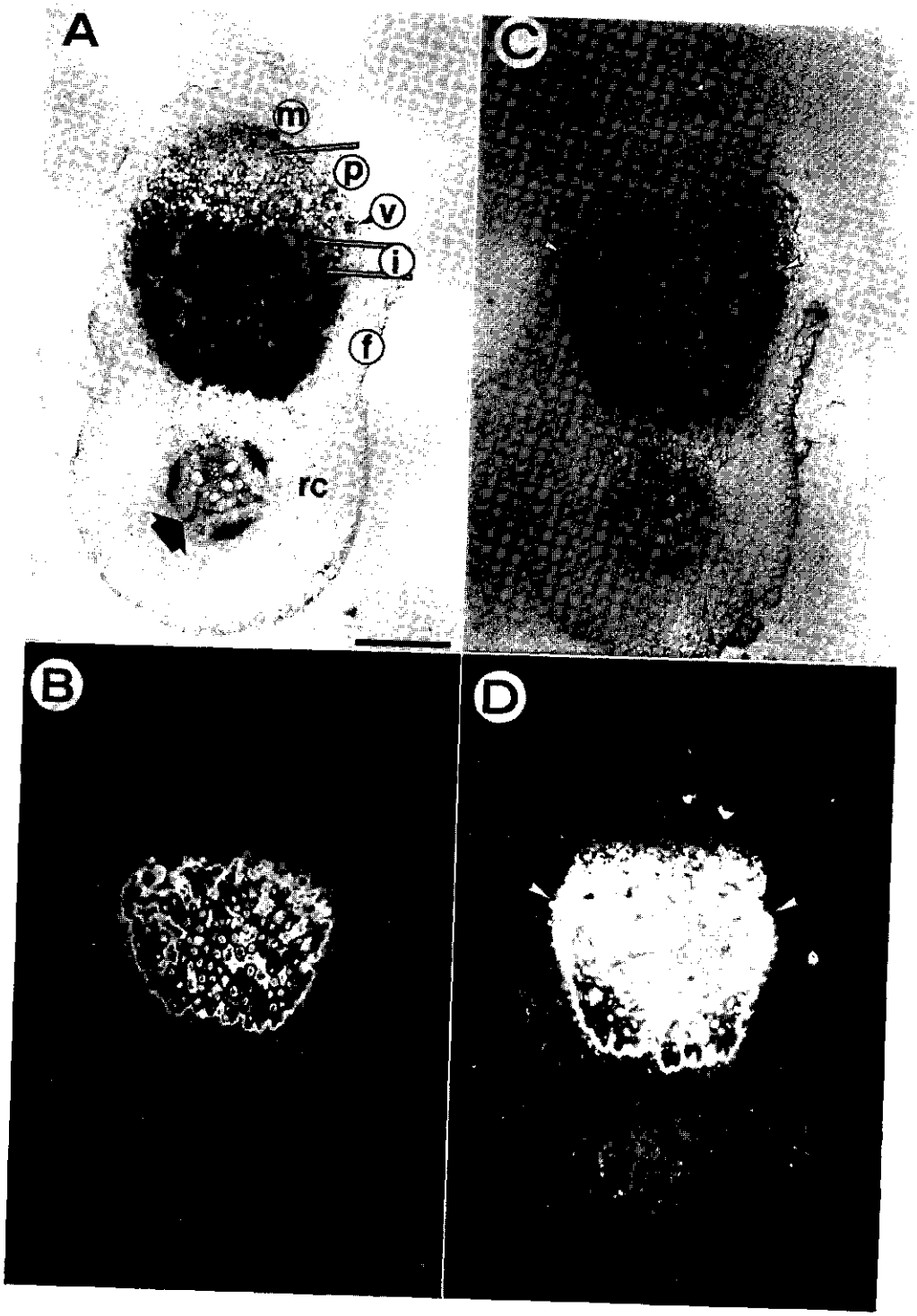
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Fig. 5. In situ localization of gene expression in indeterminate legume nodules.

In **A** and **C**, bright field microscopy was used; silver grains denoting hybridization appear in black. In **B** and **D**, darkfield microscopy was used; silver grains are visible as white dots. Due to the very high density of silver grains in some infected cells of both sections, the light scattering by darkfield illumination is impaired. **A** and **B**: Expression of a *Rhizobium leguminosarum* nitrogenase structural gene, *nifH*, in a longitudinal section of a 15-day-old pea nodule. The different zones of the developmental gradient are indicated: m, nodule meristem; p, prefixation zone; i, interzone; f, fixation zone. A senescent zone has not yet developed. *R. leguminosarum nifH* expression starts in the first cell layer of the interzone. A nodule vascular bundle (v) is indicated. An arrow points at a protoxylem pole of the root vascular bundle. The root cortex (rc) is labeled. **C** and **D**: Expression of a pea leghemoglobin (*lb*) gene in an adjacent section of the same nodule lobe. *lb* gene expression starts in the prefixation zone. The beginning of the interzone, i.e., the zone of *R. leguminosarum nifH* induction, is indicated by arrowheads. Bar = 500 μm.

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are induced [99]. The signal or mechanism that controls this developmental switch is not yet understood, although there is evidence that the O_2 concentration is involved in the induction of bacterial *nif* genes ([100]; see below).

In the nitrogen fixation zone III, the plant cells have reached their maximal size and bacteroides are fixing nitrogen. In older nodules a senescent zone IV is present. Senescence of nodule tissues has hardly been studied at the molecular level. Based on analogy to other senescent organs, it is likely that the expression of genes encoding hydrolytic enzymes like proteases and RNases will be induced in this zone. Indeed proteases, e.g., thiol proteases, have been found to be active in senescent nodules ([101,102] and references contained therein). A nodulin gene specifically expressed in senescent nodules, has been isolated from winged bean and found to encode a proteinase inhibitor [103]. Protease-inhibiting activity has also been found in the peribacteroid space of soybean nodules [104]. These data suggest that the plant has developed a system to control bacteroid senescence.

Actinorhizal symbioses

A rather diverse group of plants from eight different families have the ability to establish a symbiosis with *Frankia* bacteria resulting in actinorhizal root nodules. Up to now, about 194 actinorhizal plant species from 24 genera have been identified (for review see [105]). *Frankia* is a filamentous gram-positive actinomycetous bacterium (reviewed in [106,107]). In contrast to *Rhizobium*, *Frankia* normally grows in hyphal form, being able to form also two other specialized cell types, namely vesicles, the sites of N_2 fixation (see below), and sporangia. Although actinorhizal nodules were first described in 1829 [108], only in 1895 it was shown that they contributed to the nitrogen nutrition of the plant [109]. The identification of the microsymbiont as an actinomycete finally took place in the 1930s [110,111].

Due to their symbiosis with *Frankia*, actinorhizal plants can grow on marginal soils. They are used in soil reclamation and reforestation, for timber-, fuelwood-, and pulp production, as windbreak plants in desert agroforestry systems and also to stabilize coastal sand dunes in tropical and subtropical countries [112–120].

Frankiae have not been classed into species thus far. Physiological criteria could only be used to define two broad groups of strains [121]. As in the case of *Rhizobium*-legume symbioses, Frankiae show different degrees of host specificity, but here the attempt to use the host specificity as a taxonomic criterion has proven impracticable [122]. Meanwhile, *Frankia* strains have been isolated from several host plants and can be grown in culture (for review see [105]). During isolation, some strains require the addition of a root steroid, dipterocarpol, for initial growth stimulation [123,124]. The inability to isolate *Frankia* strains from some actinorhizal plant families, for example Datiscaceae and Coriariaceae, might reflect special requirements of these strains for growth stimulation. For the strains cultured thus far, four major host specificity groups have been defined [125]: group 1 includes those strains capable of nodulating *Alnus*, *Comptonia*, *Myrica* and *Gymnostoma*, group 2 includes strains inducing nodules on *Casuarina*, *Allocasuarina*, *Myrica* and *Gymnostoma*, group 3 includes *Frankia* strains able to nodulate *Elaeagnus*, *Hippophae*, *Shepherdia*, *Myrica* and *Gymnostoma*, and group 4 includes strains that are able to nodulate either *Alnus* and *Comptonia*, or *Casuarina* and *Allocasuarina*, or *Elaeagnus*, *Hippophae*, and *Shepherdia*, but not *Myrica* or *Gymnostoma*. Strains which do not fit into this scheme are referred to as atypical, an expression also used for strains that are not able to reinfect their host plant from whose nodules they were isolated (for review see [105]).

Induction of actinorhizal nodules

As in *Rhizobium*/legume symbioses, there are two ways known by which *Frankia* can initiate a symbiotic relationship with a compatible host plant, namely root hair infection, observed in *Alnus*, *Casuarina*, *Comptonia*, and *Myrica* [126–128] and intercellular penetration which has been reported for *Elaeagnus* and *Ceanothus* [129,130]. Also in actinorhizal symbioses, the mode of infection is plant-determined [129,131].

Root hair infection starts with the deformation of the root hairs (Fig. 2B) [132]. There is no need for direct contact between the host plant cells and *Frankia* in order to induce root hair deformation [133,134]. Therefore it has been suggested that, in analogy to the signalling between *Rhizobium* and legumes, plant root exudates may stimulate the synthesis and/or release of a diffusible "Nod" factor by *Frankia* that in turn causes root hair deformation [134]. To date, the characterization of *Frankia* "*nod* genes" has not been reported, but it has been observed that (a) factor(s) present in the supernatant of a *Frankia* culture can cause root hair deformation on *Alnus glutinosa* (M. van Ghelue, E. Løvaas, E. Ringø and B. Solheim, personal communication). This suggests that like in the *Rhizobium*/legume symbiosis, the interaction indeed is initiated by an exchange of signals between the two symbionts.

Upon root hair deformation, *Frankia* hyphae associated with deformed root hairs, initiate digestion of the primary root hair cell wall and as a response, the host plant starts to build up a cell wall-like matrix around the microsymbiont (Fig. 2B) [135]. In this way, a tubular ingrowth, termed encapsulation, is created which functionally resembles the infection thread observed in the *Rhizobium*-legume symbiosis, and like the latter, grows through cortical cells (Fig. 2B) [132]. However, no equivalent of the

infection thread matrix exists in actinorhizal symbioses, but the hyphae are surrounded by the cell wall-like material of the encapsulation, equivalent of the infection thread wall in *Rhizobium*/legume symbioses. In response to the invading microsymbiont, root cortical cells proximal to the infected root hairs start to divide and enlarge, giving rise to the so-called prenodule whose cells enlarge further upon infection by encapsulated hyphae (Fig. 2B) [136]. Thereupon, cell divisions are induced in the pericycle resulting in the formation of a nodule lobe primordium, that upon infection develops into a nodule lobe. Thus, while initially, *Frankia*, like *Rhizobium*, induces cell divisions in the nodule cortex, the final nodule primordium is formed in the root pericycle like a lateral root primordium. Like in *Rhizobium*/ legume symbioses, the primordia of actinorhizal root nodules are formed mostly opposite to a protoxylem pole of the root stele [137].

Encapsulated hyphae grow from the prenodule towards the nodule primordium, thereby again crossing cortical cells [136]. After entering the nodule primordium, *Frankia* hyphae infect part of the primordium cells. During infection, the plant plasma membrane invaginates and encapsulating material is continuously deposited around the growing hyphae (for review see [132]). Thus, like *Rhizobium*, *Frankia* is surrounded by a membrane derived from the plant plasma membrane when it is present in the host cell (Fig. 3C,D). However, in contrast to *Rhizobium*, *Frankia* bacteria remain in the infection thread-like structures and are not released endocytotically. After infection, *Frankia* hyphae grow until they occupy most of the volume of the infected cell. Then, specialized vesicles are formed in which nitrogenase is expressed (Fig. 3C,D) [138,139]. From now on, new cortical cells are formed from the nodule meristem and these become infected by hyphae progressively. In this way a nodule with an indeterminate growth pattern is formed (Fig. 4B).

The process of infection by intercellular penetration is more primitive. *Frankia* hyphae enter the root by partial digestion of the middle lamella between adjacent epidermal cells and move on strictly intercellularly [129,131], while epidermal and cortical cells secrete some pectinaceous and proteinaceous material into the intercellular space [140]. No prenodule is formed, but immediately upon intercellular colonization of the root cortex, cell divisions are induced in the root pericycle resulting in the formation of the nodule primordium. While in *Rhizobium*/legume symbioses, only a few cases of infection by crack entry are known, in actinorhizal symbioses infection by intercellular penetration seems to take place in most actinorhizal plant families except for Betulaceae, Myricaceae and Casuarinaceae.

In legumes it has been shown that before an infection thread traverses a cortical cell, a dramatic rearrangement of the cytoplasm occurs. In a normal cortical cell the cytoplasm including the nucleus, is located at the periphery of the cell. Before a cortical cell is penetrated by the infection thread, the nucleus moves to the center of the cell and the cytoplasm obtains a radial polar organization which is named preinfection thread. The preinfection thread forms the pathway that the infection thread follow on their way to the nodule primordium, and the polar organization of the cytoplasm seems to be essential to support the polar growth of the infection thread [46]. Preinfection threads are reminiscent of phragmosomes, suggesting that

the corresponding cells enter the cell cycle and become arrested in the G_2 phase. This hypothesis was confirmed by analysing the expression of cell cycle specific genes [69]. Preinfection thread formation has not been studied in actinorhizal nodulation but it seems very likely that in case of infection via root hair deformation, preinfection threads have to be formed also here. Therefore it is striking that infection via root hairs is correlated with the induction of cell divisions in the cortex, although no specific function has been assigned to the dividing cortical cells forming the prenodule. We hypothesize that in actinorhizal plants, when infection threads traverse cortical cells, preinfection thread structures have to be formed, implying that cortical cells enter the cell cycle and become arrested in the G_2 phase. However, in some cortical cells this arrest is not established, leading to cell division, although this is not functional in the infection process. This hypothesis is supported by the fact that after infection of *Alnus* by *Frankia*, irregular undulated cell walls in combination with bundled arrays of microtubules, were found in postmeristematic cells proximal to the root meristem and the nodule meristem [141], indicating nonfunctional activation of the cell cycle machinery.

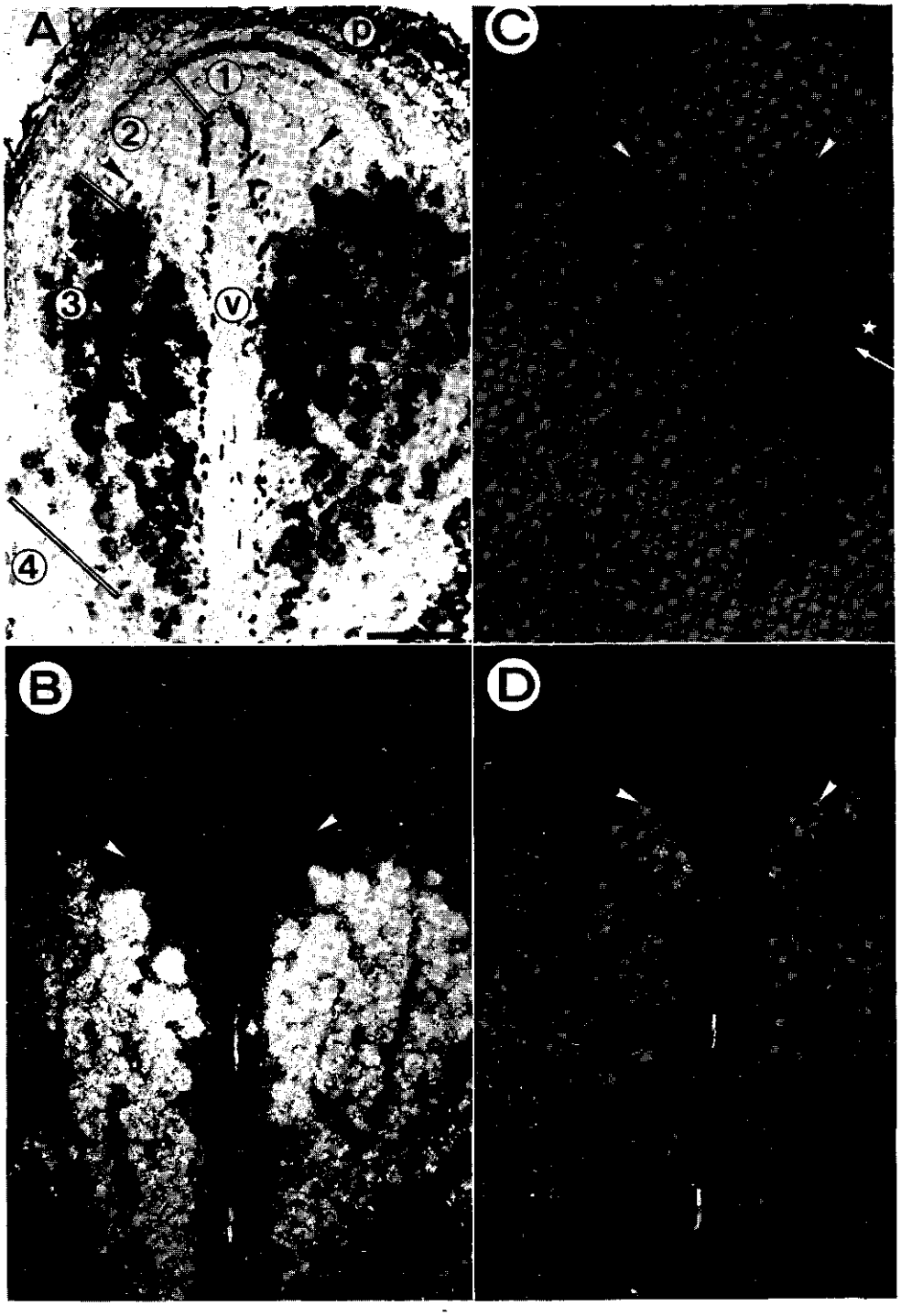
Structure of actinorhizal nodules

Actinorhizal nodules are perennial structures consisting of multiple lobes [142]. By ontogeny, as well as by tissue organization, each nodule lobe represents a modified lateral root with a central vascular cylinder. However, actinorhizal nodule lobes differ from lateral roots, in that they lack a root cap, have a superficial periderm, and contain both infected and noninfected cortical cells (Fig. 4B) (for review see [132,143]).

Like in the case of temperate legumes, actinorhizal nodule lobes have an indeterminate growth pattern due to the presence of an apical meristem that differentiates continuously in a proximal direction [132]. Depending on the developmental stage of the infected cortical cells, a zonation of the nodule lobe can be observed (Fig. 4B) [144]. Thus, starting from the distal end of the lobe, four zones can be distinguished. Zone 1, the meristematic zone, consists of small dividing cells that do not contain bacteria. Zone 2, the infection zone, corresponding to the prefixation zone in legume nodules, contains enlarging cortical cells, some of which

Fig. 6. In situ localization of gene expression in actinorhizal nodules of *Alnus glutinosa*.

In **A** and **C**, bright field microscopy was used; silver grains denoting hybridization appear in black. In **B** and **C**, darkfield microscopy and epipolarized light were used; silver grains are visible as white dots. **A** and **B**: Expression of a *Frankia* nitrogenase structural gene, *nifH*, in a longitudinal section of a nodule lobe. The different zones of the developmental gradient are indicated: 1, meristematic zone; 2, infection zone; 3, fixation zone; 4, senescence zone. Arrowheads point at infected cells of zone 2 that are not yet completely filled with hyphae and do not contain vesicles. These cells show no *Frankia nifH* expression. The central vascular bundle (v) and the periderm (p) are indicated. **C** and **D**: Expression of *A. glutinosa ag12* in an adjacent longitudinal section of the same nodule lobe. Arrowheads point to infected cells of zone 2 that are not yet completely filled with hyphae; these cells show high *ag12* expression levels. An infected cell of zone 3 showing *ag12* expression at a high level is marked by a star. An arrow points at an adjacent cell which shows little *ag12* expression. Bar = 500 µm. This is a modified version of Figure 3C-3F of Ribeiro et al. [144], reprinted with the permission of the American Society of Plant Physiologists.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are infected and in turn, enlarge more than uninfected cells while being gradually filled with hyphae from the center outward [139,145]. Once the infected cells are completely filled with hyphae, provesicles are formed as terminal swellings on hyphae or on short side branches [146]. In zone 3, the fixation zone, provesicles differentiate into vesicles. During this step of differentiation, the synthesis of nitrogenase, the enzyme responsible for the reduction of atmospheric nitrogen in ammonia, is induced [147]. The expression of the structural *nif* genes encoding nitrogenase is a marker for the shift from zone 2 to zone 3 (Fig. 6A) [144,148]. In zone 4, the senescence zone, cortical cells become senescent and the microsymbiont as well as the host cytoplasm is degraded. This zonation of the nodule cortex has been found to be applicable to nodules of *Alnus glutinosa*, *Casuarina glauca* and *Ceanothus griseus*, where infected cells are distributed over the cortex. However, in *Datisca* and *Coriaria*, where only a defined area of the cortex can be infected (see below), the developmental pattern is more complicated ([144]; K. Pawlowski and A.M. Berry, unpublished results).

Due to the structural similarities between actinorhizal nodules and lateral roots, the products of nodule-specific genes can be expected to be involved in either of three processes. First, genes whose products are involved in the developmental shift from lateral root to nodule development, would determine the difference between lateral root- and nodule meristems and therefore should be differentially expressed in the respective meristems. Second, genes whose products are involved in the infection process should be expressed in the young infected cells. Third, there could be nodule-specific genes whose products are involved in the metabolic specialization of the nodule, i.e., in the assimilation of the ammonium exported by symbiotic *Frankia*, or in the transport and synthesis of carbon sources for the bacteria. Since actinorhizal plants mostly represent woody shrubs or trees, recalcitrant to molecular biological analysis [149], their nodule-specific genes have not been examined as thoroughly as those of legumes. Only recently, actinorhiza-specific genes have been cloned from *Casuarina* [150,151] and *Alnus* [144,152,153]. One nodule-specific gene from *Alnus*, *ag12*, was found to be expressed at the highest levels in the infected cells of the infection zone 2, i.e., in cells where *nif* genes have not been induced yet (Fig. 6C,D) [144]. *Ag12* encodes a serine protease which thus seems to be involved in the infection process. Another nodule-specific gene family has been found ([154]; K. Pawlowski, C. Guan and T. Bisseling, unpublished results) showing an expression pattern similar to that of *ag12*. These genes encode glycine-rich proteins with a signal peptide indicating that they might be localized in the cell wall. Thus, the infection process and the interaction with the bacterial symbiont, appear to involve sets of nodule-specific genes. Several other genes were found to be expressed at elevated levels in *Alnus* nodules compared to roots [153]. Their products mostly were involved in nodule nitrogen and carbon metabolism, i.e., in the metabolic specialization of nodules. No member of the putative group of genes important for the developmental shift from root to nodule development has been identified yet.

Actinorhizal and legume nodule metabolism

Legume nodules as well as actinorhizal nodules have to provide a suitable environment for nitrogen fixation by the endosymbiont, i.e., they have to protect bacterial nitrogenase from O_2, supply the intracellular bacteria with carbon sources and assimilate the product of N_2 fixation, ammonium, which is exported by the bacteria. Furthermore, in the context of the complete plant, nodules represent carbon sinks and nitrogen sources: efficient transport of carbon sources to the nodules and of nitrogen sources from the nodules has to be provided. Different strategies have been adopted to fulfill these requirements that will be discussed in the following paragraphs.

Oxygen protection of bacterial nitrogenase

While nitrogenase itself is O_2 sensitive, the high amount of energy required for the nitrogenase reaction has to be generated by oxidative processes, leading to a high demand for O_2 in nodules. To meet these conflicting demands, different strategies have been developed. For legume nodules, physiological studies have shown that the nodule parenchyma forms an O_2 diffusion barrier (Fig. 4A) [155]. This, together with the high O_2 consumption rate of *Rhizobium*, leads to a low O_2 concentration in the central tissue of the nodule, while in the infected cells, high levels of the O_2-carrier protein leghemoglobin facilitate O_2 diffusion to the sites of respiration (Fig. 5C,D) [156,157]. Since in indeterminate legume nodules, the nodule parenchyma is interrupted by the meristem at the distal end of the nodule, an O_2 gradient is formed (Fig. 4A). Data on bacterial nitrogenase gene expression in the free-living state have shown that nitrogenase expression is induced by low O_2 tension [158]. It has been suggested that this type of regulation may also play a role in symbiosis, where nitrogenase gene expression starts in the first layer of interzone II–III (Fig. 5A,B). In fact, when overall O_2 concentration in alfalfa nodules was reduced by submerging the nodules in agar, the nitrogenase structural gene *nifH* was expressed also in the prefixation zone [101], confirming the role of O_2 in *nif* regulation.

O_2 is generally assumed to diffuse via the intercellular spaces, because its diffusion is about 10^4 times faster in air than in water. The O_2 diffusion barrier in the nodule parenchyma is established by cell layers in which the size of the intercellular spaces can be controlled [157], presumably by the release and uptake of intercellular water in the nodule parenchyma [159–161]. Furthermore, nodulins like *ENOD2* which are specifically expressed in the nodule parenchyma might contribute to the formation of the O_2 diffusion barrier [95].

Actinorhizal nodules are structurally rather diverse (for review see [143]). An example of this diversity are the O_2 diffusion pathways. In order to provide O_2 access to the sites of respiration, i.e., N_2-fixing *Frankia* vesicles [162] and plant mitochrondria, O_2 has to pass through the nodule periderm and reach the infected cells via intercellular spaces. To provide O_2 access through the periderm, two strategies have been developed: either the periderm is disrupted by lenticels like in some legume nodules, or agraviotropically growing nodule roots containing large air spaces, are

protruding from the lobes [163]. The O_2 concentration can affect nodule anatomy, such as causing changes in the size of lenticels in *Alnus* and *Coriaria*, in the thickness of the periderm in *Coriaria* or in the length of nodule roots in *Myrica* [164–166]. There is also variability in the arrangement of infected cells in the cortex. While the infected cells are distributed over the nodule cortex, interspersed with uninfected cells, in nodules formed by *Alnus*, *Casuarina*, *Ceanothus* and *Myrica*, in nodules formed by *Coriaria* or *Datisca* they are arranged in a continuous kidney-shaped patch at one side of the acentric stele, not interspersed with uninfected cells [166,167].

The mechanisms of O_2 protection among actinorhizal plants diverge considerably as well. In contrast to *Rhizobium*, *Frankia* can fix nitrogen also in the free-living state at atmospheric O_2 concentrations [168]. This is achieved by the location of the O_2 sensitive nitrogenase, in special vesicles which provide O_2 protection by their outer envelopes consisting of multilayered hopanoid membranes (Fig. 3C,D) [169,170]. In symbiosis the shape and position of the vesicles in the infected cells is determined by the host plant [131]. While vesicles formed in culture are spherical, in symbiosis their shape [107], envelope morphology [171], and internal structure (septate or nonseptate) (for review see [107,172]) depends on the host plant. Vesicles can also contribute to oxygen protection in symbiosis. The symbiotic vesicles have a high respiratory capacity [162], thereby further decreasing the amount of O_2 in the direct neighbourhood of nitrogenase. A different situation is found in *Casuarina-* and *Allocasuarina* symbioses. Here, *Frankia* forms atypical hyphae instead of vesicles, for nitrogen fixation in the infected cells [173]. In these symbioses, but also in nodules of *Myrica* where *Frankia* forms vesicles [174], the infected cells are surrounded by an O_2 diffusion barrier, achieved by lignification of the cell walls of the infected cells and of the adjacent uninfected cells [175–179]. Furthermore, *Casuarina*, *Allocasuarina*, and *Myrica* synthesize high amounts of hemoglobin in the infected cells [151,180–182]. Hemoglobin is homologous to leghemoglobin, and like in the legume nodules it facilitates O_2 diffusion toward the sites of respiration. Hence, in some actinorhizal symbioses, like in legumes, the plants seem to be mainly responsible for providing O_2 protection to bacterial nitrogenase. However, in contrast to legumes, in actinorhiza both partners can contribute to O_2 protection, as signified by the formation of *Frankia* vesicles in nodules.

Hydrogen metabolism

As shown above, hydrogen (H_2) production is an obligatory part of the nitrogenase mechanism; furthermore, in the absence of other reducible substrates, the total electron flux through nitrogenase is funnelled into H_2 production [183]. H_2 is a competitive inhibitor of N_2 fixation [184]. Consequently, nitrogen fixers tend to express an uptake hydrogenase to oxidize H_2 to H_2O, resulting in O_2 consumption and energy (ATP) generation. In free-living *Frankia* as well as in cultures of some rhizobia, activation of hydrogenase expression by H_2 results in an increased nitrogenase activity [185–189].

Research on the benefits of uptake hydrogenase activity for symbiotic nitrogen

fixation, has yielded inconclusive data. For legume symbioses, where the effects of isogenic strains differing only in hydrogenase activity could be examined, contradictory results were obtained. While in some cases, hydrogenase activity was beneficial for the symbiotic performance of a rhizobial strain [190–192] in others it was detrimental [193,194]. At any rate, no selection pressure favouring rhizobia which can express hydrogenase seems to exist, as signified by the fact that many rhizobial strains have been isolated which do not contain uptake hydrogenase [195–197]. Therefore, it seems likely that uptake hydrogenase activity is not important under conditions of sufficient carbon supply and O_2 protection. This hypothesis is supported by physiological studies on free-living *Azorhizobium caulinodans* ORS571, showing that hydrogenase activity is a disadvantage under conditions of O_2 limitation [198].

For *Frankia*, no isogenic strains are available, hampering studies on the role of hydrogenase activity for symbiotic nitrogen fixation (for review see [199]). However, the vast majority of *Frankia* strains isolated thus far shows hydrogenase activity ([200]; F. Tavares, U. Mattsson and A. Sellstedt, personal communication). Thus, in actinorhizal nodules where the bacteria have to contribute to O_2 protection themselves, bacterial uptake hydrogenase activity may be more important for symbiotic efficiency.

Nitrogen metabolism

In both legume and actinorhizal symbioses, ammonium, the product of nitrogen fixation, is exported by the bacteria and assimilated in the plant cytoplasm via the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway (for review see [201]). High levels of plant GS activity were found both in legume and in *A. glutinosa* nodules [201]. After ammonium assimilation, glutamate has to be metabolized into nitrogen transport forms, which depend on the plant species. In temperate legumes as well as in most actinorhizal plants examined thus far, the major nitrogen transport forms are amides, namely, glutamine and asparagine [201,202]. Tropical legumes with the exception of some trees-[203] transport ureides [201]. Most *Alnus* species and *Casuarina equisetifolia* use an ureide, citrulline, as nitrogen transport form [201,202].

In indeterminate legume nodules as well as in actinorhizal nodules of *Alnus*, ammonium assimilation as signified by GS expression seems to be confined to the infected cells and to the nodule vascular system [153,204,205]. In determinate nodules, however, where ammonium assimilation and the synthesis of ureide for nitrogen transport are spatially separated, the situation is more diverse: GS activity in soybean nodules was found both in the infected and in the uninfected cells [206], while GS expression in nodules of *Phaseolus vulgaris* was confined to the infected cells and to the nodule vascular system [207]. However, uricase (nodulin-35), which catalyzes the oxidation of uric acid to allantoin, in both soybean and *Phaseolus* nodules is localized in the specialized peroxisomes of uninfected cells only [208–210], and the activity of the enzyme catalyzing the next step in purine oxidation, allantoinase, was also confined to the uninfected cells [208]. The uninfected cells of determinate nodules, contain specialized peroxisomes where these

enzymes are localized and ureide biosynthesis takes place [211]. They form a network and are involved in transport of nitrogenous compounds to and carbon sources from the nodule vascular bundles [212]. Connected by an elaborate tubular endoplasmic reticulum system which is appressed to the specialized peroxisomes, the places of ureide biosynthesis, and continues through plasmodesmata, they constitute a more or less continuous network throughout the central tissue, facilitating the transport of nitrogenous compounds to the nodule vascular bundle [212]. In indeterminate legume nodules, however, where amides serve as nitrogen transport form, whose biosynthesis takes place in the cytoplasm, no specialized function could be assigned to the uninfected cells in the central tissue, which are fewer than in determinate nodules and do not form a network [73]. Here, efficient transport of nitrogenous compounds seems to be achieved by the presence of transfer cells in the pericycle of the nodule vascular bundles, providing an abundant surface area across which the transport can occur [213].

In actinorhizal nodules of *Alnus glutinosa*, citrulline biosynthesis seems to take place in the infected cells, since acetyl ornithine transaminase, an enzyme involved in citrulline biosynthesis, has been found to be expressed in these cells [153]. Although *Alnus* is a ureide transporter, there is no homology with determinate legume nodules, because citrulline is not synthesized via de novo ureide biosynthesis as in the case of the tropical legumes [214,215], but via ornithine [216]. Thus, the biosynthesis of citrulline does not require peroxisomal enzymes, but seems to take place at least partially in the mitochondria where ornithine carbamoyl transferase was detected [217]. No metabolic specialization of the uninfected cells of *Alnus* nodules, except for starch storage [218], could be found up to now. Transport functions in *Alnus* nodules seem to be fulfilled by the pericycle of the central vascular bundle of the nodule lobes, which consists of several layers of small cells with a dense cytoplasm, but without the cell wall structures typical for transfer cells [136,219]. Considering that in *Alnus* nodule lobes, the transport function which in legume nodules is carried out by several vascular bundles, is concentrated on the central stele, it seems likely that the proliferation of the pericycle serves to improve the transport capacities.

Carbon metabolism

Nodules need to be supplied with carbon sources for maintenance and growth, energy for N_2 fixation and for supply of acceptor molecules for assimilation of the fixed nitrogen. Shoot carbohydrate pools have been identified as the primary source for the maintenance of nodule N_2 fixation activity, also during darkness ([218,220]; reviewed for legumes in [221]), in spite of the presence of starch grains in legume [96,210] and actinorhizal nodules [218,220,222]. Assimilates are transported in the form of sucrose from source to sink tissues [223] where they are introduced into metabolism by the action of symplastic sucrose synthase, or apoplastic invertase. In mature legume nodules, high sucrose synthase activities have been detected [224], and sucrose synthase transcription has been shown to be induced in legume as well as actinorhizal nodules [153,225,226].

Analysis of nodule enzyme activities has shown that malate is the primary product of glycolysis in legume nodules, and in turn seems to be exported to the bacteroides as an energy source or to serve as an ammonium acceptor and to be metabolized to aspartate [221]. This is achieved by high activities of phosphoenolpyruvate (PEP) carboxylase, malate dehydrogenase (MDH) and aspartate transaminase (AAT) in legume nodules [221,227]. Nodulin-26 which is located in the PBM, has been suggested to mediate the transport of malate into the PBS [29], although 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 [228]. In actinorhizal nodules of *A. glutinosa*, high activities of PEP carboxylase [229] and MDH activity were found [230]. However, the identity of the carbon source provided by the plant to endosymbiotic *Frankia* is not clear yet, although a malate-aspartate-shuttle has been suggested [230]. Furthermore, it remains to be examined how far nodule carbon transport and metabolism differs between different actinorhizal symbioses.

Conclusions and future prospects

In nodules, specific needs have to be fulfilled to allow nitrogen fixation. The comparison between legume- and actinorhizal nodules shows that these requirements can be met in a variety of ways. This implies that there will be multiple possibilities to solve these problems in new nitrogen fixing systems.

The comparison of structure and development of *Rhizobium*/legume and actinorhizal nodules has revealed several differences. First, *Rhizobium*/legume nodules have a stem-like morphology with peripheral vascular system and infected cells in the central tissue, while actinorhizal nodule lobes represent modified lateral roots with a central vascular cylinder and infected cells in the cortex. Second, the two types of nodules are also developmentally different, legume nodule primordia being induced in the root cortex and actinorhizal nodule primordia in the root pericycle. Third, while rhizobia in symbiosis are released into the plant cytoplasm by a process resembling endocytosis, no such release is taking place in actinorhizal symbioses. Fourth, there is no compartment in actinorhizal nodules corresponding to the infection thread matrix in *Rhizobium*/legume systems. Thus, both types of symbioses seem fundamentally dissimilar. However, in spite of these differences there is evidence for a phylogenetic relationship between both symbioses, since the comparison of sequences of the gene encoding the large subunit of ribulosebisphosphate carboxylase from different plant species has shown that there seems to be a single phylogenetic origin of susceptibility to nitrogen-fixing root nodule symbioses in angiosperms [231]. This is supported by the fact that nodules induced by *Rhizobium* on *Parasponia* (*Ulmaceae*), the only nonlegume being able to enter a symbiosis with rhizobia [232], structurally and developmentally resemble actinorhizal nodules. Furthermore, in *Parasponia* nodules, rhizobia are not released from the infection threads, and an infection thread matrix is not discernable [233,234]. Thus, the differences between both symbioses may simply be due to the variability of ways to meet the re-

quirements for symbiotic nitrogen fixation in legumes vs. other plant families.

While previously legume nodules were considered unique organs and root nodule induction seemed to require a set of specific genes, new results have changed our view on nodule development. Proteins previously thought to be nodule-specific, have been shown to have counterparts in nonsymbiotic plant development, as it has been found for hemoglobin [28,235]. Infection thread growth during legume nodule induction has been related to a common developmental process, namely cell cycling [69]. Root hair deformation has been identified as newly induced tip growth [54]. The identification of the developmental pathways from which symbiotic processes are derived, will allow the development of strategies to engineer new systems. Hence, the possibility of transferring the ability to enter N_2-fixing symbioses to other crop plants can be considered more optimistically nowadays because of the results in recent research on nodule development.

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References

1. Heichel GH, Vance CP. Legume nitrogen: symbiotic fixation and recovery by subsequent crops. In: Helsel ZR (ed) Energy in Plant Nutrition and Pest Control. Amsterdam: Elsevier, 1987:63–80.
2. Paul EA. Towards the year 2000: Directions for future nitrogen research. In: Wilson JR (ed) Advances in Nitrogen Cycling in Agricultural Ecosystems. Wallingford, UK: CAB International, 1988:417–425.
3. Howard JB, Rees DC. Nitrogenase – a nucleotide-dependent molecular switch. Ann Rev Biochem 1994;63:235–264.
4. Shaw VK, Brill WJ. Isolation of an iron-molybdenum cofactor from nitrogenase. Proc Natl Acad Sci USA 1977;74:1493–1497.
5. Burris RH. Nitrogenases. J Biol Chem 1991;266:9339–9342.
6. Bergman B, Johansson C, Söderbäck E. The *Nostoc-Gunnera* symbiosis. New Phytologist 1992;122:379–400.
7. Elmerich C, Zimmer W, Vieille C. Associative nitrogen-fixing bacteria. In: Stacey G, Burris RH, Evans HJ (eds) Biological Nitrogen Fixation. New York: Chapman and Hall, 1992;212–258.
8. Okon Y, Labandera-Gonzalez CA. Agronomic applications of *Azospirillum* – an evaluation of 20 years worldwide field inoculation. Soil Biol Biochem 1994;26:1591–1601.
9. Khush GF, Bennett J (eds). Nodulation and Nitrogen Fixation in Rice: Potentials and Prospects. Manila: International Rice Research Institute, 1992.
10. Hellriegl H. Welche Stickstoffquellen stehen der Pflanze zu Gebote? Zeitschrift des Verbands für

die Rübenzucker-Industrie des Deutschen Reiches 1886;36:863–877.
11. Beyerinck MW. Die Bakterien der Papilionaceen-Knöllchen. Bot Zeitung 1888;46–50:725–804.
12. De Bruijn FJ. The unusual symbiosis between the diazotrophic stem-nodulating bacterium *Azorhizobium caulinodans* ORS571 and its host, the tropical legume *Sesbania rostrata*: plant and bacterial aspects. In: Kosuge T, Nester EW (eds) Plant-Microbe Interactions. New York: Macmillan, 1989;457–493.
13. Marvel DJ, Torrey JG, Ausubel FM. *Rhizobium* symbiotic genes required for nodulation of legume and nonlegume hosts. Proc Natl Acad Sci USA 1987;84:1319–1323.
14. Weeden N, Kneen BE, LaRue TA. Genetic analysis of *Sym* genes and other nodule-related genes in *Pisum sativum*. In: Gresshoff PM (ed) Nitrogen Fixation: Achievement and Objectives. New York: Chapman and Hall, 1990;323–330.
15. Caetano-Anollés G, Gresshoff PM. Plant genetic control of nodulation. Ann Rev Microbiol 1991;45:345–382.
16. Kolchinsky A, Funke R, Gresshoff PM. Dissecting molecular mechanisms of nodulation: taking a leaf from Arabidopsis. Plant Molec Biol 1994;26:549–552.
17. Carroll BJ, McNeil DL, Gresshoff PM. Mutagenesis of soybean (*Glycine max* (L.) Merr.) and the isolation of nonnodulating mutants. Plant Sci 1986;47:109–114.
18. Nutman PS. Improving nitrogen fixation in legumes by plant breeding; the relevance of host selection experiments in red clover (*Trifolium pratense* L.) and subterranean clover (*T. subterraneum* L.). Plant Soil 1984;82:285–301.
19. Häser A, Robinson DL, Duc G, Vance CP. A mutation in *Vicia faba* results in ineffective nodules with impaired bacteroid differentiation and reduced synthesis of late nodulins. J Exp Bot 1992;43:1397–1407.
20. Landau-Ellis D, Angermuller SA, Shoemaker RC, Gresshoff PM. The genetic locus controlling supernodulation in soybean (*Glycine max* L.) co-segregates tightly with a cloned molecular marker. Molec Gen Genet 1991;228:221–226.
21. Landau-Ellis D, Gresshoff PM. Supernodulating soybean mutant alleles *nts382* and *nts1007* show no recombination with the same restriction length polymorphism marker. Molec Plant Microbe Interact 1992;5:428–429.
22. Kneen BE, Weeden NF, LaRue TA. Nonnodulating mutants of *Pisum sativum* (L.) cv Sparkle. J Hered 1994;85:129–133.
23. Sánchez F, Padilla JE, Péréz H, Lara M. Control of nodulin genes in root-nodule development and metabolism. Ann Rev Plant Physiol Plant Molec Biol 1991;42:507–528.
24. Verma DPS. Signals in root nodule organogenesis and endocytosis of *Rhizobium*. Plant Cell 1992;4:373–382.
25. Franssen HJ, Vijn I, Yang W-C, Bisseling T. Developmental aspects of the *Rhizobium*-legume symbiosis. Plant Molec Biol 1992;19:89–107.
26. Mylona P, Pawlowski K, Bisseling T. Symbiotic nitrogen fixation. Plant Cell 1995;7:869–885.
27. Van Kammen A. Suggested nomenclature for plant genes involved in nodulation and symbiosis. Plant Molec Biol Report 1984;2:43–45.
28. Taylor ER, Nie XZ, MacGregor AW, Hill RD. A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. Plant Molec Biol 1994;24:853–862.
29. Miao G-H, Verma DPS. Soybean nodulin-26 gene encoding a channel protein is expressed only in the infected cells of nodules and is regulated differently in roots of homologous and heterologous plants. Plant Cell 1993;5:781–794.
30. Scheres B, Van de Wiel C, Zalensky A, Horvath B, Spaink H, Van Eck H, Zwartkruis F, Wolters AM, Gloudemans T, van Kammen A, Bisseling T. The *ENOD12* product is involved in the infection process during pea-*Rhizobium* interaction. Cell 1990;60:281–294.
31. Kouchi H, Hata S. Isolation and characterization of novel cDNAs representing genes expressed at early stages of soybean nodule development. Molec Gen Genet 1993;238:106–119.
32. Yang W-C, Katinakis P, Hendriks P, Smolders A, de Vries F, Spee J, van Kammen A, Bisseling T, Franssen H. Characterization of *GmENOD40*, a gene showing novel patterns of cell-specific

expression during soybean nodule development. Plant J 1993;3:573–585.
33. Nap JP, Bisseling T. Developmental biology of a plant-prokaryote symbiosis: the legume root nodule. Science 1990;250:948–954.
34. Becana M, Klucas RV. Oxidation and reduction of leghemoglobin in root nodules of leguminous plants. Plant Physiol 1992;98:1217–1221.
35. Fisher RF, Long SR. *Rhizobium*-plant signal exchange. Nature 1992;357:655–660.
36. Spaink HP. Rhizobial lipopolysaccharides: Answers and questions. Plant Molec Biol 1992;20:977–986.
37. Dénarié J, Cullimore J. Lipo-oligosaccharide nodulation factors: a new class of signalling molecules mediating recognition and morphogenesis. Cell 1993;74:951–954.
38. Roche P, Debellé F, Maillet F, Lerouge P, Truchet G, Dénarié J, Promé J-C. Molecular basis of symbiotic host specificity in *Rhizobium meliloti: nodH* and *nodPQ* genes encode the sulfation of lipo-oligosaccharide signals. Cell 1991;67:1131–1143.
39. Journet EP, Pichon M, Dedieu A, de Billy F, Truchet G, Barker DG. *Rhizobium meliloti* Nod factors elicit cell-specific transcription of the *ENOD12* gene in transgenic alfalfa. Plant J 1994;6:241–249.
40. Okker RJH, Schlaman HRM, Spaink HP, Lugtenberg BJJ. Function of nodulation genes of *Rhizobium*. Symbiosis 1993;14:283–295.
41. Van Rhijn P, Vanderleyden J. The *Rhizobium*-plant symbiosis. Microbiol Rev 1995;59:124–142.
42. Peters NK, Frost JW, Long SR. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science 1986;233:977–980.
43. Goethals K, Van Montagu M, Holsters M. Conserved motifs in a divergent *nod* box of *Azorhizobium caulinodans* ORS571 reveals a common structure in promoters regulated by LysR-type proteins. Proc Natl Acad Sci USA 1992;89:1646–1650.
44. Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Promé J-C, Dénarié J. Symbiotic host specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 1990;344:781–784.
45. Spaink HP, Sheely DM, van Brussel AAN, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinhold VN, Lugtenberg BJJ. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 1991;354:125–130.
46. Van Brussel AAN, Bakhuizen R, Van Spronsen PC, Spaink HP, Tak T, Lugtenberg BJJ. Induction of preinfection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium*. Science 1992;257:70–71.
47. Horvath B, Heidstra R, Lados M, Moerman M, Spaink HP, Promé J-C, Van Kammen A, Bisseling T. Induction of pea early nodulin gene expression by Nod factors of *Rhizobium*. Plant J 1993;4:727–733.
48. Vijn I, Das Neves L, Van Kammen A, Franssen H, Bisseling T. Nod factors and nodulation in plants. Science 1993;260:1764–1765.
49. Stokkermans TJW, Peters NK. *Bradyrhizobium elkanii* lipooligosaccharide signal induce complete nodule structures on *Glycine soja* Siebold et Zucc. Planta 1994;193:413–420.
50. Chandler MR, Date RA, Roughley RJ. Infection and root nodule development in *Stylosanthes* species by *Rhizobium*. J Exp Bot 1982;33:47–57.
51. Dreyfus BL, Alazard D, Dommergues YR. Stem-nodulating rhizobia. In: Klug MC, Reddy CE (eds) Current Perspectives in Microbial Ecology. Washington, DC: American Society of Microbiology, 1986;161–169.
52. De Faria SM, Hay GT, Sprent JI. Entry of rhizobia into roots of *Mimosa scabrella* Bentham occurs between epidermal cells. J Gen Microbiol 1988;134:2291–2296.
53. Fahraeus G. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J Gen Microbiol 1957;16:374–381.
54. Heidstra R, Geurts R, Franssen H, Spaink HP, Van Kammen A, Bisseling T. Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. Plant Physiol 1994;105:787–797.
55. Cook D, Dreyer D, Bonnet D, Howell M, Nony E, VandenBosch K. Transient induction of a peroxidase gene in *Medicago truncatula* precedes infection by *Rhizobium meliloti*. Plant Cell

1995;7:43–55.

56. Scheres B, Van Engelen F, Van der Knaap E, Van de Wiel C, Van Kammen A, Bisseling T. Sequential induction of nodulin gene expression in the developing pea nodule. Plant Cell 1990;2:687–700.
57. Vijn I, Martinez-Abarca F, Yang W-C, Das Neves L, Van Brussel A, van Kammen A, Bisseling T. Early nodulin gene expression during Nod factor-induced processes in *Vicia sativa*. Plant J 1995;8:111–119.
58. Goethals K, Leyman B, Van den Eede G, Van Montagu M, Holsters M. An *Azorhizobium caulinodans* ORS571 locus involved in lipopolysaccharide production and nodule formation on *Sesbania rostrata* stems and roots. J Bacteriol 1994;176:92–99.
59. Pichon M, Journet E-P, de Billy F, Dedieu A, Huguet T, Truchet G, Barker DG. *Rhizobium meliloti* elicits transient expression of the early nodulin gene *ENOD12* in the differentiating root epidermis of transgenic alfalfa. Plant Cell 1992;4:1199–1211.
60. Csanadi G, Szecsi J, Kalo P, Kiss P, Endre G, Kondorosi A, Kondorosi E, Kiss GB. *ENOD12*, an early nodulin gene, is not required for nodule formation and efficient nitrogen fixation in alfalfa. Plant Cell 1994;6:201–213.
61. Callaham D, Torrey JG. The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium*. Can J Bot 1981;59:1647–1664.
62. Bakhuizen R. The Plant Cytoskeleton in the *Rhizobium*-Legume Symbiosis. PhD Thesis, Leiden University, Leiden, The Netherlands, 1988.
63. Turgeon BG, Bauer WD. Ultrastructure of infection-thread development during the infection of soybean by *Rhizobium japonicum*. Planta 1985;163:328–349.
64. Brewin NJ. Development of the legume root nodule. Ann Rev Cell Biol 1991;7:191–226.
65. Kijne JW. The *Rhizobium* infection process. In: Stacey G, Burris RH, Evans HJ (eds) Biological Nitrogen Fixation. New York: Chapman and Hall, 1992:349–398.
66. Dart PJ. The infection process. In: Quispel A (ed) Biology of Nitrogen Fixation. Amsterdam: North Holland Publishing Company, 1974:381–429.
67. VandenBosch KA, Bradley DJ, Knox JP, Perotto S, Butcher GW, Brewin NJ. Common components of the infection thread matrix and the intercellular space identified by immunochemical analysis of pea nodules and uninfected roots. EMBO J 1989;8:335–342.
68. Rae AL, Bontante-Fasolo P, Brewin NJ. Structure and growth of infection threads in the legume symbiosis *with Rhizobium leguminosarum*. Plant J 1992;2:385–395.
69. Yang W-C, de Blank C, Meskiene I, Hirt H, Bakker J, van Kammen A, Franssen H, Bisseling T. *Rhizobium* Nod factors reactivate the cell cycle during infection and nodule primordium formation, but the cycle is only completed in primordium formation. Plant Cell 1994;6:1415–1426.
70. Newcomb W. A correlated light and electron microscopic study of symbiotic growth and differentiation in *Pisum sativum* root nodules. Can J Bot 1976;54:21630–2186.
71. Newcomb W. Nodule morphogenesis and differentiation. Int Rev Cytol 1981;13(Suppl):S247–S297.
72. Tsien HC, Dreyfus BL, Schmidt EL. Initial stages in the morphogenesis of nitrogen-fixing stem nodules of *Sesbania rostrata*. Molec Gen Genet 1983;206:291–299.
73. Schaede R. Die Knöllchen der adventiven Wasserwurzeln von *Neptunia oleracea* und ihre Bakteriensymbiose. Planta 1940;31:1–21.
74. James EK, Sprent JI, Sutherland JM, McInroy SG, Michin RT. The structure of nitrogen fixing root nodules on the aquatic mimosoid legume *Neptunia plena*. Ann Bot 1992;69:173–180.
75. Chandler MR. Some observations on infection of *Arachis hupogaea* L. by *Rhizobium*. J Exp Bot 1978;29:749–755.
76. Alazard D, Duhoux E. Development of stem nodules in a tropical forage legume, *Aeschynomene afraspera*. J Exp Bot 1990;41:1199–1206.
77. Libbenga KR, Bogers RJ. Root-nodule morphogenesis. In: Quispel A (ed) The Biology of Nitrogen Fixation. Amsterdam: North-Holland Publishing Company, 1974;430–472.
78. Asad S, Fang Y, Wycoff KL, Hirsch AM. Isolation and characterization of cDNA and genomic clones of *MsENOD40*: transcripts are detected in meristematic cells of alfalfa. Protoplasma

1994;183:10–23.

79. Matvienko M, van de Sande K, Yang W-C, van Kammen A, Bisseling T, Franssen H. Comparison of soybean and pea *ENOD40* cDNA clones representing genes expressed during both early and late stages of nodule development. Plant Molec Biol 1994;26:487–493.
80. Gresshoff PM, Delves AC. Plant genetic approaches to symbiotic nodulation and nitrogen fixation in legumes. In: Blonstein AD, King PJ (eds) A Genetic Approach to Plant Biochemistry. Heidelberg: Springer, 1986;159–206.
81. Rolfe BG, Gresshoff PM. Genetic analysis of legume nodule initiation. Ann Rev Plant Physiol Plant Molec Biol 1988;39:297–319.
82. Libbenga KR, Harkes PAA. Initial proliferation of cortical cells in the formation of root nodules in *Pisum sativum* L. Planta 1973;114:17–28.
83. Ardourel M, Demont N, Debellé F, Maillet F, de Billy F, Promé J-C, Dénarié J, Truchet G. *Rhizobium meliloti* lipooligosaccharide nodulation factors: different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. Plant Cell 1994;6:1357–1374.
84. Hirsch AM, Bhuvaneswari TV, Torrey JG, Bisseling T. Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc Natl Acad Sci USA 1989;86:1244–1248.
85. Cooper JB, Long SR. Morphogenetic rescue of *Rhizobium meliloti* nodulation mutants by *trans*-zeatin secretion. Plant Cell 1994;6:215–225.
86. Roth LE, Stacey G. Bacterium release into host cells of nitrogen-fixing soybean nodules: the symbiosome membrane comes from three sources. Euro J Cell Biol 1989;49:13–23.
87. Perotto S, Donovan N, Droback BK, Brewin NJ. Differential expression of a glycosyl inositol phospholipid antigen on the peribacteroid membrane during pea nodule development. Molec Plant-Microbe Interact 1995;8:560–568.
88. Mellor RB, Werner D. Peribacteroid membrane biogenesis in mature legume root nodules. Symbiosis 1987;3:89–114.
89. Mellor RB. Bacteroides in the *Rhizobium*-legume symbiosis inhabit a plant internal lytic compartment: implication for other microbial endosymbioses. J Exp Bot 1989;40:831–839.
90. Kimmback A, Mellor RB, Werner D. α-Mannosidase II isoenzyme in the peribacteroid space of *Glycine max* root nodules. J Exp Bot 1987;38:1373–1377.
91. Mellor RB, Mšrschel E, Werner D. Legume root response to symbiotic infection: enzymes of the peribacteroid space. Z Naturforsch 1984;39:123–125.
92. Zhang Y, Roberts DM. Expression of soybean nodulin-26 in transgenic tobacco. Targeting to the vacuolar membrane and effects on floral and seed development. Molec Biol Cell 1995;6:109–117.
93. Kammenberg EL, Brewin NJ. Expression of a cell surface antigen from *Rhizobium leguminosarum* 3841 is regulated by oxygen and pH. J Bacteriol 1989;171:4543–4548.
94. Hirsch AM, Smith CA. Effects of *Rhizobium meliloti nif* and *fix* mutants on alfalfa root nodule development. J Bacteriol 1987;169:1137–1146.
95. Van de Wiel C, Scheres B, Franssen H, van Lierop MJ, van Lammeren A, van Kammen A, Bisseling T. The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules. EMBO J 1990;9:1–7.
96. Vasse J, de Billy F, Camut S, Truchet G. Correlation between ultrastructural differentiation of bacteroides and nitrogen fixation in alfalfa nodules. J Bacteriol 1990;172:4295–4306.
97. Yang W-C, Horvath B, Hontelez J, van Kammen A, Bisseling T. In situ localization of *Rhizobium* mRNAs in pea root nodules: *nifA* and *nifH* localization. Molec Plant-Microbe Interact 1991;4:464–468.
98. De Maagd RA, Yang W-C, Goosen-de Roo L, Mulders IHM, Roest HP, Spaink HP, Bisseling T, Lugtenberg BJJ. Downregulation of expression of the *Rhizobium leguminosarum* outer membrane protein gene *ropA* occurs abruptly in interzone II–III of pea nodules and can be uncoupled from *nif* gene activation. Molec Plant-Microbe Interact 1993;7:276–281.
99. Kardailsky I, Yang W-C, Zalensky A, van Kammen A, Bisseling T. The pea late nodulin gene *PsNOD6* is homologous to the early nodulin genes *PsENOD3/14* and is expressed after

leghaemoglobin genes. Plant Molec Biol 1993;23:1029–1037.
100. Soupène E, Foussard M, Boistard P, Truchet G, Batut J. Oxygen as a key developmental regulator of *Rhizobium meliloti* N_2 fixation: gene expression within the alfalfa root nodule. Proc Natl Acad Sci USA 1995;92:3759–3763.
101. Pladys D, Rigaud J. Senescence in French bean nodules: occurrence of different proteolytic activities. Physiol Plant 1985;63:43–48.
102. Pladys D, Vance CP. Proteolysis during development and senescence of effective and plant gene-controlled ineffective alfalfa nodules. Plant Physiol 1993;103:379–384.
103. Manen JF, Simon P, van Slooten J-C, Østerås M, Frutiger S, Hughes GJ. A nodulin specifically expressed in senescent nodules of winged bean is a protease inhibitor. Plant Cell 1991;3:259–170.
104. Garbers C, Meckbach R, Mellor RB, Werner D. Protease (thermolysin) inhibition in the peribacteroid space of *Glycine max* root nodules. J Plant Physiol 1988;132:442–445.
105. Benson DR, Silvester WB. Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol Rev 1993;57:293–319.
106. Simonet P, Normand P, Hirsch AM, Akkermans ADL. The genetics of the *Frankia* actinorhizal symbiosis. In: Gresshoff PM (ed) The Molecular Biology of Symbiotic Nitrogen Fixation. Boco Raton, FL: CRC Press, 1990:77–109.
107. Baker DD, Mullin BC. Actinorhizal symbioses. In: Stacey G, Burris RH, Evans HJ (eds) Biological Nitrogen Fixation. New York: Chapman and Hall, 1992;259–292.
108. Meyen J. Über das Hervorwachsen parasitischer Gebilde aus den Wurzeln anderer Pflanzen. Flora 1829;12:49–64.
109. Hiltner L. Über die Bedeutung der Wurzelknöllchen von *Alnus glutinosa* für die Stickstoffernährung dieser Pflanze. Landwirschaft Verständ Stud 1985;46:153–161.
110. Krebber O. Untersuchungen über die Wurzelknöllchen der Erle. Arch Microbiol 1932;3:588–608.
111. Schaede R. Über die Symbionten in den Knöllchen der Erle und des Sanddornes und die cytologischen Verhältnisse in ihnen. Planta 1933;19:389–416.
112. Dawson JO. Actinorhizal plants: their use in forestry and agriculture. Outl Agric 1986;15:202–208.
113. Albouchi A. Comparative study of 2 young windbreaks of *Cupressus sempervirens* and *Casuarina glauca*. Ann Instit Nat Rech Agron Tunisie 1989;74–83.
114. Adegbehin JO, Igboanugo ABI, Omijeh JE. Potentials of agroforestry for sustainable food and wood production in the savanna areas of Nigeria. Savanna 1990;11:12–26.
115. Diem HG, Dommergues YR. Current and potential uses and management of *Casuarinaceae* in the tropics and subtropics. In: Schwintzer CR, Tjepkema JD (eds) The Biology of *Frankia* and Actinorhizal Plants. New York: Academic Press, 1990;317–342.
116. El-Lakany MH. Provenance trials of *Casuarina glauca* and *C. cunninghamiana* in Egypt. In: El-Lakany MH, Turnbull JW, Brewbaker JL (eds) Advances in Casuarina Research and Utilization. Cairo: American University in Cairo, Desert Development Center, 1990;12–22.
117. Skoupy J. The role of trees in agroforestry. Desert Cont Bull (UNEP) 1991;19:38–45.
118. Sundararaju R, Chinnathurai AK. Technology packages for reclamation and development of wastelands. Indian Forester 1992;118:609–615.
119. Puri S, Singh S, Bhushan B. Evaluation of fuelwood quality of indigenous and exotic tree species of India's semiarid region. Agroforest Syst 1994;26:123–130.
120. Sagwal SS. *Casuarina* on farm: A plant of economic importance. Indian Farming 1993;43:12–14
121. Lechevalier MP, Lechevalier HA. Genus *Frankia* Brunchorst 1886, 174^{AL}. In: Williams ST, Sharpe ME, Holt HG (eds) Bergey's Manual of Systematic Bacteriology, vol 4. Baltimore, MD: Williams and Wilkins, 1989;2410–2417.
122. Lechevalier MP, Lechevalier HA. Systematics, isolation, and culture of *Frankia*. In: Schwintzer CR, Tjepkema JD (eds) The Biology of Frankia and Actinorhizal Plants. New York: Academic Press, 1990;35–60.
123. Quispel A, Burggraaf AJP, Borsje H, Tak T. The role of lipids in the growth of *Frankia* isolates. Can J Bot 1983;61:2801–2806.
124. Quispel A, Svendsen AB, Schripsema J, Baas WJ, Erkelens C, Lugtenberg J. Identification of

dipterocarpol as isolation factor for the induction of primary isolation of *Frankia* from root nodules of *Alnus glutinosa* (L.) Gaertner. Molec Plant-Microbe Interact 1989;2:107–112.
125. Baker DD. Relationships among pure cultured strains of *Frankia* based on host specificity. Physiol Plant 1987;70:245–248.
126. Torrey JG. Initiation and development of root nodules of *Casuarina* (*Casuarinaceae*). Am J Bot 1976;63:335–344.
127. Berry AM, McIntyre L, McCully ME. Fine structure of root hair infection leading to nodulation in the *Frankia-Alnus symbiosis*. Can J Bot 1986;64:292–305.
128. Callaham D, Newcomb W, Torrey JG, Peterson RL. Root hair infection in actinomycete-induced root nodule initiation in *Casuarina*, *Myrica*, and *Comptonia*. Bot Gaz 1979;140(Suppl):S1–S9.
129. Miller IM, Baker DD. The initiation, development and structure of root nodules in *Elaeagnus angustifolia* L. (Elaeagnaceae). Protoplasma 1985;128:107–119.
130. Liu Q, Berry AM. The infection process and nodule initiation in the *Frankia-Ceanothus* root nodule symbiosis: a structural and histochemical study. Protoplasma 1991;163:82–92.
131. Racette S, Torrey JG. Root nodule initiation in *Gymnostoma* (Casuarinaceae) and *Shepherdia* (Elaeagnaceae) induced by *Frankia* strain HFPGpI1. Can J Bot 1989;67:2873–2879.
132. Berry AM, Sunell LA. The infection process and nodule development. In: Schwintzer CR, Tjepkema JD (eds) The Biology of *Frankia* and Actinorhizal Plants. New York: Academic Press, 1990;61–81.
133. Burggraaf AJP, van der Linden J, Tak T. Studies on the localization of infectible cells of *Alnus glutinosa* roots. Plant Soil 1983;74:175–188.
134. Prin Y, Rougier M. Preinfection events in the establishment of *Alnus-Frankia* symbiosis: Study of the root hair deformation step. Plant Physiol (Life Sci Adv) 1987;6:99–108.
135. Berry AM, Torrey JG. Root hair deformation in the infection process of *Alnus rubra*. Can J Microbiol 1983;61:2863–2976.
136. Burgess D, Peterson RL. Development of *Alnus japonica* root nodules after inoculation with *Frankia* strain HFPArI3. Can J Bot 1987;65:1647–1657.
137. Callaham D, Torrey JG. Prenodule formation and primary nodule development in roots of *Comptonia* (Myricaceae). Can J Microbiol 1977;51:2306–2318.
138. Mian S, Bond G. The onset of nitrogen fixation in young alder plants and its relation to differentiation in the nodular endophyte. New Phytologist 1978;80:187–192.
139. Schwintzer CR, Berry AM, Disney LD. Seasonal patterns of root nodule growth, endophyte morphology, nitrogenase activity and shoot development in *Myrica gale*. Can J Bot 1982;60:746–757.
140. Liu QQ, Berry AM. Localization and characterization of pectic polysaccharides in roots and root nodules of *Ceanothus spp.* during intercellular infection by *Frankia*. Protoplasma 1991;163:93–101.
141. Sunell LA, Berry AM. Preinfection cell wall formation in roots and developing nodules of *Alnus rubra* bong. Protoplasma 1992;168:87–93.
142. Bond G. Root-nodule symbiosis with actinomycete-like organisms. In: Quispel A (ed) The Biology of Nitrogen Fixation. Amsterdam: North-Holland Publishing Company. 1974;342–378.
143. Silvester WB, Harris SL, Tjepkema JD. Oxygen regulation and hemoglobin. In: Schwintzer CR, Tjepkema JD (eds) The Biology of *Frankia* and Actinorhizal Plants. New York: Academic Press, 1990;157–176.
144. Ribeiro A, Akkermans ADL, Van Kammen A, Bisseling T, Pawlowski K. A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. Plant Cell 1995;7:785–794.
145. Lalonde M. Immunological and ultrastructural demonstration of nodulation of the European *Alnus glutinosa* (L.) Gaertn. host plant by an actinomycetal isolate from the North American *Comptonia peregrina* (L.) Coult. root nodule. Bot Gaz 1979;140(S):35–43.
146. Fontaine MF, Lancelle SA, Torrey JG. Initiation and ontogeny of vesicles in cultured *Frankia spp.* strain HFPArI3. J Bacteriol 1984;160:921–927.
147. Huss-Danell K, Bergman B. Nitrogenase in *Frankia* from root nodules of *Alnus incana* (L.) Moench: Immunolocalization of the Fe- and MoFe-proteins during vesicle differentiation. New

Phytologist 1990;116:443–455.
148. Pawlowski K, Akkermans ADL, van Kammen A, Bisseling T. Expression of *Frankia nif* genes in actinorhizal nodules of *Alnus glutinosa*. Plant Soil 1995;170:371–376.
149. Séguin A, Lalonde M. Expression of actinorhizins in the development of the *Frankia-Alnus* symbiosis. In: Polsinelli M, Materassi R, Vincenzini M (eds) Developments in Plant and Soil Sciences: Nitrogen Fixation. Dordrecht: Kluwer Academic Publishers, 1991;602–608.
150. Fleming AI, Wittenberg JB, Wittenberg BA, Dudman WF, Appleby CA. The purification, characterization and ligand-binding kinetics of hemoglobins from root nodules of the nonleguminous *Casuarina glauca-Frankia* symbiosis. Biochim Biophys Acta 1987;911:209–220.
151. Jacobsen-Lyon K, Østergaard-Jensen E, Jørgensen J-E, Marcker KA, Peacock J, Dennis E. Symbiotic and nonsymbiotic hemoglobin genes of *Casuarina glauca*. Plant Cell 1995;7:213–223.
152. Goetting-Minesky MP, Mullin BC. Differential gene expression in an actinorhizal symbiosis: Evidence for a nodule-specific cysteine proteinase. Proc Natl Acad Sci USA 1994;91:9891–9895.
153. Pawlowski K, Guan C, Ribeiro A, van Kammen A, Akkermans ADL, Bisseling T. Genes involved in *Alnus glutinosa* nodule development. In: Kiss GB, Endre G (eds) Proceedings of the 1st European Nitrogen Fixation Conference. Szeged: Officina Press, 1994;220–224.
154. Twigg PG. Isolation of a Nodule-Specific cDNA Encoding a Putative Glycine-Rich Protein from *Alnus glutinosa*. PhD Dissertation, University of Tennessee, Knoxville, TN, USA 1993.
155. Tjepkema JD, Yocum CS. Measurement of oxygen partial pressure within soybean nodules by oxygen microelectrodes. Planta 1974;119:351–360.
156. Appleby CA. Leghemoglobin and *Rhizobium* respiration. Ann Rev Plant Physiol 1984;35:443–2478.
157. Witty JF, Minchin FR, Skøt L, Sheely JE. Nitrogen fixation and oxygen in legume root nodules. Oxford Surv Plant Cell Biol 1986;3:275–315.
158. David M, Daveran ML, Batut J, Dedieu A, Domergue O, Ghai J, Hertig C, Boistard P, Kahn D. Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. Cell 1988;54:671–683.
159. Parsons R, Day DA. Mechanism of soybean nodule adaptation to different oxygen pressures. Plant Cell Environ 1990;13:501–512.
160. Purcell LC, Sinclair TR. Soybean (*Glycine max*) nodule physical traits associated with permeability responses to oxygen. Plant Physiol 1993;103:149–156.
161. Denison RF, Kinraide TB. Oxygen-induced membrane depolarizations in legume root nodules – possible evidence for an osmoelectrical mechanism controlling nodule gas permeability. Plant Physiol 1995;108:235–240.
162. Vikman P-A. The symbiotic vesicle is a major site for respiration in *Frankia* from *Alnus incana* root nodules. Can J Microbiol 1992;38:779–784.
163. Bond G. Some features of root growth in nodulated plants of *Myrica gale* L. Ann Bot 1952;16: 467–475.
164. Silvester WB, Silvester JK, Torrey JG. Adaptation of nitrogenase to varying oxygen tension and the role of the vesicle in root nodules of *Alnus incana* subsp *rugosa*. Can J Bot 1988;66:1772–1779.
165. Silvester WB, Whitbeck J, Silvester JK, Torrey JG. Growth, nodule morphology and nitrogenase activity of *Myrica gale* grown with roots at various oxygen levels. Can J Bot 1988;66:1762–1771.
166. Silvester WB, Harris SL. Nodule structure and nitrogenase activity of *Coriaria arborea* in response to varying pO_2. Plant Soil 1989;118:97–109.
167. Hafeez F, Akkermans ADL, Chaudhary AH. Observations on the ultrastructure of *Frankia spp.* in root nodules of *Datisca cannabina* L. Plant Soil 1984;79:383–402.
168. Parsons R, Silvester WB, Harris S, Gruijters WTM, Bullivant S. *Frankia* vesicles provide inducible and absolute oxygen protection for nitrogenase. Plant Physiol 1987;83:728–731.
169. Meesters TM, Van Vliet WM, Akkermans ADL. Nitrogenase is restricted to the vesicles in *Frankia* strain EAN1pec. Physiol Plant 1987;70:267–271.
170. Berry AM, Harriot OT, Moreau RA, Osman SF, Benson DR, Jones AD. Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. Proc Natl Acad Sci USA 1993;90:6091–6094.
171. Berg RH. Symbiotic vesicle ultrastructure in high pressure-frozen, freeze-substituted actinorhizae.

Protoplasma 1994;183:37–48.
172. Newcomb W, Wood SM. Morphogenesis and fine structure of *Frankia* (Actinomycetales): the microsymbiont of nitrogen-fixing actinorhizal root nodules. Int Rev Cytol 1987;109:1–88.
173. Berg RH, McDowell L. Endophyte differentiation in *Casuarina* actinorhizae. Protoplasma 1987;136:104–117.
174. Henry MF. Étude ultrastructurale de l'endophyte présent dans les nodusités radiculaires de *Myrica gale* L. Bull Soc Bot France 1977;124:291–300.
175. Berg RH, McDowell L. Cytochemistry of the wall of infected cells in *Casuarina* actinorhizae. Can J Bot 1987;66:2038–2047.
176. Zeng S, Tjepkema JD, Berg RH. Gas diffusion pathway in nodules of *Casuarina cunninghamiana*. Plant Soil 1989;118:119–123.
177. Sellstedt A, Reddell P, Rosbrook PA, Ziehr A. The relations of haemoglobin and lignin-like compounds to acetylene reduction in symbiotic *Casuarina*. J Exp Bot 1991;42:1331–1337.
178. Zeng S, Tjepkema JD. The wall of the infected cell may be the major diffusion barrier in nodules of *Myrica gale* L. Soil Biol Biochem 1994;5:633–639.
179. Zeng S, Tjepkema JD. The resistance of the diffusion barrier in nodules of *Myrica gale* L. changes in response to temperature but not to partial pressure of O_2. Plant Physiol 1995;107:1269–1275.
180. Tjepkema JD, Asa DJ. Total and CO_2-reactive heme content of actinorhizal nodules and the roots of some nonnodulating plants. Plant Soil 1987;100:225–236.
181. Sellstedt A, Reddell P, Rosbrook PA. The occurrence of haemoglobin and hydrogenase in nodules of 12 *Casuarina-Frankia* symbiotic associations. Physiol Plant 1991;82:458–464.
182. Pathirana SM, Tjepkema JD. Purification of hemoglobin from the actinorhizal root nodules of *Myrica gale* L. Plant Physiol 1995;107:827–831.
183. Hadfield KL, Bulen WA. Adenosine triphosphate requirement of nitrogenase from *Azotobacter vinelandii*. Biochem 1969;8:5103–5108.
184. Wilson PW, Umbreit WW. Mechanism of symbiotic nitrogen fixation. III. Hydrogen as specific inhibitor. Arch Microbiol 1937;8:440–457.
185. Emerich DW, Ruiz-Argüeso T, Ching TM, Evans HJ. Hydrogen dependent nitrogenase activity and ATP-formation *in Rhizobium japonicum* bacteroides. J Bacteriol 1979;137:153–160.
186. Hanus FJ, Maier RJ, Evans HJ. Autotrophic growth of H_2-uptake-positive strains of *Rhizobium japonicum* in an atmosphere supplied with hydrogen gas. Proc Natl Acad Sci USA 1979;76:1788–1792.
187. De Vries W, Stam H, Stouthamer AH. Hydrogen oxidation and nitrogen fixation in rhizobia, with special attention focused on strain ORS571. Antonia van Leeuwenhoek 1984;52:85–96.
188. Stam H, van Verseveld H, de Vries W, Stouthamer AH. Hydrogen oxidation and efficiency of nitrogen fixation in succinate-limited chemostate cultures of *Rhizobium* ORS571. Arch Microbiol 1984;139:53–60.
189. Murry MA, Lopez MF. Interaction between hydrogenase, nitrogenase and respiratory activities in a *Frankia* isolate from *Alnus rubra*. Can J Microbiol 1989;35:636–641.
190. Evans HJ, Hanus FJ, Russell SA, Harker AR, Lambert GR, Dalton DA. Biochemical characerization and genetics of H_2 recycling in *Rhizobium*. In: Ludden PW, Burris JE (eds) Nitrogen Fixation and CO_2 Metabolism. New York: Elsevier, 1985;3–11.
191. Garg N, Garg RP, Nainawatee HS. *In planta* comparison of Hup$^+$ and isogenic Hup$^-$ *Rhizobium leguminosarum*. Indian J Exp Bot 1990;28:427–429.
192. Sajid GM, Campbell WF. Symbiotic activity in pigeon pea inoculated with wild-type Hup$^-$, Hup$^+$ and transconjugant Hup$^+$ *Rhizobium*. Tropical Agriculture 1994;7212:182–187.
193. Sørensen GM, Wyndaele R. Effect of transfer of symbiotic plasmids and of hydrogenase genes (*hup*) on symbiotic efficiency of *Rhizobium leguminosarum* strains. J Gen Microbiol 1986;132:317–324.
194. Hume DJ, Shelp BJ. Superior performance of the Hup$^-$ *Bradyrhizobium japonicum* strain 532C in Ontario soybean field trials. Can J Plant Sci 1990;70:661–666.
195. Bedmar EJ, Phillips DA. *Pisum sativum* cultivar effects on hydrogen metabolism in *Rhizobium*. Can J Bot 1983;62:1682–1686.

196. Trueisen TA, Wyndale R. Recycling efficiency in hydrogenase uptake positive strains of *Rhizobium leguminosarum*. Physiol Plant 1984;62:45–50.
197. Saini I, Chander DR, Hagpal P. Uptake hydrogenase in fast-growing strains of *Rhizobium spp.* (*Sesbania*) in relation to nitrogen fixation. J Appl Bacteriol 1987;62:449–452.
198. Boogerd FC, Ferdinandy-Van Vlerken MMA, Mawadza C, Pronk AF, Stouthamer AH, Van Verseveld HW. Nitrogen fixation and hydrogen metabolism in relation to the dissolved oxygen tension in chemostat cultures of the wild-type and a hydrogenase-negative mutant of *Azorhizobium caulinodans*. Appl Environ Microbiol 1994;60:1859–1866.
199. Huss-Danell K. The physiology of actinorhizal nodules. In: Schwintzer CR, Tjepkema JD (eds) The Biology of *Frankia* and Actinorhizal Plants. New York: Academic Press, 1990;129–156.
200. Sellstedt A, Lindblad P. Activities, occurrence, and localization of hydrogenase in free-living and symbiotic *Frankia*. Plant Physiol 1990;92:809–815.
201. Schubert KR. Products of biological nitrogen fixation in higher plants: synthesis, transport, and metabolism. Ann Rev Plant Physiol 1986;37:539–574.
202. Sellstedt A, Atkins CA. Composition of amino compounds transported in xylem of *Casuarina spp.* J Exp Bot 1991;42:1493–1497.
203. Yoneyama T, Kondo M. *Sesbania spp.*, *Aeschynomene indica* and *Crotalaria spp.* are amide-exporters. Soil Sci Plant Nutr 1990;36:689–693.
204. Hirel B, Perrot-Rechenmann C, Maudinas B, Gadal P. Glutamine synthetase in alder (*Alnus glutinosa*) root nodules. Purification, properties and cytoimmunochemical localization. Physiol Plant 1982;55:197–203.
205. Brears T, Walker EL, Coruzzi GM. A promoter sequence involved in cell-specific expression of the pea glutamine synthetase *GS3A* gene in organs of transgenic tobacco and alfalfa. Plant J 1991;1:235–244.
206. Miao G-H, Hirel B, Marsolier MC, Ridge RW, Verma DPS. Ammonia-regulated expression of a soybean gene encoding cytosolic glutamine synthetase in transgenic *Lotus corniculatus*. Plant Cell 1991;3:11–22.
207. Forde BG, Day HM, Turton JF, Wen-jun S, Cullimore JV, Oliver JE. Two glutamine synthetase genes from *Phaseolus vulgaris* L. display contrasting developmental and spatial patterns of expression in transgenic *Lotus corniculatus* plants. Plant Cell 1989;1:391–401.
208. Hanks JF, Tolbert NE, Schubert KR. Localization of enzymes of ureide biosynthesis in peroxisomes and microsomes of nodules. Plant Physiol 1981;68:65–69.
209. Nguyen T, Zelechowska M, Foster V, Bergmann H, Verma DPS. Primary structure of the soybean nodulin-35 gene encoding uricase II localized in the peroxisomes of uninfected cells of nodules. Proc Natl Acad Sci USA 1985;82:5040–5044.
210. Taté R, Patriarca EJ, Riccio A, Defez R, Iaccarino M. Development of *Phaseolus vulgaris* root nodules. Molec Plant-Microbe Interact 1994;7:582–589.
211. Vaughn KC. Structural and cytochemical characterization of three specialized peroxisome types in soybean. Physiol Plant 1985;64:1–12.
212. Selker JML. Three-dimensional organization of uninfected tissue in soybean root nodules and its relation to cell specialization in the central region. Protoplasma 1988;147:178–190.
213. Pate JS, Gunning GES, Briarty LG. Ultrastructure and functioning of the transport system of the leguminous root nodules. Planta 1969;85:11–34.
214. Schubert KR. Enzymes of purine biosynthesis and catabolism in *Glycine max*. Plant Physiol 1981;86:1115–1122.
215. Atkins CA. Ammonia assimilation and export of nitrogen from the legume nodule. In: Dilworth M, Glenn A (eds) Biology and Biochemistry of Nitrogen Fixation. Amsterdam: Elsevier, 1991;293–319.
216. Martin F, Hirel B, Gadal P. Purification and properties of ornithine carbamoyl transferase 1 from *Alnus glutinosa* root nodules. Z Pflanzenphysiol 1983;111:413–422.
217. Blom J, Roelofsen W, Akkermans ADL. Assimilation of nitrogen in root nodules of alder (*Alnus glutinosa*). New Phytologist 1981;89:321–326.
218. Wheeler CT, Lawrie AC. Nitrogen fixation in root nodules of alder and pea in relation to the supply

of photosynthetic assimilates. In: Nutman PS (ed) Symbiotic Nitrogen in Plants. Cambridge: Cambridge University Press, 1976;497–509.
219. Burgess D, Peterson RL. Effect of nutrient conditions on root nodule development in *Alnus japonica*. Can J Bot 1987;65:1658–1670.
220. Wheeler CT. The causation of the diurnal changes in nitrogen fixation in the nodules of *Alnus glutinosa*. New Phytologist 1971;70:487–495.
221. Vance CP, Heichel GH. Carbon in N_2 fixation: Limitation or exquisite adaptation. Ann Rev Plant Physiol Plant Molec Biol 1991;42:373–392.
222. Wheeler CT, Watts SH, Hillman JR. Changes in carbohydrates and nitrogenase compounds in the root nodules of *Alnus glutinosa* in relation to dormancy. New Phytologist 1983;95:209–218.
223. Pate JS. Root exudation studies on the exchange of ^{14}C-labeled organic substances between the roots and shoot of the nodulated legume. Plant Soil 1962;17:333–356.
224. Morell M, Copeland L. Sucrose synthase of soybean nodules. Plant Physiology 1985;78:149–154.
225. Thummler F, Verma DPS. Nodulin-100 of soybean is the subunit of sucrose synthase regulated by the availibility of free heme in nodules. J Biologic Chem 1987;262:14730– 14736.
226. Küster H, Frühling M, Perlick AM, Pühler A. The sucrose synthase gene is predominantly expressed in the root nodule tissue of *Vicia faba*. Molec Plant-Microbe Interact 1993;6:507–514.
227. Vance CP, Gregerson RG, Robinson DL, Miller SS, Gantt JS. Primary assimilation of nitrogen in alfalfa nodules: molecular features of the enzymes involved. Plant Sci 1994;101:51–64.
228. Weaver CD, Shower NH, Louis CF, Roberts DM. Nodulin-26, a nodule specific symbiosome membrane protein from soybean, is an ion channel. J Biologic Chem 1994;269:17858–17862.
229. McClure PR, Coker GT, Schubert KR. Carbon dioxide fixation in roots and nodules of *Alnus glutinosa*. I. Role of phosphoenolpuryvate carboxylase and carbamyl phosphate synthetase in dark CO_2 fixation, citrulline synthesis, and N_2 fixation. Plant Physiol 1983;71:652–657.
230. Akkermans ADL, Huss-Danell K, Roelofsen W. Enzymes of the tricarboxylic acid cycle and the malate-aspartate shuttle in the N_2-fixing endophyte of *Alnus glutinosa*. Physiol Plant 1981;53:289–294.
231. Soltis DE, Soltis PS, Morgan DR, Swensen SM, Mullin BC, Dowd JM, Martin PG. Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc Natl Acad Sci USA 1995;92:2647–2651.
232. Trinick MJ. Structure of nitrogen-fixing nodules formed by *Rhizobium* on roots of *Parasponia andersonii* Planch. Can J Microbiol 1979;25:565–578.
233. Lancelle SA, Torrey JG. Early development of *Rhizobium*-induced root nodules of *Parasponia rigida*. I. Infection and early nodule initiation. Protoplasma 1984;123:26–37.
234. Lancelle SA, Torrey JG. Early development of *Rhizobium*-induced root nodules of *Parasponia rigida*. II. Nodule morphogenesis and symbiotic development. Can J Bot 1984;63:25–35.
235. Christensen T, Dennis ES, Peacock JW, Landsmann J, Marcker KA. Hemoglobin genes in nonlegumes: cloning and characterization of a *Casuarina glauca* hemoglobin gene. Plant Molec Biol 1991;16:339–344.
236. Schlaman HRM, Spaink HP, Okker RJH, Lugtenberg BJJ. Subcellular localization of the *nodD* gene product in *Rhizobium leguminosarum*. J Bacteriol 1989;171:4686–4693.
237. Siqueira JO, Nair MG, Hammerschmidt R, Safir GR. Significance of phenolic compounds in plant-soil-microbial systems. Crit Rev Plant Sci 1991;10:63–121.
238. Carlson RW, Juan SJ, Bhat UR, Glushka J, Spaink HP, Wijfjes AHM, Van Brussel AAN, Stokkermans TJW, Peters NK, Stacey G. The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type-1 and type-2 strains of *Bradyrhizobium japonicum*. J Biologic Chem 1993;168:18372–18381.
239. Price NPJ, Relic B, Talmont F, Lewin A, Promé J-C, Broughton WJ. Broad-host-range *Rhizobium* species strain NGR234 secretes a family of carbamoylated, and fucosylated, nodulation signals that are *O*-acetylated or sulphated. Molec Microbiol 1992;6:3575–3584.

Chapter 2

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

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Short communication

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

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Abstract

Two nodule cDNA clones representing genes involved in *Alnus glutinosa* nitrogen metabolism were analysed. *ag11* encoded glutamine synthetase (GS), the enzyme responsible for ammonium assimilation, while *ag118* encoded acetylornithine transaminase (AOTA), an enzyme involved in the biosynthesis of citrulline, the nitrogen transport form in *Alnus*. GS mRNA was found at highest levels in root nodules, where it was present in the infected cells as well as in the cells of the pericycle of the vascular system. AOTA transcripts were found at high levels in nodules, confined to the infected cells, suggesting that in nodules of *A. glutinosa*, citrulline biosynthesis takes place mainly in the infected cells.

The major enzyme responsible for the first step in ammonium assimilation is glutamine synthetase (GS; EC 6.3.1.2) which catalyses the ATP-dependent condensation of ammonium with glutamate to yield glutamine [16]. Plant GS isoenzymes are located in the cytosol or the chloroplasts [23]. Different GS isoforms are encoded by small multigene families whose members have been shown to be differentially expressed in an organ-specific manner [2, 28, 33]. GS expression has been extensively investigated in legumes, especially in root nodules where GS plays a crucial role in the assimilation of ammonium, the product of rhizobial nitrogen fixation.

Nitrogen-fixing actinomycetes of the genus *Frankia* can induce actinorhizal nodules (actinorhizae) on the roots of several woody plants from eight different families, 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 [3]. In actinorhizal nodules as well as in legume nodules, ammonium, the product of bacterial nitrogen fixation, is assimilated via the GS/GOGAT (glutamine synthetase/glutamate synthase) pathway [31]. GS activity has been found in root nodules of *A. glutinosa*, where it was associated with the cytosol of the large inner cortical cells [4, 17].

The product of the GS/GOGAT pathway, glutamate, is further metabolized in root nodules to yield a nitrogen transport form which is brought into the xylem. The nitrogen transport form depends on the host plant. Most actinorhizal plants export amides, but *Alnus* species are citrulline exporters [31]. In plants as well as in microorganisms, citrulline is synthesized via the arginine biosynthetic pathway. The enzyme acetylornithine transaminase (AOTA; also known as acetylornithine aminotransferase, ACOAT; EC 2.6.1.11)

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y08681 (pAg11) and Y08680 (pAg118).

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Ag118    1 MTSLQYFSLNRPVFPATHLHRPGIRHLQVSACANVEVQAPSSVKKQGVSK  50
ArgD     1                                MSLQTLIEQATNPPESGSAASSPPST  26
ARG8     1                                          MFKRYLSSTSSRRFT  15

Ag118   51 EVMEAAGRVLVGTYAR.VPVVLSRGKGCKLY.DPDGREYLDLSAGIAVNV  98
ArgD    27 DSFDAS...VMSTYGR.FPLALERGAGCRVW.DTQGKEYLDFVAGIATCT  71
ARG8    16 SILEEKAFQV.TTYSRPEDLCITRGKNAKLYDDVNGKEYIDFTAGIAVTA  64

Ag118   99 LGHADSDWLRAVTEQAATLTHVSNVFY.......SIPQVELAKRLVASSF 141
ArgD    72 LGHAHPAMVEAVTRQIQKLHHVSNLYYIPEQGELAQWIIQ.......HSC 124
ARG8    65 LGHANPKVAEILHHQANKLVHSSNLYFTKECLDLSEKIVEKTKQFGGQHD 114

Ag118  142 ADRVFFSNSGTEANEAAIKFARKFQRFTRPDEKQPATEFVSFSNSFHGRT 191
ArgD   125 ADRVFFCNSGAEANEAAIKLARKYAHTVLDIEK...PIILTANASFHGRT 161
ARG8   115 ASRVFLCNSGTEANEAALKFAKHHG.IMKNPSKQ...GIVAFENSFHGRT 160

Ag118  192 MGSLALTSKENYRSPFEPVMPGVTFLEYGNIEAATQLI.....QRRKIAA 236
ArgD   162 LATITATGQAKYQKYFDPLVPGFHYVNYNDISAVEAAISELDBGDYRVAA 211
ARG8   161 MGALSVTWNSKYRTPFGDLVPHVSFLNLNDEMTKLQSYIETKKDE..IAG 208

Ag118  237 VFVEPIQGEGGVYSATKEFLYALRKACDDSGTLLVFDEVQCGLGRTGYLW 286
ArgD   212 ILIEPLQGEGGVRPGDVEYFQKLRQICDDTGILLMFDEVQVGMGRSGKLW 261
ARG8   209 LIVEPIQGEGGVFPVEVEKLTGLKKICQDNDVIVIHDEIQCGLGRSGKLW 258

Ag118  287 AHEIY..DVFPDIMTLAKPLAGGLPIGAVLVTERVASAITYGDHGTTFAG 334
ArgD   262 GYEYLGVE..FDIFTSAKGLGGGIPIGA.MMSKKFCDVFQPGEHASTFGG 308
ARG8   259 AHAYLPSEAHPDIFTSAKALGNGFPIAATIVNEKVNNALRVGDEGTTYGG 308

Ag118  335 GPLVCKAALTVLDKILRPGFLASVSEKGHYFKEMLINKLGG.NSHVREVR 383
ARGD   309 NPFACGVALAVCQTLERENILQNVQDRGEQLRSGLRAIAAKYPHELTEVR 358
ARG8   309 NPLACSVSNYVLDTIADEAFLKQVSKKSDILQKRLREIQAKYPNQIKTIR 358

Ag118  384 GVGLIVGIELD....VSASPLVNACLNSGLLVLTAGKGNVVRIVPPLIIT 429
ArgD   359 GWGLINGLELAADIPLTAADVVKAAINEGLLLVPAGPK.VVRFVPPLIVT 407
ARG8   359 GKGLM....LGAEFVEPPTEVIKKARELGLLIITAGKS.TVRFVPALTIE 403

Ag118  430 EQELEKAAEILLQCLPALDRHG 451
ArgD   408 EAEINTALKLLEKALATVTA   427
ARG8   404DELIEEGMDAFEKAIEAVYA    423
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Figure 1. AOTA amino acid sequence comparisons. Comparison between the amino acid sequence of Ag118 and the sequences of *Anabaena* AOTA (ArgD) [13] and yeast AOTA (ARG8) [15]. Sequence data were analysed using the programs of the Wisconsin Genetics Computer Group (GCG) [9]. Gaps were introduced to optimize the alignment. Identical amino acids are given in bold type.

catalyses the fourth step in this pathway, the conversion of N-acetyl-γ-glutamate semialdehyde to N-acetylornithine [22, 7, 8].

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

To analyse nitrogen metabolism in actinorhizal nodules, an *A. glutinosa* nodule cDNA library was screened differentially with nodule and root cDNA, respectively [29]. 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 ([29], data not shown). The ends of the cDNA clones were sequenced [30] and the deduced amino acid sequences were used for homology searches in the nucleotide sequence databases of the National Center of Biotechnology Information (NCBI), National Library of Medicine, NIH, in Bethesda, MD [1]. Two clones encoding products that were 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 from roots and nodules of soybean (95% amino acid similarity; data not shown) [24] and to a nodule-enhanced cytosolic GS from alfalfa (93%; data not shown) [34]. Because of these homologies and the fact that Ag11 does not contain a transit peptide sequence at the N-terminus (data not shown), it probably represents a cytosolic GS isoform and was termed AgGS1. In previous physiological studies by Hirel *et al.* [17], two

GS isoenzymes have been reported in *Alnus glutinosa* nodules, a major isoform and minor isoform. The major one showed the same chromatographic behaviour as, and probably was identical with, root GS [17]. Up to now, the data available do not allow to conclude which isoform AgGS1 represents.

The insert of the cDNA clone pAg118 encoded a 451 amino acid polypeptide, homologous to acetylornithine transaminase (AOTA) encoded by the *argD* gene of *Anabaena* (43%; Fig. 1) [13] and by the *ARG8* gene from *Saccharomyces cerevisiae* (42%; Fig. 1) [15]. Only in the N-terminal regions, which probably represent transit peptides for specific subcellular localization, amino acid sequences were divergent. These data strongly suggested that Ag118 represents an AOTA. It is known that yeast AOTA (*ARG8*) and mammalian ornithine aminotransferase (OAT) are all located in the mitochondrial matrix [25, 15]. This would suggest that Ag118 is also located in the mitochondria. The N-terminus of Ag118 fulfils the criteria for mitochondrial transit peptides and for the N-termini of mature mitochondrial proteins [38]. The mitochondrial localization of AOTA agrees with cytochemical data on the localization of ornithine carbamyl transferase, another enzyme involved in citrulline biosynthesis which was found in the mitochondria of host cells in *Alnus* root nodules [32], indicating that citrulline is synthesized in the mitochondria.

To analyse the organization and complexity of GS and AOTA genes in *A. glutinosa*, DNA gel 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 DNA gel blot hybridization results indicate that there are at least two members in the GS gene family of *A. glutinosa* (Fig. 2A), while the hybridization with *ag118* leads to a more complicated pattern, containing several weaker hybridizing bands (Fig. 2B). These bands might represent genes encoding other aminotransferases, since Ag118 also showed homology to human [18] and *Drosophila ananassae* OATs (GenBank accession number dbjID50331; data not shown), supporting the hypothesis of an evolutionary relationship between AOTAs and OATs [15].

Expression of GS and AOTA

RNA gel blot hybridization was performed to check the organ-specific expression of GS and AOTA genes using

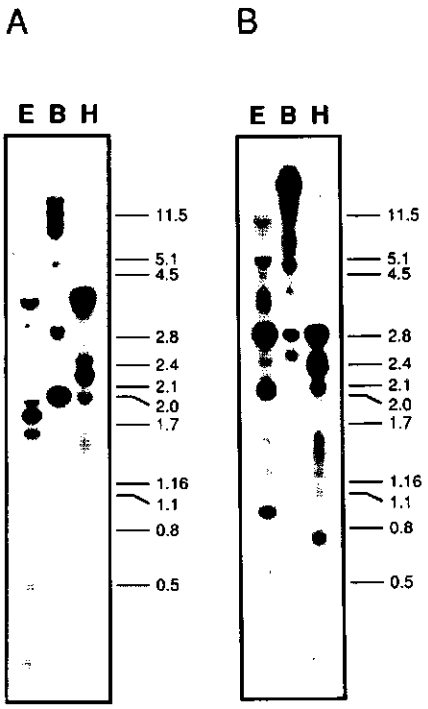


Figure 2. DNA gel blot hybridization analysis. DNA gel blots containing total DNA of *Alnus glutinosa* [29] digested with *Eco*RI (E), *Bam*HI (B) and *Hin*dIII (H) were hybridized with ^{32}P-labelled inserts of pAg11 (**A**) and pAg118 (**B**), respectively. Hybridization conditions have been described elsewhere [29]. The *ag11* cDNA contains an *Eco*RI, a *Hin*dIII and two *Bam*HI sites. The *ag118* cDNA contains a *Hin*dIII site.

RNA isolated from seedling roots and cotyledons harvested 2–3 weeks after germination, nodules harvested 5–8 weeks after infection, shoot tips, male and female flowers, and developing fruits harvested in April, June, and September 1994, respectively. 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.

The expression patterns of *ag11* and *ag118* in the nodule were determined by *in situ* hybridization of longitudinal and cross-sections of *A. glutinosa* nod-

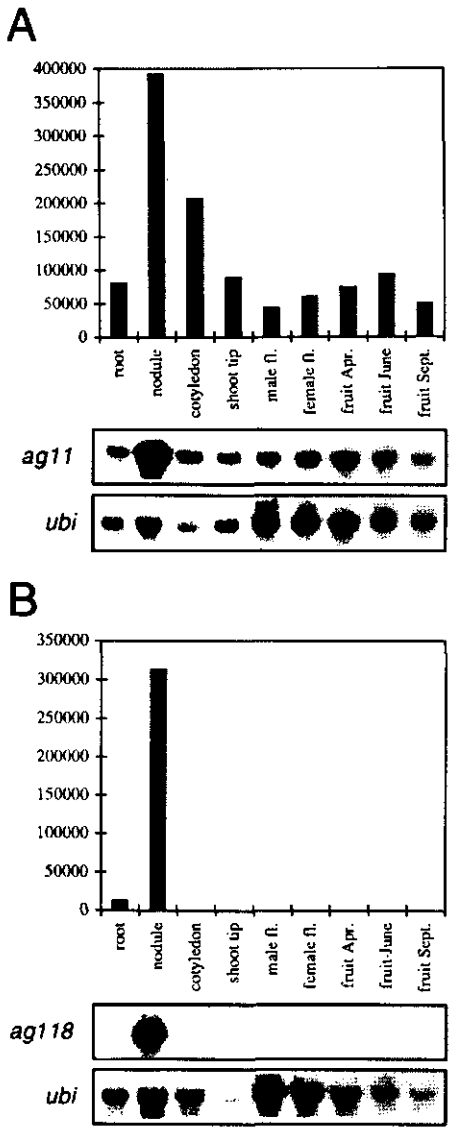
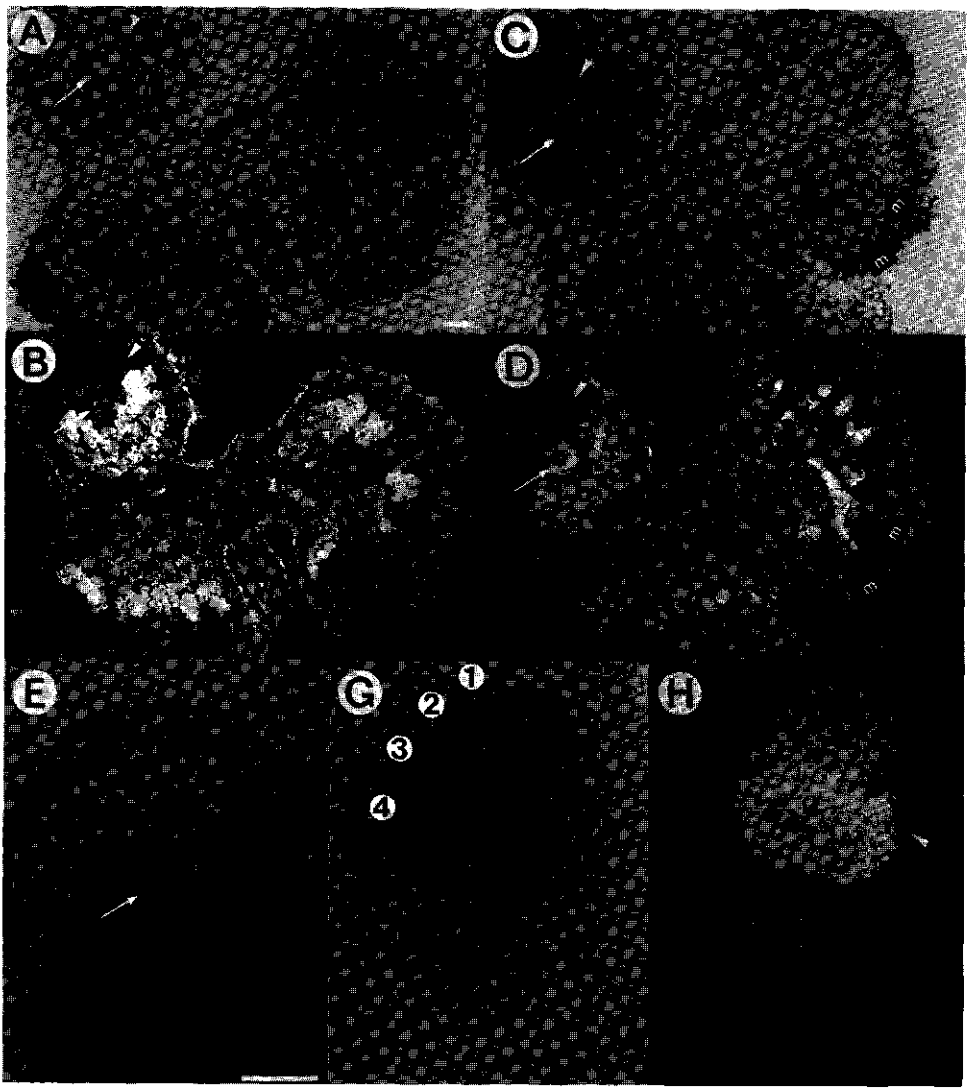


Figure 3. RNA gel blot hybridization analysis. RNA gel blots containing about 10 μg total RNA [27] per slot were hybridized with ^{32}P-labelled inserts of pAg11 (**A**) and pAg118 (**B**), respectively. Hybridization conditions have been described elsewhere [29]. The amount of mRNA on the filters was determined by hybridization with a soybean ubiquitin probe [19]. Signal was quantified by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Expression levels are shown as relative area units calculated from comparison with ubiquitin expression. RNA was isolated from roots, nodules, cotyledons, shoottips, male flowers, female flowers, and from immature fruits harvested April, June, and September, respectively.

ules with ^{35}S-labelled antisense and sense RNA probes, respectively [35]. For a marker of the developmental gradient of the infected cells, *in situ* hybridizations with antisense RNA of the nitrogenase structural gene *nifH* from *Frankia* HFPArI3 were performed on adjacent sections [29]. 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 system 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 system (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).

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 from indeterminate nodules of pea (*Pisum sativum* ssp. *sativum* L. cv. Rondo) and from determinate nodules of *Phaseolus vulgaris* L. cv. Negro-Jamapa [36] were hybridized with *P. vulgaris* nodule-specific GS (gln-γ) [2] antisense and sense 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 system (Figs. 4G/H). These results are consistent with those of promoter analysis of pea *GS3A* in transgenic alfalfa nodules [5]. In *P. vulgaris* nodules, *gln-γ* transcripts were confined to the infected cells (Figs. 4I/J), confirming the results of Forde *et al.* [14]. Thus, while the study of nodule GS gene expression using promoter-GUS fusions in heterologous legumes can yield misleading results [21], the data obtained for pea and *P. vulgaris* could be confirmed using *in situ* hybridization.



Conclusions

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. Thus, it is very probable that the assimilation of ammonium exported by symbiotic *Frankia*, and the biosynthesis of the nitrogen

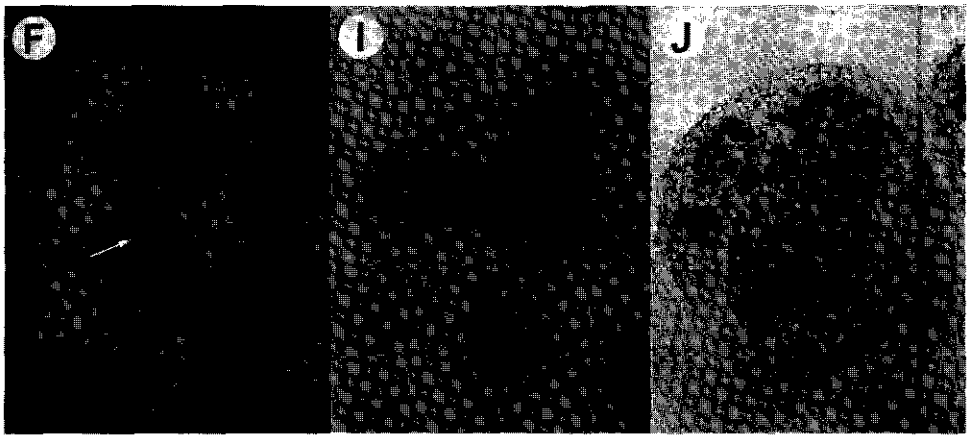


Figure 4. In situ localization of GS and AOTA mRNA. In bright-field micrographs (**A**, **C**, **E**, **G**, **I**, **J**), silver grains denoting hybridization are visible as black dots. In dark-field micrographs (**B**, **D**, **F**, **H**), silver grains appear as white dots. Panel (**F**) is a dark field photograph taken under epipolarized light to visualize silver grains in particular. 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 with 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 with T7 RNA polymerase. For *P. vulgaris gln-γ*, a 360 bp *Eco*RI/*Bam*HI fragment of the coding region [14] 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 with T3 RNA polymerase. The production of *Frankia nifH* antisense RNA has been described elsewhere [29]. **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 system (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 system (white arrow). **G/H** Longitudinal section of a 18-day-old pea nodule hybridized with *P. vulgaris GS*, antisense RNA. The zonation of the nodule according to Vasse *et al.* [37] 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*, 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*, 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**.

transport form, citrulline, occurs mainly in the infected cells of *A. glutinosa* nodules. Citrulline would then have to be exported from the infected cells to the xylem elements.

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, as shown by the presence of high amounts of mitochondria, and by high levels of sucrose synthase expression [6, 36]. The fact that GS transcripts were found in the pericycle of the nodule vascular system 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. Glutamate is enriched in the stem xylem compared to nodules [4]. An assimilation product 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 [20] who have estimated that a nitrogenous solute can be catabolized and reassimilated five or more times before ending up in the seed. Thus, we postulate that nitrogenous solutes are degraded and ammonium is reassimilated in the vascular pericycle of *Alnus* nodules in the course of transport to the xylem. Also in legume nodules, GS expression is found in the nodule vascular system, indicating that reassimilation of ammonium occurs in the course of the transport of nitrogenous solutes to the xylem [14, 5, 34] (Fig. 4G/H; Fig. 4I/J). Reassimilation of ammonium seems to be involved in nitrogen transport in general, since GS expression has been detected in the phloem of stems, leaves, and roots [11].

All legume nodules examined thus far have in common that GS gene expression in the central tissue is controlled developmentally as well as metabolically, while the different modes of regulation usually are applied to different GS genes [16]. The fact that GS expression is always induced before the onset of nitrogen fixation and the export of ammonium by bacteroids [26, 14, 24, 34] (Fig. 4G/H; Fig. 4I/J), and the fact that GS is expressed in ineffective, i.e. not nitrogen-fixing nodules, indicate developmental control. Metabolic control, i.e. induction by ammonium, has been shown directly for a soybean GS gene [24]. Furthermore, the fact that GS expression is consistently much lower in ineffective than in effective nodules [39, 10, 12] indicates that legumes contain GS genes which are expressed in nodules under metabolic control. This is different from the situation in actinorhizal nodules of *A. glutinosa*, where GS induction does not seem to precede the onset of bacterial nitrogen fixation. 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. Thus, developmental control of GS expression seems to be lacking in the infected cells of *A. glutinosa* nodules.

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References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic local alignment search tool. J Mol Biol 215: 403–410 (1990).
2. Bennett MJ, Lightfoot DA, Cullimore JV: cDNA sequences and differential expression of the gene encoding the glutamine sythetase polypeptide of *Phaseolus vulgaris* L. Plant Mol Biol 12: 553–565 (1989).
3. Benson DR, Silvester WB: Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol Rev 57: 293–319 (1993).
4. Blom J, Roelofsen W, Akkermans ADL: Assimilation of nitrogen in root nodules of alder (*Alnus glutinosa*). New Phytol 89: 321–326 (1981).
5. Brears T, Walker EL, Coruzzi GM: A promoter involved in cell-specific expression of the glutamine synhetase *GS3A* gene in organs of transgenic tobacco and alfalfa. Plant J 1: 235–244 (1991).
6. Burgess D, Peterson RL: Development of *Alnus japonica* root nodules after inoculation with *Frankia* strain HFPArI3. Can J Bot 65: 1647–1657 (1987).
7. Cunin R, Glansdorff N, Piérard A, Stalon V: Biosynthesis and metabolism of arginine in bacteria. Microbiol Rev 50: 314–343 (1986).
8. Davis R: Compartmental and regulatory mechanisms in the arginine pathway of *Neurospora crassa* and *Saccharomyces cerevisiae*. Microbiol Rev 50: 280–313 (1986).
9. Devereux J, Haeberli P, Smithies O: A comprehensive set of sequence analysis programs for the VAX. Nucl Acids Res 12: 387–395 (1984).
10. Dunn K, Dickstein R, Burnett BK, Peterman TK, Thoidis G, Goodman HM, Ausubel FM: Developmental regulation of nodule-specific genes in alfalfa root nodules. Mol Plant-Microbe Interact 1: 66–74 (1988).
11. Edwards JE, Walker EL, Coruzzi GM: Cell-specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase. Proc Natl Acad Sci USA 87: 3459–3463 (1990).
12. Egli MA, Larson RJ, Hruschka WR, Vance CP: Synthesis of nodulins and nodule-enhanced polypeptides by plant gene-controlled ineffective alfalfa nodules. J Exp Bot 42: 969–977 (1991).
13. Floriano B, Herrero A, Flores E: Analysis of expression of the *argC* and *argD* genes in the cyanobacterium *Anabaena* sp. strain PCC 7120. J Bact 176: 6397–6401 (1994).
14. Forde BG, Day HM, Turton JF, Shen WJ, Cullimore JV, Oliver JE: Two glutamine synthetase genes from *Phaseolus vulgaris* L. display contrasting developmental and spatial patterns of expression in transgenic *Lotus corniculatus* plants. Plant Cell 1: 391–401 (1989).

15. Heimberg H, Boyen A, Crabeel M, Glansdorff N: *Escherichia coli* and *Saccharomyces cerevisiae* acetylornithine aminotransferase: evolutionary relationship with ornithine aminotransferase. Gene 90: 69–78 (1990).
16. Hirel B, Miao GH, Verma DPS: Metabolic and developmental control of glutamine synthetase genes in legumes and nonlegume plants. In: Verma DPS (ed) Control of Plant Gene Expression, pp. 443–458. CRC Press, Boca Raton, FL (1993).
17. Hirel B, Perrot-Rechenmann C, Maudinas B, Gadal P: Glutamine synthetase in alder (*Alnus glutinosa*) root nodules. Purification, properties and cytoimmunochemical localization. Physiol Plant 55: 197–203 (1982).
18. Inana G, Totsuka S, Redmond M, Dougherty T, Nagie J, Shiono T, Ohura T, Kominami E, Katunuma N: Molecular cloning of human ornithine aminotransferase mRNA. Proc Natl Acad Sci USA 83: 1203–1207 (1986).
19. Kouchi H, Hata S: Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol Gen Genet 238: 106–119 (1993).
20. Lea PJ, Miflin BJ: Transport and metabolism of asparagine and other nitrogen compounds within the plant. In: Miflin BJ (ed) The Biochemistry of Plants, vol. 5, pp. 569–608. Academic Press, New York (1980).
21. Marsolier MC, Carrayol E, Hirel B: Multiple functions of promoter sequences involved in organ-specific expression and ammonium regylation of a cytosolic soybean glutamine synthetase gene in transgenic *Lotus corniculatus*. Plant J 3: 405–414 (1993).
22. McKay G, Shargool PD: Biosynthesis of ornithine from glutamate in higher plant tissues. Plant Sci Lett 9: 189-193 (1977).
23. McNally S, Hirel B: Glutamine synthetase isoforms in higher plants. Physiol Vég 21: 761–774 (1983).
24. Miao GH, Hirel B, Marsolier MC, Ridge RW, Verma DP: Ammonium-regulated expression of a soybean gene encoding cytosolic glutamine synthetase in transgenic *Lotus corniculatus*. Plant Cell 3: 11–22 (1991).
25. Mueckler MM, Pitot HC: Sequence of the precursor to rat ornithine aminotransferase deduced from a cDNA clone. Biol Chem 260: 12993–12997 (1985).
26. Padilla JE, Campos F, Conde V, Lara M, Sánchez F: Nodule-specific glutamine synthetase is expressed before the onset of nitrogen fixation in *Phaseolus vulgaris* L. Plant Mol Biol 9: 65–74 (1987).
27. Pawlowski K, Kunze R, de Vries S and Bisseling T: Isolation of total, poly(A) and polysomal RNA from plant tisues. In: Gelvin SB, Schilperoort RA (eds) Plant Molecular Biology Manual, 2nd ed. D5: 1–13 Kluwer Academic Publishers, Dordrecht, Netherlands (1994).
28. Peterman TK, Goodman HM: The glutamine synthetase gene family of *Arabidopsis thaliana*: light-regulation and differential expression in leaves, roots and seeds. Mol Gen Genet 230: 145–154 (1991).
29. Ribeiro A, Akkermans ADL, van Kammen A, Bisseling T, Pawlowski K: A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. Plant Cell 7: 785–794 (1995).
30. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).
31. Schubert KR: Products of biological nitrogen fixation in higher plants: synthesis, transport and metabolism. Annu Rev Plant Physiol 37: 539–574 (1986).
32. Scott A, Gardner IC, McNally SF: Localization of citrulline synthesis in the alder root nodule and its implication in nitrogen fixation. Plant Cell Rep 1: 21–22 (1981).
33. Stanford AC, Larsen K, Barker DG, Cullimore JV: Differential expression within the glutamine synthetase gene family of the model legume *Medicago truncatula*. Plant Physiol 103: 73–81 (1993).
34. Temple SJ, Heard J, Ganter G, Dunn K, Sengupta-Gopalan C: Characterization of a nodule-enhanced glutamine synthetase from alfalfa: nucleotide sequence, *in situ* localization, and transcript analysis. Mol Plant-Microbe Interact 8: 218–227 (1995).
35. Van de Wiel C, Scheres B, Franssen HJ, van Lierop MJ, van Lammeren A, van Kammen A, Bisseling T: The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules. EMBO J 9: 1–7 (1990).
36. Van Ghelue M, Ribeiro A, Solheim B, Akkermans ADL, Bisseling T, Pawlowski K: Sucrose synthase and enolase expression in actinorhizal nodules of *Alnus glutinosa*: comparison with legume nodules. Mol Gen Genet 250: 437–446 (1996).
37. Vasse J, de Billy F, Camut S, Truchet G: Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J Bact 172: 4259–4306 (1990).
38. Von Heijne G, Steppuhn J, Herrmann RG: Domain structure of mitochondrial and chloroplast targeting peptides. Eur J Biochem 180: 535–545 (1989).
39. Werner D, Morschel E, Stripf R, Winchenbach B: Development of nodules of *Glycine max* infected with an ineffective strain of *Rhizobium japonicum*. Planta 147: 320–329 (1980).

Chapter 3

Sucrose synthase and enolase expression in actinorhizal nodules of *Alnus glutinosa*: comparison with legume nodules

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Abstract Two different types of nitrogen-fixing root nodules are known – actinorhizal nodules induced by *Frankia* and legume nodules induced by rhizobia. While legume nodules show a stem-like structure with peripheral vascular bundles, actinorhizal nodule lobes resemble modified lateral roots with a central vascular bundle. To compare carbon metabolism in legume and actinorhizal nodules, sucrose synthase and enolase cDNA clones were isolated from a cDNA library, obtained from actinorhizal nodules of *Alnus glutinosa*. The expression of the corresponding genes was markedly enhanced in nodules compared to roots. In situ hybridization showed that, in nodules, both sucrose synthase and enolase were expressed at high levels in the infected cortical cells as well as in the pericycle of the central vascular bundle of a nodule lobe. Legume sucrose synthase expression was studied in indeterminate nodules from pea and determinate nodules from *Phaseolus vulgaris* by using *in situ* hybridization.

Key words *Alnus glutinosa* · Actinorhiza · Nodule · Sucrose synthase · Enolase

Introduction

Symbiotic nitrogen fixation in legume as well as actinorhizal root nodules is dependent on the supply of carbohydrates from the host plant, which has to provide substrates for plant and bacterial respiration (Akkermans et al. 1981; Vance and Heichel 1991) and acceptor molecules for the assimilation of fixed nitrogen (Schubert 1986). Thus, efficient transport and metabolism of carbon sources is essential for nitrogen-fixing root nodules. The host plant also has to provide carbohydrates for nodule starch biosynthesis. Indeterminate legume nodules contain starch in both infected and uninfected cells (Vasse et al. 1990), and starch grains are present in the uninfected cells of determinate legume nodules and actinorhizal nodules (Wheeler and Lawrie 1976; Taté et al. 1994). The main transport form of photosynthate from source to sink tissues in plants is sucrose, which is transported from the shoot to the nodules (Pate 1962). Sucrose degradation can be initiated by the activity of apoplastic invertase or symplastic sucrose synthase (Morell and Copeland 1984, 1985). The latter appears to be primarily used in mature legume nodules (Kouchi et al. 1988; Anthon and Emmerich 1990). Sucrose synthase (UDP-glucose: D-fructose-2-glucosyl-transferase, EC 2.4.1.13) catalyzes the cleavage of sucrose to D-fructose and UDP-glucose (Akazawa and Okamoto 1980). At the sites of sucrose synthase activity in nodules, either starch biosynthesis or assimilate degradation for ammonium acceptor molecules or respiratory substrates should be taking place.

If the products of sucrose synthase reaction are not used for starch biosynthesis, but provide carbon sources for respiration or acceptor molecules for ammonium assimilation, UDP-fructose has to be metabolized to phosphoenolpyruvate (PEP). Subsequent carbon dioxide fixation by PEP carboxylase leads to the formation of dicarboxylic acids which can then be reduced to malate. High PEP carboxylase activities have been detected in both legume and actinorhizal nodules (Vance and Heichel 1991; McClure et al. 1983). PEP formation involves the action of enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) in the last step. Thus, high enolase activities are expected in

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nodules as well. At the sites of enolase activity, sucrose should be used for the production of acceptor molecules for ammonium assimilation or of substrates for plant and bacterial energy production, but not for starch biosynthesis.

While metabolism and development of legume nodules have been studied extensively, actinorhizal nodules have received less attention up to now (Mylona et al. 1995; Benson and Silvester 1993). Actinorhizal root nodules are formed by several dicotyledonous plant species upon infection by actinomycetous bacteria of the genus *Frankia* (Benson and Silvester 1993). The tissue organization of actinorhizal nodule lobes resembles that of lateral roots without root caps and with infected cells in the expanded cortex (Berry and Sunell 1990). While they are dissimilar from nodules induced by (*Azo*-, *Brady*-)*Rhizobium* on legume roots (Mylona et al. 1995), their structure is very similar to that of nodules induced by (*Brady*-)*Rhizobium* on *Parasponia*, the only non-legume nodulated by *Rhizobium* (Trinick 1979). Recently, a single phyletic origin for susceptibility to nitrogen-fixing symbioses in angiosperms has been suggested (Soltis et al. 1995). Thus, both types of nitrogen-fixing symbioses seem to be related.

In order to compare the carbon metabolism in legume and actinorhizal nodules, sucrose synthase expression was analysed by *in situ* hybridization in actinorhizal nodules of *Alnus glutinosa* and in *Rhizobium*-induced nodules of pea and *Phaseolus vulgaris*, respectively. The situation in actinorhizal nodules was further examined by analysis of enolase expression.

Materials and methods

Plant and bacterial growth conditions

Alnus glutinosa seeds were collected from a local source (Weerribben, The Netherlands). Growth conditions have been described elsewhere (Ribeiro et al. 1995). Nodules were harvested 5–13 weeks after infection, depending on the growth state of the plants. For isolation of root RNA, seedling roots were collected from uninfected plantlets 2 to 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. Cotyledons were harvested 2 to 3 weeks after germination. *A. glutinosa* flowers were collected from a local stand (Wageningen, The Netherlands) in March 1994. Developing fruits were collected from the same stand in April, June, and September 1994, respectively. *Pisum sativum* ssp. *sativum* L. cv. Rondo was grown in gravel trays and inoculated with *Rhizobium leguminosarum* bv *viciae* 248 as described by Bisseling et al. (1978). *Phaseolus vulgaris* L. cv. negro Jamapa was inoculated with *R. leguminosarum* bv. *phaseoli* strain CE330 (Diebold and Noel 1989) grown in YEM medium (Bhuvaneswari et al. 1980). Legume nodules were harvested 2–3 weeks after infection.

Isolation of DNA and RNA

DNA and RNA were isolated from *A. glutinosa* leaves as described by Ribeiro et al. (1995). Total RNA was isolated from legumes 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.

Construction and screening of a cDNA library

A cDNA library was custom-made by Stratagene (La Jolla, Calif., USA) in λZAPII from poly(A) RNA of *A. glutinosa* nodules harvested 5–8 weeks after infection. Differential screening of this library has been described by Ribeiro et al. (1995).

Cloning and sequencing procedures

DNA manipulations were carried out as described by Sambrook et al. (1989). The nucleotide sequences were determined by the dideoxy chain-termination method (Sanger et al. 1977) using an automatic sequencer (Applied Biosystems, Foster City, Mass., USA; Model 373A). Sequence data were analyzed using the programs of the Wisconsin Genetics Computer Group (Devereux et al. 1984).

Southern and Northern hybridization conditions

Total RNA was denatured in dimethyl sulfoxide/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 (New England Nuclear, Beverly, Mass., USA) filters (RNA). Northern hybridizations were performed in buffer containing 50% formamide at 42° C (Sambrook et al. 1989). Filters were washed at 65° C with decreasing salt concentrations down to 0.5 × SSC, 0.1% SDS, but in case of a heterologous probe with 2 × SSC, 0.1% SDS.

In situ hybridization

Fixation and hybridization conditions have been described elsewhere (Ribeiro et al. 1995). 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* sucrose synthase sense and antisense probes, an *Xho*I deletion derivative of pAgSus1 containing 922 bp of the 5′ end of the cDNA was linearized with *Xho*I and transcribed with T7 RNA polymerase (sense) or linearized with *Bam*HI and transcribed with T3 RNA polymerase (antisense), respectively. For *A. glutinosa* enolase sense and antisense probes, a 660 bp *Hin*dIII-*Pst*I fragment containing the 5′ part of the cDNA was subcloned in pBluescript KS$^+$ and linearized with *Hin*dIII and transcribed with T7 RNA polymerase (sense) or linearized with *Bam*HI and transcribed with T3 RNA polymerase (antisense), respectively. For *Vicia faba* sucrose synthase, a derivative of the cDNA clone containing the first 595 bp of the coding region and 40 bp of the 5′ nontranslated region in pSVB30 (Küster et al. 1993) was used. The plasmid was linearized with *Bam*HI and sense RNA was produced using T3 RNA polymerase, or linearized with *Hin*dIII and antisense RNA was produced using T7 RNA polymerase. For legume in situ hybridizations, 6×10^5 cpm/slide were used. For *A. glutinosa* in situ hybridizations, 3×10^6 cpm/slide were used.

After washing, the slides were coated with microautoradiography emulsion LM-1 (Amersham, UK) and exposed for 4 weeks at 4° C. They were developed in Kodak (Rochester, NY, USA) D19 developer for 5 min and fixed in Kodak fixer. Sections were counterstained with 0.02% ruthenium red and 0.025% toluidine blue O for 5 min each and mounted with DePeX (BDH Laboratory Supplies, Poole, UK).

Results

Isolation of sucrose synthase and enolase cDNAs from an *A. glutinosa* nodule cDNA library

A cDNA library constructed from poly(A) RNA from *A. glutinosa* nodules was screened differentially with nodule and root cDNA, respectively, in order to isolate cDNA clones corresponding to nodule-specific or nodule-enhanced genes. Twelve positive clones were isolated. Southern hybridization on blots containing genomic DNA from *Frankia* HFPArI3 and *A. glutinosa*, respectively, was performed to prove that the cDNAs were plant-encoded. The nodule-specific/nodule-enhanced nature of these cDNA clones was verified by Northern hybridization with RNA from roots and nodules (data not shown). Two cDNA clones representing nodule-enhanced genes, pAg3-1 and pAg144, were found to encode polypeptides involved in nodule carbon metabolism and selected for further analysis. Sequencing showed that the insert of pAg3-1 contained a 2412 bp open reading frame (ORF) encoding a 91 600 dalton protein representing sucrose synthase, since it showed 84.2% amino acid sequence identity with the sucrose synthase of *Vicia faba* (Küster et al. 1993; EMBL accession number X92378). According to the nomenclature for plant sucrose synthases (Hannah et al. 1994), this clone was renamed pAgSus1. Sequencing of pAg144 revealed a 1323 bp ORF encoding a 47 600 dalton protein which was identified as enolase (EMBL accession number X92377). This clone was renamed pAgPgh1 (Cushman et al. 1994). Based on the fact that it displayed 87.5% amino acid sequence identity with the cytosolic enolase of *Arabidopsis thaliana* and because its N-terminus did not show any homology to the chloroplast target sequence, AgPgh1 can be assumed to encode a cytoplasmic, not a plastidic enolase (Van der Straeten et al. 1991). Four of the five cysteine residues of plant enolases pointed out by Van der Straeten et al. (1991) are found also in AgPgh1 (data not shown). Southern hybridization suggested that both *A. glutinosa* sucrose synthase and enolase are encoded by small gene families (Fig. 1A and B).

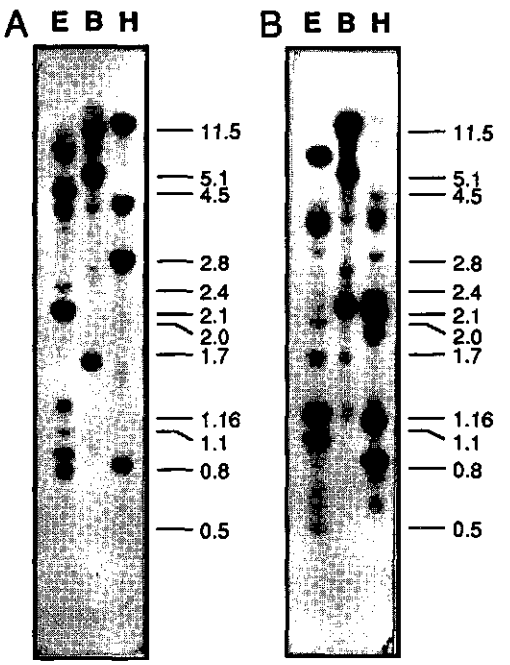


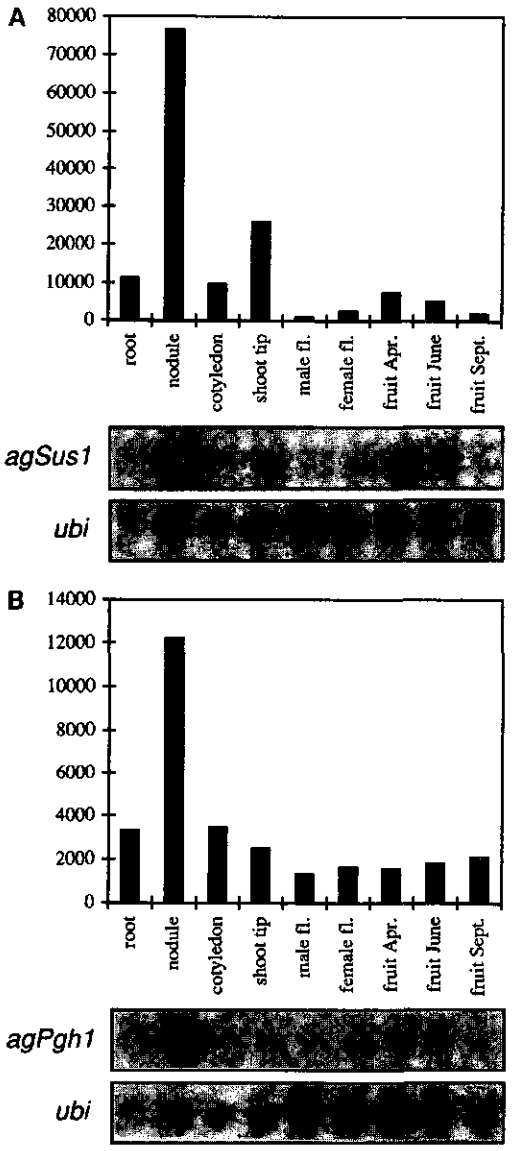
Fig. 1A, B Sucrose synthase and enolase seem to be encoded by small gene families. Southern blots containing genomic DNA of *Alnus glutinosa* digested with *Eco*RI (E), *Bam*HI (B), and *Hin*dIII (H), respectively, were hybridized with the inserts of pAgSus1 (sucrose synthase; **A**) and pAgPgh1 (enolase; **B**). Stringent washing conditions (65° C; 0.5 × SSC, 0.1% SDS) were used.

Expression of sucrose synthase and enolase in different organs of *A. glutinosa*

The expression of sucrose synthase and enolase genes in seedling roots, nodules, shoot tips, cotyledons, male and female flowers and immature fruits of *A. glutinosa* was examined by Northern blot hybridization. Both genes showed considerably higher levels of expression in nodules compared to roots (six to nine times for sucrose synthase, four to 5.5 times for enolase; Fig. 2A, B). Enolase was expressed at a higher level in roots than in shoot tips, while sucrose synthase showed higher expression in shoot tips than in roots. This is consistent with the fact that although glycolytic activity is expected to be higher in non-photosynthetic tissues (Goodwin and Mercer 1983), young leaves and the shoot meristem represent a sink tissue for photosynthate transport, and thus should contain relatively high sucrose synthase activities (Sonnewald and Willmitzer 1992). However, sucrose synthase and enolase expression levels in the different organs do not vary in parallel. This might be explained by posttranslational regulation of sucrose synthase or enolase activity. An activation of pre-existing enzymes has been suggested as a control for glycolytic activity in general (Solomos and Laties 1974), and anaerobic induction of glycolytic enzymes has been shown to take place at the level of both transcription and translation (Hake et al. 1985).

Expression of sucrose synthase and enolase in nodules of *A. glutinosa* in comparison to nitrogenase expression by the microsymbiont

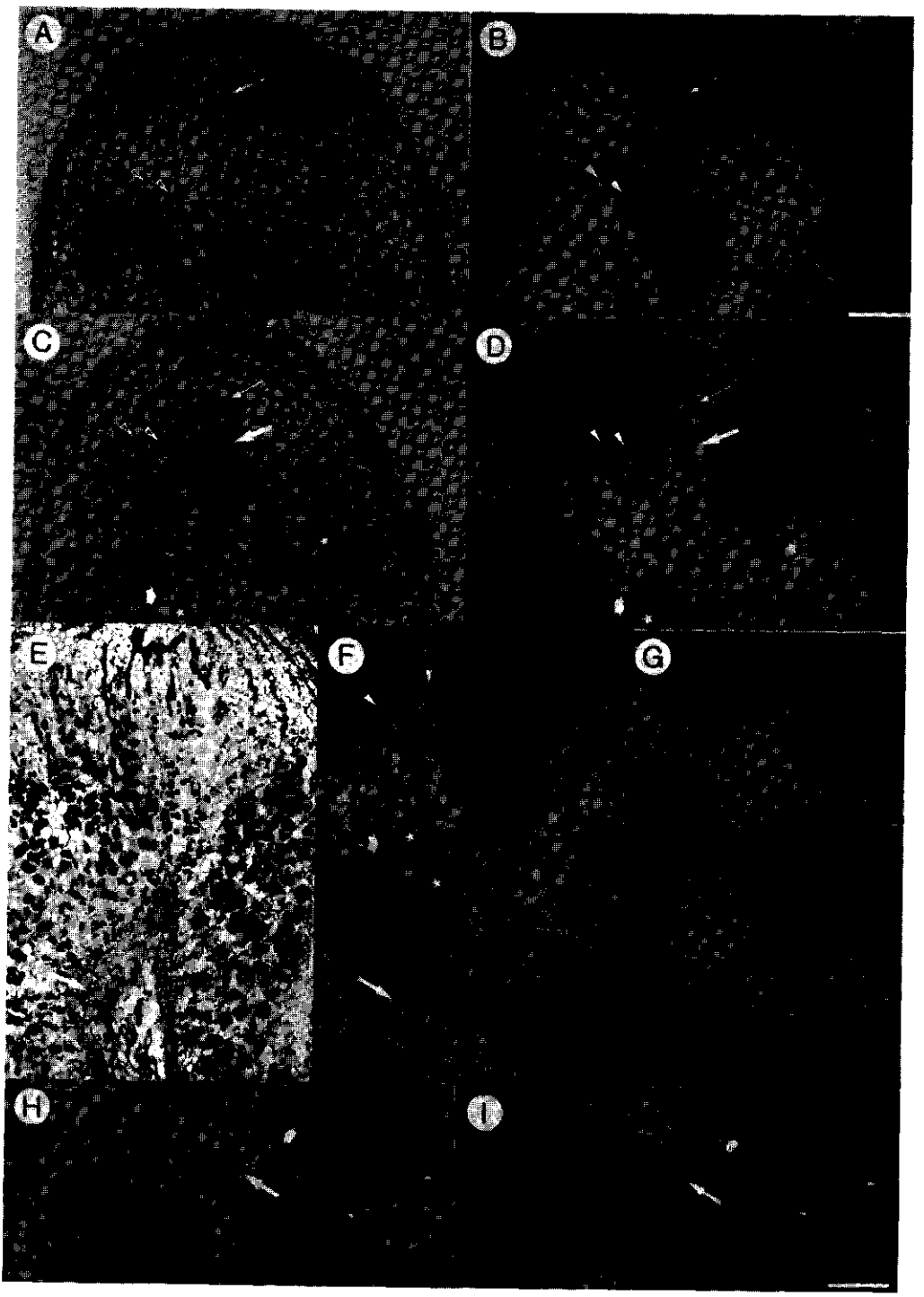
Expression of sucrose synthase and enolase in *A. glutinosa* nodules was analysed by *in situ* hybridization of



longitudinal nodule sections with ^{35}S-labeled antisense RNA. Hybridization with *Frankia nifH* was used to visualize the onset of nitrogen fixation (Fig. 3A, B; Pawlowski et al. 1995; Ribeiro et al. 1995). *A. glutinosa* nodules can be divided into four zones: (1) the meristem at the tip of the nodule lobe, (2) the infection zone where cortical cells enlarge and some are infected by *Frankia* hyphae, becoming filled with hyphae before they start to form vesicles, (3) the nitrogen fixation zone where bacterial nitrogenase is expressed in fully differentiated vesicles and nitrogen fixation takes place; and (4) the zone of senescence where endosymbiont material and plant cytoplasm are degraded. *Frankia nifH* expression, i.e. nitrogenase production and nitrogen fixation, only takes place in zone 3 (Huss-Danell and Bergman 1990; Pawlowski et al. 1995; Ribeiro et al. 1995). The results showed that sucrose synthase and enolase genes were expressed at high levels in the infected cells and in the pericycle of the central vascular bundle of the nodule lobe, but not in the uninfected cortical cells (Fig. 3C, D; E, F). Expression of sucrose synthase and enolase genes in the infected cells is detectable in zone 2, and thus precedes the onset of *nifH*

Fig. 2 Expression of (**A**) sucrose synthase (*agSus1*) and (**B**) enolase (*agPgh1*) in different organs of *A. glutinosa*. RNA was isolated from roots, nodules, cotyledons, shoot tips, male flowers, female flowers, immature fruits harvested in April, immature fruits harvested in June, and from immature fruits harvested in September. Northern blots containing about 10 µg total RNA per slot were hybridized with the inserts of pAgSus1 and pAgPgh1, respectively. Afterwards, the amount of mRNA on the filters was determined by hybridization with a soybean ubiquitin probe (Kouchi and Hata 1993). In all cases, the hybridization signal was detected by phosphoimaging and quantified by software (ImageQuant) provided by the manufacturer (Molecular Dynamics). Based on ubiquitin expression, the relative amounts of mRNA per slot were calculated. Relative area units denoting expression of *agSus1* and *agPgh1* were calibrated according to the amounts of mRNA.

Fig. 3A–I Localization of *Frankia nifH* and *A. glutinosa* sucrose synthase and enolase transcripts in longitudinal sections of *A. glutinosa* roots nodules harvested 12 weeks after infection. **A, C, E, H** and **I** represent bright field micrographs, **B, D, F, G** and **J** represent dark field/epipolarization micrographs in which silver grains are visible as yellowish dots. **A, B** Expression of *Frankia nifH*. *Arrowheads* indicate infected cells in zone 2 (see Results) which are not yet completely filled with hyphae, i.e. they do not contain vesicles and *Frankia* does not express *nifH*. *White arrows* indicate the nodule meristem. **C, D** Expression of sucrose synthase (*agSus1*). The pericycle of the nodule vascular bundle, where *agSus1* is expressed, is indicated by *long thick arrows*. A *thin arrow* points to the nodule meristem. *Arrowheads* point out infected cells of zone 2 which are not yet completely filled with hyphae, i.e. do not contain vesicles, but already express sucrose synthase. Two infected cells expressing sucrose synthase are marked by *stars*, while an uninfected cell not showing sucrose synthase expression is indicated by a *short arrow*. The starch grains in the uninfected cortical cells, which do not express sucrose synthase, are visible as tiny light blue dots. **E, F** Expression of enolase (*agPgh1*). *Arrowheads* point out infected cells of zone 2 expressing *agPgh1*. A *long white arrow* indicates the pericycle. Infected cells of zone 3 that express *agPgh1* are marked by *stars*. *Short white arrows* point out an uninfected cell of zone 3 not expressing *agPgh1*. **G** Hybridization of an adjacent section with a sense RNA probe. No accumulation of silver grains can be seen in any tissue. **H, I** Detail of sucrose synthase expression in the area near to the base of the nodule lobe (switch from nitrogen fixation zone 3 to senescence zone 4). While some cells still contain undegraded *Frankia* and express sucrose synthase (short arrow), other cells not yet showing degradation of the endosymbiont have switched sucrose synthase expression off (long arrow). Arrowheads point out starch grains in uninfected cells. Bar in **B** = 500 µm; bar in **I** = 250 µm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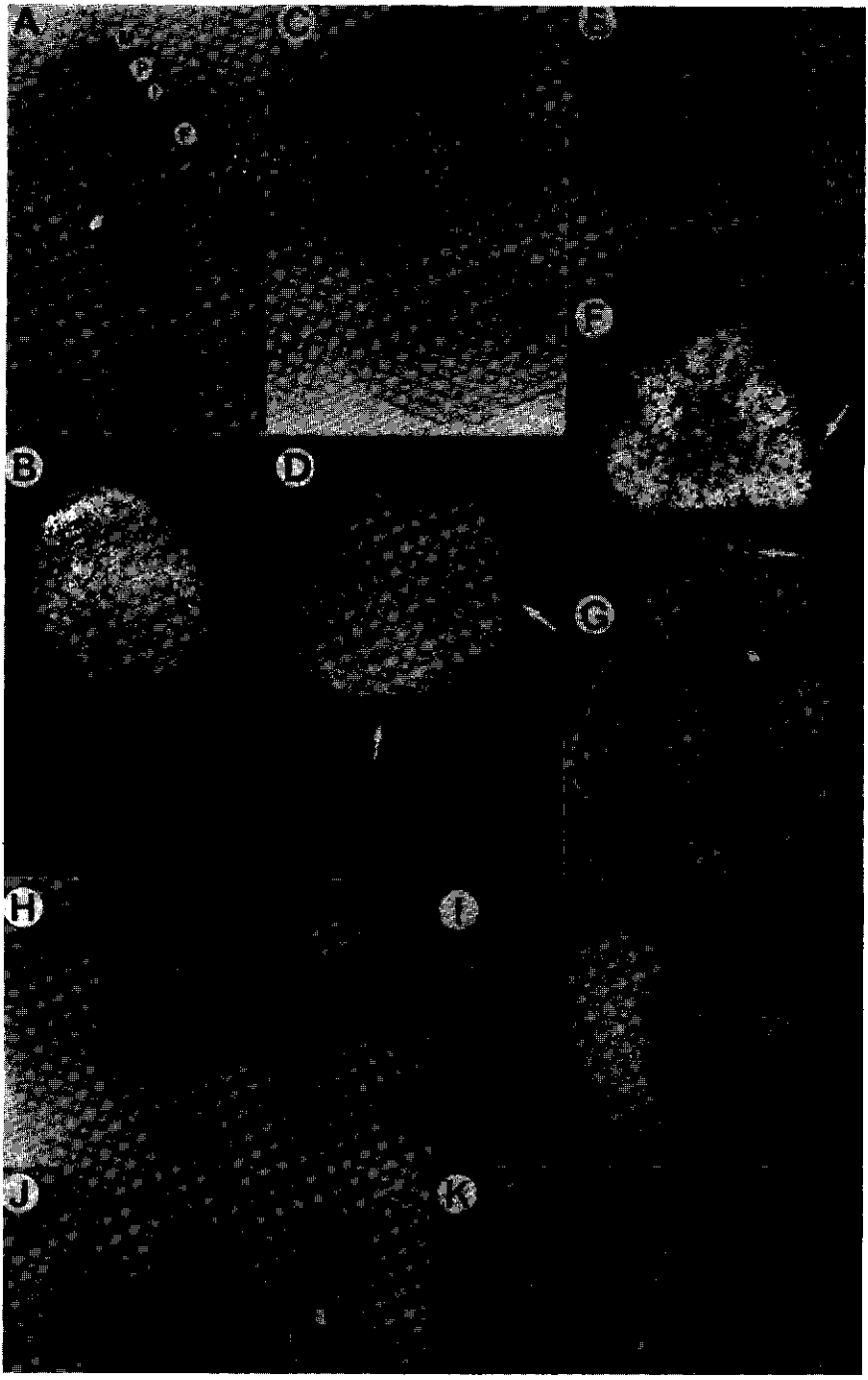
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expression (Fig. 3A–D, E, F), but increased upon onset of *nifH* expression in zone 3. No hybridization was detected by sense RNA probes, as shown in Fig. 3G. Sucrose synthase (Fig. 3H, I) as well as enolase (data not shown) expression was switched off before the endosymbiont material was degraded by the plant in the course of senescence, as has also been shown for the expression of *Frankia nifH* (Pawlowski et al. 1995). No expression of sucrose synthase was detected in the uninfected cells containing starch grains (Fig. 3C, D).

Expression of sucrose synthase in *Rhizobium*-induced nodules of *Pisum sativum* and *Phaseolus vulgaris*

To compare the situation in actinorhizal nodules with that in legume nodules, *in situ* hybridization of legume sucrose synthase was performed. *V. faba* sucrose synthase (Küster et al. 1993) antisense RNA was hybridized to longitudinal sections of mature indeterminate nodules formed by pea and of determinate nodules formed by *P. vulgaris*. The use of a heterologous probe should allow detection of the expression of different members of the sucrose synthase gene families in pea and *P. vulgaris*. The results are shown in Fig. 4. The central tissue of an indeterminate nodule is divided into meristem, prefixation zone, interzone and fixation zone (Fig. 4A) as described by Vasse et al. (1990) for alfalfa and applied by Franssen et al. (1992) to pea. In the indeterminate pea nodules, sucrose synthase was found to be expressed in the central tissue from the nodule meristem to the fixation zone (Fig. 4A, B). Sucrose synthase mRNA was also detected in the uninfected cells of the central tissue, i.e. in the nodule meristem and in the uninfected cells of the fixation zone (Fig. 4A, B). Transcripts were also found in the nodule vascular system, but at significantly lower levels than in the central tissue (Fig. 4C, D). In mature determinate nodules (harvested 21 days after infection) formed by *P. vulgaris*, sucrose synthase was expressed in infected and uninfected cells and in the nodule vascular system (Fig. 4E, F). Starch grains were found only in uninfected cells (Taté et al. 1994; Fig. 4G). Expression in uninfected cells containing starch grains was lower than in the infected cells (Fig. 4K). In young determinate nodules (harvested 14 days after inoculation), sucrose synthase was expressed in the nodule vascular system and all over the central tissue including the boundary layers (Fig. 4H, I). Expression in uninfected cells containing starch grains was higher than in infected cells (Fig. 4J).

Fig. 4A–K Localization of sucrose synthase mRNA in sections of determinate and indeterminate legume root nodules. **A**, **C**, **E** and **H** represent bright field micrographs; **B**, **D**, **F** and **I** dark-field micrographs (silver grains are visible as white dots); **G** represents a dark field photograph taken with epipolarized light; **J** and **K** represent photographs taken under phase contrast and epipolarized light (silver grains are visible as light green dots). **A** and **B** Expression of sucrose synthase in an 18-day-old pea nodule. The different zones of the nodule (Vasse et al. 1990; Franssen et al. 1992) are marked: M, meristematic zone; P, prefixation zone; I, interzone; F, fixation zone. Sucrose synthase expression can be seen all over the central tissue. An *arrow* points to a cluster of highly vacuolated uninfected cells which also express sucrose synthase. **C** and **D** Expression of sucrose synthase in a 21-day-old pea nodule. *Arrows* indicate the nodule vascular bundles expressing sucrose synthase. Elongated infected cells at the end of the fixation zone indicate the beginning of senescence (Newcomb 1981). **E** and **F** Expression of sucrose synthase in a 21-day-old *Phaseolus vulgaris* nodule. *Thin arrows* point out nodule vascular bundles expressing sucrose synthase. A *thick arrow* points out uninfected cells in the central tissue that do not express sucrose synthase. **G** Under epipolarized light, the starch grains in the uninfected cells of the central tissue, in the boundary cell layers and in the cells of the root cortex at the nodule base are visible as *light blue dots*. An *arrow* indicates a boundary layer cell containing starch grains. **H** and **I** Expression of sucrose synthase in a 14-day-old *P. vulgaris* nodule (white nodule). **A** *thick black arrow* points at uninfected cells of the central tissue expressing sucrose synthase. **J** Detail of **H**: in a 14-day-old *P. vulgaris* nodule, sucrose synthase expression in the uninfected cells containing starch grains (*black arrow*) is higher than in the infected cells (*white arrow*). Because the uninfected cells are highly vacuolated, silver grains denoting sucrose synthase expression are concentrated around the starch grains. b, boundary layer cell; c, cortical cell; i, infected cell; u, uninfected cell. **K** Detail of **E**: in a 21-day-old *P. vulgaris* nodule, sucrose synthase expression in the infected cells (*white arrow*) is higher than in the uninfected cells containing starch grains (*black arrow*). Bar in **A** = 500 μm; bar in **J** = 50 μm

Discussion

cDNAs encoding sucrose synthase and enolase from *A. glutinosa* nodules were cloned and characterized. They were found to be expressed in the infected cells and in the pericycle of the nodule vascular bundles, as well as in the nodule meristem. For comparison, expression of sucrose synthase was also studied in determinate and indeterminate legume nodules, where it represents a nodulin (Thummler and Verma 1987; Küster et al. 1993). Here, sucrose synthase was found to be expressed in the infected and uninfected cells of the central tissue, as well as in the vascular tissue. It was also expressed in the apical meristem and the preinfection zone of indeterminate nodules.

Sucrose synthase activity has been correlated with cell wall biosynthesis (Chourey et al. 1991), with phloem loading and unloading (Nolte and Koch 1993; Martin et al. 1993), and sink strength (Martin et al. 1993). Furthermore, breakdown of sucrose is necessary for starch biosynthesis. Thus, expression of sucrose synthase is expected in the meristem, the infected cells of the prefixation zone of legume nodules and of the infection zone of actinorhizal nodules, respectively, and in the phloem of vascular tissue. To produce acceptor molecules for ammonium assimilation and carbon sources for the bacteria, sucrose synthase activity is also expected in the infected cells of the fixation zone; and for starch biosynthesis sucrose synthase activity is

needed in the uninfected cells in determinate legume nodules (Taté et al. 1994) and actinorhizal nodules (Wheeler 1976; Wheeler et al. 1983). The results obtained meet all these expectations, except that no sucrose synthase mRNA was detected in the uninfected cells of *A. glutinosa* nodules (Fig. 3C, D). Furthermore, the fact that the expression patterns of enolase and sucrose synthase are identical suggests that the products of the sucrose synthase reaction enter glycolysis and are not used for starch biosynthesis. This may be due to the fact that, as shown by acridine orange staining, the RNA content of the uninfected cells is much lower than that of infected cells (data not shown), and thus sucrose synthase mRNA levels in the uninfected cells could easily be below the limit of detection by *in situ* hybridization. In any case, the experiments have shown that expression levels of sucrose synthase are much higher in infected than in uninfected cortical cells of *A. glutinosa* nodules.

The levels of sucrose synthase mRNA in the pericycle of *A. glutinosa* nodules were higher than in the vascular system of legume nodules, when compared with the expression in the respective infected cells. Whereas in legume nodules the central tissue containing the infected cells is surrounded by a network of vascular bundles containing transfer cells to improve transport activity (Walsh et al. 1989), actinorhizal nodule lobes only contain one central vascular bundle. An unusual multilayered pericycle consisting of small cells with a dense cytoplasm like that found in *A. glutinosa* nodules was reported previously for nodules of *A. japonica* (Burgess and Peterson 1987a). Burgess and Peterson (1987b) have found numerous mitochondria in the pericycle cells, signifying high glycolytic activities, and proposed that this tissue mediates the transport of nitrogen and carbon sources between vascular bundle and infected cortical cells, although it does not contain transfer cells. It is likely that the high levels of sucrose synthase and enolase expression in the pericycle of *A. glutinosa* nodule vascular bundles reflect the activity of transport processes in these cells.

Our results obtained with determinate nodules of *P. vulgaris* suggest that, before nitrogen fixation, starch biosynthesis is responsible for the major part of nodule sucrose consumption, as indicated by the higher levels of sucrose synthase mRNA in uninfected than in infected cells. However, after the onset of nitrogen fixation, higher sucrose synthase levels are found in the infected cells. These results confirm physiological studies on sucrose synthase activities in protoplasts of infected and uninfected cells of mature soybean nodules (Kouchi et al. 1988). However, contradictory results have been obtained in immunocytochemical studies on the localization of sucrose synthase in mature soybean nodules, where more sucrose synthase protein was found in the uninfected than in the infected cells of the central tissue (Gordon et al. 1992; Zammit and Copeland 1993). Furthermore, in immature soybean nodules, sucrose breakdown for starch biosynthesis seems mainly to be catalyzed by invertase (Morell and Copeland 1984; Anthon and Emmerich 1990). However, determinate nodules of soybean and *P. vulgaris* might differ in sucrose synthase and invertase expression, as they have already been shown to differ in glutamine synthetase (GS) expression: while in soybean nodules, GS is expressed in both infected and uninfected cells (Miao et al. 1991), in mature nodules of *P. vulgaris*, GS expression is confined to the infected cells (Forde et al. 1989).

The starch stored in the uninfected cortical cells of the perennial nodules of *A. glutinosa* (Wheeler and Lawrie 1976) seems to be destined to support spring flush growth, comparable to the function of carbohydrates stored in roots (Kozlowski and Keller 1966; Wheeler et al. 1983). The purpose of starch accumulation in legume nodules is largely unknown (Vance and Heichel 1991). Amyloplasts are dramatically enriched in the infected cells of the interzone of indeterminate legume nodules (Vasse et al. 1990; Franssen et al. 1992), while in determinate legume nodules they are confined to the uninfected cells of the central tissue and the boundary layers (Price et al. 1984; Taté et al. 1994). Increased starch accumulation has been correlated with ineffective or less effective symbioses (Lin et al. 1988; Malek et al. 1988). Large amyloplasts are also present in bacteria-free indeterminate nodules formed spontaneously on alfalfa (Joshi et al. 1991) and white clover (Blauenfeldt et al. 1994). Here the amyloplasts are located in the expanded cells of the central tissue, not in the small interstitial cells, indicating that these large cells, corresponding to the infected cells in functional nodules, dominate the sugar sink. Therefore it is striking that, in immature determinate nodules, sucrose synthase expression was higher in uninfected cells involved in starch storage than in infected cells (Fig. 4J, K), suggesting that before the onset of nitrogen fixation, more photosynthates are channeled into nodule starch biosynthesis than into the infection process. This fact is compatible with data on amyloplasts in bacteria free nodules indicating that carbon transport is an inherent part of nodule ontogeny and independent of *Rhizobium* (Joshi et al. 1993).

Altogether, the most striking difference between sucrose synthase expression in legume nodules and actinorhizal nodules of *A. glutinosa* is the fact that sucrose synthase expression could not be detected in the starch-containing uninfected cells of the latter. It remains to be examined whether this is a sensitivity problem due to the low metabolic activity of the uninfected cells, or whether invertase instead of sucrose synthase is responsible for the breakdown of sucrose for starch biosynthesis, as has been suggested for soybean nodules (Morell and Copeland 1984; Anthon and Emmerich 1990).

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References

Akazawa T, Okamoto K (1980) Biosynthesis and metabolism of sucrose. Biochem Plants 3:199–220

Akkermans ADL, Huss-Danell K, Roelofsen W (1981) Enzymes of the tricarboxylic acid cycle and the malate-aspartate shuttle in the N_2-fixing endophyte of *Alnus glutinosa*. Physiol Plant 53:289–294

Anthon GE, Emmerich DW (1990) Developmental regulation of enzymes of sucrose and hexose metabolism in effective and ineffective soybean nodules. Plant Physiol 92:346–351

Benson DR, Silvester WB (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol Rev 57:293–319

Berry AM, Sunnel LA (1990) The infection process and nodule development. In Schwintzer CR, Tjepkema JD (eds) The biology of *Frankia* and actinorhizal plants. Academic Press, New York, pp 61–81

Bisseling T, van den Bos RC, van Kammen A (1978) The effect of ammonium nitrate on the synthesis of nitrogenase and the concentration of leghemoglobin in pea root nodules induced by *Rhizobium leguminosarum*. Biochim Biophys Acta 539:1–11

Bhuvaneswari TV, Turgeon BG, Bauer WD (1980) Early events in the infection of soybean (*Glycine max* L. Merr) by *Rhizobium japonicum*. Plant Physiol 66:1027–1031

Blauenfeldt J, Joshi OA, Gresshoff PM, Caetano-Anollés G (1994) Nodulation of white clover (*Trifolium repens*) in the absence of *Rhizobium*. Protoplasma 179:106–110

Burgess D, Peterson RL (1987a) Development of *Alnus japonica* root nodules after inoculation with *Frankia* strain HFPArI3. Can J Bot 65:1647–1657

Burgess D, Peterson RL (1987b) Effect of nutrient conditions on root nodule development in *Alnus japonica*. Can J Bot 65:1658–1670

Chourey PS, Taliercio EW, Kane EJ (1991) Tissue-specific expression and anaerobically induced posttranscriptional modulation of sucrose synthase in *Sorghum bicolor* M. Plant Physiol 96:485–490

Cushman JC, Gietl C, Lepiniec L, Gadal P, Izui K (1994) Genes of malate and pyruvate metabolism. Plant Mol Biol Rep 12:S43–S44

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12:387–395

Diebold R, Noel KD (1989) *Rhizobium leguminosarum* exopolysaccharide mutants: biochemical and genetic analysis and symbiotic behaviour on three hosts. J Bacteriol 171:4821–4830

Forde BG, Day HM, Turton JF, Shen W-C, Cullimore JV, Oliver JE (1989) Two glutamine synthetase genes from *Phaseolus vulgaris* L. display contrasting developmental and spatial patterns of expression in transgenic *Lotus corniculatus* plants. Plant Cell 1:391–401

Franssen HJ, Vijn I, Yang W-C, Bisseling T (1992) Developmental aspects of the *Rhizobium*-legume symbiosis. Plant Mol Biol 19:89–107

Goodwin TW, Mercer EI (1983) Introduction to plant biochemistry (2nd edn). Pergamon Press, Oxford

Gordon AJ, Thomas BJ, Reynolds PHS (1992) Localization of sucrose synthase in soybean root nodules. New Phytol 122:35–44

Hake S, Kelley PM, Taylor WC, Freeling M (1985) Coordinate induction of alcohol dehydrogenase I, aldolase, and other anaerobic RNAs in maize. J Biol Chem 260:5050–5054

Hannah LC, Frommer W, Su J-C, Chourey P, Park W (1994) Sucrose synthases. Plant Mol Biol Rep 12, S72

Huss-Danell K, Bergman B (1990) Nitrogenase in *Frankia* from root nodules of *Alnus incana* (L.) Moench: immunolocalization of the Fe- and MoFe proteins during vesicle differentiation. New Phytol 116:443–455

Joshi PA, Caetano-Anollés G, Graham ET, Gresshoff PM (1991) Ontogeny and ultrastructure of spontaneous nodules in alfalfa (*Medicago sativa*). Protoplasma 162:1–11

Joshi PA, Caetano-Anollés G, Graham ET, Gresshoff PM (1993) Ultrastructure of transfer cells in spontaneous nodules of alfalfa (*Medicago sativa*). Protoplasma 172:64–76

Kozlowski TT, Keller T (1966) Food relations of woody plants. Bot Rev 32:2939

Kouchi H, Hata S (1993) Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol Gen Genet 238:106–119

Kouchi H, Katsuhiko F, Katagiri H, Minamisawa K, Tajima S (1988) Isolation and enzymological characterization of infected and uninfected cell protoplasts from root nodules of *Glycine max*. Physiol Plant 73:327–334

Küster H, Frühling M, Perlick AM, Pühler A (1993) The sucrose synthase gene is predominantly expressed in the root nodule tissue of *Vicia faba*. Mol Plant-Microbe Interact 6:507–514

Lin J, Walsh KB, Canvin DT, Layzell DB (1988) Structural and physiological bases for effectivity of soybean nodules formed by fast-growing and slow-growing bacteria. Can J Bot 66:526–534

Malek W (1988) Microscopic structure of ineffective alfalfa nodules formed by auxotrophic mutants of *Rhizobium meliloti*. J Basic Microbiol 28:651–658

Martin T, Frommer WB, Salanoubat M, Willmitzer L (1993) Expression of an *Arabidopsis* sucrose synthase gene indicates a role in metabolization of sucrose both during phloem loading and in sink organs. Plant J 4:367–377

McClure PR, Cocker III GT, Schubert KR (1983) Carbon dioxide fixation in roots and nodules of *Alnus glutinosa*. Plant Physiol 771:652–657

Miao G-H, Hirel B, Marsolier MC, Ridge RW, Verma DPS (1991) Ammonia-regulated expression of a soybean gene encoding cytosolic glutamine synthetase in transgenic *Lotus corniculatus*. Plant Cell 3:11–22

Morell M, Copeland L (1984) Enzymes of sucrose breakdown in soybean nodules. Alkaline invertase. Plant Physiol 74:149–154

Morell M, Copeland L (1985) Sucrose synthase of soybean nodules. Plant Physiol 78:149–154

Mylona P, Pawlowski K, Bisseling T (1995) Symbiotic nitrogen fixation. Plant Cell 7:869–885

Newcomb W (1981) Nodule morphogenesis and differentiation. In Giles KL, Atherly AG (eds) Biology of the *Rhizobiaceae*. Int Rev Cytol Supp 13, Academic Press, New York, pp 61–81

Nolte KD, Koch KE (1993) Companion-cell specific localization of sucrose synthase in zones of phloem loading and unloading. Plant Physiol 101:899–905

Pate JS (1962) Root exudation studies on the exchange of ^{14}C-labeled organic substances between the roots and shoot of the nodulated legume. Plant Soil 17:333–356

Pawlowski K, Akkermans ADL, van Kammen A, Bisseling T (1995) Expression of bacterial *nif* genes in actinorhizal nodules of *Alnus glutinosa*. Plant and Soil 170:371–376

Pawlowski K, Kunze R, de Vries S, Bisseling T (1994) Isolation of total, poly(A) and polysomal RNA from plant tissues. In Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual D5, (2nd edn). Kluwer Academic Publishers, Dordrecht, pp 1–13

Price GD, Mohaptra SS, Gresshoff PM (1984) Structure of nodules formed by *Rhizobium* strain ANU289 in the nonlegume *Parasponia* and the legume siratro (*Macroptilium atropurpureum*). Bot Gaz 145:444–451

Ribeiro A, Akkermans ADL, van Kammen A, Bisseling T, Pawlowski K (1995) A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. Plant Cell 7:785–794

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Schubert KR (1986) Products of biological nitrogen fixation in higher plants: synthesis, transport, and metabolism. Annu Rev Plant Physiol 37:539–574

Solomos T, Laties GG (1974) Similarities between the actions of ethylene and cyanide in initiating the climacteric and ripening of avocados. Plant Physiol 54:506–511

Soltis DE, Soltis PS, Morgan DR, Swensen SM, Mullin BC, Dowd JM, Martin PG (1995) Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc Natl Acad Sci USA 92:2647–2651

Sonnewald U, Willmitzer L (1992) Molecular approaches to sink-source interactions. Plant Physiol 99:1267–1270

Tatè R, Patriarca EJ, Riccio A, Defez R, Iaccarino M (1994) Development of *Phaseolus vulgaris* root nodules. Mol Plant-Microbe Interact 7:582–589

Thummler F, Verma DPS (1987) Nodulin-100 of soybean is the subunit of sucrose synthase regulated by the availibility of free heme in nodules. J Biol Chem 262:14730–14736

Trinick MG (1979) Structure of nitrogen-fixing nodules formed by *Rhizobium* on roots of *Parasponia andersonii*. Appl Env Microbiol 55:2046–2055

Van der Straeten D, Rodrigues-Pousada RA, Goodman HM, Van Montagu M (1991) Plant enolase: gene structure, expression, and evolution. Plant Cell 3:719–735

Vance CP, Heichel GH (1991) Carbon in N_2 fixation: limitation or exquisite adaptation? Annu Rev Plant Physiol Plant Mol Biol 42:373–392

Vasse JM, De Billy F, Camut S, Truchet G (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J Bacteriol 172: 4295–4306

Walsh KB, McCully BE, Canny MJ (1989) Vascular transport and soybean nodule function: nodule xylem is a blind alley, not a throughway. Plant Cell Env 12:395–405

Wheeler CT, Lawrie AC (1976) Nitrogen fixation in root nodules of alder and pea in relation to the supply of photosynthetic assimilates. In: Nutman PS (ed) Symbiotic nitrogen fixation in plants. Cambridge University Press, Cambridge, pp 497–509

Wheeler CT, Watts SH, Hillman JR (1983) Changes in carbohydrates and nitrogenase compounds in the root nodules of *Alnus glutinosa* in relation to dormancy. New Phytol 95:209–218

Zammit A, Copeland L (1993) Immunocytochemical localisation of nodule-specific sucrose synthase in soybean nodules. Aust J Plant Physiol 22:25–32

Chapter 4

Identification of *agthi1*, whose product is involved in biosynthesis of the thiamine precursor thiazole, in actinorhizal nodules of *Alnus glutinosa*

Ana Ribeiro, Uta Praekelt, Antoon D.L. Akkermans, Peter A. Meacock, Ab. van Kammen, Ton Bisseling and Katharina Pawlowski. The Plant Journal, Vol. 10 (2), 361-368, 1996.

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SHORT COMMUNICATION

Identification of *agthi1*, whose product is involved in biosynthesis of the thiamine precursor thiazole, in actinorhizal nodules of *Alnus glutinosa*

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Summary

A cDNA clone, pAgthi1, encoding a homologue of yeast Thi4, which is involved in thiazole biosynthesis, was isolated from a library made from poly(A) RNA from actinorhizal nodules of *Alnus glutinosa* by differential screening with nodule and root cDNA, respectively. The corresponding gene, *agthi1*, was shown to be expressed at high levels in nodules and shoot tips of *A. glutinosa*, while having low expression levels in roots, flowers, and developing fruits. The function of AgThi1 was demonstrated by yeast complementation studies, in which AgThi1 was able to rescue a yeast *thi4* mutant when fused to the yeast Thi4 signal peptide. In *A. glutinosa* nodules, high levels of *agthi1* mRNA were detected in the infected cortical cells and in the pericycle of the nodule vascular system. A homologue of this cDNA, *ara6/tz*, was identified in *Arabidopsis thaliana*. *ara6* maps in a region of chromosome 5 of *Arabidopsis* containing the *tz* locus which is consistent with the fact that *ara6* transcription is disturbed in two *tz* mutant lines. *ara6/tz* is expressed at high levels in chloroplast-containing parenchymatic cells of leaves, inflorescence shoots and flowers of *Arabidopsis*, and at lower levels in the vascular system.

Introduction

The formation of actinorhizal nodules is induced by actinomycetous bacteria of the genus *Frankia* on the roots of several dicotyledonous species belonging to eight different plant families, mostly trees or woody shrubs (Benson and Silvester, 1993). These nodules are perennial structures consisting of multiple lobes. Single lobes represent modified lateral roots without root caps, and their cortical cells can be infected by *Frankia* (Berry and Sunell, 1990). Thus, their structure is different from that of legume nodules, which represent stem-like organs with a peripheral vascular system and infected cells in the central tissue. In spite of the structural similarity between actinorhizal nodules and lateral roots, nodule-specific genes are expressed in the actinorhizal plants *Alnus* and *Casuarina*, for example, the symbiotic hemoglobin of *Casuarina glauca* (Fleming *et al.*, 1987; Jacobsen-Lyon *et al.*, 1995) and two genes of *Alnus glutinosa*, one encoding a serine protease most likely involved in the infection process (Ribeiro *et al.*, 1995) and the other encoding a cysteine protease which might be involved in nodule senescence (Goetting-Minesky and Mullin, 1994). Several nodule-enhanced genes, whose products are mostly involved in nodule metabolism, such as sucrose synthase and enolase (Van Ghelue *et al.*, 1996), and glutamine synthetase (Pawlowski *et al.*, 1994a) have also been identified in *A. glutinosa*. Since most actinorhizal plants represent trees or woody shrubs, they are recalcitrant to molecular biological and genetic analysis. Therefore, for the functional analysis of most of the actinorhiza-specific cDNAs, model systems have to be used. Such systems include yeast and *Arabidopsis* which have already been efficiently used to study gene function in other plant systems (see e.g. Cheon *et al.*, 1993; Langridge, 1994).

In this paper we report the isolation of an *A. glutinosa* nodule-enhanced cDNA, a homologue of yeast *thi4* which has been strongly suggested to encode an enzyme involved in the biosynthesis of thiazole (4-methyl-5-β-hydroxy-ethyl-thiazole), one of the two precursors of thiamine (Praekelt and Meacock, 1992; Praekelt *et al.*, 1994). Thiamine, or vitamin B1, is a co-factor in both glycolysis and the Calvin cycle. In nitrogen-fixing root nodules, the assimilation of ammonium exported by the endosymbiont to the plant cytoplasm requires high plant glycolytic activities in order to provide energy and carbon backbones. Thus, not only high levels of transcription of genes encoding glycolytic enzymes like enolase (Van Ghelue *et al.*, 1996) but also of genes encoding enzymes involved in the synthesis of

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glycolytic co-factors like thiamine can be expected in nodules. The functional characterization of the *A. glutinosa* yeast *thi4*-homologous cDNA using yeast complementation and characterization of its homologue in *Arabidopsis* is described.

Results

Isolation and characterization of nodule-specific/nodule-enhanced cDNAs from Alnus glutinosa

An *A. glutinosa* nodule cDNA library was screened differentially with nodule versus root cDNA. Ten clones hybridizing with nodule but not or only weakly with root cDNA were purified and characterized. Using hybridization with Southern blots containing DNA from *A. glutinosa* and *Frankia* HFPArI3, respectively, it was confirmed that their inserts were derived from *A. glutinosa*. Hybridization with RNA gel blots containing RNA from different organs of *A. glutinosa*, was performed to confirm the nodule-specific/nodule-enhanced nature of the cDNAs. One cDNA, *ag6*, hybridizing to a mRNA present at low levels in roots and flowers and at high level in shoot tips and nodules of *A. glutinosa* (Figure 1), was selected for further analysis. Southern analysis suggested that Ag6 was encoded by a small gene family of *A. glutinosa* (data not shown). A full-length cDNA of *ag6* was isolated from the cDNA library.

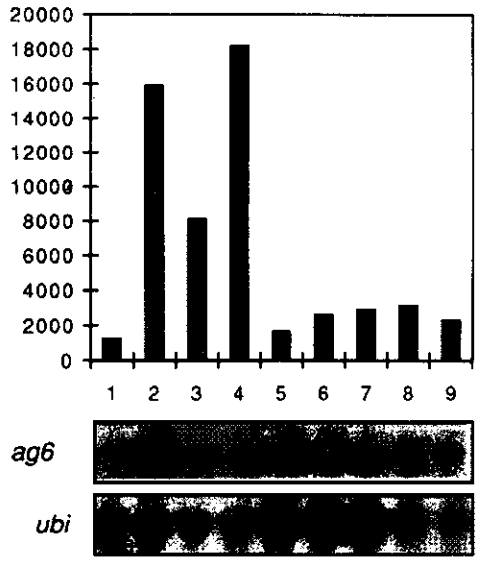


Figure 1. Expression of *agthi1* in different organs of *A. glutinosa*.
Lanes: 1, roots; 2, nodules; 3, shoot tips; 4, cotyledons; 5, male flowers; 6 female flowers; 7, immature fruits collected in April; 8, immature fruits collected in June; 9, immature fruits collected in September.
RNA gel blots containing about 10 µg of total RNA per slot were hybridized against *agthi1*. 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, Sunnyvale, CA, USA). Expression levels of *agthi1* are shown as relative area units calculated from comparison with ubiquitin expression.

Sequencing of ag6 *and identification of a homologue in* Arabidopsis thaliana

The DNA sequence of a full-length cDNA clone from *ag6* was determined (EMBL accession number X97434) and the encoded amino acid sequence was used for a homology search in the DNA data bases of the National Center of Biotechnology Information. A homologue of *ag6* was found amongst the randomly sequenced *Arabidopsis* cDNA clones (GenBank accession number T04141). This homologue was designated *ara6*. The expression of *ara6* in different organs of *Arabidopsis* was analysed by RNA gel blot hybridizations, and *ara6* was shown to be expressed in all organs at similar levels, with the exception of roots where no expression was detected (data not shown). Because a second *Arabidopsis* cDNA (GenBank accession number U17589; Machado *et al.*, 1996) showing 99% identity to the partial sequence of *ara6* was found in the data bases later, the full-length sequence of *ara6* was not determined. The 1% difference in the two *A. thaliana* sequences, T04141 and U17589 is probably due to the fact that they originate from different plant ecotypes. The proteins encoded by *ag6* and *ara6* showed high homology with Thi4 (Mol1) from yeast, which has been strongly suggested to represent an enzyme involved in the thiazole biosynthetic pathway (Praekelt and Meacock, 1992; Praekelt *et al.*, 1994; data not shown) and with Sti35, a protein encoded by a stress-induced gene from *Fusarium* sp. (Cho *et al.*, 1990), which although identified as a stress-inducible protein, is not excluded from having a function in thiamine biosynthesis (Praekelt *et al.*, 1994). In spite of the strong homology between the four proteins, Ag6 and Ara6 contain at their N-termini a signal peptide for plastidic targeting (Gavel and Von Heijne, 1990a), while both yeast Thi4 and *Fusarium* Sti35 contain an N-terminal signal peptide for mitochondrial targeting (Gavel and Von Heijne, 1990b; data not shown).

Yeast complementation studies: ag6 *is the functional* thi4 *homologue*

To prove that in fact *ag6* is a functional *thi4* homologue yeast complementation experiments were performed. In a preliminary experiment, the full-length cDNA *ag6*, when expressed in a yeast *thi4* mutant strain, did not restore thiamine-independent growth. However, since Ag6 and Thi4 contain non-homologous signal peptides, this lack of complementation could be due to problems with protein

targeting. Therefore, constructs expressing the mature part of Ag6 fused to the signal peptide of yeast Thi4 were constructed in the centromeric yeast vector YCP50 carrying the *ura* gene, designated pUP57a (sense) and pUP57b (antisense). The uracil auxotrophic yeast strain T3/3 which contains a transposon insertion in *thi4* was transformed with pUP57a and pUP57b. Transformants were selected on yeast medium lacking uracil, but containing thiamine. For testing complementation, transformants were streaked on medium with and without thiamine, respectively. T3/3(pUP57a) showed normal growth after 3 days on both media. T3/3(pUP57b) only grew on medium containing thiamine. Thus, pUP57a could complement the yeast *thi4* mutant, restoring wild-type-like thiamine-independent growth.

These results showed that Ag6 is the functional homologue of the yeast Thi4. Since a maize homologue of *ag6* and yeast *thi4* has been named *thi1* (Belanger *et al.*, 1995), *ag6* was renamed *agthi1*.

RFLP mapping: ara6 *maps at the* tz *locus of* Arabidopsis thaliana

In order to see whether *ara6* maps to a locus defined by a mutation, RFLP mapping was performed using the mapping population of recombinant inbred lines of Lister and Dean (1993). Restriction of DNA from the *Arabidopsis* ecotypes Columbia and Landsberg *erecta* with *Cla*I yielded an RFLP marker. *ara6* was shown to map on chromosome 5 between the markers m435 and g2368. On the integrated map of *Arabidopsis* (Hauge *et al.*, 1993; Koornneef, 1994), the *tz* (thiazole requirement) locus (Feenstra, 1964) has been located between these two markers. Because of the amino acid homology with yeast Thi4 and since *agthi1* can complement yeast thiazole mutants, we assumed that *ara6* might be the *tz* gene of *Arabidopsis*.

Therefore, RNA was isolated from rosette leaves of five different *tz* mutants and *A. thaliana* ecotype Landsberg *erecta* wild-type and analysed for *ara6* expression by RNA gel blot hybridization. As can be seen in Figure 2, one of those mutants, CS3375 (Redei, 1965), showed a strong reduction of *ara6* expression. Furthermore, another one, CS3573 (Redei, 1965), contained two *ara6* transcripts of different size, indicating a mutation affecting the start of transcription. These data strongly suggest that *ara6* is the transcript of the *tz* gene of *Arabidopsis*.

In situ *localization of* agthi1 *and* ara6/tz *mRNA*

The expression pattern of *agthi1* in root nodules of *A. glutinosa* was determined by *in situ* hybridization of longitudinal sections and cross-sections of nodules with ^{35}S-labeled antisense and sense RNA, respectively. As a marker for the developmental gradient of the infected cell,

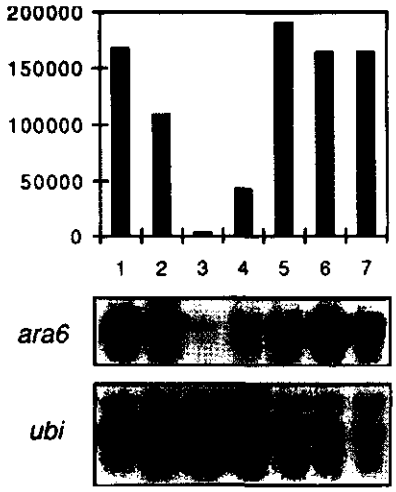
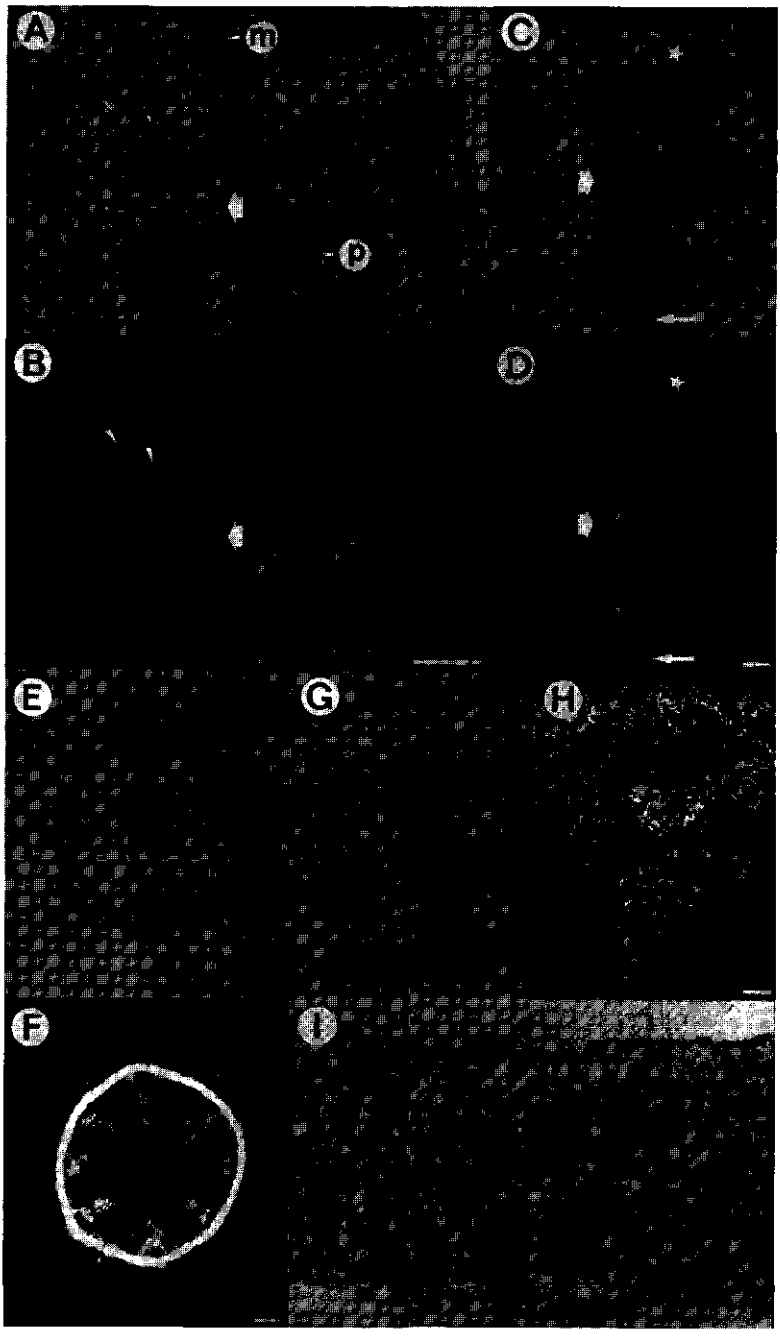


Figure 2. Expression of *ara6/tz* in rosette leaves of wild-type (ecotype Landsberg *erecta*) and *tz* mutants of *A. thaliana*.
An RNA gel blot containing about 10 µg total RNA per slot was hybridized against *ara6/tz*. Expression levels of *ara6/tz* were quantified as described in the legend of Figure 1. Lanes: 1, ecotype Landsberg *erecta*; 2, CS3311 (*gl1-1* in ecotype Estland; background for *tz-31* mutation in CS3573; Redei, 1967); 3, CS3375 (*tz-1*; Redei, 1965); 4, CS3573 (*tz-31, gl1-1*; Redei, 1965); 5, CS3574 (*tz-2, gl1-2*; Redei, 1965); 6, CS3581 (*tz-31, gl1-2*; Redei, 1965); 7, CS3590 (*tz* mutant in ecotype Columbia; Redei, 1965). Numbers represent seed stock numbers from the Arabidopsis Biological Resource Center at Ohio State University (Columbus, OH).

in situ hybridizations with antisense RNA of the nitrogenase structural gene *nifH* from *Frankia* HFPArI3 were performed in parallel (Pawlowski *et al.*, 1995; Ribeiro *et al.*, 1995). The results are shown in Figure 3(a) and (b). *agthi1* 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. *agthi1* mRNA was present in young infected cells incompletely filled with *Frankia* hyphae, but the highest levels of *agthi1* mRNA were found in mature infected cells containing nitrogen-fixing *Frankia* vesicles (Figure 3a and 3b). That means that *agthi1* expression in the infected cells preceded *nifH* expression by *Frankia* (Ribeiro *et al.*, 1995). In the distal part of the nodule vascular bundle, where no infection of the neighbouring cortical cells can be seen, expression of *agthi1* in the pericycle of the stele was not detectable (Figure 3c and 3d). No *agthi1* mRNA was found in senescent infected cells, and no signal was found in hybridizations with sense RNA (data not shown).

ara6/tz expression was analysed by *in situ* hybridization of sections of inflorescence shoots, leaves and flowers of *Arabidopsis* with ^{35}S-labeled sense and antisense RNA. *ara6/tz* was found to be expressed to high levels in the parenchymatic leaf cells containing chloroplasts, but not in the epidermis (Figure 3i). In inflorescence stem sections, high expression of *ara6/tz* was detected in chloroplast-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containing parenchymatic cortical cells and lower expression levels were found in the vascular parenchyma (Figure 3e and f and g and h). As with leaf sections, no expression was found in epidermal cells. Also in flower organs, *ara6/tz* was expressed in the chloroplast-containing parenchymatic cells and not in the epidermis (data not shown). No signal was found in hybridizations with sense RNA (data not shown).

Discussion

We have shown that the gene *agthi1* (*ag6*), which is expressed at markedly elevated levels in actinorhizal nodules of *A. glutinosa*, is the functional homologue of the yeast *thi4* gene, which has been suggested to encode an enzyme involved in the biosynthesis of the thiamine precursor, thiazole. Thiamine, or vitamin B1, contains two cyclic moieties, 4-methyl-5-β-hydroxy-ethylthiazole (thiazole) and 2-methyl-4-amino-5-hydroxy-methylpyrimidine (hydroxymethylpyrimidine). In higher plants, thiazole is very likely synthesized in plastids from 1-deoxy-D-*threo*-2-pentulose, tyrosine and cysteine (Julliard and Douce, 1991), which is essentially the same pathway as has been proposed for *E. coli* (Vander Horn *et al.*, 1993). However, it is still unclear which step of this pathway is catalysed by yeast Thi4. Amino acid sequence comparison data, RFLP mapping results, and the fact that the expression of the homologous *Arabidopsis* gene, *ara6/tz*, is impaired in some *Arabidopsis tz* mutants, strongly suggest that *ara6/tz* also represents a functional homologue of the yeast *thi4* gene. A maize homologue of yeast *thi4*, named *thi1*, has been shown to encode a protein able to complement the yeast *thi4* mutant as well (Belanger *et al.*, 1995). Thus, thiazole biosynthesis, in spite of involving different amino acid precursors in yeast and higher plants (Julliard and Douce, 1991; White and Spenser, 1982), seems to proceed via the same pathway in both systems.

While in higher plants, thiamine biosynthesis is taking place in plastids (Belanger *et al.*, 1995; Julliard and Douce, 1991), in yeast the pathway seems to take place in the mitochondria, since the N-terminal sequence of Thi4 resembles a mitochondrial targeting signal (Bedwell *et al.*, 1989). Interestingly, Belanger *et al.* (1995) report that maize *thi1* was able to complement a yeast *thi4* mutant, though not restoring wild-type-like growth, while *agthi1* needed the signal peptide of yeast *thi4* in order to complement the yeast *thi4* mutant. Probably, the plastidic signal peptide of maize Thi1 managed to confer some targeting to yeast mitochondria, as has also been shown for for the signal peptide of the small subunit of Rubisco from *Chlamydomonas* (Hurt *et al.*, 1986), while the corresponding signal peptide from AgThi1 did not.

Since thiamine is a co-factor of both glycolysis and the Calvin cycle, plant *thi1* genes are expected to be expressed in cells where high activities of either photosynthesis or glycolysis are present. Consistently, *agthi1*, *ara6/tz* and maize *thi1* (Belanger *et al.*, 1995) show very low expression levels in roots, while being expressed at high levels in photosynthetic plant organs. *In situ* hybridization has shown that high levels of *ara6/tz* expression are confined to the chloroplast-containing parenchymatic cells, and at somewhat lower levels in the vascular tissue. In *A. glutinosa* nodules, *agthi1* was found to be expressed in the infected cortical cells and in the pericycle of the nodule vascular bundle. These expression patterns suggest that energy-demanding processes are taking place in the cells expressing *agthi1* and *ara6/tz*, namely, photosynthesis in the chloroplast-containing cells, ammonium assimilation in the infected nodule cortical cells, and transport processes, for example, phloem loading, in the vascular system, respectively. This interpretation is consistent with the fact that high levels of sucrose synthase and enolase expression (Van Ghelue *et al.*, 1996) are found in the same cells that express *agthi1*.

Thus, thiamine biosynthesis in higher plants seems to be correlated with glycolytic and Calvin cycle activity.

Figure 3. *In situ* localization of *agthi1* mRNA in root nodules of *A. glutinosa* and of *ara6/tz* mRNA in inflorescence stems and leaves of *A. thaliana* ecotype Landsberg *erecta*.

In (a), (c), (e), (g), and (i) bright field microscopy was used. Silver grains denoting hybridization are visible as black dots. In (b) and (d), dark field microscopy and epipolarized light was used. Silver grains are visible as yellow dots. In (f) and (h), dark field microscopy was used, and silver grains appear as white dots.

(a) and (b) Expression of *A. glutinosa agthi1* in a longitudinal section of a nodule lobe. Expression can be found in infected cells which are not yet completely filled with *Frankia* hyphae (marked by arrowheads), but is stronger in infected cells containing nitrogen-fixing *Frankia* vesicles (marked by white arrow) and in the pericycle of the vascular bundle. m, meristem; p, pericycle.

(c) and (d) Detail of the central stele of a nodule lobe. *agthi1* expression is confined to the pericycle (short arrow); no expression is found in the phloem (long arrow). In the distal part of the nodule, where no cells are infected in the cortex, *agthi1* expression in the pericycle (marked by an asterisk) is below the detection level.

(e) and (f) Expression of *A. thaliana ara6/tz* in an inflorescence stem cross-section. Strong signal can be seen in the chloroplast-containing cells of the stem parenchyma (p). Weaker expression is found in the vascular bundles. No expression is found in the pith and in the epidermis (e).

(g) and (h) Detail of the cross-section of an inflorescence stem. Expression of *ara6* in the vascular parenchyma. e, epidermal cell; p, phloem parenchyma; s, sclerenchyma; x, xylem parenchyma.

(i) Expression of *ara6/tz* in a leaf section. *ara6/tz* is expressed at high levels in the parenchymatic cells, while no expression is found in the epidermis. e, epidermal cell.

Bars in (b) and (f) = 500 μm; bars in (d) and (h) = 100 μm.

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 h light, 8 h dark. Growth and infection conditions have been described elsewhere (Ribeiro *et al.*, 1995). 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. *A. glutinosa* flowers were collected from a local stand (Wageningen, The Netherlands) in March 1994. Developing fruits from *A. glutinosa* were collected from the same stand in April, June, and September 1994. *Arabidopsis thaliana* was grown in a greenhouse. Seeds were germinated on filter paper wetted with sterile distilled water for 1 day at 4°C and for 2 days at 21°C in the dark before being transferred to soil. Seeds of *tz* mutants were germinated on filter paper wetted with 1 mM thiamine. *tz* mutant plants were watered with 1 mM thiamine once per week.

Isolation of DNA and RNA

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

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. The differential screening has been described elsewhere (Ribeiro *et al.*, 1995).

Cloning and sequencing procedures

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 analysed using the programs of the Wisconsin Genetics Computer Group (Devereux *et al.*, 1984). Data base searches were performed using the BLAST algorithm (Altschul *et al.*, 1990) in the nucleotide sequence data bases of the National Center of Biotechnology Information (NCBI), National Library of Medicine, NIH, in Bethesda, MD.

DNA and RNA gel blot hybridization conditions

Total RNA was denatured in dimethyl sulphoxide/glyoxal and separated 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 (New England Nuclear, Beverly, MA, USA) filters (RNA) or to Amersham Hybond N^+ (Amersham, UK) filters (DNA). RNA gel blot 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 0.5 × SSC, 0.1% SDS. For heterologous hybridizations, filters were washed with 2 × SSC, 0.1% SDS.

Yeast complementation

For growth of yeast, the medium described by Wickerham (1951) was used. Supplements were added according to the auxotrophies of the yeast strains used. For expressing the complete *ag6* cDNA in yeast, it was excised from pBluescript SK^- using *Sal*I and *Bam*HI and cloned in sense and antisense orientation into the *Sal*I/*Bam*HI-digested yeast expression vector pG3 which carries the yeast *trp* gene (Schena *et al.*, 1991). The constructs contained the *Alnus* cDNA under the control of the yeast glyceraldehyde phosphate dehydrogenase (GPD) promoter and the phosphoglycerate kinase (PGK) terminator. Vector and constructs were transformed into a haploid disrupted spore of the diploid *thi1::ura3* disruption strain 842 described by Praekelt and Meacock (1992). Transformants were selected on medium lacking tryptophan.

For the construction of vectors expressing Ag6 fused to the signal peptide of yeast Thi4, the yeast *thi4* promoter (1 kb) plus N-terminus of the *thi4* coding region and the *thi4* terminator (1 kb) were amplified by PCR using primers 5'-CCGAATTCAACCTAATG-ACTGTTCTATA-3' and 5'-CCGGATCCGTCAGATAAGCAGTGA-GTAC-3' for promoter/N-terminus and 5'-CCGGATCCGCTTTTT-AATGAGGTATGCTT-3' and 5'-CCGGTCGACGAGCTCGGTACCCG-GAGATCC-3' for the terminator and cloned between the *Eco*RI and *Sal*I sites of the centromeric yeast vector YCP50 which contains the yeast *ura* gene. The *ag6* cDNA was amplified by PCR using the 5' primer 5'-AAGGATCCCCGCCGTACGATCTGAAAGC-3' and the 3' primer 5'-CCGGATCCCTAAATATTTCCGGCATT-3' and cloned in sense and antisense orientation into the internal *Bam*HI site between the *thi1* promoter plus N-terminus and the terminator. These constructs were designated pUP57a (sense) and pUP57b (antisense), respectively. The junction sequence between the yeast N-terminus and the *Alnus* Ag6 sequence is as follows: (THI4 amino acid position 25) CLSDGGSPPYDLKAF (Ag6 amino acid position 60). The constructs were used to transform T3/3, a yeast strain containing a transposon insertion in *thi4* (*Mat*α, *his3–Δ200, ura3–167, trp1–Δ, leu2–Δ, GAL, thi4::Ty–H3HIS3;* Byrne *et al.*, unpublished).

In situ *hybridization*

Tissue fixation and hybridization conditions have been described by Ribeiro *et al.* (1995). For the *Frankia nifH* riboprobe, pFnifH1 (Ribeiro *et al.*, 1995) was linearized with *Eco*RI and antisense RNA was transcribed using T7 RNA polymerase. For *A. glutinosa agthi1* sense and antisense riboprobes, the original cDNA clone comprising positions 67–1341 of the full-length sequence was linearized with *Xba*I and transcribed with T3 RNA polymerase (antisense) or linearized with *Hind*III and transcribed with T7 RNA polymerase (sense), respectively. For *A. thaliana ara6/tz* sense and antisense riboprobes, a roughly 0.5 kb *Hind*III fragment of the cDNA clone was subcloned in pBluescript KS^+ (direction of transcription same as for β-galactosidase) and linearized with *Xho*I and transcribed with T7 RNA polymerase (antisense) or linearized with *Bam*HI and transcribed with T3 RNA polymerase (sense), respectively.

After washing, the slides were coated with microautoradiography emulsion LM-1 (Amersham, UK) and exposed for 4 weeks at 4°C. They were developed in Kodak (Rochester, NY, USA) D19 developer for 5 minutes 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 DePeX (BDH Laboratory supplies, Poole, UK).

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Bedwell, D.M., Strobel, S.A., Yun, K., Jongeward, G.D. and Emr, S. (1989) Sequence and structural requirements of a mitochondrial protein import signal defined by saturation cassette mutagenesis. *Mol. Cell. Biol.* **9**, 1014–1025.

Belanger, F.C., Leustek, T., Chu, B. and Kriz, A.L. (1995) Evidence for the thiamine biosynthetic pathway in higher plant plastids and its developmental regulation. *Plant Mol. Biol.* **29**, 809–821.

Benson, D.R. and Silvester, W.B. (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol. Rev.* **57**, 293–319.

Berry, A.M. and Sunnell, L.A. (1990) The infection process and nodule development. In *The Biology of* Frankia *and Actinorhizal Plants* (Schwintzer, C.R. and Tjepkema, J.D., eds). New York: Academic Press, pp. 61–81.

Cheon, C.-I., Lee, N.-G., Siddique, A.-B.M., Bal, A.K. and Verma, D.P.S. (1993) Roles of plant homologs of Rab1p and Rab7p in the biogenesis of the peribacteroid membrane, a subcellular compartment formed *de novo* during root nodule symbiosis. *EMBO J.* **12**, 4125–4135.

Choi, G.H., Marek, E.T., Schardia, C.L., Richey, M.G., Chang, S. and Smith, D.A. (1990) *sti35*, a stress-responsive gene in *Fusarium* spp. *J. Bacteriol.* **172**, 4522–4528.

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* **12**, 387–395.

Feenstra, W.J. (1964) Isolation of nutritional mutants in *Arabidopsis thaliana*. *Genetica*, **35**, 259–269.

Fleming, A.I., Wittenberg, J.B., Wittenberg, B.A., Dudman, W.F. and Appelby, C.A. (1987) The purification, characterization and ligand-binding kinetics of hemoglobins from root nodules of the non-leguminous *Casuarina glauca-Frankia* symbiosis. *Biochim. Biophys. Acta*, **911**, 209–220.

Gavel, Y. and von Heijne, G. (1990a) A conserved cleavage-site motif in chloroplast transit peptides. *FEBS Lett.* **261**, 455–458.

Gavel, Y. and von Heijne, G. (1990b) Cleavage-site motifs in mitochondrial targeting peptides. *Prot. Eng.* **4**, 33–37.

Goetting-Minesky, M.P. and Mullin, B.C. (1994) Differential gene expression in an actinorhizal symbiosis: Evidence for a nodule-specific cysteine proteinase. *Proc. Natl Acad. Sci. USA*, **91**, 9891–9895.

Hauge, B.M., Hanley, S.M., Cartinhour, S., Cherry, J.M., Koorneef, M., Stam, P., Chang, C., Kempin, S., Medrano, L. and Meyerowitz, E.M. (1993) An integrated genetic/RFLP map of the *Arabidopsis thaliana* genome. *Plant J.* **3**, 745–754.

Hurt, E.C., Soltanifar, N., Goldschmidt-Clermont, M., Rochaix, J.-D. and Schatz, G. (1986) The cleavable pre-sequence of an imported chloroplast protein directs attached polypeptides into yeast mitochondria. *EMBO J.* 5, 1343–1350.

Jacobsen-Lyon, K., Jensen, E.Ø., Jørgensen, J.-E., Marcker, K.A., Peacock, J. and Dennis, E. (1995) Symbiotic and non-symbiotic hemoglobin genes from *Casuarina glauca*. *Plant Cell*, **7**, 213–223.

Julliard, J.-H. and Douce, R. (1991) Biosynthesis of the thiazole moiety of thiamin (vitamin B1) in higher plant chloroplasts. *Proc. Natl Acad. Sci. USA*, **88**, 2042–2045.

Koornneef, M. (1994) Arabidopsis Genetics. In *Arabidopsis* (Meyerowitz, E.M. and Smerville, C.R., eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 89–120.

Kouchi, H. and Hata, S. (1993) Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. *Mol. Gen. Genet.* **238**, 106–119.

Langridge, J. (1994) *Arabidopsis thaliana*, a plant. *Drosophila Bioassays*, **16**, 775–778.

Lister, C. and Dean, C. (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. *Plant J.* **4**, 745–750.

Machado, C.R., Costa de Oliveira, R.L., Boiteux, S., Praekelt, U.M., Meacock, P.A. and Menck, C.F.M. (1996) *Thi1*, a thiamine biosynthetic gene in *Arabidopsis thaliana*, complements bacterial defects in DNA repair. *Plant Mol. Biol.*, in press.

Pawlowski, K., Guan, C., Ribeiro, A., Van Kammen, A., Akkermans, A.D.L. and Bisseling, T. (1994a) Genes involved in *Alnus glutinosa* nodule development. In *Proc. First European Nitrogen Fixation Conference* (Kiss, G.B. and Endre, G., eds). Szeged, Hungary: Officina Press, pp. 220–224.

Pawlowski, K., Kunze, R., de Vries, S. and Bisseling, T. (1994b) Isolation of total, poly(A) and polysomal RNA from plant tissues. In *Plant Molecular Biology Manual* D5, 2nd Edn (Gelvin, S.B. and Schilperoort, R.A., eds). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 1–13.

Pawlowski, K., Akkermans, A.D.L., Van Kammen, A. and Bisseling, T. (1995) Expression of *Frankia nif* genes in actinorhizal nodules of *Alnus glutinosa*. *Plant Soil*, **170**, 371–376.

Praekelt, U.M. and Meacock, P.A. (1992) *MOL1*, a *Saccharomyces cerevisiae* gene that is highly expressed in early stationary phase during growth on molasses. *Yeast*, **8**, 699–710.

Praekelt, U.M., Byrne, K.L. and Meacock, P.A. (1994) Regulation of *THI4(MOL1)*, a thiamine-biosynthetic gene of *Saccharomyces cerevisiae*. *Yeast*, **10**, 481–490.

Redei, G.P. (1965) Genetic blocks in the thiamine synthesis of the angiosperm *Arabidopsis*. *Am. J. Bot.* **52**, 834–841.

Redei, G.P. (1967) Genetic estimate of cellular autarky. *Experientia*, **23**, 584.

Ribeiro, A., Akkermans, A.D.L., Van Kammen, A., Bisseling, T. and Pawlowski, K. (1995) A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. *Plant Cell*, **7**, 785–794.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

Schena, M., Lloyd, A.M. and Davis, R.W. (1991) A steroid-inducible gene expression system for plant cells. *Proc. Natl Acad. Sci. USA*, **88**, 10 421–10 425.

Tai, T. and Tanksley, S. (1990) A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. *Plant Mol. Biol. Rep.* **8**, 297–303.

Vander Horn, P.B., Backstrom, A.D., Stewart, V. and Begley, T.P. (1993) Structural genes for thiamine biosynthetic enzymes (*thiCEFGH*) in *Escherichia coli* K-12. *J. Bacteriol.* **175**, 982–992.

Van Ghelue, M., Ribeiro, A., Solheim, B., Akkermans, A.D.L., Bisseling, T. and Pawlowski, K. (1996) Sucrose synthase and enolase expression in actinorhizal nodules of *Alnus glutinosa*: comparison with legume nodules. *Mol. Gen. Genet.* **250**, 437–446.

White, R.L. and Spenser, I.D. (1982) Thiamine biosynthesis in yeast. *J. Am. Chem. Soc.* 104, 4934–4943.

Wickerham, L.J. (1951) Taxonomy of yeast. *U.S. Dept. Agric. Tech. Bull.* **1029**, 11–56.

EMBL Data Library accession number X97434 (*agthi1*).

Chapter 5

A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development

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A Nodule-Specific Gene Encoding a Subtilisin-Like Protease Is Expressed in Early Stages of Actinorhizal Nodule Development

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To identify genes specifically expressed during early stages of actinorhizal nodule development, a cDNA library made from poly(A) RNA from root nodules of *Alnus glutinosa* was screened differentially with nodule and root cDNA, respectively. Seven nodule-enhanced and four nodule-specific cDNA clones were isolated. By using in situ hybridization, two of the nodule-specific cDNAs were shown to be expressed at the highest levels in infected cells before the onset of nitrogen fixation; one of them, *ag12* (*A. glutinosa*), was examined in detail. Sequencing showed that *ag12* codes for a serine protease of the subtilisin (EC 3.4.21.14) family. Subtilisins previously appeared to be limited to microorganisms. However, subtilisin-like serine proteases have recently been found in archaebacteria, fungi, and yeasts as well as in mammals; a plant subtilisin has also been sequenced. In yeast and mammals, subtilases are responsible for processing peptide hormones. A homolog of *ag12*, *ara12*, was identified in Arabidopsis; it was expressed in all organs, and its expression levels were highest during silique development. Hence, our study shows that subtilases are also involved in both symbiotic and nonsymbiotic processes in plant development.

INTRODUCTION

Actinorhizal root nodules are induced by actinomycetes of the genus *Frankia* on several woody dicotyledonous plant species belonging to eight different plant families (Benson and Silvester, 1993). The tissue organization of single lobes of these nodules resembles that of lateral roots in that they contain a central vascular bundle. However, they lack root caps and have both infected and uninfected cortical cells (Berry and Sunell, 1990). The formation of these lobes is initiated in the pericycle. The structure of actinorhizal nodules is dissimilar to nodules induced by *Azorhizobium*, *Bradyrhizobium*, or *Rhizobium* on legume roots (Hirsch, 1992), which are initiated in the root cortex of the plant and contain peripheral vascular bundles, yet similar to that of nodules induced by *Bradyrhizobium* or *Rhizobium* on *Parasponia*, the only non-legume nodulated by *Rhizobium* (Trinick, 1979).

Similar to nodules formed on temperate legumes, actinorhizal nodule lobes have an indeterminate growth pattern due to the presence of an apical meristem that differentiates continuously in a proximal direction (Berry and Sunell, 1990; Hirsch, 1992). A part of the new cortical cells formed by the meristem is subsequently infected by bacterial hyphae. This continuous production and infection of new cells leads to a zonation of the nodule lobe. Thus, starting from the apical meristem, we can distinguish four zones. The meristematic zone (zone 1) consists of small dividing cells that do not contain bacteria. The prefixation zone (zone 2) contains enlarging cortical cells. Some of them are infected and in turn enlarge more than uninfected cells, while being gradually filled with hyphae from the center outward (Lalonde, 1979; Schwintzer et al., 1982; Berry and Sunell, 1990). When they are completely filled with hyphae, provesicles are formed; they represent terminal swellings on hyphae or on short side branches (Fontaine et al., 1984). In the fixation zone (zone 3) in the course of the differentiation of provesicles into vesicles, bacterial *nif* (nitrogen fixation) gene expression is induced and nitrogenase is produced (Huss-Danell and Bergman, 1990). Nitrogenase catalyzes the reduction of atmospheric nitrogen into ammonia. The induction of *nif* gene expression, for example, of the nitrogenase structural gene *nifH*, is a suitable marker for the transition of zone 2 to zone 3 and can be easily detected by in situ hybridization (Pawlowski et al., 1995). The senescent zone (zone 4) is characterized by the occurrence of cortical cell senescence and degradation of the host cytoplasm as well as of the microsymbiont (Berry and Sunell, 1990). Thus, in each mature nodule lobe, a gradient of developmental stages is present, beginning with uninfected meristematic cells and finishing with senescent cells.

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In *Rhizobium*-induced legume nodules, several nodule-specific genes, the so-called nodulin genes (van Kammen, 1984), have been identified (for reviews, see Franssen et al., 1992; Hirsch, 1992). By using in situ hybridization with indeterminate nodules of pea and alfalfa, Scheres et al. demonstrated that early and late nodulin genes are expressed in subsequent zones of the nodule (1990a). Late nodulin genes, which are expressed at the onset of bacterial nitrogen fixation, seem to be involved in nodule functions. Examples include leghemoglobin (reviewed by Appleby et al., 1988), glutamine synthetase (Cullimore and Bennett, 1988), and sucrose synthase (Thummler and Verma, 1987; Küster et al., 1993) genes. Early nodulin genes, which are expressed before the onset of nitrogen fixation, have been implicated either in infection or in building up the nodule structure. Such genes include *ENOD2* (Van de Wiel et al., 1990), *ENOD12* (Scheres et al., 1990b), and the *Medicago truncatula* proline-rich protein gene *MtPRP4* (Wilson et al., 1994).

Because of the structural similarity between actinorhizal nodules and lateral roots, we asked whether nodule-specific genes are also expressed during early stages of actinorhizal nodule development or whether for actinorhiza, nodule-specific gene expression is only necessary for nodule functioning, for example, to support carbon supply to the bacteria and assimilation and metabolism of the ammonium produced by bacterial nitrogen fixation. To answer this question, we isolated nodule-specific cDNAs from a cDNA library made from *Alnus glutinosa* root nodules, focusing specifically on cDNAs representing genes expressed during early stages of nodule development.

RESULTS

Isolation and Characterization of Nodule-Specific cDNAs

To obtain cDNA clones corresponding to genes involved in nodule development, $\sim 8 \times 10^5$ clones of an *A. glutinosa* nodule cDNA library were differentially screened with nodule and root cDNA, respectively. The nodule-specific/enhanced nature of the isolated cDNA clones was confirmed by RNA gel blot hybridization, showing that seven different nodule-enhanced and four different nodule-specific clones had been purified. DNA gel blot hybridization was performed to confirm that the isolated cDNA clones were not derived from *Frankia*.

To determine which of the isolated clones represented a gene expressed during an early stage of development, in situ hybridization studies were conducted using the bacterial nitrogenase structural gene *nifH* as a marker for the developmental stage of infected cortical cells. These studies revealed that two of the nodule-specific clones, pAg12-1 and pAg164, represent genes expressed before *nif* gene expression. *ag164* (*A. glutinosa*) encodes a glycine-rich protein and has been found to be highly homologous to pAgNt84 (Mullin et al., 1993; Twigg, 1993); therefore, it was not studied in greater detail. The second clone, pAg12-1, was selected for further analysis.

By using RNA gel blot hybridization analysis, a 2.5-kb transcript of *ag12* was found in nodules but not in roots, cotyledons, male or female flowers, and developing fruits (Figure 1A). However, a low level of transcript was detected in shoot tips. To find a full-length cDNA clone, eight different cDNA clones were isolated from the library and the ends of their inserts were sequenced. They were derived from two different but highly homologous mRNAs, *ag12-1* and *ag12-2* (95% identity in the first 980 bp of the coding region; 64.6% identity in 92 bp of the 3′ nontranslated region). DNA gel blot hybridization with the inserts of pAg12-1 and pAg12-2 confirmed these results, revealing two to six hybridizing bands in the genome of *A. glutinosa* (Figure 2). Thus, Ag12 is encoded by a small gene family. In situ hybridization was performed using a fragment of the coding region derived from pAg12-1 (see Methods); this fragment detected both mRNAs. One full-length clone representing the other member of the family was designated pAg12-2, and it was selected for sequencing.

Localization of *ag12* mRNA in *A. glutinosa* Nodules

The expression pattern of *ag12* in *A. glutinosa* root nodules was determined by in situ hybridization of longitudinal and cross-sections of nodules with ^{35}S-labeled antisense and sense RNAs, respectively. To obtain a reference point for the developmental stage of the infected cell, in situ hybridizations with antisense RNA of the nitrogenase structural gene *nifH* from *Frankia* were performed on adjacent sections (Pawlowski et al., 1995). The results showed that *ag12* was expressed at high levels in the visibly infected cells of zone 2 (Figures 3A and 3B). These cells had not yet completely filled with hyphal material and did not yet express *nifH* (Figures 3C and 3D). A longer exposure showed that *ag12* was also expressed in the infected cells of zone 3, although at lower levels (Figures 3E and 3F). In Figures 3C to 3F, *ag12* was expressed at high levels in the young infected cells of zone 2; at the onset of *nifH* expression, however (beginning of zone 3; Figures 3C and 3D), the level of *ag12* transcript was reduced markedly (see detail in Figure 3G). In the mature infected cells of zone 3, *ag12* was expressed at different levels (Figures 3E and 3F), although always lower than in zone 2 and higher than in the area of zone 3 where *nifH* was induced. No expression of *ag12* was found in senescent, infected cells of zone 4 (Figure 3H). Thus, *ag12* expression was induced to high levels upon infection of cortical cells in zone 2. Subsequently, expression levels were markedly reduced at the onset of nitrogen fixation. This meant that infected cells had passed through a phase where *ag12* expression was undetectable. The level of *ag12* mRNA then increased again and was maintained at irregular levels. In some of the mature infected cells, *ag12* expression was barely detectable. At the onset of senescence (zone 4), *ag12* expression was switched off.

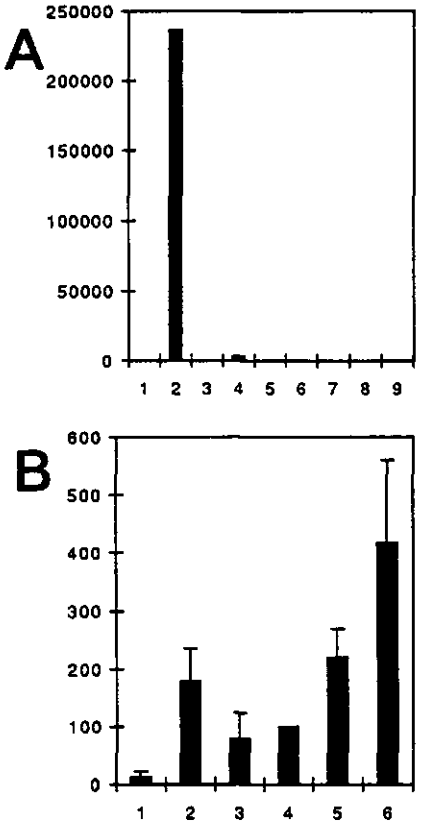

Figure 1. RNA Gel Blot Hybridization Analysis.

(A) Expression of *ag12* in different organs of *A. glutinosa*.
Bar 1, roots; bar 2, nodules; bar 3, cotyledons; bar 4, shoot tips; bar 5, male flowers; bar 6, female flowers; bar 7, immature fruits collected in April; bar 8, immature fruits collected in June; bar 9, immature fruits collected in September.
(B) Expression of *ara12* in different organs of Arabidopsis.
Bar 1, roots; bar 2, rosette leaves; bar 3, cauline leaves; bar 4, inflorescence stems; bar 5, flowers; bar 6, immature siliques.
RNA gel blots containing ~10 μg of total RNA per slot were hybridized against *ag12* and *ara12*, respectively. Afterward, the amount of mRNA on the filters was determined by a hybridization with a soybean ubiquitin probe (Kouchi and Hata, 1993). The signal was quantified using a PhosphorImager. The results were calculated as the percentage of expression in nodules in **(A)** and as a percentage of expression in inflorescence stems in **(B)**, respectively. The average results of three independent experiments were combined in one diagram. Standard deviations are given.

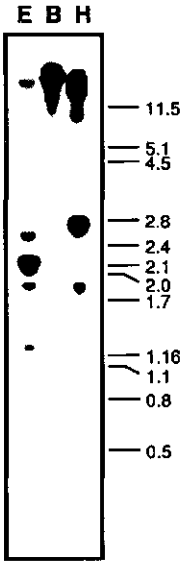

Figure 2. Ag12 Is Encoded by a Small Gene Family.

A DNA gel blot containing genomic DNA of *A. glutinosa* digested with EcoRI (E), BamHI (B), and HindIII (H), respectively, was hybridized with the insert of pAg12-2. *ag12-1* and *ag12-2* cDNAs contain one internal EcoRI site each but no HindIII or BamHI sites.

ag12 Encodes a Serine Protease and Has a Homolog in Arabidopsis

The DNA sequence of the insert of pAg12-2 was determined (EMBL accession number X85975). The 2435-bp cDNA contains an open reading frame encoding a protein of 761 amino acids and with an isoelectric point of 7.25 (Figure 4). The amino acid sequence was used for homology searches in the sequence data bases of the National Center of Biotechnology Information, and a homolog was found among the randomly sequenced Arabidopsis cDNA clones (GenBank accession number T04180; clone 2E4T7P). It was designated *ara12*. RNA gel blot analysis showed that *ara12* hybridizes with a 2.5-kb mRNA present in all organs of Arabidopsis; however, transcript levels varied, with the highest levels being in immature siliques (Figure 1B). The DNA sequence of *ara12* was determined (EMBL accession number X85974). Because 2E4T7P is not a full-length cDNA clone, the 5′ region was amplified by polymerase chain reaction as described in Methods. However, the amplification product was still not full length. Sequence comparison showed that *ara12* encodes a polypeptide with 61% homology to the polypeptide encoded by *ag12-2* (Figure 4).

Both Ag12 and Ara12 show significant homology to prokaryotic serine proteases of the subtilisin family (Barr, 1991; Siezen

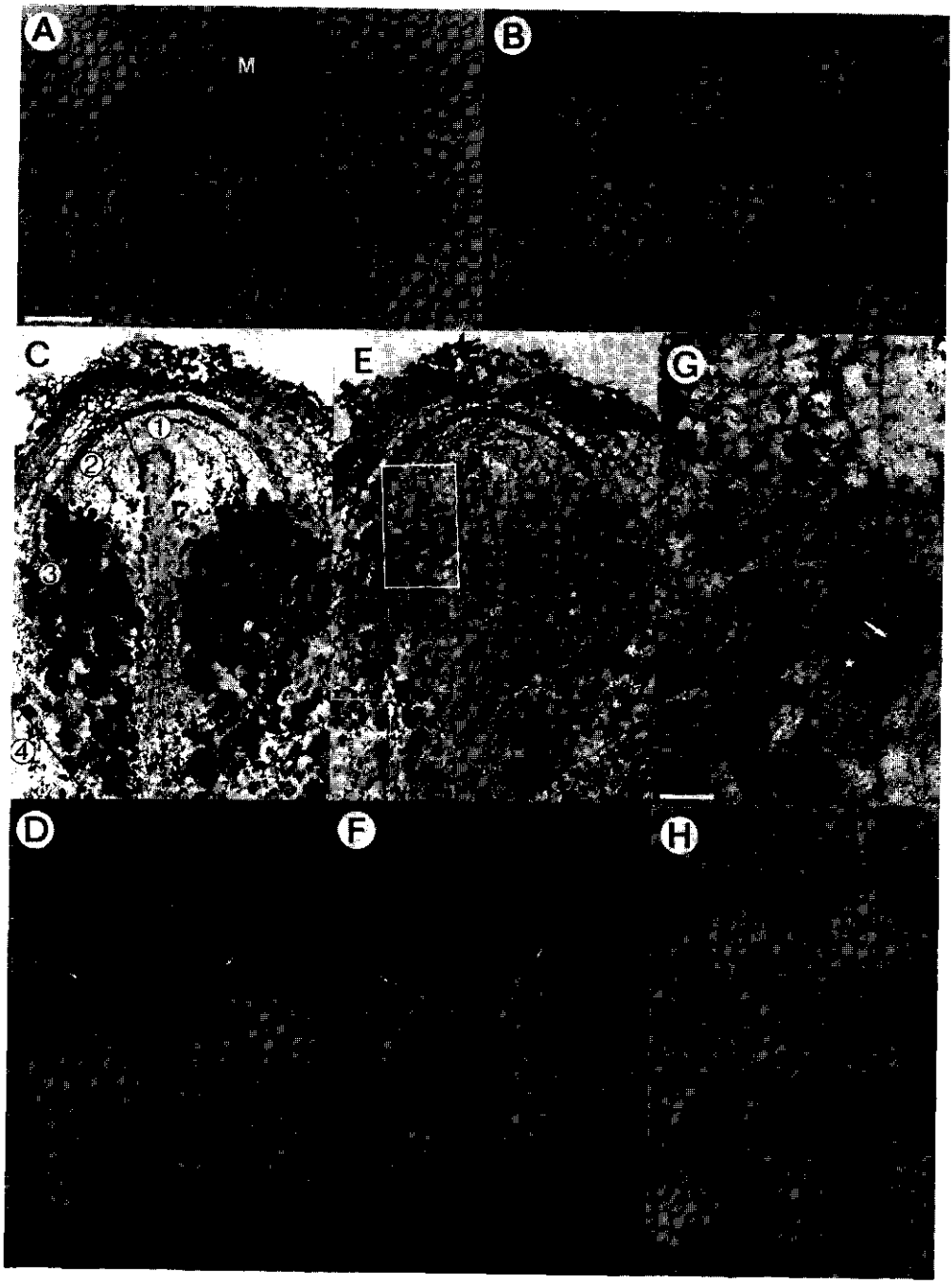

Figure 3. Localization of *Frankia nifH* and *A. glutinosa ag12* in Sections of *A. glutinosa* Nodules.

et al., 1991). Homology is particularly high around the four residues forming the active site of subtilases (Barr, 1991; Siezen et al., 1991; Figures 4 and 5). Subtilases are produced as preproenzymes, therefore remaining inactive until secreted and processed (Siezen et al., 1991). The start of the mature proteins encoded by *ag12-2* and *ara12* has been deduced from sequence comparisons with other subtilases (R. Siezen, personal communication; Yamagata et al., 1994; Figure 4); the putative transmembrane signal peptide cleavage site was determined according to the method of von Heijne (1986).

DISCUSSION

Nodule-Specific Gene Expression in *A. glutinosa*

Altogether, four nodule-specific and seven nodule-enhanced cDNAs were isolated. Two of the nodule-specific cDNA clones, pAg12-1 (Figure 3) and pAg164 (data not shown), were shown to represent genes expressed at high levels in infected cells before the onset of bacterial *nif* gene expression. Thus, in spite of their structural similarity with lateral roots, the early stages of actinorhizal nodule development appeared to involve the expression of nodule-specific genes. However, the expression of these early nodulin genes in the infected cells implied that they were most likely involved in the interaction with the bacterial symbiont. In legume nodules, several early nodulin genes are expressed specifically in cells not infected by *Rhizobium*. For example, *ENOD2* is expressed in the nodule parenchyma (van de Wiel et al., 1990), *MtPRP4* is expressed in the meristem of *M. truncatula* nodules (Wilson et al., 1994), and soybean *GmENOD40* is expressed in the nodule vascular tissue and, to a lower level, in the uninfected cells of soybean nodules (Kouchi and Hata, 1993; Yang et al., 1993). Thus, cells not infected by *Rhizobium* also show nodule-specific differentiation in legume nodules. However, in actinorhizal nodules the infected cells seem to be the only specialized cell type. Thus, to date we have found no evidence that the formation of the actinorhizal nodule structure itself requires the expression of nodule-specific genes. The change of the developmental program from roots to actinorhizal nodule lobes may involve only subtle changes in gene expression.

The other nodule-specific or nodule-enhanced cDNAs so isolated represent genes that are expressed at later stages of *Alnus* nodule development. Sequencing has shown that most of them encode enzymes involved in the carbon and nitrogen metabolism of the nodule (Pawlowski et al., 1994a). Thus, as in legumes, the metabolic specialization of actinorhizal nodules comprises high expression levels of genes whose products are involved in glycolysis and nitrogen assimilation.

A Subtilase Involved in Nodule Development

We showed that Ag12 and Ara12 represent subtilisin-like proteases based on amino acid sequence conservation (Barr, 1991; Siezen et al., 1991). For a long time, many have thought that the subtilisin-like proteases are unique to prokaryotes. However, more recently, subtilases have been found in fungi and in yeast; here, they are involved in processing the mating pheromone and killer toxin precursors (Julius et al., 1984; Mizuno et al., 1988). In turn, subtilases have been identified in higher eukaryotes such as mammals, insects, *Mollusca*, and nematodes; here, they are involved in proprotein processing. In these organisms, several subtilisins are responsible for processing peptide hormone and pheromone precursors, cleaving at sites comprised of pairs of basic amino acid residues (dibasic sites; reviewed by Barr, 1991). There is also evidence that a subtilase is involved in processing the cuticle collagens of

Figure 3. (continued).

In **(A)**, **(C)**, **(E)**, **(G)**, and **(H)**, bright-field microscopy was used; silver grains denoting hybridization appear as black dots. In **(B)**, **(D)**, and **(F)**, dark-field microscopy and epipolarized light were used; silver grains are visible as yellow dots. Slides were exposed for 4 weeks in **(C)** to **(H)** or for 2 weeks in **(A)** and **(B)**.

(A) and **(B)** Expression of *ag12* in a longitudinal section of a nodule lobe. After exposure for 2 weeks, only the hybridization in infected cells of zone 2 can be seen. M, meristematic zone.

(C) and **(D)** Expression of *Frankia nifH*. The four different zones described in the Introduction are marked. Arrowheads point to infected cells of zone 2 that are not yet completely filled with hyphae and do not contain vesicles. These cells show no *Frankia nifH* expression.

(E) and **(F)** Expression of *ag12* in adjacent longitudinal sections of the same nodule lobe. Arrowheads point to infected cells of zone 2 that are not yet completely filled with hyphae, that is, not containing vesicles; these cells show high *ag12* expression levels. Arrows point to two infected cells of zone 3 in **(E)**; one cell shows *ag12* expression at a high level, the other shows little expression. The areas magnified in panels **(G)** and **(H)** are indicated by white boxes.

(G) Detailed view of **(E)** (zones 2 and 3): reduction of *ag12* expression at the shift from zone 2 to zone 3. An infected cell of zone 2 showing high *ag12* expression is indicated by an arrow. An adjacent cell of zone 3 (indicated by a star) contains *Frankia* vesicles and shows significantly reduced expression of *ag12*.

(H) Detailed view of **(E)** (zones 3 and 4): no expression of *ag12* in senescent cells. S, senescent cell.

Bar in **(A)** = 500 μm; bar in **(G)** = 100 μm.

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                                   *
Ag12   MKLYNGICLPYLFLFASCICLAL.EAASTSMEKSTYIVHMDKSHMPKAFT
Ara12  .........AFFLLL....CLGFCEVESSSSDQGTYIVHMAKSQTPSSFD

Ag12   SHHWWYSSIVDCLNSEKPTTSSFVYTYNHVLHGFSASLSHQELDTLRESP
Ara12  LHSHWYDSSLRSI.SD...SAELLYTYENAIHGFSTRLTQEEADSLMTQP

                  -> mature enzyme                 *
Ag12   GFVSAYRDRNATLDTTHTPRFLSLN.PTGGLWP.ASNYGEDVIIGVIDSG
Ara12  GVISVLPEHRYELHTTRTPLFLGLDEHTADLFPEAGSYS.DVVVGVLDTG

Ag12   VWPESDSFKDDGMTAQVPARWKGICSRE.GFNSSMHCNSKLIGARYFNKG.
Ara12  VWPESKSYSDEGF.GPIPSSWKGGCEAGTNFTASLCNRKLIGARFFARGY

                                *
Ag12   ..IMAAIPNATFSMNSARDTLGHGTHTASTAAGNYVNGASYFGYGKGTAR
Ara12  ESTWGPIDESKESR.SPRDDDGHGTHTSSTAAGSVVEGASLLGYASGTAR

Ag12   GIAPRARVAVYKVTWPEGRYTSDVLAGIDQAIADGVDVISISLGYDGVP.
Ara12  GML.HA.LAVYKVCWLGGCFSSDILAAIDKAIADNVNVLSMSLG.GGMSD

                                *
Ag12   LYEDPIAIASFAAMEKGVVVSTSAGNAGPFFGNHHEGIPWVLTVAAGNID
Ara12  YYRDGVAIGAFAAMERGILVSCSAGNAGPSSSSLSNVAPWITTVGAGTLD

Ag12   RSFAGTLTLGNDQTITGWTMFPASAIES.SQLVYNKTISACNSTE....
Ara12  RDFPALAILGNGKNFTGVSLFKGEALPDKLLPFIY..AGNASWATNGNLC

Ag12   ....LLSDAVYS.VVICEAITPIYAQI...DAITRSNVAGAILISNHTKL
Ara12  MTGTLIPEKVKGKIVMCD..RGINARVQKGDVVKAAGGVGMILANTAANG

Ag12   FELGGGVS.CPCLVISPKDAAALIK.YAKTDEFPLAGLKFQETITGTKPA
Ara12  EELVADAHLLPATTVGEK.AGDIIRKYVTTDPNPTASISILGTVVGVKPS

Ag12   PAVAYYSSRGPSPSYPGILKPDVMAPGSLVLASWIPNEATAQIGTNVYLS
Ara12  PVVAAFSSRGPNSITPNILKPDLIAPGVNILAAW..TGAAGPTG....LA

                                *
Ag12   S.....HYWMVSGTSMACPHASGVAALLKAARPEWSPAAIRSAMMTTANP
Ara12  SDSRRVEFNIISGTSMSCPHVSGLAALLKSVHPEWSPAAIRSALMTTAY.

Ag12   LDNTLNPIHENGKK.FHLA.....SPLAMGAGHIDPNRALDPGLVYDATP
Ara12  ..RTYK....DGKPLLDIATGKPSTPFDHGAGEVSPTTATNPGLIYDLTT

Ag12   QDYINLLCSMNYNKAQILAIVRSDSYTCSNDPS.....SDLNYPSFIAFH
Ara12  EDYLGFLCALNYTSPQIRSVSRRN.YTC..DPSKSYSVADLNYPSF.AVN

Ag12   NSTCRRSVNTFQRTVTNVGDGAATYKATVTA.PKDSRVIVSPQTLAFGSK
Ara12  VDGA..GAYKYTATVTSVG.GAGTYSVKVTSETTGVKISVEPAVLNFKEA

Ag12   YEKQSYNLTIINFTRD.TERKDI.SFGALVWANENGKHVRSPIVVSPLR
Ara12  NEKKSYTVT...FTVDSSKPSGSNSFGSIEWS..DGKHVVGSPVAIS..W

Ag12   IND
Ara12  T
```

Figure 4. Sequence Comparison of Ag12 and Ara12.

Conserved amino acids are shown in boldface type. The putative signal peptide cleavage site (between positions 28 and 29 in Ag12) is marked with a star. The start of the putative mature enzyme (position 114 in Ag12) is indicated. The amino acids forming the active site of subtilases (D at 145, H at 216, N at 316, and S at 537; positions in the Ag12 sequence) are marked by black dots. Gaps have been introduced to optimize the alignment.

Caenorhabditis elegans (Yang and Kramer, 1994). In plants, however, only physiological evidence was available to substantiate the existence of subtilisins (Rudenskaya, 1994; Schaller and Ryan, 1994) until a subtilisin-like protease was sequenced from melon fruits (Yamagata et al., 1994).

Both *ag12* and *ara12* expression levels were particularly high in early stages of development, that is, in early infection stages of actinorhizal nodules and during silique development in Arabidopsis. Thus, it is likely that both enzymes play a role in protein processing and not in degradation. The presence of a putative signal peptide in the sequence of Ag12 and Ara12 indicates that both enzymes are probably active in the extracellular space. In the case of Ag12, which was only expressed in infected cells, the site of activity could be the space between invaginated plasma membrane and bacteria. Thus, Ag12 might be involved in processing a protein that is part of the cell wall–like matrix material surrounding the bacteria or in processing another protein with an undetermined function.

In contrast to subtilases, cysteine proteases and aspartic proteases have been studied extensively in plants and have been implicated in several degradative processes in plant development, for example, seed germination (Tormakangas et al., 1991; Cejudo et al., 1992; Cervantes et al., 1994), fruit development (Lin et al., 1993), senescence (Granell et al., 1992), and wilting (Williams et al., 1994). Analysis of protease activity during legume nodule development has revealed cysteine protease activities appearing during nodule senescence in nodules of French bean involved in degradation of leghemoglobin and bacteroids (Pladys and Rigaud, 1985; Pladys et al., 1991). A nodule-specific cysteine protease has been identified in *A. glutinosa* (Goetting-Minesky and Mullin, 1994). This protease might be involved in actinorhizal nodule senescence because cysteine proteases are also involved in senescence processes in other systems (Granell et al., 1992). Pladys and Rigaud (1985)

```
AVPRCA    IIDDGV    DNHGTACA    AGNGNE    GTSSACPGA

BASBPN    VIDSGI    NSHGTHVA    AGNEGT    GTSMASPHV
LLPRTP    VIDSGI    EQHGMHVA    AGNSGS    GTSMASPFI

TAPROK    VIDTGI    NGHGTHCA    AGNNNA    GTSMATPHV
MACDPA    IIDTGI    HGHGTHCA    AGNDNR    GTSMATPHI
THPRB1    VVDSGI    LGHGTHVS    AGNGDS    GTSMATPHV

Ag12      VIDSGV    LGHGTHTA    AGNAGP    GTSMACPHA
Ara12     VLDTGV    DGHGTHTS    AGNAGP    GTSMSCPHA

SCKEX2    IVDDGL    DYHGTRCA    SGNGGT    GTSAAAPLA

DMFUR1    ILDDGL    NRHGTRCA    SGNGGR    GTSASAPLA
HSFUR     ILDDGI    NRHGTRCA    SGNGGR    GTSASAPLA
```

Figure 5. Sequence Conservation around the Amino Acids Forming the Active Site of Subtilases.

Subtilisins from different organisms are compared. The list begins with a cyanobacterial sequence, followed by two bacterial sequences, three sequences from fungi, the two plant sequences described in this study, a sequence from yeast, and two sequences from animals (Drosophila and humans, respectively). The last three sequences comprise proteinases processing hormone precursors. Amino acids conserved in at least five organisms are set in boldface. The four amino acids forming the active site (D, H, N, and S) are outlined. AVPRCA, calcium-dependent proteases from *Anabaena* sp (Maldener et al., 1991); BASBPN, subtilisin of *Bacillus amyloliquefaciens* (Wells et al., 1983); LLPRTP, subtilisin of *Lactococcus lactis* (Vos et al., 1989); TAPROK, proteinase K from *Tritirachium album* (Gunkel and Gassen, 1989); MACDPA, cuticle-degrading protease from *Metarhizium anisopliae* (St. Leger et al., 1992); THPRB1, alkaline protease from *Trichoderma harzianum* (Geremia et al., 1993); SCKEX2, yeast KEX2 endopeptidase (Mizuno et al., 1988); DMFUR1, furin from Drosophila (Roebroek et al., 1991); HSFUR, human furin (Barr et al., 1991).

also found serine protease activity during legume nodule functioning, but the enzyme was not characterized further.

Ag12 and Ara12 in Symbiotic and Nonsymbiotic Plant Development

ag12 was expressed at high levels only in nodules of *A. glutinosa*; here, it probably was involved in the interaction with the endosymbiont. However, it also was expressed at low levels in *A. glutinosa* shoot tips, that is, during nonsymbiotic plant development, which is consistent with the occurrence of the homologous *ara12* in a nonsymbiotic plant like Arabidopsis.

Legume nodulin genes are thought to be derived from the duplication of genes involved in nonsymbiotic processes (Nap and Bisseling, 1990), as is the case for leghemoglobins (Taylor et al., 1994) and nodulin-26 (Miao and Verma, 1993), or to be genes recruited from other developmental programs, as is the case for *ENOD12* (Scheres et al., 1990b) and *ENOD40* (Kouchi and Hata, 1993; Yang et al., 1993). The first has also been shown to have occurred in actinorhiza because, in *Casuarina glauca*, a small family of hemoglobin genes has been found that is exclusively expressed in nodules (Fleming et al., 1987); a distinct hemoglobin gene has also been found that is expressed in roots and at a lower level in leaves, stems, and nodules (Christensen et al., 1991; Jacobsen-Lyon et al., 1995).

It is not clear whether the same *ag12* genes are expressed in shoot tips and in nodules of *A. glutinosa* or whether different members of the gene family are expressed in these two organs. Therefore, based on our data, it cannot be determined whether there is a nodule-specific member of the *ag12* family. Similar to legume nodular genes, in *A. glutinosa* a nodule-specific gene was either recruited or duplicated from nonsymbiotic development. Two lines of evidence suggest that subtilases are involved in several processes in plant development: first, *ara12* is expressed in every organ of Arabidopsis; and second, sequences with ~65% homology to *ara12* are found among the randomly sequenced Arabidopsis cDNAs (GenBank accession numbers T21798 and T22184). In the case of *ag12*, we propose that a proprotein-processing subtilase involved in nonsymbiotic plant development has acquired a specific function in the interaction with symbiotic *Frankia*.

METHODS

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 hr of light and 8 hr of darkness. Seeds were germinated in trays containing sterile gravel wetted with sterile tap water. After 3 weeks, the seedlings were transferred to sterile gravel wetted with quarter-strength Hoagland's 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 to 13 weeks after infection. Nodules for in situ hybridization were grown in a hydroponic tank with quarter-strength Hoagland's solution. Male and female flowers of *A. glutinosa* were collected from a local stand (Wageningen, The Netherlands) in March 1994. Developing fruits were collected from the same stand in April, June, and September 1994.

Arabidopsis thaliana ecotype Landsberg *erecta* was grown in a greenhouse in pot soil. Seeds were germinated on filter paper wetted with tap water for 1 day at 4°C and for 2 days at 21°C in the dark before transfer to pot soil. Arabidopsis roots were obtained by germinating seeds and cultivating in liquid LS medium (Linsmaier and Skoog, 1965) with 2% sucrose on a shaking incubator at 21°C in the light. Rosette leaves were harvested before bolting; stems, cauline leaves, and flowers were harvested shortly after flowering. Siliques were harvested 2 to 3 weeks after flowering.

Isolation of DNA and RNA

For isolation of root RNA, seedling roots were collected from uninfected plantlets 2 to 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 to 13 weeks after infection.

DNA was isolated from *A. glutinosa* leaves according to Dellaporta et al. (1983), with some modifications: Polyclar AT (Serva, Heidelberg, Germany) was added during grinding, and to remove further polyphenols, an extraction with Polyclar AT was performed before isopropanol precipitation. Afterward, the DNA was purified by a CsCl gradient. DNA from *Frankia* HFPArI3 was isolated from cultures grown in P medium with 0.2% casamino acids. Cells were resuspended in 1.75 mL of 0.3 M sucrose, 120 mM EDTA, 25 mM Tris-HCl, pH 8.0. Fifty milligrams of lysozyme and 30 mg of achromopeptidase (Sigma) were added, and lysis took place at 37°C for 30 min. Four hundred and seventy microliters of 5 M NaCl and 375 μL of 10% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide in 0.7 M NaCl were added, and the mixture was incubated at 65°C for 30 min. The DNA was extracted with chloroform and phenol-chloroform and precipitated with isopropanol, followed by an ethanol precipitation. Total RNA was isolated from *A. glutinosa*, as described by Pawlowski et al. (1994b). 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 Arabidopsis, as described by Kiedrowski et al. (1992).

Construction and Screening of a cDNA Library

A cDNA library from poly(A) RNA of *A. glutinosa* nodules harvested 5 to 8 weeks after infection was custom-made by Stratagene in λZapII. Probes for the differential screening of this library were prepared from RNA isolated from *A. glutinosa* nodules and roots, respectively. Complementary DNA was produced from 10 μg of total RNA using reverse transcriptase (Amersham) and amplified using a random primed DNA labeling protocol (Feinberg and Vogelstein, 1983) in the presence of α-^{32}P-dATP. Plaques (10^5) were plated and amplified in situ after transfer to Amersham Hybond filters (Amersham), as described by Sambrook et al. (1989). After hybridization in buffer without formamide (Sambrook et al., 1989), filters were washed at 65°C with decreasing salt concentrations down to 0.5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, followed by a washing step in 0.5 × SSC, 4% SDS,

to remove contaminants due to remnants of bacterial debris. For isolation of full-length cDNA clones, no in situ amplification of phages on the filters was performed. In a second screening, minipreparation DNA from 224 library plasmid clones was digested with EcoRI and subjected to gel electrophoresis. DNA gel blots were hybridized with nodule and root cDNA, respectively. Clones whose inserts were only hybridizing against nodule cDNA were selected for further analysis.

Cloning and Sequencing Procedures

DNA manipulations were carried out as described by Sambrook et al. (1989). Deletion clones were constructed using the exonuclease III kit from Promega. The nucleotide sequences were determined using the dideoxy chain termination method (Sanger et al., 1977) and an automatic sequencer (model 373A; Applied Biosystems, Foster City, CA). Sequence data were analyzed using the programs of the Genetics Computer Group (Devereux et al., 1984). Data base searches were performed using the BLAST algorithm (Altschul et al., 1990) in the *nucleotide sequence data bases of the National Center of Biotechnology* Information, National Library of Medicine, and National Institutes of Health.

DNA and RNA Gel Blots and Hybridization Conditions

Total RNA was denatured in dimethyl sulfoxide/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 (New England Nuclear Research, Beverly, MA) filters (RNA) or to Amersham Hybond N+ (Amersham) filters (DNA). RNA gel blot hybridizations were performed in buffer containing 50% formamide at 42°C (Sambrook et al., 1989). DNA gel blot 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 to 0.5 × SSC, 0.1% SDS, and, in the case of a heterologous probe, with 2 × SSC, 0.1% SDS. Hybridization signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In Situ Hybridization

Tissues were fixed in 100 mM sodium phosphate, pH 7.2, containing 4% paraformaldehyde and 0.25% glutaraldehyde for 4 hr under vacuum, dehydrated via a graded ethanol series, and embedded in Paraclean (Klinipath, Duiven, The Netherlands). Sections (7 μm thick) were dried on polylysine-coated slides at 37°C overnight, deparaffinized with xylene, and rehydrated via a graded ethanol series. Hybridization pretreatment, hybridization, and washing were performed essentially as described by Cox and Goldberg (1988) and adapted by van de Wiel et al. (1990). Washing stringency, RNase concentration, and DTT concentrations in the washing buffers were increased.

For the *Frankia* HFPArI3 *nifH* probe, a 387-bp SstI internal fragment of *nifH* from pFQ148 (Simonet et al., 1988) was subcloned in pBluescript SK− (Stratagene), yielding pFnifH1. This plasmid was linearized with EcoRI, and antisense RNA was transcribed using T7 RNA polymerase. For *A. glutinosa ag12-1* sense and antisense probes, a plasmid containing 212 to 685 bp of the cDNA in pBluescript SK− was linearized using HindIII and transcribed with T3 RNA polymerase (antisense) or linearized with BamHI and transcribed with T7 RNA polymerase (sense), respectively. After washing, the slides were coated with microautoradiography emulsion LM-1 (Amersham) and exposed for 4 weeks at 4°C. They were developed in Kodak D19 developer (Eastman Kodak, Rochester, NY) for 5 min and fixed in Kodak fix. Sections were counterstained with 0.02% ruthenium red and 0.025% toluidine blue O for 5 min each and mounted with DePeX (BDH Laboratory Supplies, Poole, United Kingdom).

Polymerase Chain Reaction

Taq polymerase (SuperTaq) was obtained from Sphaero Q (Leiden, The Netherlands) and used according to the instructions provided by the manufacturer. Using oligonucleotide 5′-GGTGTGTAGCTCGTAACGG-3′ and the T3 primer (Bethesda Research Laboratory, Gaithersburg, MD) on the λPRL2 cDNA library of Arabidopsis (T. Newman, Ohio State University, Columbus, OH), fragments of 250 to 600 bp were amplified. Fragments of 400 to 600 bp were subcloned and sequenced.

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REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. **215,** 403–410.

Appleby, C.A., Bogusz, D., Dennis, E.S., and Peacock, W.J. (1986). A role for haemoglobin in all plant roots? Plant Cell Environ. **11,** 359–367.

Barr P.J. (1991). Mammalian subtilisins: The long-sought dibasic processing endoproteases. Cell **66,** 1–3.

Barr, P.J., Mason, O.B., Landsberg, K.E., Wong, P.A., Kiefer, M.C., and Brake, A.J. (1991). cDNA and gene structure for a human subtilisin-like protease with cleavage specificity for paired basic amino acid residues. DNA Cell Biol. **10,** 319–328.

Benson, D.R., and Silvester, W.B. (1993). Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol. Rev. **57,** 293–319.

Berry, A.M., and Sunell, L.A. (1990). The infection process and nodule development. In The Biology of *Frankia* and Actinorhizal Plants, C.R. Schwintzer and J.D. Tjepkema, eds (New York: Academic Press), pp. 61–81.

Berry, A.M., and Torrey, J.G. (1979). Isolation and characterization *in vivo* and *in vitro* of an actinomycetous endophyte from *Alnus rubra* Bong. In Symbiotic Nitrogen Fixation in the Management of Temperate Forests, J.C. Gordon, D.T. Wheeler, and D.A. Perry, eds (Corvallis, OR: Oregon State University), pp. 69–84.

Cejudo, F.J., Murphy, G., Chinoy, C., and Baulcombe, D.C. (1992). A gibberellin-regulated gene from wheat with sequence homology to cathepsin B of mammalian cells. Plant J. **2**, 937–948.

Cervantes, E., Rodriguez, A., and Nicolas, G. (1994). Ethylene regulates the expression of a cysteine proteinase during germination of chickpea (*Cicer arietinum* L.). Plant Mol. Biol. **25**, 207–215.

Christensen, T., Dennis, E.S., Peacock, J.W., Landsmann, J., and Marcker, K.A. (1991). Hemoglobin genes in non-legumes: Cloning and characterization of a *Casuarina glauca* hemoglobin gene. Plant Mol. Biol. **16**, 339–344.

Cox, K.H., and Goldberg, R.B. (1988). Analysis of plant gene expression. In Plant Molecular Biology: A Practical Approach, C.H. Shaw, ed (Oxford, England: IRL Press), pp. 1–34.

Cullimore, J.V., and Bennett, M.J. (1988). The molecular biology and biochemistry of plant glutamine synthetase from root nodules of *Phaseolus vulgaris* L. and other legumes. J. Plant Physiol. **132**, 581–587.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. **1**, 19–21.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**, 387–395.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**, 6–13.

Fleming, A.I., Wittenberg, J.B., Wittenberg, B.A., Dudman, W.F., and Appleby, C.A. (1987). The purification, characterization and ligand-binding kinetics of hemoglobins from root nodules of the non-leguminous *Casuarina glauca*-*Frankia* symbiosis. Biochim. Biophys. Acta **911**, 209–220.

Fontaine, M.S., Lancelle, S.A., and Torrey, J.G. (1984). Initiation and ontogeny of vesicles in cultured *Frankia* sp. strain HFPArI3. J. Bacteriol. **160**, 921–927.

Franssen, H.J., Vijn, I., Yang, W.C., and Bisseling, T. (1992). Developmental aspects of the *Rhizobium*/legume symbiosis. Plant Mol. Biol. **19**, 89–107.

Geremia, R.A., Goldman, G.H., Jacobs, D., Ardiles, W., Vila, S.B., Van Montagu, M., and Herrera-Estrella, A. (1993). Molecular characterization of the proteinase-encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. Mol. Microbiol. **8**, 603–613.

Goetting-Minesky, P., and Mullin, B.C. (1994). Differential gene expression in an actinorhizal symbiosis: Evidence for a nodule-specific cysteine proteinase. Proc. Natl. Acad. Sci. USA **91**, 9891–9895.

Granell, A., Harris, N., Pisabarro, A.G., and Carbonell, J. (1992). Temporal and spatial expression of a thiolprotease gene during pea ovary senescence, and its regulation by gibberellin. Plant J. **2**, 907–915.

Gunkel, F.A., and Gassen, H.G. (1989). Proteinase K from *Tritirachium album* Limber: Characterization of the chromosomal gene and expression of the cDNA in *Escherichia coli*. Eur. J. Biochem. **179**, 185–194.

Hirsch, A.M. (1992). Developmental biology of legume nodulation. New Phytol. **122**, 211–237.

Hoagland, D.R., and Arnon, D.T. (1938). The water-culture method for growing plants without soil. California Agriculture Experiment Station Circular 347. (Berkeley, CA: University of California).

Huss-Danell, K., and Bergman, B. (1990). Nitrogenase in *Frankia* from root nodules of *Alnus incana* (L.) Moench: Immunolocalization of the Fe- and MoFe proteins during vesicle differentiation. New Phytol. **116**, 443–455.

Jacobsen-Lyon, K., Jensen, E.Ø., Jørgensen, J.-E., Marcker, K.A., Peacock, W.J., and Dennis, E.S. (1995). Symbiotic and nonsymbiotic hemoglobin genes of *Casuarina glauca*. Plant Cell **7**, 213–223.

Julius, D., Brake, A., Blair, L., Kunisawa, R., and Thorner, J. (1984). Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-α-factor. Cell **37**, 1075–1089.

Kiedrowski, S., Kawalleck, P., Hahlbrock, K., Somssich, I.E., and Dangl, J.L. (1992). Rapid activation of a novel plant defense gene is strictly dependent on the *Arabidopsis RPM1* disease resistance locus. EMBO J. **11**, 4677–4684.

Kouchi, H., and Hata, S. (1993). Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol. Gen. Genet. **238**, 106–119.

Küster, H., Frühling, M., Perlick, A.M., and Pühler, A. (1993). The sucrose synthase gene is predominantly expressed in the root nodule tissue of *Vicia faba*. Mol. Plant-Microbe Interact. **6**, 507–514.

Lalonde, M. (1979). Techniques and observations of the nitrogen fixing *Alnus* root nodule symbiosis. In Recent Advances in Biological Nitrogen Fixation, N.S. Subba Rao, ed (New Delhi, India: Oxford University Press and IBH), pp. 421–434.

Lin, E., Burns, D.J., and Gardner, R.C. (1993). Fruit developmental regulation of the kiwifruit actinidin promoter is conserved in transgenic petunia plants. Plant Mol. Biol. **23**, 489–499.

Linsmaier, E., and Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. **18**, 100–127.

Maldener, I., Lockau, W., Cai, Y.P., and Wolk, C.P. (1991). Calcium-dependent protease of the cyanobacterium *Anabaena*: Molecular cloning and expression of the gene in *Escherichia coli*, sequencing and site-directed mutagenesis. Mol. Gen. Genet. **225**, 113–120.

Meesters, T.M., van Genesen, S.T., and Akkermans, A.D.L. (1985). Growth, acetylene reduction activity and localization of nitrogenase in relation to vesicle formation in *Frankia* strains Cc1.17 and Cp1.2. Arch. Microbiol. **143**, 137–142.

Miao, G.-H., and Verma, D.P.S. (1993). Soybean nodulin-26 gene encoding a channel protein is expressed only in the infected cells of nodules and is regulated differently in roots of homologous and heterologous plants. Plant Cell **5**, 781–794.

Mizuno, K., Nakamura, T., Ohshima, T., Tanaka, S., and Matsuo, H. (1988). Yeast KEX2 gene encodes an endopeptidase homologous to subtilisin-like serine proteases. Biochem. Biophys. Res. Commun. **156**, 246–254.

Mullin, B.C., Goetting-Minesky, P., and Twigg, P. (1993). Differential gene expression in the development of actinorhizal root nodules. In New Horizons in Nitrogen Fixation, R. Palacios, J. Mora, and W.E. Newton, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 309–314.

Nap, J.-P., and Bisseling, T. (1990). The roots of nodulins. Physiol. Plant. **79**, 407–414.

Pawlowski, A., Guan, C., Ribeiro, A., van Kammen, A., Akkermans, A.D.L., and Bisseling, T. (1994a). Genes involved in *Alnus glutinosa* nodule development. In Proceedings of the First European Nitrogen Fixation Conference, G.B. Kiss and G. Endre, eds (Szeged, Hungary: Officina Press), pp. 220–224.

Pawlowski, K., Kunze, R., de Vries, S., and Bisseling, T. (1994b). Isolation of total, poly(A) and polysomal RNA from plant tissues. In Plant Molecular Biology Manual, D5, 2nd ed, S.B. Gelvin and R.A. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1–13.

Pawlowski, K., Akkermans, A.D.L., van Kammen, A., and Bisseling, T. (1995). Expression of *Frankia nif* genes in actinorhizal nodules of *Alnus glutinosa*. Plant and Soil, in press.

Pladys, D., and Rigaud, J. (1985). Senescence in French bean nodules: Occurrence of different proteolytic activities. Physiol. Plant. **63**, 43–48.

Pladys, D., Dimitrijevic, L., and Rigaud, J. (1991). Localization of a protease in protoplast preparations in infected cells of French bean nodules. Plant Physiol. **97**, 1174–1180.

Roebroek, A.J., Pauli, I.G., Zhang, Y., and van de Ven, W.J. (1991). cDNA sequence of a *Drosophila melanogaster* gene, *Dfur1*, encoding a protein structurally related to the subtilisin-like proprotein processing enzyme furin. FEBS Lett. **289**, 133–137.

Rudenskaya, G.N. (1994). New subfamilies of subtilisins. Bioorganicheskaya Khimiya **20**, 475–484.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**, 5463–5467.

Schaller, A., and Ryan, C.A. (1994). Identification of a 50-kDa systemin-binding protein in tomato plasma membranes having Kex2p-like properties. Proc. Natl. Acad. Sci. USA **91**, 11802–11806.

Scheres, B., van Engelen, F., van der Knaap, E., van de Wiel, C., van Kammen, A., and Bisseling, T. (1990a). Sequential induction of nodulin gene expression in the developing pea nodule. Plant Cell **2**, 687–700.

Scheres, B., van de Wiel, C., Zalensky, A., Horvath, B., Spaink, H., van Eck, H., Zwartkruis, F., Wolters, A.M., Gloudemans, T., van Kammen, A., and Bisseling, T. (1990b). The ENOD12 gene product is involved in the infection process during the pea-*Rhizobium* interaction. Cell **60**, 281–294.

Schwintzer, C.R., Berry, A.M., and Disney, L.D. (1982). Seasonal patterns of root nodule growth, endophyte morphology, nitrogenase activity, and shoot development in *Myrica gale*. Can. J. Bot. **60**, 746–757.

Siezen, R.J., de Vos, W.M., Leunissen, J.A.M., and Dijstra, B.W. (1991). Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. Protein Eng. **4**, 719–737.

Simonet, P., Normand, P., and Bardin, R. (1988). Heterologous hybridization of *Frankia* DNA to *Rhizobium meliloti* and *Klebsiella pneumoniae nif* genes. FEMS Microbiol. Lett. **55**, 141–146.

St. Leger, R.J., Frank, D.C., Roberts, D.W., and Staples, R.C. (1992). Molecular cloning and regulatory analysis of the cuticle-degrading-protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. Eur. J. Biochem. **204**, 991–1001.

Taylor, E.R., Nie, X.Z., MacGregor, A.W., and Hill, R.D. (1994). A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. Plant Mol. Biol. **24**, 853–862.

Thummler, F., and Verma, D.P.S. (1987). Nodulin-100 of soybean is the subunit of sucrose synthase regulated by the availability of free heme in nodules. J. Biol. Chem. **262**, 14730–14736.

Tormakangas, K., Runeberg-Roos, P., Ostman, A., Tilgmann, C., Sarkkinen, P., Kervinen, J., Mikola L., and Kalkkinen, N. (1991). Aspartic proteinase from barley seeds is related to animal cathepsin. Adv. Exp. Med. Biol. **306**, 355–359.

Trinick, M.G. (1979). Structure of nitrogen-fixing nodules formed by *Rhizobium* on roots of *Parasponia andersonii*. Appl. Environ. Microbiol. **55**, 2046–2055.

Twigg, P.G. (1993). Isolation of a Nodule-Specific cDNA Encoding a Putative Glycine-Rich Protein from *Alnus glutinosa*. PhD Dissertation (Knoxville, TN: University of Tennessee).

van de Wiel, C., Scheres, B., Franssen, H.J., van Lierop, M.J., van Lammeren, A., van Kammen, A., and Bisseling, T. (1990). The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules. EMBO J. **9**, 1–7.

van Kammen, A. (1984). Suggested nomenclature for plant genes involved in nodulation and symbiosis. Plant Mol. Biol. Rep. **2**, 43–45.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**, 4683–4690.

Vos, P., Simons, G., Siezen, R.J., and de Vos, W.M. (1989). Primary structure and organization of the gene for a procaryotic, cell envelope-located serine proteinase. J. Biol. Chem. **264**, 13579–13585.

Wells, J.A., Ferrari, E., Henner, D.J., Estell, D.A., and Chen, E.Y. (1983). Cloning, sequencing, and secretion of *Bacillus amyloliquefaciens* subtilisin in *Bacillus subtilis*. Nucleic Acids Res. **11**, 7911–7925.

Williams, J., Bulman, M., Huttly, A., Phillips, A., and Neill, S. (1994). Characterization of a cDNA from *Arabidopsis thaliana* encoding a potential thiol protease whose expression is induced independently by wilting and abscisic acid. Plant Mol. Biol. **25**, 259–270.

Wilson, R.C., Long, F., Maruoka, E.M., and Cooper, J.B. (1994). A new proline-rich early nodulin from *Medicago truncatula* is highly expressed in nodule meristematic cells. Plant Cell **6**, 1265–1275.

Yamagata, H., Masuzawa, T., Nagaoka, Y., Ohnishi, T., and Iwasaki, T. (1994). Cucumisin, a serine protease from melon fruits, shares structural homology with subtilisin and is generated from a large precursor. J. Biol. Chem. **169**, 32725–32731.

Yang, J., and Kramer, J.M. (1994). In vitro mutagenesis of *Caenorhabditis elegans* cuticle collagens identifies a potential subtilisin-like protease cleavage site and demonstrates that carboxyl domain disulfide bonding is required for normal function but not assembly. Mol. Cell. Biol. **14**, 2722–2730.

Yang, W.-C., Katinakis, P., Hendriks, P., Smolders, A., de Vries, F., Spee, J., van Kammen, A., Bisseling, T., and Franssen, H. (1993). Characterization of *GmENOD40*, a gene showing novel patterns of cell-specific expression during soybean nodule development. Plant J. **3**, 573–585.

Chapter 6

Expression of *cg12*, a gene encoding a nodule-specific subtilisin-like protease, in actinorhizal nodules of *Casuarina glauca*

Ana Ribeiro, Laurent Laplaze, Hugo Ramirez, Antoon D.L. Akkermans, Ab. van Kammen, Ton Bisseling and Katharina Pawlowski, in preparation.

Expression of *cg12*, a gene encoding a nodule-specific subtilisin-like protease, in actinorhizal nodules of *Casuarina glauca*

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Abstract

We have cloned a cDNA from *Casuarina glauca* nodules representing the homolog of an *Alnus glutinosa* gene, *ag12*, encoding a nodule-specific subtilisin-like protease. RNA hybridization analysis has shown that, like *ag12*, *cg12* is expressed at high levels in nodules, but not in roots. *In situ* hybridization showed that it is expressed in the young infected cells of the nodule where *Frankia* does not yet fix nitrogen. The occurence of these subtilases during infection of cortical cells in two different types of actinorhizal nodules supports phylogenetic data which indicate that the symbiosis of Casuarinaceae, *Alnus*, and Myricaceae has a common evolutionary origin.

Introduction

Actinomycetes of the genus *Frankia* can enter a root symbiosis with several woody dicotyledonous plant species, belonging to eight different plant families, commonly called actinorhizal plants. The result of this symbiosis is the formation of root nodules where *Frankia* is fixing atmospheric nitrogen inside plant cells. Actinorhizal root nodules are coralloid structures consisting of multiple lobes, each of which resembles a modified lateral root without a root cap and with a superficial periderm, a central vascular bundle, and both infected and uninfected cortical cells (Benson and Silvester, 1993; Pawlowski et al., 1996).

Actinorhizal nodule lobes have an indeterminate growth pattern, due to the presence of an apical meristem, resulting in a zonation of each lobe. Thus, starting from the apical meristem, we can distinguish four zones (Ribeiro et al., 1995). The meristematic zone (zone 1) consists of small dividing cells that do not contain bacteria. The prefixation zone (zone 2) contains enlarging cortical cells, some of which become gradually infected with hyphae from the center outward (Schwintzer et al., 1982; Berry and Sunell, 1990). In the nitrogen fixation zone (zone 3) *Frankia* vesicles have formed, bacterial *nif* (nitrogen fixation) genes are expressed, and nitrogen fixation takes place (Fontaine et al., 1984). In the senescence zone (zone 4) nitrogen fixation stops and both plant and bacterial material is degraded (Berry and Sunell, 1990).

We were interested to study whether plant genes involved in the process of cortical cell infection by *Frankia* are conserved among actinorhizal symbioses. Previously, two nodule-specific gene families whose expression correlates with this process have been identified in *Alnus glutinosa* (Ribeiro et al., 1995; Pawlowski et al., 1996). One of them encodes glycine- and histidine-rich metal-binding proteins (Twigg, 1993; Pawlowski et al., 1996). The other one, *ag12*, encodes subtilisin-like proteases (Ribeiro et al.,1995). The presence of a N-terminal signal peptide in both types of proteins suggests that they are targeted to the vacuole or to the extracellular space. As a first step to assess the general importance of Ag12-like proteases in actinorhizal symbioses, a homolog was cloned from *Casuarina glauca*.

Molecular phylogeny suggests that nitrogen fixing root nodule symbiosis in *Alnus* (Betulaceae), *Casuarina* (Casuarinaceae), and *Myrica* (Myricaceae) has a common evolutionary origin (Figure 1; Swensen, 1996). This is supported by symbiosis-related features; for example, only plants belonging to branch c (Figure 1) are infected intracellularly by *Frankia* via root hairs. Root hair infection starts with the deformation of root hairs (reviewed by Pawlowski et al., 1996). After that, *Frankia* hyphae enter root hairs, encapsulated in a cell wall-like matrix of plant origin. Initially, *Frankia* induces cell divisions

in the root outer cortex, giving rise to the prenodule. Upon infection of prenodule cells with encapsulated hyphae, cell divisions are induced in the root pericycle resulting in the formation of the nodule lobe primordium, which after infection develops into a nodule lobe. In the actinorhizal plants belonging to branch a (Figure 1), no prenodule is formed, and *Frankia* reaches the nodule primordium cells via penetration of the root epidermis and intercellular colonization of the root cortex. In all actinorhizal nodules, upon infection of a plant cell, *Frankia* hyphae branch until they occupy most of the volume of the infected cell. This process is accomplished by the invagination of the plant plasma membrane and continuous deposition of encapsulating material.

Because of the phylogenetic relationship and the similarity of the infection processes of *Alnus* and *Casuarina*, the latter was selected to study whether an *ag12* homolog is involved in process of cortical cell infection by *Frankia* in this plant.

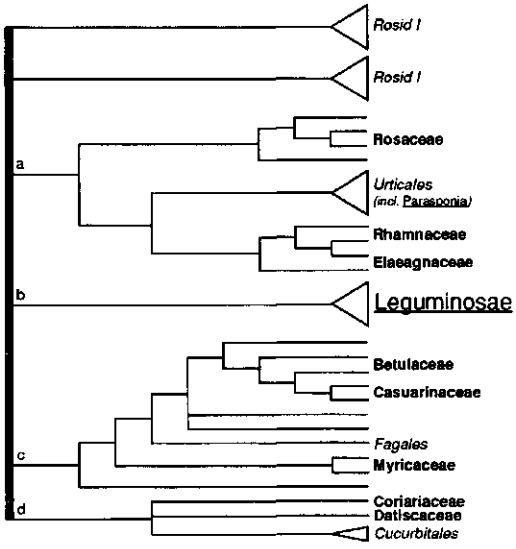

Figure 1: Part of a strict consensus tree summarizing the major clades found in phylogenetic analysis of 99 *rbcL* sequences (modified after Soltis et al., 1995). The thick black line denotes the nitrogen-fixing clade. Four clades (a-d) within this clade contain symbiotic species. Families containing species entering a symbiosis with *Frankia* strains are given in bold print, families containing species interacting with rhizobia are underlined. The figure was kindly provided by S. Swensen (Ithaca College, NY, USA).

Results

Isolation and sequencing of *cg12*

For the isolation of the *ag12* homolog from *Casuarina glauca*, degenerate primers were designed (see Materials and Methods) on the basis of amino acid sequences conserved between two subtilisin-like proteases from higher plants, *A. glutinosa* Ag12 and *Arabidopsis thaliana* Ara12 (Ribeiro et al., 1995). By reverse-transcription and PCR, a 0.8-kb DNA fragment was amplified from RNA isolated from *C. glauca* nodules, which in turn was cloned and sequenced. Sequence comparison showed that the cloned fragment represents the homolog of *ag12* (Figure 2). Therefore, the clone was designated pCg12.

```
Cg12 MAEFAAMEKGVLVSTSAGNEGPFFGNLHNGIPWVLTVAGGTVDRSFAGTL
Ag12 IASFAAMEKGVVVSTSAGNAGPFFGNMHNGIPWVLTVAAGNIDRSFAGTL

Cg12 TLGNDQIITGWTLFPASAVIQNLPLVYDKNISACNSPELLSEAIYTIIIC
Ag12 TLGNDQTITGWTMFPASAIIESSQLVYNKTISACNSTELLSDAVYSVVIC

Cg12 EQARSIRDQIDSLARSNVVGAILISNNTNSSEL.GEVTCPCLVISPKDAE
Ag12 EAITPIYAQIDAITRSNVAGAILISNHTKLFELGGGVSCPCLVISPKDAA

Cg12 AVIKYANFNEIAFASMKFQKTFLGAKPAPAVASYTSRGPSPSYPGVLKPD
Ag12 ALIKYAKTDEFPLAGLKFQETITGTKPAPAVAYYSSRGPSPSYPGILKPD

Cg12 VMAPGSQILAAWVPTDATAQIGTNVYLSSHYNMVSGTSMACPHASGIAAL
Ag12 VMAPGSLVLASWIPNEATAQIGTNVYLSSHYNMVSGTSMACPHASGVAAL

Cg12 LKAAHPEWSPAATSS
Ag12 LKAAHPEWSPAAIRS
```

Figure 2: Sequence comparison of Ag12 (amino acid residues 302 to 566) and Cg12. Conserved amino acids are shown in bold print.

Expression of *cg12*

RNA gel blot hybridization analysis

Northern blots with RNA isolated from *C. glauca* roots and nodules were prepared and hybridized with *cg12*. A 2.5-kb transcript was detected in RNA of nodules but not of roots (Figure 3), thus confirming the nodule-specific nature of *cg12*. The size of the *cg12* transcript was similiar to that of its homologs in *A. glutinosa* and *Arabidopsis thaliana*. This supports that *cg12* is the homolog of *ag12* from *A. glutinosa*.

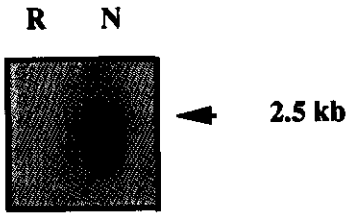

Figure 3: RNA gel blot hybridization analysis: Expression of *cg12* in roots and nodules of *Casuarina glauca*. R, roots; N, nodules.

RNA gel blots containing about 10 µg of total RNA per slot were hybridized with *cg12*. The signal was detected by using a Phospholmager.

Localization of cg12 *mRNA*

The expression pattern of *cg12* in *C. glauca* root nodules was determined by *in situ* hybridization of longitudinal- and cross-sections of nodules with ^{35}S-labelled antisense and sense RNA probes. To obtain a reference point for the developmental stage of the infected cell, *in situ* hybridizations with antisense RNA of the nitrogenase structural gene *nifH* from *Frankia* were performed on adjacent sections (Pawlowski et al., 1995). The results showed that, similarly to *ag12*, *cg12* was expressed at highest levels in the young infected cells of zone 2, where *Frankia* does not express *nifH* (Figure 4), and at lower and irregular levels in the infected cells of zone 3, where *Frankia* does express *nifH* (data not shown). No expression of *cg12* was detected in other nodule cells.

Figure 4: Localization of *Frankia nifH* and *C. glauca cg12* mRNA in sections of *C. glauca* nodules. Bright-field microscopy was used; silver grains denoting hybridization appear as black dots. Slides were exposed for 4 weeks at 4^0C and stained with Ruthenium red and Toluidine Blue after developing. **(A)** *Frankia nifH* expression denotes nitrogen-fixing infected cells. **(B)** *cg12* expression is detected in young infected cells where *Frankia* does not yet express *nifH* (arrowheads); in this section, *cg12* expression was below the detection limit in the infected cells that do express *nifH* (arrows).

Discussion

Based on sequence and expression analysis, *cg12* represents the *C. glauca* homolog of *A. glutinosa*, *ag12*, which is proposed to encode a protein involved in the infection of nodule cortical cells by *Frankia* (Ribeiro et al., 1995). Ag12 and Cg12 represent subtilisin-like proteases.

Subtilases have been identified in a wide variety of organisms, where they can have either degradative or processing functions. According to Gluschankof and Fuller (1994), all processing proteases of the subtilisin family contain a so-called P-domain that is missing in degradative proteases. Based on these data, both Ag12 and Cg12 would fall in the degradative class since they miss the P-domain (data not shown). However, since both *ag12* and *cg12* and their homolog in *Arabidopsis*, *ara12* (Ribeiro et al., 1995), are expressed at particularly high levels in early stages of development, i.e. in early stages of infection in actinorhizal nodules and during the beginning of silique development in *Arabidopsis*, it seems more likely that these enzymes play a role in protein processing and not in degradation. Up to now neither bacterial- nor plant subtilases with the features of a processing enzyme have been identified. Neverthless, there is evidence for the existence of processing proteases in plants, since a fungal peptide toxin was correctly processed when expressed in tobacco (Kinal et al., 1995). Thus, it might be possible that the presence of the P-domain in the class of processing subtilases is related to phylogeny and not just to function. In this case, plant subtilases without this domain might still have processing functions.

Phylogenetic analysis suggests that actinorhizal Myricaceae, Casuarinaceae, and Betulaceae (subclade c; Figure 1) have a common symbiotic ancestor (Swensen, 1996). Although the infection process and the spatial distribution of the nodule cells are similar in all these three plant families, there are differences concerning the nodule oxygen protection/diffusion pathways and *Frankia* differentiation (reviewed by Pawlowski et al., 1996), which might suggest that the symbiotic syndrome developed independently in Betulaceae and Casuarinaceae and Myricaceae, respectively (Swensen, 1996). However, the conservation of Ag12/Cg12, together with the fact that so far all the attempts to isolate homologous genes from legume nodules of *Vicia sativa* as well as from actinorhizal nodules of *Datisca glomerata* have failed (data not shown), might be a further indication that indeed, the symbiotic syndrome in *Alnus*,*Casuarina*, and *Myrica* had a common evolutionary origin.

Methods

Plant and bacterial growth conditions

Casuarina glauca seeds were provided by the Desert Development Center (Egypt). The plants were grown in a greenhouse at temperatures ranging from 25 to 30 ^{0}C, under 14 hr of light and 10 hr of darkness. Seeds were germinated in trays containing a mixture of sand and vermiculite (1:1). After four months the seedlings were transfered to hydroponic culture with quarter-strength Hoagland's solution (Hoagland and Arnon, 1938) supplemented with 17 mg/l $(NH_4)_2SO_4$. Nutrient solution was changed weekly. One week before inocculation the plants were deprived of nitrogen. Four month-old plants were infected with 1:3/1:4 diluted dispersed culture of *Frankia* Thr (Girgis et al., 1990) grown in BAP-PCM medium (Fontaine et al., 1986). Nodules were harvested 3 to 4 weeks after infection. Actinorhizal nodules of *Datisca glomerata* nodules were kindly provided by A.M. Berry (UC Davis, CA, USA). Legume nodules of *Vicia sativa* were obtained as described by Vijn et al. (1995).

Isolation of RNA

For isolation of root RNA, seedling roots were collected from uninfected plantlets 4 to 5 months after germination. For the isolation of nodule RNA, 3 to 4 week-old nodules were used. Total RNA was isolated from *C. glauca* and *D. glomerata* as described by Pawlowski et al. (1994). Total RNA from *Vicia sativa* nodules were isolated as described by Govers et al. (1985).

Reverse transcription and Polymerase chain reaction (RT-PCR)

AMV reverse transcriptase was obtained from Gibco BRL (Gaithersburg, MD). cDNA was synthesized from total RNA isolated from *C. glauca* nodules as described by the manufacturer. For the PCR, Taq polymerase was obtained from Gibco BRL (Gaithersburg, MD) and used according to the instructions provided by the manufacturer. Using oligonucleotides 5'-GCIATIG(G/C)II(G/C)ITT(T/C)GCI GCIATGG-3' (Forward) and 5'-GCI(G/C)(A/T)IC(G/T)IGTIGCIGCIGGI(G/C)(A/T) CCA-3' (Reverse) a 0.8 kb *cg12* fragment was amplified from *C. glauca* nodule cDNA during 35 cycles (94 ^{0}C, 1.5 min; 42 ^{0}C, 1.5 min; 72 ^{0}C, 1.5 min).

Cloning and sequencing procedures

The 800 bp DNA fragment amplified by PCR was subcloned into pGEM-T[1] using a Kit from Promega (Madison, WI) for subcloning PCR products, yielding pCg12. The nucleotide sequence was determined as described by Ribeiro et al. (1995).

In situ hybridization

In situ hybridization experiments were performed as described by Ribeiro et al. (1995). For probe preparation, pCg12 was linearized with *Sst*II and transcribed with T7 RNA polymerase (antisense) or linearized with *Pst*I and transcribed with T3 RNA polymerase (sense)

RNA gel blots and hybridization conditions

Northern blots with nodule- and root RNA were prepared and hybridized as described by Ribeiro et al. (1995). For RNA blot hybridization the insert of pCg12 was cut out using *Pst*I and *Sst*II.

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We thank Tony van Kampen for DNA sequencing, Didier Bogusz and Emile Duhoux (ORSTOM-Genetrop, Montepellier, France) for providing *Casuarina glauca* material, Alison M. Berry (UC Davis, CA, USA) for providing *Datisca glomerata* nodules, and Susan Swensen (Biology Department, Ithaca College, New York) for providing Figure 1. This work was supported by a WOTRO (Netherland's Foundation for the Advancement of Tropical Research) fellowship to A.R., by a ORSTOM fellowship to L.L., and by CONACyT felowship to H.R. KP and T.B. also acknowledge support by the Dutch Organization for Scientific Research (NWO).

References

Benson, D.R. and Silvester, W.B. (1993). Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol. Rev. 57, 293-319.

Berry, A.M. and Sunell, L.A. (1990). The infection process and nodule development. In The Biology of Frankia and Actinorhizal Plants, C.R. Schwintzer and J.D. Tjepkema, eds (New York: Academic Press), pp. 61-81.

Fontaine, M.S., Lancelle, S.A., and Torrey, J.G. (1984). Initiation and ontogeny of vesicles in cultured *Frankia* sp. strain HFPArI3. J. Bacteriol. 160, J. Bacteriol. 160, 921-927.

Fontaine, M.S., Young, P.H., and Torrey, J.G. (1986). Effect of long-term preservation of *Frankia* strains on infectivity, effectivity and *in vitro* nitrogenase activity. Appl. Environ. Microbiol. 51, 694-698.

Girgis, Z.M.G., Ishac, Z.Y., El-Haddad, M., Saleh, A.E., Diem, H.G., and Dommergues, R.Y. (1990). First report on isolation and culture of effective *Casuarina*-compatible strain of *Frankia* from Egypt. In Advance in *Casuarina* research and utilization, H.M. El-Lakany, W.J. Turnbull, and J.L. Brewbaker, eds (Cairo: Desert Development Center, A.U.C.), pp. 156-164.

Gluschankof, P., and Fuller, R.S. (1994). A C-terminal domain conserved in precursor processing proteases is required for intramolecular N-terminal maturation of pro-Kex2 protease. EMBO J. 13 (10), 2280-2288.

Govers, F., Gloudemans, T., Moerman, M., Van Kammen, A., and Bisseling, T. (1985). Expression of plant genes during the development of pea root nodules. EMBO J. 4, 861-867.

Hoagland, D.R., and Arnon, D.T. (1938). The water-culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ. 347. (Berkeley, CA: University of California).

Kinal, H., Park, C.-M., Berry, J.O., Koltin, Y., and Bruenn, J.A. (1995). Processing and secretion of a virally encoded antifungal toxin in transgenic tobacco plants: evidence for a Kex2p pathway in plants. Plant Cell 7, 677-688.

Pawlowski, K., Kunze, R., de Vries, S., and Bisseling, T. (1994). Isolation of total, poly(A) and polysomal RNA from plant tissues. In Plant Molecular Biology Manual, D5, 2nd edition, S.B. Gelvin, and R.A. Schilperoort, eds (Dordrecht: Kluwer Academic Publishers), pp. 1-13.

Pawlowski, K., Akkermans, A.D.L., van Kammen, A., and Bisseling, T. (1995). Expression of *Frankia nif* genes in actinorhizal nodules of *Alnus glutinosa*. Plant and Soil, 170, 371-376.

Pawlowski, K., Ribeiro, A., and Bisseling, T. (1996). Nitrogen fixing root nodule symbioses: legume nodules and actinorhizal nodules. In Biotechnology Annual Review Part II, M.R. El-Gewely, ed (Amsterdam: Elsevier Science b.v.) pp. 151-184.

Ribeiro, A., Akkermans, A.D.L., van Kammen, A., Bisseling, T., and Pawlowski, K. (1995). A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. Plant Cell 7, 785-794.

Schwintzer, C.R., Berry, A.M., and Disney, L.D. (1982). Seasonal patterns of root nodule growth, endophyte morphology, nitrogenase activity, and shoot development in *Myrica gale*. Can. J. Bot. 60, 746-757

Soltis, D.E., Soltis, P.S., Morgan, D.R., Swensen, S.M., Mullin, B.C., Dowd, J.M., and Martin, P.G. (1995). Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc. Natl. Acad. Sci. USA 92, 2647-2651.

Swensen, S.M. (1996). The evolution of actinorhizal symbioses: Evidence for multiple origins of the symbiotic association. Am. J. Bot. 83, 1503-1512.

Twigg, P.G. (1993). Isolation of a nodule-specific cDNA encoding a putative glycine-rich protein from *Alnus glutinosa*. PhD Dissertation, Knoxville, TN: University of Tennessee.

Vijn, I., Martinez-Abarca, F., Yang, W.-C., Das Neves, L., Van Brussel, A., Van Kammen, A., and Bisseling, T. (1995). Early nodulin gene expression during Nod factor-induced processes in *Vicia sativa*. Plant J. 8, 111-119.

Chapter 7

Concluding Remarks

Nitrogen-fixing root nodule symbioses

Two groups of plants, legumes and actinorhizal plants, are able to enter root nodule symbioses with nitrogen-fixing soil bacteria, rhizobia and *Frankia*, respectively.

Actinorhizal and legume nodules are dissimilar in their structure as well as in several aspects of their development (chapter 1). First, while legume nodules have a stem-like morphology, actinorhizal nodule lobes represent modified lateral roots. Second, legume nodules are formed by mitotic reactivation of root cortical cells, while actinorhizal nodule formation is initiated in the root pericycle. Third, in most legumes, bacteria are released by an endocytosis-like process into the cytoplasm of plant cells, whereas in actinorhizal nodules the plant membrane surrounding *Frankia* remains attached to the plasmamembrane, and no endocytosis is observed. Interestingly, nodules of *Parasponia*, the only non-legume nodulated by rhizobia, structurally and developmentally resemble actinorhizal nodules.

In spite of the differences between actinorhizal and legume symbioses, the two systems share common aspects especially with respect to nodule functioning. For example, in both legume and actinorhizal nodules, ammonium, the product of nitrogen fixation, is exported by the bacteria and assimilated in the plant cytoplasm via the GS (glutamine synthetase)/GOGAT (glutamate synthase) pathway (Schubert, 1986; chapter 2). Moreover, sucrose synthase is implied in carbon metabolism in both cases (chapter 3). In some actinorhizal plants, the mechanism of nodule oxygen transport involves a nodule-specific oxygen-binding protein, hemoglobin, similar to that (leghemoglobin) found in legume nodules (Jacobsen-Lyon et al., 1995). In addition to the nodulin (leg)hemoglobin, both systems involve several other nodulin genes. In legumes these genes have been classified into early and late nodulin genes (Nap and Bisseling, 1990a). Late nodulin genes are expressed at the onset of bacterial nitrogen fixation, and seem to be involved in nodule functioning. Early nodulin genes are expressed before the onset of nitrogen fixation, and are probably involved in either infection or in building up the nodule structure. Also in actinorhizal nodule formation early nodulin genes appear to play a role. Examples are, *ag12/cg12* (chapters 5 and 6), and *ag164* (Guan et al., 1997), which are both expressed in young infected cells, prior to bacterial nitrogen fixation, and have been suggested to be involved in the infection process. Ag12/Cg12 are subtilisin-like proteases that are among the first to be identified in plants. The role of *cg12* in early stages of nodule development has been confirmed by showing that it is also expressed in prenodule cells in *Casuarina glauca* (Ana Ribeiro, Laurent Laplaze and Katharina Pawlowski, unpublished data). The prenodule is a cluster of dividing cortical cells and as such it is similar to the legume nodule primordium, although it does not contribute to the actinorhizal nodule lobe

structure. Up to now early nodulins common to both rhizobial and actinorhizal symbiosis have not been identified.

Achievements in molecular studies of actinorhizal symbioses

Due to their woody nature, actinorhizal plants were for many years recalcitrant to molecular biological and genetic analysis. The achievements described in this thesis, together with the recent results of others working on the molecular aspects of actinorhizal symbioses (Goetting-Minesky and Mullin, 1994; Jacobsen-Lyon et al., 1995; Guan, 1996; Gherbi et al., 1996; Franche et al., 1997; Diouf et al., 1995; A.M. Berry, unpublished results) show that meanwhile, actinorhizal plants can be examined using standard molecular biological techniques such as isolation of nucleic acids, *in situ* hybridization, and plant transformation.

In the course of this work, we have also shown that the lack of a genetic system for actinorhizal plants can be compensated by using model systems like *Arabidopsis* and yeast to analyse gene functions (chapter 4). Many of the actinorhizal nodule-specific/enhanced genes identified thus far have homologs in non-symbiotic plants like *Arabidopsis* (chapter 4 and 5; Guan et al., 1997). This, together with the fact that quick mapping procedures and an integrated genetic map for *Arabidopsis* are available, can be useful to analyse the function of the *Arabidopsis* homologs of actinorhizal genes. Furthermore, mapping analyses can be complemented with phenotype studies, since a suitable transformation system is also available for *Arabidopsis*. Because some actinorhizal nodulin genes have also homologs in yeast (chapter 4), from which site-directed mutants can be constructed easily, complementation studies can be performed to ascertain gene function (chapter 4).

Due to these achievements, the different aspects of actinorhizal nodulation can now be studied in more detail.

Molecular phylogenetic data

Molecular phylogenetic analyses, based on chloroplast *rbcL* gene sequences, suggest that all plants able to form nitrogen fixing root nodules, with either *Frankia* or rhizobia as microsymbiont, belong to a single branch of the rosid I clade (Soltis et al., 1995). Thus, a single genetic predisposition to enter a root nodule symbiosis seems to have arisen at one point in evolution. Based on it, both rhizobial and actinorhizal symbioses seem to have developed three to four times independently (Doyle, 1994; Swensen, 1996), and plant

families containing members able to enter a root nodule nitrogen-fixing symbiosis are classified into four different groups, i.e., subclades (Soltis et al., 1995; Swensen, 1996). Three of these subclades contain actinorhizal plants, and one contains the legumes (Figure 1 in chapter 6). Interestingly, *Parasponia*, the only non-legume nodulated by rhizobia, whose nodules are actinorhiza-like, groups together with actinorhizal plants in one of the subclades. This classification, based on *rbcL* gene sequences, is supported by some aspects of the symbioses. For example, all plants in subclade a (Figure 1 in chapter 6) are infected intercellularly, though by different microsymbionts; the plants in subclade c are all are infected intracellularly by *Frankia*; and only the plants from subclade d form nodules displaying a special pattern of infected cells in the cortex of the nodule. Thus, the diversity of nodule development and infection between the different groups of plants can be seen as a result of independent acquisition of symbiotic properties in the course of evolution.

Future prospects

Altogether molecular phylogeny has shown that plants entering a root nodule symbiosis with nitrogen-fixing bacteria (*Frankia* or rhizobia) are closely related, and the plants of the rosid I clade most probably share a common feature which allowed them to develop an interaction with *Frankia* or rhizobia. Additionally, molecular phylogeny and cytology suggest that common principles are underlying the independently developed nodule formation processes. Therefore, parallel studies with the different systems will be useful to get more insight about the properties determining the capacity to develop nitrogen-fixing root nodule symbioses. Such insight might be important in the attempts to transfer the ability to enter nitrogen-fixing root nodule symbiosis to plant species outside the rosid I clade.

As previously mentioned, apart from hemoglobin and some nodulins involved in nodule carbon- and nitrogen metabolism, genes in common to rhizobial and actinorhizal nodules have not yet been identified. However, all nodulins examined to date have been shown to be either recruited or duplicated from non-symbiotic plant development (Nap and Bisseling, 1990b; Jacobsen-Lyon et al., 1995), and homologs of nodulins have been found in non-symbiotic plants such as *Arabidopsis*. These facts could be exploited to study genes shared by the two types of nodules and to determine their role in the symbiotic relationship. This might be one of the strategies to identify the common aspects that make legumes and actinorhizal plants susceptible to the symbiotic interaction with *Rhizobium* and *Frankia*. In addition, comparative studies at the physiological, cytological, and molecular biological level, could be useful to study the divergences among the different root nodule nitrogen fixing symbiotic systems and provide insight in the different strategies used by plants to achieve symbiotic nitrogen

fixation. Recently, *in situ* hybridization with GS antisense RNA revealed that ammonium assimilation in actinorhizal nodules of *Datisca glomerata* takes place in the uninfected cells (A.M. Berry and K. Pawlowski, unpublished data). This expression pattern is completely different from that found in actinorhizal nodules of *Alnus* (chapter 2) and *Casuarina* (A.M. Berry and K. Pawlowski, unpublished data) as well as in legume nodules (chapter 2). Such results exemplify the potential of comparative studies, that are now possible due to the availability of cloned genes.

References

Diouf, D., Gherbi, H., Prin, Y., Franche, C., Duhoux, E., and Bogusz, D. (1995). Hairy root nodulation of *Casuarina glauca*: A system for the study of symbiotic gene expression in an actinorhizal tree. Mol. Plant-Microbe Interact. 8, 325-349.

Doyle, J.J. (1994). Phylogeny of the legume family: An approach to understanding the origins of nodulation. Annu. Rev. Ecol. Syst. 25, 325-349.

Franche, C., Diouf, D., Le, Q.V., Bogusz, D., N'Diaye, A., Gherbi, H., Gobe, C., and Duhoux, E. (1997). Genetic transformation of the actinorhizal tree *Allocasuarina verticillata* by *Agrobacterium tumefaciens*. Plant J., in press.

Gherbi, H., Duhoux, E., Franche, C., Pawlowski, K., Berry, A., and Bogusz, D. (1997). Cloning of a full-length symbiotic hemoglobin cDNA and *in situ* localization of the corresponding mRNA in *Casuarina glauca* root nodules. Physiol. Plant., in press.

Goetting-Minesky, M.P., and Mullin, B.C. (1994). Differential gene expression in actinorhizal symbiosis: Evidence for a nodule-specific cysteine proteinase. Proc. Natl. Acad. Sci. USA 91, 9891-9895.

Guan, C.-H. (1996). Plant gene expression in actinorhizal nodules of *Alnus glutinosa*, Department of Molecular Biology, Wageningen Agricultural University, Ph.D. thesis.

Guan, C.-H, Pawlowski, K., and Bisseling, T. (1997). Interaction between *Frankia* and actinorhizal plants. In Subcellular Biochemistry: Plant Microbe Interactions, B.B. Biswas and H.K. Das, eds (London, UK: Plenum Publishing Company), in press.

Jacobsen-Lyon, K., Jensen, E.Ø., Jørgensen, J.-E., Marcker, K.A., Peacock, W.J., and Dennis, E.S. (1995). Symbiotic and nonsymbiotic hemoglobin genes of *Casuarina glauca*. Plant Cell 7, 213-223.

Nap, J.-P., and Bisseling, T. (1990). Developmental biology of a plant-prokaryote symbiosis: The legume root nodule. Science 250, 948-954.

Nap, J.-P., and Bisseling, T. (1990). The roots of nodulins. Physiol. Plant. 79, 407-414.

Schubert, K.R. (1986). Products of biological nitrogen fixation in higher plants: synthesis, transport and metabolism. Ann. Rev. Plant Physiol. 37, 539-574.

Soltis, D.E., Soltis, P.S., Morgan, D.R., Swensen, S.M., Mullin, B.C., Dowd, J.M., and Martin, P.G. (1995). Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc. Natl. Acad. Sci. USA 92, 2647-2651.

Swensen, S.M. (1996). The evolution of actinorhizal symbioses: Evidence for multiple origins of the symbiotic association. Am. J. Bot. 83, 1503-1512.

Summary

Samenvatting

Actinorhiza-knollen worden, door actinomyceten van het genus *Frankia*, geïnduceerd op de wortels van verschillende dicotyle plantesoorten, behorende tot acht verschillende families. Elke knol bestaat uit gemodificeerde zijwortels ("lobes") zonder wortelmutsje, met een centrale vaatbundel en met geïnfecteerde en niet-geïnfecteerde cortexcellen.

Om plantegenen te isoleren die betrokken zijn bij het vormen van een actinorhiza-knol hebben we een cDNA bank van *Alnus glutinosa* knollen differentieel gescreend met respectivelijk knol en wortel cDNA. Verschillende cDNA's, die genen vertegenwoordigen die op een hoger nivo in de knol tot expressie komen dan in de wortel (bepaald met RNA blot hybridisatie), zijn geïsoleerd en gesequenceerd. De plaats van expressie van de corresponderende mRNA's in de knol was onderzocht met in situ hybridisatie. Wanneer het nodig was, zijn model systemen als gist en *Arabidopsis* gebruikt om de functie van de gecodeerde eiwitten te analyseren.

Twee van de geïsoleerde cDNA cloons waren sucrose synthase en enolase, enzymen betrokken bij koolstof metabolisme. De hiermee overeenkomende genen komen in alle planteweefsels tot expressie, maar op een duidelijk hoger nivo in knollen. In situ hybridisatie liet zien dat in knollen beide genen sterk tot expressie kwamen in de geïnfecteerde cortexcellen en in de pericykel van de vaatbundel.

Een andere cDNA cloon, pAgthi1, codeerde voor een eiwit homoloog aan gist Thi4, wat een rol speelt in thiazol biosynthese. Het hiermee overeenkomende gen, *agthi1*, kwam sterk tot expressie in de knol en in de scheutapex van *A. glutinosa*, terwijl het zwak tot expressie kwam in wortels, bloemen en ontwikkelende vruchten. In knollen was *agthi1* mRNA gelocaliseerd in de geïnfecteerde cortexcellen en in de pericykel van de vaatbundel. Een homoloog gen, *ara6/tz* is geïdentificeerd in *Arabidopsis thaliana*. *ara6/tz* mapt in een gebied van chromosoom 5 van Arabidopsis wat het *tz* locus bevat. Dit komt overeen met de waarnemingen dat *ara6/tz* transcriptie verminderd was in 2 van 5 *Arabidopsis tz* mutant lijnen. *ara6/tz* komt sterk tot expressie in chloroplast bevattende parenchym cellen van bladeren, scheuten van de bloeiwijze en bloemen van *Arabidopsis*, en minder sterk in het vaatweefsel. De functie van AgThi1 is aangetoond door complementatie studies in gist, waarbij AgThi1 een gist *thi4* mutant kon redden.

cDNA's zijn geïsoleerd die kodeeren voor glutamine synthetase (GS) en acetylornithine transaminase (AOTA), beiden betrokken bij stikstofmetabolisme. GS is het verantwoordelijke enzym voor ammonium assimilatie, terwijl AOTA betrokken is bij de biosynthese van

citrulline, de vorm waarin stikstof getransporteerd wordt in *Alnus*. GS mRNA is gevonden in alle weefsels die getest zijn, met het hoogste nivo in knollen, waar het aanwezig is in de geïnfecteerde cellen en in de cellen van de pericykel van het vaatweefsel. AOTA mRNA is op zeer laag nivo in wortels en op hoog nivo in knollen gedetecteerd, waar expressie beperkt was tot geïnfecteerde cellen. Deze data suggereren dat in *A. glutinosa* knollen ammonium assimilatie plaats vindt in de geinfecteerde cellen en in de pericykel van het vaatweefsel, en citrulline biosynthese voornamelijk in de geïnfecteerde cellen. Ammonium assimilatie in de pericykel is waarschijnlijk gerelateerd aan stikstoftransport.

Een van de weinige knol specifike genen, d.w.z. genen die niet tot expressie komen in wortels, *ag12*, kwam het sterkst tot expressie in geïnfecteerde cellen, voor de start van stikstof fixatie. Sequentie analyse liet zien dat *ag12* kodeert voor een serine protease van de subtilisine (EC 3.4.21.14) familie. Een gen homoloog aan *ag12*, *ara12*, is geïdentificeerd in *Arabidopsis*. *ara12* kwam in alle organen tot expressie, met de sterkste expressie tijdens de vroege silique ontwikkeling. Om het belang van dit protease in andere actinorhiza symbiosen vast te stellen is het expressiepatroon van een homoloog gen, *cg12*, onderzocht in knollen van *Casuarina glauca* en er werd gevonden dat het vergelijkbaar was met dat van *ag12*. Deze resultaten worden bediscussieerd met het oog op de phylogenetische relatie tussen *Alnus* en *Casuarina*.

Summary

Actinorhizal multilobe nodules are induced by actinomycetes of the genus *Frankia* on the roots of several dicotyledenous species belonging to eight different plant families. Each nodule lobe is a modified lateral root, without a root cap, with a central vascular cylinder, and with infected and uninfected cortical cells.

To isolate plant genes involved in the establishment of an actinorhizal symbioses, we have differentially screened an *A. glutinosa* nodule cDNA library with nodule- and root cDNA, respectively. Several cDNAs, representing genes expressed at elevated levels in nodules compared to roots, as determined by RNA gel blot analysis, were isolated and sequenced. The localization of the corresponding mRNAs in the nodule was examined by *in situ* hybridization. Whenever necessary, model systems such as yeast and *Arabidopsis* were used to analyse the functions of the encoded proteins.

Two of the isolated cDNA clones corresponded to sucrose synthase and enolase, enzymes involved in carbon metabolism. The corresponding genes were expressed in all plant tissues but at markedly elevated levels in nodules. *In situ* hybridization showed that in nodules, both sucrose synthase and enolase were expressed at high levels in the infected cortical cells as well as in the pericycle of the vascular bundle.

Another cDNA clone, pAgthi1, was shown to encode a homolog of yeast Thi4, which is involved in thiazole biosynthesis. The corresponding gene, *agthi1*, was found to be expressed at high levels in nodules and shoot tips of *A. glutinosa*, while being expressed at low levels in roots, flowers, and developing fruits. In nodules, *agthi1* mRNA was localized in the infected cortical cells and in the pericycle of the nodule vascular system. A homolog of this gene, *ara6/tz*, was identified in *Arabidopsis thaliana*. *ara6/tz* maps in a region of chromosome 5 of *Arabidopsis* containing the *tz* locus. This is consistent with the observations that *ara6/tz* transcription was impaired in two out of five *Arabidopsis tz* mutant lines. *ara6/tz* is expressed at high levels in chloroplast-containing parenchymatic cells of leaves, inflorescence shoots and flowers of *Arabidopsis*, and at lower levels in the vascular system. The function of AgThi1 was demonstrated by yeast complementation studies, in which AgThi1 was able to rescue a yeast *thi4* mutant.

cDNAs encoding glutamine synthetase (GS) and acetylornithine transaminase (AOTA), both involved in nitrogen metabolism, were isolated. GS is the enzyme responsible for ammonium assimlation, while AOTA is involved in the biosynthesis of citrulline, the nitrogen transport form in *Alnus*. GS mRNA was found in all tissues tested with the highest levels in nodules,

where it was present in the infected cells as well as in the cells of the pericycle of the vascular system. AOTA transcripts were detected at very low levels in roots and at high levels in nodules, where it was confined to the infected cells. These data suggested that in *A. glutinosa* nodules, ammonium assimilation takes place in both the infected cells and in the pericycle of the vascular system, and citrulline biosynthesis occurs mainly in the infected cells. Ammonium assimilation in the pericycle is likely to be related to nitrogen transport.

One of the few nodule-specific genes, i.e., genes that are not expressed in roots, *ag12*, was shown to be expressed in nodules at the highest levels in infected cells before the onset of nitrogen fixation. Sequencing showed that *ag12* encodes a serine protease of the subtilisin (EC 3.4.21.14) family. A homolog of *ag12*, *ara12*, was identified in *Arabidopsis*. *ara12* was expressed in all organs, with the highest expression levels in the beginning of silique development. To assess the importance of this protease in other actinorhizal symbioses, the expression pattern of its homolog, *cg12*, was examined in nodules of *Casuarina glauca* and found to be similar to that of *ag12*. These results are discussed in view of the phylogenetical relationship of *Alnus* and *Casuarina*.

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Curriculum vitae

I was born in ex-Lourenço Marques, now Maputo, Mozambique on March 22, 1967. From 1972 to 1977 I enjoyed my primary school and after that I spent seven years in high school. From 1985 to 1990 I studied Agronomy in the Department of Crop Production and Protection, Faculty of Agronomy and Forestry, Eduardo Mondlane University, in Maputo, Mozambique. In the period April 1991-March 1993 I did the M.Sc. course in Biotechnology in the Agricultural University, Wageningen, The Netherlands, under the supervision of Dr. Rommert van den Bos and Prof. Dr. Ab van Kammen. My M.Sc. thesis, entitled "Isolation and characterization of nodule-specific/enhanced cDNA clones from *Alnus glutinosa* nodules induced by *Frankia* ArI3", was done in the Depatment of Molecular Biology, of the same university, under supervision of Dr. Katharina Pawlowski and Dr. Ton Bisseling. In August 1993 I started my Ph.D. studies in the Department of Molecular Biology, Wageningen Agricultural University, having Dr. Katharina Pawlowski and Dr. Ton Bisseling as my co-promoters, and Prof. Dr. Ab van Kammen as my promoter.