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Symbiosis between *Frankia* and actinorhizal plants: Root nodules of non-legumes

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In actinorhizal symbioses, filamentous nitrogen-fixing soil bacteria of the genus *Frankia* induce the formation of nodules on the roots of a diverse group of dicotyledonous plants representing trees or woody shrubs, with one exception, *Datisca glomerata*. In the nodules, *Frankia* fixes nitrogen and exports the products to the plant cytoplasm, while being supplied with carbon sources by the host. Possibly due to the diversity of the host plants, actinorhizal nodules show considerable variability with regard to structure, oxygen protection mechanisms and physiology. Actinorhizal and legume-rhizobia symbioses are evolutionary related and share several features.

Keywords: Actinorhizal symbioses, *Alnus*, *Casuarina*, *Datisca glomerata*, *Frankia*

Two types of root nodule symbioses, namely rhizobial-legume and Frankia-actinorhizal plant, exist between nitrogen-fixing soil bacteria and higher plants. In both cases, the macrosymbionts develop special organs, the root nodules, to host the microsymbionts. Within nodule cells, the microsymbionts form the nitrogenase enzyme complex that catalyzes the reduction of dinitrogen. In actinorhizal symbioses, the microsymbionts are *Frankia* strains, filamentous, Gram-positive actinomycetes, that interact with dicotyledonous plants from eight different families, collectively called actinorhizal plants[1].

In contrast to legume nodules, actinorhizal nodules are coralloid structures composed of modified lateral roots without root caps, with a central vascular system and infected cells in the expanded cortex. Nodule primordia are formed in the root pericycle like lateral root primordia. Interestingly, nodules of *Parasponia*, the only non-legume infected by rhizobia, resemble actinorhizal nodules developmentally and structurally.

Actinorhizal plants

More than 200 species of dicotyledonous plants, mostly trees or woody shrubs, that are distributed in 24 genera belonging to eight different families can enter actinorhizal symbioses[1] (Table 1). The host plants do not include important crop species and therefore have not been examined to the same extent as legume symbioses. However, owing to their symbiosis, actinorhizal plants are capable of growing on marginal soils and therefore have been exploited in erosion control, soil reclamation, agroforestry and dune stabilization, as well as in fuel wood, pulp and timber production. For instance, Casuarinaceae are utilized in stabilizing desert and coastal dunes (i.e. in shelter belts) and in the reclamation of salt-affected soil (e.g., *Casuarina equisetifolia* is very salt tolerant[2,3]) or in intercropping systems[4]. Though primarily native of the southern hemisphere (Australian and the Indo-Pacific areas), the range of distribution of some genera has been extended considerably by artificial dissemination. Casuarinaceae are typical angiosperm trees with distinctive foliage of deciduous, jointed needle-like branchlets with reduced scale-like leaves organized in whorls, an adaptation to survival in arid climates[5]. Due to its high calorific value, *C. equisetifolia* wood is used as a fuel in India and China[3]. Another actinorhizal species, *Coriaria nepalensis*, a deciduous shrub, has been successfully used in erosion control[6]. *Alnus* species are used as nurse crops[7], for soil reclamation[8] and for timber and pulp[9].

One actinorhizal species, in particular, that has the potential to become a multipurpose crop is *Hippophae rhamnoides* (sea buckthorn), a dioecious shrub or small tree, the growth pattern and height of which varies with geographical location. The fruits are rich in vitamins and trace elements, and the seed oil, rich in unsaturated fatty acids, has interesting light absorption and emollient properties[10]. It also contains high concentrations of antioxidants[11]. In fact, medicinal use of sea buckthorn has been recorded in

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Table 1 — List of dicotyledonous plants that can enter actinorhizal symbioses

Order/Sub-class	Family	Genera
Higher Hamamelidae	Betulaceae	*Alnus*
	Casuarinaceae	*Casuarina, Allocasuarina, Gymnostoma, Ceuthostoma*
	Myricaceae	*Myrica, Comptonia*
Rosales	Rosaceae	*Cercocarpus, Chamaebatia, Cowania, Dryas, Purshia*
	Rhamnaceae	*Ceanothus, Colletia, Discaria, Kenthrothamnus, Retanilla, Talguenea, Trevoa*
	Elaeagnaceae	*Elaeagnus, Hippophae, Shepherdia*
Cucurbitales		
	Coriariaceae	*Coriaria*
	Datiscaceae	*Datisca*

the Tibetan "rGyud Bzi" as early as in the eighth century[12]. The only drawback of sea buckthorn is that fruit harvest is very labour-intensive (because of the thorns to which the plant owes its name) and does not lend itself to mechanization[13]. Breeding programs for sea buckthorn exist in several countries.

Research on actinorhizal symbioses has been hampered by the fact that actinorhizal plants, with one suffruticose exception (*Datisca glomerata*), represent trees or woody shrubs not very amenable to molecular biological analyses and due to their long generation times, with the exception of *Datisca glomerata* (six months), are absolutely unsuited for genetic analyses. Furthermore, so far only two actinorhizal species, *Allocasuarina verticillata*[14] and *Casuarina glauca*[15] can be transformed.

Actinorhizal microsymbionts: Frankia strains

Unlike most rhizobia, *Frankia* strains can grow on dinitrogen as the sole nitrogen source in the free-living state. When nitrogen limitation occurs under microaerobic conditions, nitrogenase is formed in *Frankia* hyphae[16]. Under nitrogen limitation and aerobic conditions, *Frankia* strains form spherical vesicles at the ends of hyphae or short side-hyphae (Fig. 1A). In these special organs, the nitrogenase enzyme complex is formed and nitrogen fixation takes place. The vesicles are surrounded by multi-layered envelopes containing bacterial steroid lipids called hopanoids[17-19]. Since the number of layers is correlated with the oxygen tension, it is assumed that they act as an oxygen diffusion barrier[18].

Nitrogen-fixing vesicles formed in culture are invariably spherical. *In planta,* however, shape, septation and subcellular localization of *Frankia* vesicles depend on the host plant (e.g., spherical septate vesicles at the periphery of the infected cell in *Alnus*[20] (Fig. 1B) and lanceolate vesicles which point at the central vacuole, forming a ring around it in

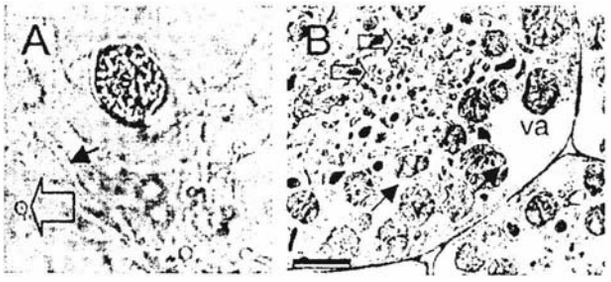

Fig. 1 — *Frankia* in culture and *in planta*. (A) *Frankia* culture grown in nitrogen-free medium. A small arrow points at a hypha, a broad arrow points at a vesicle; s, sporangium. (B) Electron micrograph of part of an infected cell from an *Alnus glutinosa* nodule. h, hyphae; v, vesicle; s, septae. Bar = 5 μm (courtesy of Dr. K. Demchenko).

Datisca and *Coriaria*[21,22]). A single *Frankia* strain can form different types of vesicles in different host plants[23]. Hence, the differentiation of *Frankia* vesicles in symbiosis can be considered a symbiosis-specific differentiation comparable to that of the bacteroids in legume symbioses. The frequency of vesicles in infected plant cells is much higher than that in culture[24] (Figs 1A *versus* 1B), indicating another regulatory effect of the plant host. *Frankia* can also form a third type of specialized structures, namely multilocular sporangia[25]. On the basis of the presence or absence of sporangia within root nodules, *Frankia* strains can be classified as spore$^+$ or spore$^-$, respectively. Sporangia formed *in planta* are very similar to those formed in culture[26]. To date, the presence of sporangia has only been reported in plants of the genera *Alnus, Myrica* and *Comptonia*, i.e. in some higher Hamamelidae. Within these genera, however, the presence or absence of sporulation in nodules seems largely controlled by the *Frankia* strain, rather than by the host plant[25].

Until now, it has not been possible to cultivate the microsymbionts of all actinorhizal plants. No nitrogen-fixing *Frankia* strains could be isolated from

nodules of Rosaceae, *Datisca, Coriaria* or *Ceanothus* species, nor was it possible to infect these plants with cultured *Frankia* strains. Similarly, no spore$^+$ *Frankia* strains have ever been successfully isolated from nodules[25]. In several cases, actinomycetous bacteria could be isolated from surface-sterilized nodules of *Datisca, Coriaria* or *Ceanothus species*, but these bacteria could not fix nitrogen or re-infect the host plant on their own[27,28]. On the other hand, PCR methods now allow the identification of *Frankia* strains in nodules as well as in soil, without the need to cultivate them[29]. Hence, it could be shown that actinorhizal nodules can contain multiple bacterial strains, comprising not only different *Frankia* strains but also, in the outer cortex, non-nitrogen-fixing related actinomycetes that cannot reinfect the host plant (called 'atypical strains')[28,30].

Frankia taxonomy is in a transitory stage. Most *Frankia* strains are still referred to by acronyms based on plant of origin, or by strain identification numbers using a coding system devised in 1983 at a meeting in Madison, Wisconsin[31]. Only in one case (*F. alni*[29]), a specific name has found entry into literature. Since host specificity is complex and strains isolated from a particular host plant may not re-infect the host but may instead be able to infect other host plants[32,33], a naming system based on host specificity like the one developed for rhizobia would be impractical. Molecular systematics based on 16S and 23S rDNA sequences as well as sequences of protein-coding genes (*glnII*) and of intergenic spacer regions (*nifH-D* or *nifD-K*), or PCR-RFLP studies based on these sequences, has led to the emendation of three groups of infective *Frankia* strains and one group of 'atypical strains' (Fig. 2[34-38]). Group III contains only non-culturable strains. The problems involved with handling unculturable strains, and strains that do not reinfect the plant they were isolated from, as well as the fact that it is still impossible to transform *Frankia*, have hampered research on actinorhizal symbiosis even more than the woody nature of the host plants.

Actinorhizal nodule structure

Actinorhizal nodules are composed of multiple modified lateral roots, surrounded by a superficial periderm, with infected cells in the expanded cortex (Fig. 3). Due to the activity of the apical meristem, the infected cells are arranged in a developmental gradient[39]. Close to the meristem in the infection zone, some cortical cells become infected by *Frankia* hyphae which branch and gradually fill the cells. During this process, the bacterial hyphae are separated from the cytoplasm by the invaginated plasma membrane of the infected cell. Once a cell is filled with *Frankia* hyphae, vesicles develop and nitrogen fixation starts[40], marking the shift to the fixation zone. After some time, symbiotic nitrogen fixation stops, and intracellular *Frankia* is degraded by the plant cell in the zone of senescence. Actinorhizal nodules are perennial organs and can reach fist-size. In a several years old nodule, only the outer part contains nitrogen-fixing infected cells, while most of the volume is taken up by the zone of senescence.

Nodule aeration can be facilitated by lenticels (e.g. in *Alnus* (Fig. 3D), *Datisca* or *Coriaria*) or nodule roots in plants whose roots are often submerged in water (e.g. in *Casuarina* (Fig. 3E), *Myrica* or *Datisca*[41]). Nodule roots contain large air spaces in their cortex and grow ageotropically from the tips of nodule lobes[42]. They show determinate growth; the length of nodule roots depends on the oxygen tension[43].

Like in legume-rhizobia symbioses, in actinorhizal plants too only cells formed after signal exchange

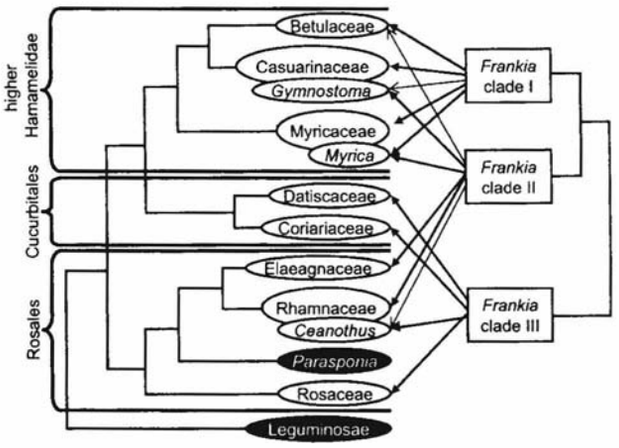

Fig. 2 — Simplified scheme of the phylogenetic relationship between groups of actinorhizal plants and *Frankia* strains, based on recent literature[34,139,151]. Groups of plants infected by rhizobia are added for overview and labeled by inverse print. Thick arrows connect *Frankia* clades with the groups of host plants members of the clades they are commonly associated with. Thin arrows indicate that members of that *Frankia* clade have been isolated from, or detected in an effective or ineffective nodule of a member of the plant group at least once. There is host specificity within the *Frankia* clades, i.e., not all members of a *Frankia* clade can nodulate all plants associated with that clade. Up to now, members of *Frankia* clade III have not been cultured. Some actinorhizal genera (*Gymnostoma, Myrica* and *Ceanothus*) differ in microsymbiont specificity from the rest of the family as indicated.

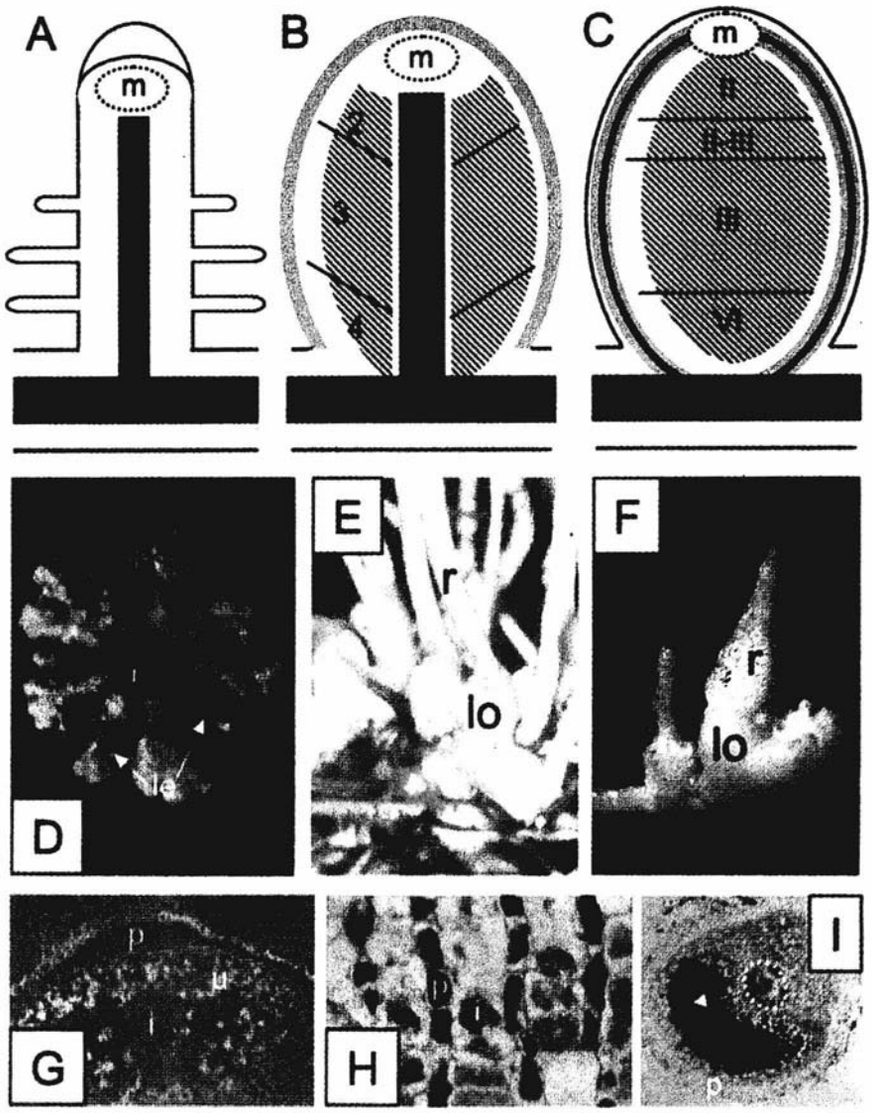

Fig. 3 — Structure of actinorhizal nodules. Schematic representation of the structure of a lateral root (A), an actinorhizal nodule lobe (B) and an indeterminate legume nodule (C). The vascular system is labelled in black. Calyptra and root hairs are shown. m, meristem. Due to the activity of the apical meristem (m), the nodule cortex can be divided into the infection zone (2), where cortical cells become gradually filled with *Frankia* hyphae, and afterwards, vesicles differentiate (with the exception of *(Allo-)Casuarina* nodules), the fixation zone (3) where nitrogen is fixed by *Frankia* in the infected cells, and the senescence zone (4), where *Frankia* hyphae and vesicles are degraded. Nodule lobes are surrounded by a periderm which is shown in dark gray. The organization of the inner tissue of an indeterminate legume nodule is based on Vasse *et al.*[184]. The inner tissue can be divided in the prefixation zone (II), where infection threads infect nodule cells and bacteroids differentiate within peribacteroid membranes, the interzone zone (II-III), where bacteroids have developed and nitrogen fixation starts, the fixation zone (III) and the zone of senescence (IV). (D, E, F) Actinorhizal nodules in overview. Photographs of mature nodules of (D) *Alnus glutinosa*; (E) *Casuarina glauca* and (F) *Datisca glomerata* are shown. le, lenticel; lo, nodule lobe; r, nodule root. (G, H, I) Details of nodule structure. (G) Lenticel seen in a longitudinal section through an *Alnus glutinosa* nodule lobe, stained with Ruthenium Red and Toluidine Blue and photographed under fluorescent light. Several layers of polyphenol-filled cells (p) are under the rupture in the periderm. Starch grains can be seen in the uninfected (u), but not in the infected cells (i). (H) In *Alnus* and *Casuarina*, the nodule cortex contains layers of polyphenol-containing cells (p) as can be seen in the longitudinal section of the infection zone of a nodule lobe from *Alnus glutinosa*. That polyphenol-filled cells and infected cells (i) alternate like here, is not the rule; often, there are three or more layers of infected cells between layers of polyphenol-filled cells. (I) In this Toluidine Blue-stained cross section of a nodule lobe from *Datisca glomerata*, it can be seen that the infected cells are not distributed over the cortex interspersed with uninfected cells as in other actinorhizal nodules, but form a continuous kidney-shaped patch on one side of the acentric stele (surrounded by a dotted line). In these nodules, infected cells retain a large central vacuole (arrow). *Datisca* nodules are surrounded by a multi-layered periderm (p). As in *Alnus* (see panel D), the uninfected cortical cells contain large starch grains. Panel (D) by courtesy of A.D.L. Akkermans, panel (E) of D. Bogusz.

with the microsymbiont can stably internalize bacteria. Hence, infected nodule cells seem to carry a specific differentiation. The organization of infected cells in the nodule cortex differs aomng actinorhizal plant genera. In most cases they are interspersed with uninfected cells (Figs 3G and 3H) but in *Datisca* and *Coriaria* they form a continuous patch, that appears kidney-shaped in cross-section, on one side of the acentric stele (Figs 3I and 3H). In *Casuarina* and *Alnus*, cells containing vacuoles filled with phenolics form continuous files from the apex to the base of the nodule lobe, separating layers of infected and uninfected phenolic-free cortical cells[44,45] (Fig. 3H). These files are organized in concentric layers. In each layer, *Frankia* grows acropetally without ever crossing the files of phenolic-filled cells except in the youngest part of the nodule where not all cells of the file are already filled with phenolics. In *Alnus*, the number of cortical cell layers in between phenolic-filled layers depends on the growth conditions. Mostly, every third or fifth cortical cell layer consists of phenolic-filled cells, but sometimes, layers of infected cells and layers of phenolic-filled cells alternate. In plant-pathogen interactions, layers or tissues of phenolic-filled cells seem to serve as pathogen barriers[46,47]. In ineffective, i.e. non-nitrogen fixing *A. glutinosa* nodules that are induced by incompatible *Frankia* strains[48,49], the distribution of phenolic-containing infected cells is irregular. In these nodules, phenolics are regularly found within infected cells, a phenomenon rarely observed in effective nodules, suggesting that the production of phenolics may be part of a defense response of the plant, an idea supported by the fact that in these nodules, endosymbiont degradation starts soon after the infected cells have been filled with *Frankia* hyphae[50].

The oxygen dilemma of nitrogen fixation

While the nitrogenase enzyme complex is irreversibly denatured by oxygen, the process of biological nitrogen fixation requires high amounts of ATP that have to be won by respiratory processes. This leads to the oxygen dilemma of nitrogen fixation-nodules have to be well aerated but nitrogenase has to be protected from oxygen. Diverse strategies are available to achieve oxygen protection: (1) an oxygen diffusion barrier can surround the tissue containing infected cells, or each infected cell individually; (2) oxygen binding proteins (hemoglobins) in infected cells can transport oxygen to the sites of respiration while keeping it away from those of nitrogen fixation; (3) elevated respiration can remove oxygen from the infected cells; (4) *Frankia* can form vesicles not only for nitrogen fixation under aerobic conditions in the free-living state but also *in planta*. In legumes, strategies (1) and (2) are combined, in that a variable oxygen diffusion barrier consisting of layers of cells lacking intercellular spaces surrounds the inner tissue of the nodules that contains the infected cells, while allowing oxygen access to the peripheral vascular system[51]. The combination of strategies deployed in actinorhizal nodules depends on the host plant genus. For instance, *Casuarina* nodules resemble legume nodules in that *Frankia* does not form vesicles[52], infected cells contain large amounts of a nodule-specific hemoglobin[53,54], and the walls of infected cells are lignified[55]. In nodules of *Alnus,* the microsymbiont takes part in oxygen protection of nitrogenase. First, the number of the layers of *Frankia* vesicle envelopes is correlated with the oxygen tension[56,57], and second, the vesicles are sites of high respiratory activity[58]. Recently, it has been found that *Frankia* contains a hemoglobin[59] similar to that in *Nostoc*[60] which may play a role of oxygen protection of nitrogenase.

Infection mechanisms

Frankia hyphae can enter plant roots either intracellularly via root hairs, or intercellularly between root epidermal cells. Like in legume-rhizobia symbioses, the infection mechanism in actinorhizal symbioses is determined by the host plant species. Members of the higher Hamamelidae (Betulaceae, Casuarinaceae and Myricaceae) are infected intracellularly, while in all other actinorhizal plants infection seems to take place intercellularly.

Intracellular infection

Intracellular infection of actinorhizal plants starts with the induction of root hair deformation by factors secreted by *Frankia* strains, the chemical nature of which is still unknown[61] (Fig. 4A). In contrast to legume symbioses, bacterial factors inducing root hair deformation on actinorhizal plants are not specific to their microsymbionts, but are also produced by other soil microbes[62]. When a hypha is trapped in a root hair curl, local hydrolysis of the root hair cell wall takes place, the plasma membrane invaginates and an infection thread-like structure, the so-called encapsulation, develops by which the hypha enters the root[42,63]. In contrast to legume infection threads, no equivalent of the infection thread matrix exists in

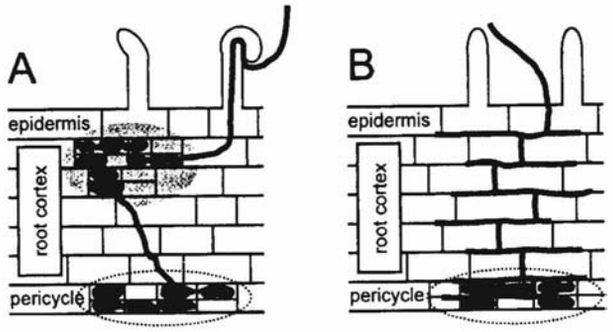

Fig. 4 — Infection mechanisms. (A) Intracellular infection. Root hairs deform in response to the presence of an unknown compound exuded by *Frankia* in the rhizosphere. A *Frankia* hypha enters the plant through an infection thread-like structure formed in a curled root hair. Simultaneously, cell divisions are induced in the outer cortex and the infection thread-like structure (encapsulated hypha) grows toward these dividing cells and infects them, i.e., fills them from the centre outward by extensive branching. The dividing cortical cells represent the prenodule (labelled in gray). Now, the formation of the nodule primordium is induced in the pericycle opposite to a protoxylem pole, and *Frankia* hyphae in infection thread-like structures grow from the prenodule to the nodule primordium and infect primordium cells. The nodule primordium is marked by a dotted line. (B) Intercellular infection. *Frankia* hyphae penetrate the root epidermis between epidermal cells and colonize the root cortex intercellularly. Cell divisions are induced in the root pericycle, leading to the formation of a nodule primordium. *Frankia* hyphae infect nodule primordium cells from the apoplast. The nodule primordium is marked by a dotted line.

infection thread-like structures formed by actinorhizal plants; *Frankia* hyphae are embedded in cell wall-like material (the encapsulation) which corresponds to the infection thread wall of legumes. Concomitantly, cell divisions are induced in the outer cortex of the root. The infection thread-like structure grows across cells to the dividing cortical cells and infects some of them. This leads to the formation of a cortex-based structure, called the prenodule, consisting of infected and newly formed uninfected cells.

Like legume infection threads, infection thread-like structures require the formation of pre-infection thread structures in root cortical cells they are about to cross[64,65]. Infection of cells occurs by intense branching of infection thread-like structures within the cells, which are being filled with *Frankia* hyphae from the center outward[66-68]. Once an infected cell is filled with branched hyphae in infection-thread like structures, vesicles are formed, nitrogenase is produced and nitrogen fixation starts[40,67]. Infected and uninfected prenodule cells have been shown to carry the same differentiation as their counterparts in mature nodules; hence, the prenodule can be considered as a primitive symbiotic organ[69].

While the prenodule develops, cell divisions are induced in the pericycle of the root vascular system that lead to the formation of a nodule primordium[69]. Depending on the host plant species, more than one nodule primordium can be induced per prenodule[70]. Hyphae in infection thread-like structures grow from the prenodule to the nodule primordium, again by cell-to-cell passage, and infect primordium cells. Each nodule primordium develops into a nodule lobe. Interestingly, the induction of nodules on roots of *Parasponia* sp. by rhizobia more or less follows this mechanism, except that rhizobia enter the root intercellularly[71,72].

Intercellular infection

During intercellular infection, *Frankia* hyphae enter the root between epidermal cells and start colonizing the root cortex[73-76] (Fig. 4B). Neighbouring host cells secrete cell wall-like material rich in protein and pectin into the apoplast, probably facilitating bacterial growth[77]. Simultaneously, and prior to contact with bacteria, pericycle cells start to divide, leading to the formation of a nodule primordium. When *Frankia* hyphae reach primordium cells, they are internalized in branching infection-thread like structures. In intercellularly infected plants, nodule cells are always infected from the apoplast; infection threads do not grow from one cell to another. Once the infected cells have been filled with encapsulated *Frankia* hyphae from the center outward, vesicles are formed and nitrogen fixation starts.

In most host plant genera whose microsymbiont has not yet been cultured (Rosaceae, *Datisca* and *Coriaria*) infection mechanisms have not been characterized. However, two ways exist to distinguish between intra- and intercellularly infected nodules. First, prenodules occur only during intracellular infection. Second, since in intracellularly infected plants, infection threads grow by cell-to-cell passage, their nodules contain files of infected cells that can be separated by files of uninfected cells (Fig. 3H). Such infection patterns are never found in intercellularly infected nodules. Based on these criteria, the above mentioned host plants can be concluded to be infected by the intercellular pathway.

Nutrient-dependent regulation and autoregulation of nodulation

In nodulation the plant partner has to provide the bacteria with energy both for its general metabolism and for nitrogen fixation. In the presence of combined

nitrogen in the soil, this represents a waste of energy. Phosphate availability is a crucial factor: since nodulated plants have higher phosphate requirements than non-nodulated plants, nodulation can be detrimental under conditions of both nitrate and phosphate deprivation[24,68,78,79]. To ensure that bacterial colonization is kept in a beneficial range, the plant has to restrict the number of nodules which may form at its roots. This process, the regulation of nodulation, has been examined in great detail in legumes. The inhibition of nodule-induction or -development by already existing nodules, which limits the number of root nodules per plant, is called autoregulation of nodulation, in contrast to the inhibition of nodulation by the presence of combined nitrogen (N-inhibition). Both processes have been studied in detail in legumes, where N-inhibition- and/or autoregulation-deficient mutants are also available[80,81]. The lack of such mutants is a great detriment to the research on autoregulation in actinorhizal plants. Interestingly, regulatory mechanisms seem to differ between plants forming determinate and indeterminate nodules[82]. During autoregulation in legumes, a signal moves from the root system to the shoot. Increased root nodulation is detected in shoots via an increase of the root-derived signal, in response to which the shoot produces an inhibitor that is translocated to the root and inhibits further nodule development. Recently, a receptor kinase involved in the perception of the root-derived signal in shoots during both autoregulation and nitrate inhibition of nodulation, has been identified in three different legume species[83].

The analysis of the (auto-)regulation of nodulation in actinorhizal plants has been slowed down by the lack of available plant mutants. Inhibition of nodulation by combined nitrogen has been shown in many genera of actinorhizal plants[84]and seems to be independent of the infection mechanism. Similarly, phosphate seems to have a positive effect on nodulation in both intracellularly and intercellularly infected actinorhizal plants[85,86]. This similarity is compounded by the fact that like for intracellular infection, which is assumed to occur in the zone of root hair extension, susceptibility for intercellular infection seems to occur only in a transient window[87,88].

There seems to exist a difference between the autoregulatory mechanisms of intracellularly (*Alnus*[89]) and intercellularly (*Discaria*[88]) infected actinorhizal plants. In *Alnus,* a temporary release of N-inhibition in the presence of *Frankia* led to new infections, while in *Discaria,* it led to an increase of the biomass of existing nodules. Nevertheless, in both cases, a shoot regulatory factor and a root regulatory factor are supposed to interact[84].

The receptor kinases involved in N-inhibition and autoregulation of legume nodulation are closely related to CLAVATA1 from *Arabidopsis*, a receptor kinase involved in cell fate determination in shoot apical meristems[90], likely to have evolved by duplication of an ancestral CLAVATA1-like gene[83], and controls not only nodule, but also lateral root meristems. For this reason and due to the involvement of similar root and shoot factors in N-inhibition/autoregulation in legumes and actinorhizal plants, it is tempting to speculate that the basic mechanisms that were recruited in legumes to control nodulation were also adapted in actinorhizal plants.

Nodule metabolism

Nitrogen metabolism

In legume-rhizobia symbioses, intracellular bacteroid export the fixed nitrogen in the form of ammonia or alanine[91]. In the first case, ammonia is protonated to ammonium in the acidic environment of the peribacteroid space, taken up actively by a plant ammonium transporter and assimilated in the glutamine synthetase (GS)-glutamate synthase (GOGAT) pathway[92]. Analyses have shown that either GS expression is confined to the infected cells[93,94], or it is expressed all over the inner tissue, i.e., the infected and uninfected cells of the nodules[95]. Furthermore, export of an assimilated form of fixed nitrogen, alanine, by bacteroids has been reported for legume nodules[96], but its role *in planta* remains controversial[97,98]. Experiments with incorporation of $^{15}N_2$ into amino acids support the assimilation via the GS-GOGAT pathway in *Alnus glutinosa* and *Myrica gale*[24]. The fact that GS was not found in *Frankia* isolated from *Alnus incana* nodules[99] indicates that in this plant too, the fixed nitrogen is exported in the form of ammonia and assimilated in the plant cytoplasm. This is supported by the finding that GS transcription is induced in nodules of *Alnus glutinosa*, specifically in the infected cells[94]. However, the presence of an additional nitrogen export form in the *Alnus-Frankia* symbiosis, or the existence of a different pathway of nitrogen assimilation in other actinorhizal plants, has not been disproven. Preliminary results indicate that the situation in *Datisca glomerata* nodules is different from that in

Alnus nodules[100]. For a summary of the nitrogen transport compounds in actinorhizal plants see Huss-Danell[101]

Carbon metabolism

Nodules represent strong carbon sinks, requiring photoassimilates for growth and maintenance as well as for the energy-demanding processes of nitrogen-fixation, -assimilation and -transport. The form of photoassimilates most commonly used in long distance transport in the plant phloem is sucrose. In sink organs, sucrose can be cleaved either by sucrose synthase or by one of the invertase isoforms. For legumes, the fact that a pea much deficient in nodule sucrose synthase activity does not support bacterial nitrogen fixation in root nodules[102]indicates that here, sucrose synthase plays the main role in introducing sucrose into nodule metabolism, although invertases are also active in nodules[103,104]. Induction of sucrose synthase was also found in actinorhizal nodules of *Alnus glutinosa*[105] but the roles of invertases in nodule carbon metabolism are yet to be examined. At any rate, the carbon transport forms in the phloem of most actinorhizal plants have not been examined, leaving the possibility that different enzymes are involved in regulating nodule carbon sink strength. For instance, in Rosaceae, symplastic phloem loading seems to be the rule, leading to a broader range of translocated carbohydrates[106,107] the metabolism of which would include additional enzymes.

In the free-living state, *Frankia* strains grow well on short chain fatty acids (acetate and propionate), variably on succinate, malate or pyruvate, and poorly or not at all on different sugars[1]. The question arises as to which carbon source is delivered to symbiotic *Frankia* by their actinorhizal host plants. In legume-rhizobia symbioses, the microsymbionts receive carbon sources from the plants in the form of dicarboxylic acids[108], implying a similar situation in actinorhizal symbioses. Studies on the metabolism of symbiotic *Frankia* are performed using so-called vesicle clusters isolated from nodules, mainly of *Alnus* and also of *Hippophae* and *Datisca*. Vesicle clusters are prepared from homogenates of root nodules and consist of symbiotic vesicles together with a part of their subtending hyphae[109,110]. Vesicle clusters from *Alnus* spp. have an aerobic metabolism[111]. Succinate, as well as a combination of malate, glutamate and NAD, stimulated respiration[112-114]. The tricarboxylic acid cycle enzymes isocitrate dehydrogenase, succinate dehydrogenase, fumarase and malate dehydrogenase were active in vesicle clusters[112]. Although enyzme activities supporting the operation of a malate-aspartate shuttle between *Alnus* and *Frankia* were also found[112,113], the existence of such a shuttle has not been proved, and the enzyme activities could also be explained in terms of a passive uptake of substrates[24]. Similarly, CO_2 fixation observed in actinorhizal nodules might be due to metabolism of dicarboxylic acids, or to reactions associated with ammonia assimilation[101,115].

An alternative hypothesis would be that symbiotic *Frankia* is supplied with hexoses by the host plant. The fact that the sugars sucrose, trehalose, maltose, glucose and fructose stimulate respiration in vesicle clusters from *Alnus rubra*[116] is consistent with, but does not prove, the hexose hypothesis since metabolism of hexoses in symbiotic *Frankia* could simply reflect the ability to metabolize *Frankia*'s own storage carbohydrates, namely glycogen and trehalose[117]. In fact, glycogen has been found in root nodules in hyphae and developing vesicles[118]. In summary, the carbon sources supplied to symbiotic *Frankia* have not yet been identified.

Uptake hydrogenase

During nitrogen fixation, some of the ATP and reductant is always used in a side reaction of nitrogenase for the reduction of H^+ to H_2. Since H_2 is an inhibitor of nitrogenase, oxidation of H_2 to H^+ by uptake hydrogenase (Hup) might be expected to be beneficial for the symbiosis, first to prevent H_2 accumulation, and second to recover reductant or ATP consumed during H_2 production. However, in legume symbioses where isogenic Hup^+ and Hup^- rhizobial strains are available, no significant difference in symbiotic efficiency could be found between the two types of strains. With a single exception, all actinorhizal symbioses studied have shown hydrogenase activity[119,120]. This is somewhat unfortunate because the Hup^- phenotype can be useful in field experiments. First, in Hup^- symbioses, H_2 evolution-which is easily detectable-signifies nitrogenase activity, i.e. nitrogen fixation. Second, in Hup^- symbioses, acetylene reduction activity can be converted directly into nitrogen fixation activity provided the relative efficiency of nitrogenase is known[121,122]. The Hup^- exception is a $spore^+$ strain, the so-called 'local source of *Frankia*' first described by Sellstedt and Huss-Danell[123]. In cultured *Frankia* CpI1, hydrogenase was found in hyphae as well as in vesicles[124,125] making it unclear whether it has a specific role in nitrogen fixation at all.

Molecular biology of actinorhizal plants

In legumes, the study of genes induced during nodule development, summarily called nodulin genes, has been very helpful for the understanding of the symbiosis. In general, genes expressed at significantly higher levels in nodules than in roots encode products involved in (a) nodule metabolism, i.e. enzymes, (b) the internalization of the microsymbiont, i.e. apoplastic proteins specific to infected cells or infection thread-containing cells, (c) the developmental difference between nodules and roots and (d) the signal exchange between macro- and microsymbiont.

Initially, the term "nodulin gene" was coined for genes exclusively expressed in legume nodules[126]. However, several so-called nodulin genes were found out later to be also expressed in plant organs other than nodules[127], or to be in fact expressed in roots, though at levels only to be detected by RT-PCR methods. Nowadays, the term "nodulin gene" means that the corresponding gene is expressed at elevated levels in nodules compared to roots. In this review, the term "nodule-specific genes" is used to refer to those genes for which no expression in roots could be detected by RNA gel blot hybridization analysis, and "nodule-enhanced genes" for genes whose expression is detected in roots and nodules by RNA gel blot hybridization, though at higher levels in nodules.

Differential screening of actinorhizal nodule cDNA libraries with nodule versus root cDNA has been performed for two higher Hamamelidae (*Alnus glutinosa* and *Casuarina glauca*[39,128]), one member of the Rosales (*Elaeagnus umbellata*[129]) and one member of the Cucurbitales (*Datisca glomerata*[130]). The nodule-specific genes identified are summarized in Table 2. Five nodule-specific genes were found in *Alnus,* one encoding an enzyme involved in fatty acid metabolism, one encoding a plasma membrane transporter and the others encoding putative apoplastic proteins. In *Casuarina,* a gene encoding symbiotic hemoglobin, equivalent to leghemoglobins from legumes, as well as homologues of two of the nodule-specific genes encoding apoplastic proteins identified in *Alnus*[131,132] was found. For one of the latter genes, expression patterns were compared in nodules of *Alnus* and *Casuarina* and found to be identical[39,69,131]. In *Elaeagnus*, a nodule-specific gene encoding an acidic chitinase was identified[133]. In *Datisca*, the homologue of a nodule-specific gene with unknown function in soybean[134]and *Medicago sativa*[135] was found to be expressed in a nodule-specific manner[136]. This is so far the only instance of homologous nodule-specific genes in legumes and actinorhizal plants. The expression patterns of the homologues in soybean and *Datisca* showed some overlap-in both cases, expression was found in the nodule meristem and in mature infected cells-but the *Datisca* homologue was also expressed in the vascular system, in cortical cells during infection by *Frankia*, and in the nodule periderm while the soybean homologue was not[134,136]. The occurrence of homologues in several non-symbiotic plant species, e.g., maize and rice[136], as well as the expression in the nodule meristem in both soybean and *Datisca,* implies that the gene product has a general function in organogenesis, and that the differences in expression patterns between the legume soybean and the actinorhizal *Datisca* is related to the developmental differences of both types of nodules. Another nodule-specific transcript from *Datisca* turned out to represent an incompletely spliced version of rubisco activase mRNA[130]. This is intriguing for two reasons. First, rubisco activase expression is controlled on the level of splicing, a fact not known previously. Second, the signal transduction pathway that leads to the

Table 2—Genes expressed specifically in actinorhizal nodules

Plant species	Gene name	Gene product	Reference
Alnus glutinosa	*AgNOD-CP*	cysteine proteinase	182
	Ag12	serine proteinase (subtilase)	39
	AgNt164, AgNt84: AgGHRPs	small glycine- and histidine-rich putative cell wall protein	140
	AgMTR1	plasma membrane transporter	157
	Ag135	enzyme involved in fatty acid metabolism	183
Casuarina glauca	*CgHbSym*	leghemoglobin	54, 128
	Cg12	serine proteinase (subtilase), homologue of Ag12	131
	CgGHRP	small glycine- and histidine-rich putative cell wall protein	132
Elaeagnus umbellata	*EuNOD-CHIT1*	acidic chitinase	133
Datisca glomerata	*dg93*	unknown function	136

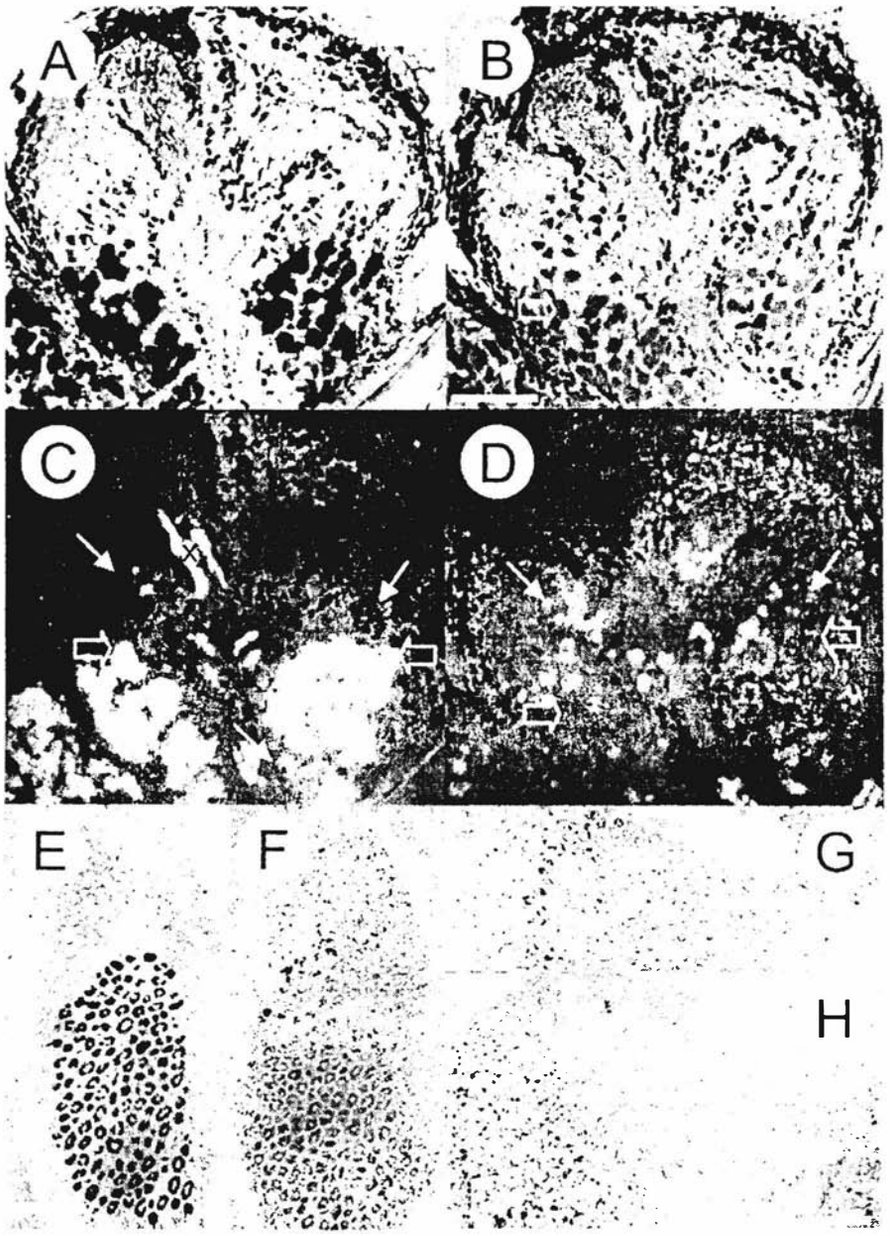

Fig. 5 — Visualization of gene expression patterns and subcellular localization of mRNA by *in situ* hybridization of nodule sections with ^{35}S-labeled antisense RNA. Panels A, B, E-H represent bright field micrographs, silver grains denoting hybridization are visible as black dots. Panels C and D represent dark field micrographs, silver grains appear as black dots. The bars denote 200 μm. (A-D) Plant *ag12* and *Frankia nifH* expression in *Alnus glutinosa* nodules[39]. Adjacent 7μm thick nodule sections were hybridized with *Frankia nifH* (A, C) and *Alnus ag12* antisense RNA (B, D), respectively. Thin arrows point at cortical cells in the process of infection that express *ag12*. Broad arrows point at infected cortical cells fully filled with *Frankia* material. Here, *Frankia* expresses *nifH* and nitrogen fixation takes place, while *ag12* expression levels are reduced. m, meristem. x, xylem elements show bright autofluorescence. (E-H) Plant rubisco activase and *Frankia nifH* expression in *Datisca glomerata* nodules[130]. (E, F) Adjacent nodule sections were hybridized with *Frankia nifH* (E) and *Datisca* rubisco activase antisense RNA (F), respectively. The infected cells are surrounded by cortical cells containing large amyloplasts (a). Thin arrows point at the youngest infected cells that are fully filled with *Frankia* material and express *nifH*. Broad arrows point at cells the nuclei of which contain rubisco activase mRNA. This includes uninfected cortical cells (see also broad arrow in G). Cortical cells become multinucleate upon infection. Panel (G) (developmental gradient from left to right) shows that the highest amounts of rubisco activase are present in youngest layers of infected cortical cells that are filled completely with *Frankia* material. Panel (H) shows that rubisco activase expression can be detected only at the infected side of a nodule lobe, though not only in infected cells. No silver grain accumulation is found in nuclei on the uninfected side of the vascular systems of two branching nodule lobes (ellipse). m, meristem; v, vascular system.

induction of rubisco activase transcription is active in *Datisca* nodules. Hence, the components light- or sugar- dependent signal transduction pathways of leaf may have been recruited for the control of nodule development.

Nodule-enhanced genes, as expected, mostly encode enzymes involved in nodule metabolism. Glutamine synthetase (GS) in *Alnus, Casuarina* and *Datisca*[94,100,137], sucrose synthase and enolase in *Alnus*[105], chalcone synthase in *Casuarina*[44] are encoded by nodule-enhanced genes, reflecting the high activity of ammonium assimilation in nodules, the generally enhanced sugar metabolic activity of nodules as compared to roots and accumulation of polyphenols in nodules, respectively. Other nodule-enhanced genes encode structural proteins, like an acidic cell wall protein in senescent infected cells and the vascular system of *Alnus*[138] and a *Datisca* homologue of one of the nodule-specific genes encoding apoplastic proteins of *Alnus* and *Casuarina*, namely an glycine- and histidine-rich cell wall protein[139]. Polyubiquitin and a basic chitinase in *Elaeagnus*[129,133] and a PR10 protein gene of *Datisca*[139] are also expressed in a nodule-enhanced manner.

The products of genes encoding putative apoplastic proteins and expressed specifically in the infected cells are assumed to play a role in the internalization of the microsymbiont, either as part of the extracellular matrix that embeds *Frankia* in infection thread-like structures, or by modifying a compound of this matrix. The subtilases of *Alnus* and *Casuarina,* and the small glycine- and histidine-rich proteins of *Alnus* fall into this group[39,131,140] (Table 2). Both gene families are expressed at high levels in young infected cells that are in the process of being filled with branching *Frankia* hyphae. The latter have been suggested to represent a part of the plant-derived matrix embedding intracellular *Frankia*; in this matrix they might have a role in the transport of metal ions to the microsymbiont[140]. Interestingly, the *Datisca* gene encoding their homologue is not specific to infected cells, nor to nodules, being expressed also in the periderm of nodules and roots[139], i.e. in cells with suberized walls. It is tempting to speculate that a cell wall protein evolved for the interaction with aliphatic substances was recruited for the matrix embedding *Frankia* in infected cells.

The role of the nodule-specific subtilases of *Alnus* and *Casuarina* is less clear. Based on their substrate specificity, subtilases can be classed into degrading and processing enzymes[141]. If the nodule-specific subtilases belong to this class they might play a role in the cell wall reconstruction that is required during growth and branching infection thread-like structures. The processing subtilases are highly substrate-specific and are involved in protein maturation or production of peptide hormones. Several plant subtilases seem to belong to this class, for instance SBP50 that was suggested to be involved in prosystemin maturation[142]. Thus, the subtilase nodulins might be involved in the maturation of cell wall proteins, or in the production of signal factors at the interface between micro- and macrosymbiont.

The question arises whether the protein composition of the cell wall-like material in branching infection thread-like structures in infected cells is different from that of infection thread-like structures by which *Frankia* enters the root during intracellular infection. The fact that only cells formed after signal exchange with *Frankia* can be infected by branching infection thread-like structures, while infection thread-like structures can grow through root cortical cells indicates that the cells of the prenodule and nodule primordium carry a specific differentiation not found in root cortical cells. Unfortunately, the data available so far do not provide an answer to this question. Both cell types express the nodule-specific subtilase in *Casuarina,* as evidenced by experiments with promoter-GUS fusions in transgenic plants showing that the nodule-specific subtilase promoter is already active in root hairs containing infection threads[143]. The expression in root cortical cells containing infection thread-like structures, however, is too low to be detected by *in situ* hybridization[131], and the expression patterns of most other nodulin genes have not been examined by promoter-GUS fusions in transgenic plants.

The nodule-specific acidic chitinase from *Elaeagnus* is strongly expressed in the meristem and less strongly expressed in the periderm, outer cortex and uninfected cells of the fixation zone[133]. Chitinase expression has also been analysed in legume nodules[144-146], but until now, no chitinase gene has been found to be expressed in legume nodule meristems. However, two chitinase promoters have been characterized that are active in lateral root meristems[144,147]. Independent evidence also suggests that chitinases are involved in plant development[148]. It is possible that the expression of chitinase genes is one of the factors that distinguish legume nodule meristems from the meristems of actinorhizal nodule lobes, as the expression of a chitinase-promoter GUS

fusion could be used to distinguish between root and nodule meristems of white clover[144], but further research is needed before such a conclusion can be drawn.

Evolution of nitrogen-fixing root nodule symbioses

Phylogenetic analysis has shown that all plants able to enter a root nodule symbiosis group in a single clade (Rosid I), i.e. they can be traced back to a common ancestor[149]. However, most Rosid I species are not able to form root nodules. Within the Rosid I clade, rhizobial symbioses are supposed to have evolved four times independently, namely three times among legumes and once for *Parasponia*[150]. Similarly, four independent origins have been suggested for actinorhizal symbioses[151] (Fig. 2). Alternatively, it could be assumed that originally, all Rosid I plants were able to enter root nodule symbioses, but many of them lost this ability during evolution. Several sets of physiological and molecular biological data support the concept of a common origin but independent evolution of groups of root nodule symbioses, e.g., there is evidence for conservation of infected cell-specific transcription factors between legumes and actinorhizal plants.

Transcription factors involved in root nodule cell differentiation and their evolutionary implications

As mentioned above, while infection threads can grow through root cortical cells, only cells formed after signal exchange with the microsymbiont can be infected by branching infection thread-like structures, leading to nitrogen fixation in intracellular *Frankia*. The same is true for rhizobial symbioses. Thus, infected cells carry a specific differentiation that allows the stable internalization of, and the nutrient exchange with, a bacterial microsymbiont. Experiments using transgenic plants containing transcriptional fusions of promoters of nodule-specific genes with the GUS reporter gene have hinted at common features of cells infected by rhizobia and *Frankia*, respectively.

A GUS fusion with the promoter of the gene encoding the nodule infected cell-specific symbiotic hemoglobin of *Casuarina glauca* was expressed specifically in the infected cells of nodules of the legume *Lotus corniculatus*[54]. Similarly, a soybean leghemoglobin promoter-GUS fusion was expressed specifically in the infected cells of actinorhizal *Allocasuarina verticillata* nodules[152], although the infected cells are not at morphologically equivalent positions in both types of nodules. Hence, infected cell-specific transcription factors are conserved between legumes and *(Allo-)Casuarina*.

In the same vein, the expression pattern of a GUS fusion of the hemoglobin promoter from *Parasponia andersonii* (Ulmaceae), the only non legume able to enter a symbiosis with rhizobia, was conserved in the actinorhizal *Allocasuarina*[152]. However, in *Lotus*, the *Parasponia* hemoglobin promoter was more active in uninfected than in infected cells of the inner tissue[153]. This result seems to contradict the conservation of infected cell-specific transcription factors. On the other hand, the *Parasponia* hemoglobin promoter is not nodule-specific, but also active during non-symbiotic development, and in nodules it is not only expressed in infected, but in uninfected cortical cells, though at much lower levels[154,155]. Its expression pattern in *Lotus* may be due to signals for non-symbiotic expression.

Altogether, these data suggest that there is conservation of infected cell-specific transcription factors in root nodule-forming plants, suggesting a common origin for the specific differentiation that enabled the stable integration of the microsymbiont. However, with regard to the detailed differentiation of infected cells, preliminary data on GUS fusions with actinorhizal promoters of nodule-specific genes other than (leg-)hemoglobin do not point at conserved transcription factors for specific stages of the development of infected cells, indicating that the mechanisms evolved independently[156]. Another set of data pointing in the same direction is connected to the induction of rubisco activase transcription in *Datisca glomerata* nodules[130]. In nodules of *Alnus glutinosa*, no rubisco activase message could be detected[157]. It is not likely that the regulation of a basic photosynthetic gene as conserved as rubisco activase differs between closely related plants. Therefore, the induction of rubisco activase transcription in *Datisca*, but not in *Alnus* nodules, indicates that different signal transduction pathways have been recruited for the control of nodule development in different groups of actinorhizal plants. These data support the idea that based on the common precondition acquired by the Rosid I ancestor, the nodulation syndrome evolved independently in the different symbiotic subgroups of Rosid I[150,151].

There is evidence indicating a connection between legumes and actinorhizal Casuarinaceae that does not include *Parasponia*. Three hemoglobin genes/gene families are found in symbiotic as well as non-

symbiotic plants[158]. Family III represents truncated hemoglobins similar to those found in bacteria and protozoa[159]. Leghemoglobins as well as the symbiotic hemoglobin from *Casuarina glauca* belong to family II, while the non-symbiotic hemoglobins of legumes and *Casuarina* belong to family I[54,160,161]. In contrast, the hemoglobin found in nodules as well as in vegetative organs of *Parasponia andersonii* is a member of family I; family II hemoglobins have not been identified in *Parasponia*[53,158]. This fact, in combination with the conserved specificity of the respective promoters from legumes and *Casuarina*, may indicate that legumes and actinorhizal higher Hamamelidae (Casuarinaceae, Betulaceae and Myricaceae; Fig. 2) are more closely related to each other than to the other nitrogen-fixing groups[152]. This is supported by the comparison of expression patterns of other genes in nodules[139].

Different groups of root nodule-forming plants

Altogether, there are striking differences among actinorhizal nodules formed by plants from different subclades of Rosid I. The host range of *Frankia* is ostensibly wider than that of rhizobia, including plants from eight different families, which may be due to its ability to fix nitrogen in the free-living state, that resulted in more independence from the host plants. However, there are far more plant genera whose members are able to enter rhizobial, rather than actinorhizal symbioses. Was an actinorhizal symbiosis an impediment to further evolution of the host plant species? In legumes, all nodules wherein bacteroids are retained in infection threads and not internalized via an endocytotic process are formed by tree species. This is particularly obvious in the nodule structure of different members-arborescent, shrubby and herbaceous-of the legume genus *Chamaecrista*[162]. The structure of *Frankia* (Fig. 1) necessitates a symbiosis with persistent infection threads. The internalization of microsymbionts by continuous invagination of the plasma membrane without complete endocytosis has to counteract the turgor of the plant cell, putting high demands on the cytoskeleton, and perhaps requiring some stabilization of the cell walls of infected cells as given in lignified tissues. In contrast, the endocytotic internalization of rhizobia in peribacteroid membranes allows turgor control of the symbiosome by aquaporins in the peribacteroid membranes[163]. In the only intracellular symbiosis between a higher plant and the filamentous cyanobacterium *Nostoc*, the nitrogen-fixing *Gunnera* symbiosis, the microsymbiont is internalized by a complete endocytotic process[164]. While in arbuscular mycorrhizal symbioses, branching fungal hyphae are internalized in root cortical cells in an incomplete endocytotic process like in the case of *Frankia* hyphae; arbuscules only have a life time of a few days[165]. It is tempting to speculate that this restriction, the necessity for persistent infection threads that required a stabilization of the walls of infected cells, impaired the distribution of the actinorhizal symbiosis.

Features required for root nodule symbioses

As mentioned above, phylogenetic data suggest that 50-100 million years ago, the ancestor of the Rosid I clade had acquired a property based on which a root nodule symbiosis could, but not necessarily did, develop[166]. This raises the question about the nature of that property. Root nodule symbioses require (a) the controlled uptake of a microsymbiont in the plant root, (b) the concomitant suppression of plant defense, (c) the stable integration of the microsymbiont into plant cells, and (d) the induction of the formation of a lateral root organ. These processes are already realized in the arbuscular mycorrhizal (AM) symbiosis between roots of plants of all taxa and fungi of the order Glomales[165]. AM symbioses date back at least 400 million years and may even have been a prerequisite to terrestrial plant life[165,167].

Hyphae of AM fungi invade plant cells and form branched structures called arbuscules or hyphal coils, surrounded by the invaginated plant plasma membrane. In some cases, AM fungi can induce the formation of lateral root-like structures, so-called myconodules, on the roots of some tropical trees including those of legume species and one actinorhizal species[168,169]. Myconodules resemble single-lobed actinorhizal nodules but have only a short life-time[169], which might be seen in context with the short life-time of fungal arbuscules in root cortical cells in general.

The analysis of legume mutants deficient in nodulation has shown that rhizobial and AM symbioses involve common components in plant signal transduction. Several legume mutants have been identified that are affected in early stages of both symbioses[170]. Thus, in the evolution of nitrogen-fixing root nodule symbioses, mechanisms that had evolved earlier for fungal symbioses may have been exploited. In this context, the chitinaceous nature of the rhizobial Nod factors has led to the speculation that perhaps,

nitrogen-fixing bacteria have copied fungal signal molecules. However, there is evidence that plants themselves can form Nod factor-like signal molecules[171,172]. In spite of the phylogenetic relationship between both symbioses, *Frankia* Nod factors do not seem to have a structure similar to that of rhizobial Nod factors[61]. Yet, the fact that a fungus, *Penicillium nodositatum*, can exploit the actinorhizal intracellular infection pathway of *Alnus* to form parasitic myconodules[173-175] indicates that *Frankia* Nod factors can also be formed by fungi.

The evidence that the integration of bacteria into plant cells and the induction of a lateral root organ are functionally related suggests that both mechanisms may have evolved concomitantly. The formation of infection threads requires root cortical cells arrested in the G1 phase of the cell cycle to re-enter the cell cycle and get re-arrested in the G2 phase[176]. Hence, bacteria have to induce plant cell cycling prior to infection thread growth and the induction of nodule primordium formation-complete cell cycling-might be a side effect of the induction of infection thread growth. Laplaze *et al.*[69] have suggested that prenodule-like structures, i.e., foci of newly formed cortical cells containing bacteria in branching infection threads (Fig. 4A), have been the origins of root nodule symbioses. In this context, the induction of lateral root organs by the microsymbionts is a later addition enabling the plant to improve the removal of the fixed nitrogen. In all plant families that include nodulating species, except for legumes, the formation of nodule primordia is induced in the pericycle similar to that for lateral root primordia. It has been proposed that the stem-like organisation of legume nodules is a result of their induction in the root cortex rather than the pericycle, and is due to the predisposition of legumes to form lateral root storage organs, a tendency that is not present in other symbiotic plant families[177]. The developmental relationship between lateral roots and legume nodules is confirmed by the fact that some legumes can form intermediates between lateral roots and nodules under certain conditions[178].

Outlook

Further research is needed to understand the evolution of root nodule symbioses. The trait acquired by the ancestor of root nodule-forming plants that formed the basis for the ability to establish a root nodule symbiosis, remains to be identified[149]. Once this trait is known, it will be possible to transfer nitrogen fixing root nodule symbioses to agriculturally important plants of other families. The broader variety of actinorhizal in comparison to legume symbioses implies that in spite of their lower agricultural importance, their analysis might be more promising with regard to finding the common trait than that of legumes. At present the most urgent objectives on the bacterial side of actinorhizal research are the establishment of a transformation method for *Frankia*, the characterization of *Frankia* Nod factors and to find culture conditions for members of *Frankia* Clade III (Fig. 2). On the plant side, many questions require the availability of symbiotic mutants of actinorhizal plants. The only actinorhizal plant that has a short enough generation time to be suited for genetic analysis is *Datisca glomerata* with a generation time of six months[179]. Alternatively, the generation time of the transformable actinorhizal trees, *Casuarina glauca* or *Allocasuarina verticillata*, might be artificially shortened by constitutive expression of the *Arabidopsis* genes *LEAFY* or *APETALA1* which promote flowering[180]. *C. glauca* might be especially suited for this purpose due to its small genome; its genome size is in the range of that of *Arabidopsis*[181].

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