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Symbiotic Signaling in Actinorhizal Symbioses

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Abstract: Actinorhizal symbioses are mutualistic associations between plants belonging to eight angiosperm families and soil bacteria of the genus *Frankia*. These interactions lead to the formation of new root organs, actinorhizal nodules, where the bacteria are hosted and fix atmospheric nitrogen thus providing the plant with an almost unlimited source of nitrogen for its nutrition. It involves an elaborate signaling between both partners of the symbiosis. In recent years, our knowledge of this signaling pathway has increased tremendously thanks to a series of technical breakthroughs including the sequencing of three *Frankia* genomes [1] and the implementation of RNA silencing technology for two actinorhizal species. In this review, we describe all these recent advances, current researches on symbiotic signaling in actinorhizal symbioses and give some potential future research directions.

Keywords: Actinomycete, auxin, legume, mycorrhiza, biological nitrogen fixation, *Rhizobium*, symbiosis, SYMRK.

1. INTRODUCTION

Plant growth is often limited by the amount of available nitrogen. Only some prokaryotes are able to use the dinitrogen that comprises about 80% of the atmosphere as a nitrogen source through a process known as nitrogen fixation. Some plants have therefore evolved the capability to associate with some nitrogen-fixing bacteria to benefit from this source of nitrogen. The most complex of such associations lead to the formation of new root organs called nodules where the bacteria are hosted in specialized plant cells and fix nitrogen. Root-nodule symbioses occur in legumes with rhizobia bacteria and in plants belonging to eight angiosperm families collectively called actinorhizal plants that associate with soil actinomycetes belonging to the genus *Frankia*. Interestingly, molecular analyses have shown a common evolutionary origin of root nodulation symbioses [2]. While the legume-rhizobia symbiosis has received a lot of attention because of the economical importance of several legume species (such as soybean), actinorhizal symbioses have been little studied. However, in most cases, the rates of nitrogen fixation in actinorhizal plants are comparable to those found in legumes and they play very important ecological roles in plant ecosystems.

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Recent studies in legumes have tremendously increased our understanding of the symbiotic signaling molecules and transduction pathways. In the legume-rhizobia interaction, one of the key factors mediating recognition between the plant and the bacteria are lipochitooligosaccharides called Nod factors [3]. The perception of Nod factors induces a series of well-characterized responses in the host plant root [4]. Extensive mutant screenings performed in legumes led to the identification of several loci involved in this signaling cascade, and recently most of the corresponding genes were identified by map-based approaches [4]. In comparison, very little is known about the signaling mechanisms leading to actinorhizal symbioses formation. All actinorhizal plants except *Datisca* are woody shrubs or trees and are therefore recalcitrant to genetic approaches. However, recent progresses including the development of *Frankia* and actinorhizal plants genomics [1,5-7] have opened new avenues to identify the components involved in the symbiotic dialogue in both partners [8]. The aim of this review is to summarize our current knowledge of the signaling pathways involved in pre-infection and infection in actinorhizal symbioses.

2. SYMBIOTIC PREINFECTION SIGNALING MOLECULES

The symbiotic interaction starts by the exchange of symbiotic signals in the soil between the plant and the bacteria. This molecular dialogue involves signaling molecules that are responsible for the specific recognition of the plant host and its endosymbiont.

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2.1. Specificity in Plant-Bacteria Recognition

In general, *Frankia* strains are much more promiscuous than rhizobia and no *Frankia* strain specific to a single host plant species has been described to date [9]. Nevertheless host specificity is present at different levels and a broad correspondence can be defined between the phylogenies of *Frankia* strains and actinorhizal plants Fig. (**1**). *Frankia* strains can be grouped in three related clusters, and members of each cluster show distinct host ranges [10]. Strains belonging to cluster I (="Alnus" cluster) were isolated from plants belonging to the Fagales clade and show a high level of host specificity, as they are only able to interact with plants belonging to this clade. A subgroup within this cluster, the "Casuarina" strains, appears to have evolved even higher levels of specificity as members of this subgroup are only able to nodulate two *Casuarinaceae* genera, *Casuarina* and *Allocasuarina* in natural conditions [11]. Strains belonging to cluster III (="Elaeagnus" cluster) have a wider host range and can interact with plants belonging to five families within two distant plant clades, the Rosales and the Fagales. The third group of *Frankia* (= cluster II or "uncultured") has not yet been isolated in pure culture, but cross-inoculation experiments performed with crushed nodules also suggest a broad host range for members of this cluster that nodulate plants belonging to 4 families within the Rosales and Cucurbitales clades. On the plant side, most actinorhizal species are nodulated by few *Frankia* strains belonging to the same cluster but a few genera like *Myrica* (*Myricaceae*), *Ceanothus* (*Rhamnaceae*) and *Gymnostoma* (*Casuarinaceae*) are highly promiscuous and accept a wide variety of *Frankia* strains from distinct clusters [9].

The existence of these cross inoculation groups is expected to be the result of specific recognition events taking place at the molecular level. Different families of signal molecules or alternatively different chemical substitutions on the same chemical backbone as in the rhizobia/legume symbiosis would result in specific recognition by receptors of the host plant of a particular group of symbionts [12]. Promiscuous host plants like *Myrica* or *Gymnostoma* would recognize a common feature whereas specific recognition of a special decoration would be needed in the case of narrow-range species like *Casuarina*. Strains that are able to nodulate highly divergent plant species would be able to synthesize multiple recognition signals, as is the case for the broad host spectrum *Rhizobium* strain NGR234 [13].

In addition to *Frankia*, actinorhizal nodules can accommodate other actinomycetes that cannot infect the host plant on their own [14, 15] and also saprophytic fungi like *Penicillium nodositatum* [16-18]. Interestingly *Gymnostoma*, the "promiscuous" genus of the *Casuarinaceae* family, is also able to form nodules that contain arbuscular mycorrhizal fungi [19]. The induction of actinorhizal nodules and the capacity of actinorhizal plants to accommodate microbes intracellularly seems therefore to be less strictly controlled than for legume nodules, suggesting that a wide variety of organisms are able to mimic the symbiotic signaling molecules recognized by plant receptors.

2.2. Plant Signals: a Role of Flavonoids?

Flavonoids are a diverse group of secondary metabolites derived from the phenylpropanoid pathway. They are widespread throughout the plant kingdom, from mosses to angiosperms, where they are involved in a wide range of biological processes such as flower and fruit pigmentation, UV radiation protection, pollen germination, cell cycle regulation, and as signal and defense compounds in interactions with beneficial and pathogenic micro-organisms [20,21]. They are involved in early steps of arbuscular mycorrhizal symbiosis [22] and are supposed to be implicated in cyanobacterial-plant symbiosis [23]. In the nitrogen-fixing symbiosis between legumes and rhizobia, flavonoids are involved as plant chemotactic signals to rhizobia and as activators of *nod* gene expression, which lead to the biosynthesis of lipochitooligosaccharides (Nod Factors), the bacterial symbiotic signals [24].

In actinorhizal symbioses, some evidence of chemoattraction and proliferation of *Frankia* bacteria has also been reported in the rhizosphere of several species [23], but direct

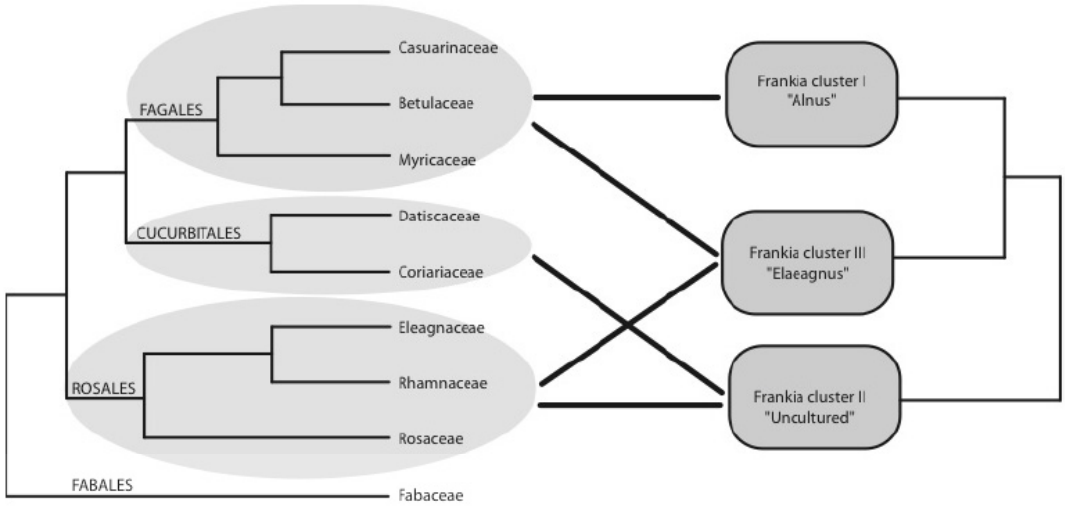

Fig. (1). Phylogenetic relationships between plant families containing actinorhizal species (grey background) and the corresponding *Frankia* strains. Black lines represent compatible interactions leading to nodulation.

evidence of flavonoids as early signals exchanged between plant and *Frankia* is still lacking. Several studies have reported an implication of these molecules during plant-*Frankia* interactions. Benoit and Berry [25] have reported that flavonoid-like compounds extracted from *Alnus rubra* seeds affect nodulation. These results were confirmed by Hughes *et al.* [26] who observed that flavonols (e.g., quercetin and kaempferol) contained in *Alnus glutinosa* root exudates were able to enhance or decrease the level of nodulation. More recently, the analysis of a *C. glauca* root and nodule expressed sequence-tag (EST) database led to the identification of several genes involved in the flavonoid biosynthesis pathway [5]. Interestingly, an important accumulation of transcripts corresponding to a putative isoflavone reductase gene was observed shortly after inoculation by *Frankia* and is associated with the presence of specific isoflavonoid compounds in early inoculated plants, thus suggesting a possible role of isoflavonoids as signals in the *C. glauca-Frankia* symbiosis (our laboratory, unpublished results). Altogether, these data suggest that flavonoids act as plant signals in actinorhizal symbioses and might determine the symbiotic specificity in actinorhizal symbioses. In order to test that, we are currently using a RNA interference (RNAi) strategy to generate plants impaired in flavonoid biosynthesis through down regulation of a gene encoding a chalcone synthase, the first enzyme in this biosynthetic pathway, and look at the effects on symbiosis formation.

2.3. Bacterial Signal Molecules

The identification of Nod factors in the 1990s was a huge step in the elucidation of the molecular dialogue between rhizobia and legumes and a strong focus on Nod factor research yielded a huge amount of information on the way Nod factors are synthesized, exported and recognized by plant receptors, and the resulting signaling cascades and gene networks implicated in the plant cell. Until recently, Nod factors were considered to be the universal signaling molecule enabling the recognition of rhizobia by legumes. Surprisingly, the sequencing of the genome of a photosynthetic *Bradyrhizobium* revealed that this strain lacks the canonical *nod* genes needed to synthesize Nod factors, and does not need Nod factors to nodulate its host, the tropical legume *Aeschynomenae sensitiva* [27], proving that in this case the symbiotic recognition is mediated by a different kind of molecule. This view was confirmed on *Lotus japonicus* where functional nodules were obtained in mutant plants inoculated with bacteria unable to synthesize Nod factors [28]. At least two signaling pathways leading to nodulation are supposed to exist in legumes, an evolutionarily recent one dependent on Nod factors needed for epidermal and cortical responses such as root hair curling and the formation of infection threads, and an ancestral one independent of Nod factors acting at later stages when rhizobial cells become internalized in the cortical cells [28].

In the case of actinorhizal plants infected through the intracellular infection pathway Fig. (**2**), the first visible plant response to *Frankia* is an extensive deformation of root hairs that occurs in the zone of root hair elongation within the first 24 h. Root hair deformation also occurs in response to supernatants of *Frankia* cultures even at dilutions of 10^{-5} [29]. In contrast to rhizobia that require the presence of flavonoids produced by the host plant roots to synthesize Nod factors, the production of *Frankia* root hair deforming factor (RDF) is not affected by the presence of root exudates in the culture media [29].

Attempts were made to isolate the *Frankia* RDF using similar approaches than the ones used to identify rhizobial Nod factors [30]. Partial purification of the RDF was achieved using a root hair deformation assay on *Alnus* [31]. This *Frankia* RDF was found to be heat stable, hydrophilic, resistant to a chitinase treatment and relatively small [31]. The last two properties are not shared with lipo-chito-oligosaccharidic Nod factors, suggesting a different chemical nature for the *Frankia* RDF [31]. In rhizobia, enzymes involved in the biosynthesis of Nod factors are encoded by the *nod* genes that are clustered in specific zones called "symbiotic islands". The recent sequencing of three *Frankia* genomes revealed the lack of the core *nodA* gene, thus suggesting that *Frankia* might not be able to synthesize signal molecules similar to nod factors [1]. Moreover, *nod* genes homologues in *Frankia* are only distantly related to rhizobial nod genes, are not clustered within a symbiotic island and their expression is not induced under symbiotic conditions [1,7] further supporting the idea that they are not involved in synthesizing a symbiotic Nod-like factor.

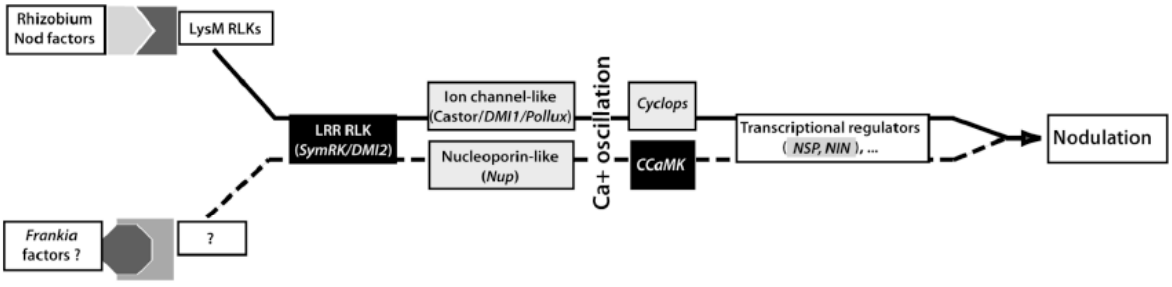

Fig. (2). Simplified model of the Nod symbiotic signaling pathway. In the legume-rhizobia symbiosis, Nod factors synthesized by rhizobia are recognized by LysM receptors activating a calcium-dependent signaling pathway, the Nod pathway that leads to nodulation. In actinorhizal symbioses, a functional equivalent of the rhizobial Nod factor (not yet identified) is supposed to bind to a receptor and activate a Nod-like signaling pathway. Some molecular determinants of this putative signaling cascade have been characterized in actinorhizal plants and shown to play to similar role as their orthologs in legumes (SYMRK, CCaMK - black boxes). Grey boxes correspond to genes identified among *C. glauca* ESTs that may be part of this signaling pathway but are not yet characterized.

2.4. Future Strategies to Find Signaling Molecules

Numerous attempts have been made to purify the *Frankia* RDF, but until now its chemical nature remains elusive, in part due to the inherent difficulties of the root hair deformation assay. Furthermore, it has been shown that root hair deformation is induced by non-compatible *Frankia* strains [29] and even by numerous non-symbiotic soil bacteria [32], raising the question on whether the RDF is central to the symbiotic process. In addition, the root hair deformation assay is not suitable for most actinorhizal plants since only Fagales are infected through the intracellular infection pathway.

An alternative or complementary approach to the root-hair deformation bioassay is the establishment of a biological assay based on plant genes that are transcribed specifically in response to the interaction with *Frankia*. This approach is particularly suited for *C. glauca*, where transgenic plants expressing promoter:GUS fusions can be generated using *A. tumefaciens* [33] and promoters of several symbiotic genes are well characterized [34]. Furthermore, large homogeneous populations can be generated by clonal propagation [35]. In an attempt to find promoters activated specifically during the pre-infection steps, we looked for genes that are strongly expressed prior to bacterial infection in Legumes. Among these are *MtEnod11* and *PsEnod12B*, two genes from *M. truncatula* and *Pisum sativum* respectively, strongly transcribed in infected cells but also a few hours after rhizobial inoculation and after incubation with rhizobial Nod factors. In *C. glauca* a strong activation of these promoters is observed in infected cells, but not prior to infection or after the incubation with the supernatants of *Frankia* cultures, suggesting that the activation of *ProMtEnod11* and *ProPsEnod12B* in cells infected by symbiotic bacteria is conserved in actinorhizal plants, whereas this is not the case for the NF-dependent preinfection expression [34,36].

In the past few years, global approaches allowed the generation of huge amounts of data. On the plant side, EST libraries were developed in *C. glauca* [5] providing extensive lists of genes with a potential implication in the actinorhizal symbiosis. Among the candidate genes that could be used as symbiotic markers of pre-infection events is *CgNIN,* a gene from *C. glauca. CgNIN* encodes a putative transcription factor showing 63 % similarity with *LjNIN*, a gene from *L. japonicus*. *LjNIN* is essential to the symbiosis and expressed a few hours after rhizobial inoculation [37]. Quantitative PCR experiments have shown a strong expression of *CgNIN* in *C. glauca* nodules (Our laboratory, unpublished results). Plants carrying a *ProCgNIN::GUS* construct are being generated and will be used to isolate diffusible signaling molecules produced by *Frankia* if *ProCgNIN* is strongly and specifically activated at pre-infection stages. Transcriptome studies were performed on *C. glauca* and *A. glutinosa* (Hocher *et al.*, manuscript in preparation) the global analysis of genes expressed in the early stages of the interaction will probably provide more candidate genes suitable for this kind of approach.

On the bacterial side, the sequencing of three *Frankia* genomes [1] and the subsequent analysis of *Frankia* secretome, transcriptome and proteome [7,38,39] have also yielded vast amounts of data and enabled the identification of several proteins that may play a role in the symbiotic signaling process. Unfortunately, performing a functional analysis on the corresponding genes remains a very difficult task in the absence of a stable transformation protocol for *Frankia* [40]. Another option is to use the fact that some non-*Frankia* bacteria isolated from actinorhizal nodules seem to be able to mimic *Frankia* symbiotic signals and to induce nodule formation in the host plant. Some of these bacteria are actinomycetes and can be genetically manipulated. A genetic screen could be performed on this bacteria to identify genes involved in the biosynthesis of symbiotic signals.

3. PERCEPTION AND TRANSDUCTION OF THE PREINFECTION SYMBIOTIC SIGNAL(S)

In actinorhizal symbioses, due to the lack of genetic tools on both the bacterial and plant sides, basic mechanisms involved in perception and transduction of *Frankia* signals are still poorly understood. In contrast, in the legume-rhizobia symbiosis, signal exchanges between the two partners have been well studied. The perception of compatible rhizobial Nod factors induces a series of responses in the root including root hair curling, ion flux changes, calcium spiking, membrane depolarization, cytoskeletal modifications and activation of cell division [41,42]. Genetic studies using extensive mutant screening and map-based cloning on the model legume species *Lotus japonicus* and *Medicago truncatula* are unraveling the molecular mechanisms of the symbiotic signaling pathway. On the top of the signal transduction pathway are 2 receptor-like serine/threonine kinases with a LysM domain (LjNFR1&5/MtNFP&MtLyk). LysM-RLKs are required to trigger the early responses to Nod factors such as root hair deformation, Ca^{2+} influx and Ca^{2+} oscillations [43]. Point mutations and domain swapping experiments suggest that the recognition of Nod factors is mediated by the LysM domains present in these proteins [44]. Other genes acting downstream Nod factors perception include the putative cation channels *DMI1*/*Castor/Pollux* [45,46], the LRR receptor kinases *DMI2/SYMRK* [47,48], the calcium/calmodulin dependant kinases *DMI3/CCaMK*, the nucleoporins *Nup133* and *Nup85* and *IPD3/CYCLOPS* and transcriptional regulators such as members of the GRAS family *NSP1* and *NSP2*, nodule inception (*NIN*) and *AP2-ERF* (*ERN*) transcription factors. Cytokinin receptors *CRE1/HK1* have also recently been shown to be specifically involved in the nodule organogenesis [49,50].

As mentioned before, the universality of the Nod factors dependent signaling pathway has been reassessed by some recent findings on photosynthetic bradyrhizobia [27] and by genetic engineering of *Lotus* mutants [28], suggesting the existence of alternative signaling pathways in the legume-rhizobia symbiosis.

3.1. A Common Symbiotic Signaling Element Required for Arbuscular Mycorrhization, Actinorhizal and Legume Nodulation

Analysis of the symbiotic legume mutants in the context of arbuscular mycorrhization (AM) has lead to the interesting finding that several genes are also required for AM formation [51]. This discovery suggests the existence of a

common symbiotic set of genes that are shared among plant endosymbioses. At present, 7 common genes (*SYMRK/ DMI2, DMI1/Pollux & Castor, Nup85, Nup133, CCaMK/ DMI3 and IPD3/CYCLOPS*) are required for both rhizobial and AM symbioses [52]. AM symbioses are formed between most land plant species (about 80%) and fungi of the phylum Glomeromycota. The AM symbiosis is more ancient than nitrogen-fixing root nodule symbioses. It can be traced back to at least 400 million years [53] while root nodule symbioses appeared 50-100 years ago [54]. Although, legume and actinorhizal nodules differ in their ontogeny and structure, studies based on *rbcL* gene sequence analysis have shown that all plants able to enter a root nodule symbiosis belong to the same clade (Fabids) suggesting that their common ancestor evolved a predisposition for symbiosis 70 MY ago [2]. The molecular bases of this predisposition are so far unknown, but at least part of the genetic program leading to nodulation might be derived from the more ancestral program allowing most extant plants to accommodate AM fungi [54].

Recent results have shown that actinorhizal symbioses also depend on at least one element of the common Nod and Myc signaling pathway. The receptor kinase SYMRK has been isolated from the actinorhizal specie *C. glauca*. Knockdown of *SYMRK* by RNA interference led to inhibition of nodulation and mycorrhization when plants were inoculated with compatible *Frankia* bacteria and *Glomus intraradices* fungi respectively [8]. In addition, we demonstrated that *CgSYMRK* is functionally equivalent to legume *SYMRK* by successfully complementing the *ljsymrk* mutant for both nodulation and mycorrhization [8]. Similar results were obtained with the *SYMRK* gene from another actinorhizal plant, *Datisca glomerata* [55]. *SYMRK* is therefore a common signaling element shared between AM, legume-rhizobia and actinorhizal symbioses, supporting the hypothesis that the capacity to accommodate N_2-fixing bacteria evolved at least partly from the more ancient AM genetic program. Complementation studies of *ljsymrk* using *SYMRK* genes isolated in non-nodulating species have shown an interesting feature: all the genes tested so far are able to complement the lack of mycorrhization; genes where two LRR motifs are present (= those from rice and tomato) are unable to complement the nodulation, but genes with three LRR motifs (= the ones from *Tropaelum majus* and all nodulating plants) [55]. The appearance of this additional LRR motif in SYMRK might be one of the evolutionary events that enabled members of the Fabid clade to accommodate symbiotic N_2-fixing bacteria.

3.2. Beyond SYMRK: is there a Signaling Pathway Shared by All Endosymbioses?

Since SYMRK plays a central role in AM, legume and actinorhizal symbioses, other genes belonging to the Nod signaling pathway may also be required for the three symbioses. As mentioned above, legume mutant analyses revealed the existence up to now of 7 genes involved in both rhizobial and AM symbioses. These seven genes are also probably involved in the actinorhizal signaling. An analysis performed on *C. glauca* and *A. glutinosa* EST databases allowed the identification of several genes showing high percentages of similarity with legume genes involved in the nod signaling pathway Fig. (**2**); [5]. Moreover, a global expression analysis performed using DNA chips on nodules and non infected roots demonstrated that most of these genes are expressed in roots and nodules, with a higher expression in nodules compared to roots (Hocher *et al.*, manuscript in preparation). An in-depth characterization of two of these genes (=*CgCCaMK* and *CgNIN*) is underway. In legumes, CCamK/DMI3 is involved in the transduction of Ca^{2+} oscillations that have been shown to be a key component of the Nod signaling pathway [42,56]. The potential involvement of *CgCCaMK* in the interaction between *C. glauca* and *Frankia* suggests that Ca^{2+} oscillations may also occur during the actinorhizal association. To what extent the role of genes intervening at the very early stages is also conserved between the three endosymbioses remains to be elucidated. In legumes the recognition of compatible bacteria is mediated by LysM-RLKs [43]. Genes similar to legume LysM-RLKs can be found among *C. glauca* and *A. glutinosa* EST libraries but their involvement in the perception of *Frankia* has yet to be examined.

3.3. Looking for Signal Transduction Elements Specific to the Actinorhizal Program

Despite the fact that root nodule symbioses share common genetic bases, there are probably unique molecular elements characteristic of actinorhizal symbioses. *Frankia* is phylogenetically distant from rhizobia and is able to interact with 8 angiosperm families, whereas rhizobia can nodulate species belonging only to the *Fabaceae* family (with the exception of *Parasponia* sp). Moreover, the symbiotic signal molecule(s) in *Frankia* seem to be chemically different from Nod Factors and would therefore be perceived by different receptors. The recent availability of genomic tools and databases in *C. glauca* and *A. glutinosa* (Hocher *et al.*, manuscript in preparation) will provide new means to identify genes acting in the symbiotic signaling pathway in actinorhizal plants.

4. SYMBIOTIC SIGNALING DURING PLANT CELL INFECTION

Two modes of infections of actinorhizal plants by *Frankia* have been described: intracellular (root hair) infection and intercellular infection Fig. (**3**). During intracellular infection, root hairs become deformed in response to *Frankia* signals. Trapped *Frankia* hyphae penetrate and grow basipetally inside the root hair while being encapsulated by the host-derived membrane and a thin cell wall Fig. (**3**); [57]. *Frankia* remains intracellular while it progresses in the root cortex and invades first some prenodule and then nodule cells Fig. (**3**). During intercellular infection, root hairs do not deform or branch, a prenodule is not formed and growth of *Frankia* in infected roots is through intercellular spaces. *Frankia* hyphae become intracellular when they invade the young nodule primordium Fig. (**3**); [57].

4.1. Conservation of Infection-Related Gene Regulation between Rhizobial and Actinorhizal Symbioses

Little is known about the mechanisms that control the infection of plant cells by endosymbiotic microorganisms. *Cg12*, a symbiotic gene characterized in *C. glauca* encoding

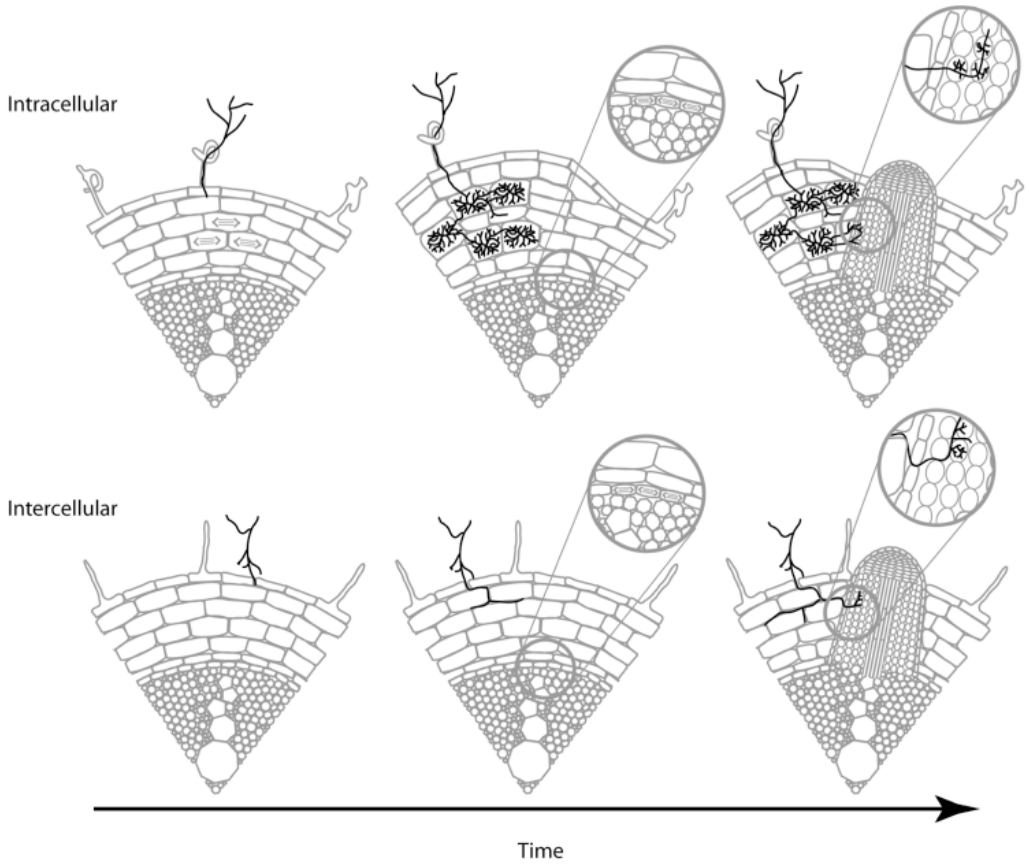

Fig. (3). Description of the two modes of infection of actinorhizal plants.

a subtilisin-like serine protease, is specifically expressed after inoculation with *Frankia* [58]. Using *C. glauca* plants expressing *ProCg12::GUS* and *ProCg12::GFP* fusions we showed that the *Cg12* promoter is active in *Frankia*-infected root hairs, and in root and nodule cortical cells containing *Frankia* hyphae [59]. *Cg12* is specifically expressed during the infection by *Frankia* and is not induced during AM formation in *C. glauca* or in response to *Frankia* diffusible signals [59]. When the *ProCg12::GUS* and *ProCg12::GFP* constructs were introduced in the model legume *M. truncatula*, the *Cg12* promoter was activated exclusively in cells infected by *Sinorhizobium meliloti*, indicating that both symbioses share common gene regulation mechanisms during bacterial intracellular infection [60]. Similarly, the expression of the legume *MtEnod11* and *PsEnod12B* genes during endosymbiotic infection is conserved in *C. glauca* [34,36]. Recently, the *ProCg12::GUS* and *ProCg12::GFP* constructs were also introduced in *Discaria trinervis*, an actinorhizal plant belonging to the Rosale clade and infected through the intercellular pathway. Interestingly, activation of the *Cg12* promoter was found in cells surrounding the intercellular *Frankia* hyphae and in nodule cells infected intracellularly by the bacteria (our laboratory, unpublished results). This suggests that the intercellular and intracellular infection pathway share molecular mechanisms and that the bacterial signals involved could be similar.

Cg12 is an excellent marker of symbiotic bacterial infection in plants belonging to three symbiotic clades and could be used to isolate the signaling molecules involved in bacterial infection using a strategy similar to the one used to identify lyso-phosphatidyl choline as a key signaling molecule involved in the AM symbiosis [61].

4.2. A Role for Auxin during Plant Cell Infection by *Frankia*?

Frankia produces phytohormones which could play a role in the symbiotic interaction. Natural auxins such indole-3-acetic acid (IAA) and phenylacetic acid (PAA) are produced by *Frankia in vitro*. Wheeler and colleagues [62] demonstrated that IAA was a product of tryptophan metabolism in *Frankia*, however its rate of synthesis and catabolism were apparently slower than in *Rhizobium* thus contributing a small amount to IAA content of the nodule. PAA has been suggested to play a special role in nodule formation in *Alnus glutinosa* [63]. Phenylacetic hopanetetrol is a *Frankia* specific lipid, present in the envelope of vesicle. Hammad and colleagues [63] proposed that PAA only bound by an ester link to the hopanetetrol basic unit would be released and affect nodule formation. Various strains of *Frankia* were shown to release PAA in culture medium at a concentration of about 10^{-5} to 10^{-6} M [63]. The hopanetetrol basic unit would be supplied by the remobilization of neosynthesized hopanoids in *Frankia* during the early stages of symbiosis and, later, senescent *Frankia* could remobilize PAA to help maintain permanent synthesis of nodule tissues [63].

Recent investigations from our laboratory have linked auxin with infection of plant cells by *Frankia*. The auxin influx inhibitor 1-naphtoxy acetic acid (1-NOA) perturbs

actinorhizal nodule formation suggesting that auxin influx carriers play a role in the infection process [64]. Two auxin influx carrier homologues (AUX1-LAX) genes were isolated in *Casuarina glauca* and were found to share high levels of deduced protein sequence identity with Arabidopsis AUX-LAX proteins. Through complementation of the Arabidopsis *aux1* mutant, only one (*CgAUX1*) was functionally equivalent to AUX1 [64]. Studies in the spatial and temporal expression pattern of the *ProCgAUX1::GUS* reporter construct in *C. glauca* found that *CgAUX1* is expressed in plant cells infected by *Frankia* throughout the course of actinorhizal nodule formation [64]. In addition, *CgAUX1* expression was also detected in the vascular tissues in non-infected and infected roots and in nodules [64]. Altogether, these results indicate that CgAUX1 is involved in the intracellular infection of *C. glauca* cells by *Frankia*. Interestingly, *CgAUX1* is not expressed in *C. glauca* cell infected by the endosymbiotic AM fungus *Glomus intraradices* [65]. This indicates that the expression of *CgAUX1* is not a general response to intracellular infection by an endosymbiotic microorganism. It was hypothesized that *CgAUX1* expression allows the entry and perception of *Frankia*-produced auxin and restricting it to infected plant cells [65].

More recently, we found that *C. glauca* nodules contain more auxin (both IAA and PAA) than non-infected roots. Using immunolocalization techniques, these molecules were found to accumulate specifically in *C. glauca* nodule cells infected by *Frankia* [66]. This is in agreement with the recent finding that the auxin-responsive *EuNOD-ARP1* gene in *Frankia*-infected cells in *Eleagnus umbellata* actinorhizal nodules [67]. We found that this specific accumulation of auxin is driven by *in planta* auxin production by *Frankia* and the expression pattern of plant auxin influx and efflux carriers that ensure that auxin is strictly localized to the infected cells. Indeed, we found that a PIN1-like auxin efflux carrier was present in uninfected cells surrounding *Frankia*-infected cells in *C. glauca* nodules Fig. (**4**); [66]. This arrangement limits the auxin response to plant cells that are infected by *Frankia*. Nodules on *Casuarina* plants treated with 10 □M NPA appeared smaller in size compared to non-treated NPA plants (our laboratory, unpublished data) confirming the involvement of auxin efflux carriers in nodule formation. All together, these data point to a role of auxin in the infection of plant cells by *Frankia* in actinorhizal symbioses. This auxin in infected cells (alone or in synergy with a symbiotic signal) would induce changes in gene expression, cell metabolism or other things to promote the establishment of the intracellular symbiosis. For instance, it was speculated that auxin could induce genes encoding cell wall remodeling enzymes necessary for infection by *Frankia* [64,65].

5. CONCLUSIONS

Actinorhizal plants play an important ecological role in plant communities and are widely used in agroforestry, in reforestation programs, for soil rehabilitation and as a source of timber or firewood in tropical and subtropical countries [35]. Their capacity to act as pioneer species and colonize poor or degraded soils comes in large part from their ability to enter nitrogen-fixing root nodule symbioses with the soil actinomycete *Frankia*. A better understanding of the signaling pathways leading to the establishment of these symbioses could lead to the optimization of inoculation techniques and could therefore have a huge impact on the use of these plants.

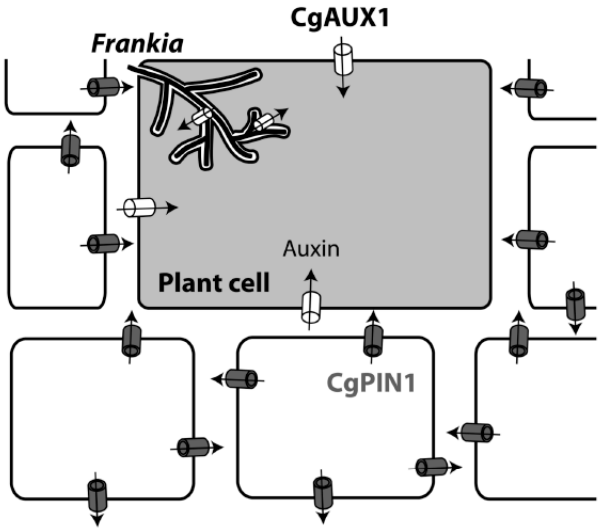

Fig. (4). Model of auxin transport in symbiotic cortical tissues of *C. glauca* nodules. An auxin influx carrier (CgAUX1) is produced in plant cells infected by *Frankia* [64] and a PIN1-like auxin efflux carrier is present in uninfected cells surrounding the infected cells [66]. Computer simulations indicate that this specific pattern of transporters activity leads to auxin accumulation in infected plant cells. This was confirmed by auxin immunolocalization.

Currently, little is known about the signaling events that lead to actinorhizal symbioses formation. However, tremendous progress happened in recent years and new tools such as *Frankia* genomes and transcriptomes [1,7] and the availability of functional genomics tools in actinorhizal plants [5,6,8,55] are opening new possibilities to identify and characterize the genes involved in signaling between the two partners during actinorhizal symbioses. New strategies are being developed to identify and isolate *Frankia* symbiotic diffusible symbiotic signals while new components of the perception and transduction pathways are being characterized in the actinorhizal plants such as *C. glauca*. This should hopefully allow us to decipher the signal exchange between the two partners. Comparing these signaling mechanisms with those involved in legume-rhizobia and AM symbioses should shed a new light on the evolution of endosymbioses that are important contributors to plant nutrition.

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