

Signalling in actinorhizal root nodule symbioses

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Abstract Plants able to establish a nitrogen-fixing root nodule symbiosis with the actinobacterium *Frankia* are called actinorhizal. These interactions lead to the formation of new root organs, called actinorhizal nodules, where the bacteria are hosted intracellularly and fix atmospheric nitrogen thus providing the plant with an almost unlimited source of nitrogen for its nutrition. Like other symbiotic interactions, actinorhizal nodulation involves elaborate signalling between both partners of the symbiosis, leading to specific recognition between the plant and its compatible microbial partner, its accommodation inside plant cells and the development of functional root nodules. Actinorhizal nodulation shares many features with rhizobial nodulation but our knowledge

on the molecular mechanisms involved in actinorhizal nodulation remains very scarce. However recent technical achievements for several actinorhizal species are allowing major discoveries in this field. In this review, we provide an outline on signalling molecules involved at different stages of actinorhizal nodule formation and the corresponding signalling pathways and gene networks.

Keywords Actinobacteria · Biological nitrogen fixation · Symbiosis · Nodulation factors · Nodulation · Actinorhizal species · Microbe-host signalling

Introduction

To cope with the rarity of assimilable nitrogen, a relatively small group of plants is able to form efficient symbiotic associations with soil bacteria capable of converting atmospheric dinitrogen into ammonium which can be used by plants thus covering their nitrogen needs. N_2 -fixing associations are restricted to four closely related orders: Fabales, Fagales, Cucurbitales and Rosales, all belonging to the clade of Fabids. These plants have developed highly sophisticated systems for housing bacterial diazotrophs in specialized root organs, called nodules. Two kinds of associations lead to the formation of root nodule symbiosis (RNS): those involving most legumes and

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Parasponia with proteobacteria collectively called rhizobia, and those involving a phylogenetically diverse group called actinorhizal plants able to interact with the actinobacteria *Frankia*. If the legumes/rhizobia symbiosis is largely known, the symbiotic association between *Frankia* and actinorhizal plants still remains poorly understood. However, phylogenomic studies confirmed that legumes and actinorhizal plants evolved from a common ancestor characterized by a “predisposition” to form symbiotic root nodules (Soltis et al. 1995; Doyle 2011; Griesmann et al. 2018) and actinorhizal symbioses emerged thus recently as original systems to explore developmental strategies to form nitrogen-fixing nodules.

Actinorhizal symbioses involve over 200 perennial species, mainly trees or shrubs with long generation times (Wall 2000) and only few of them are cultivated. In contrast to most rhizobia, the nitrogen-fixing symbiotic partners *Frankia* spp. are able to fix atmospheric nitrogen in soils or in symbiosis (*in planta*). Molecular phylogenetic approaches have identified four major *Frankia* lineages that have distinct plant host ranges (Tisa et al. 2016). However *Frankia* remains recalcitrant to stable genetic transformation making actinorhizal symbiosis difficult to study. The recent development of genetic transformation protocols for several actinorhizal species and huge amount of data generated with omics approaches have greatly expanded our knowledge on the interaction between *Frankia* and actinorhizal plants (Svistoonoff et al. 2014; Tisa et al. 2016; Gherbi et al. 2018). In this review, we report an inventory of the main recent results related to signalling molecules and gene networks involved in actinorhizal signalling.

Pre-infection signalling

The establishment of symbiotic relationships requires communication between the partners leading to a specific interaction. This recognition is a crucial step that is necessary for the selection of compatible microbes and to avoid entry of the numerous pathogens present in the soil. In most legumes the early signalling events involve the secretion of flavonoids by the host plant which act as chemotactic signals to rhizobia and induce the synthesis of lipochito-oligosaccharides called the Nod factors (NFs) that signal back to the plant (Oldroyd 2013).

Chemoattraction and proliferation of *Frankia* bacteria was also reported in the rhizosphere of different actinorhizal species (Vessey et al. 2004) but direct evidence of flavonoids as early plant signals is still lacking. The presence of host plant root exudates increases the growth of *Frankia* and favours the infection and nodulation process (Gabbarini and Wall 2008; Gabbarini and Wall 2011; Beauchemin et al. 2012; Ktari et al. 2017a). Among the molecules found in root exudates from actinorhizal plants are flavonoids, which were shown to have strong impact on nodulation of *Casuarina glauca* (Auguy et al. 2011; Abdel-Lateif et al. 2013). Moreover, flavonoids extracted from seeds of *Myrica gale* (Fagales) were shown to promote *Frankia* growth and nitrogen fixation only in compatible strains (Popovici et al. 2010, 2011).

On the bacterial side, several studies have investigated the presence of functional equivalents of rhizobial NFs in *Frankia*. In rhizobia, NF biosynthesis is dependent on *nodABC* genes (Masson-Boivin et al. 2009). Regarding *Frankia*, it was shown more than two decades ago that *Frankia alni* DNA cannot complement rhizobial nod mutants (C  r  monie et al. 1998). The absence of close homologs of *nodABC* in this *Frankia* strain was demonstrated when the first *Frankia* genomes became available (Normand et al. 2007). Similar results were found for 35 other sequenced genomes (Tisa et al. 2016). Distant homologs of *nodB* and *nodC* were often detected in those genomes but at least in *F. alni*, the expression of these genes is not induced under symbiotic conditions (Alloisio et al. 2010). Chitin oligomers similar to rhizobial NFs are not detected in *F. alni* culture supernatant and purified rhizobial NFs fail to elicit the symbiotic responses in *Alnus glutinosa* and *C. glauca* (Ghelue et al. 1997; C  r  monie et al. 1999; Svistoonoff et al. 2010; Chabaud et al. 2016). Taken together these observations suggest that at least in the case of *C. glauca* and *A. glutinosa*, early actinorhizal signalling is not dependant on canonical *nodABC* genes or molecules closely related to rhizobial NFs. Canonical *nodABC* genes have however recently been found in the genome of one isolated strain, *Frankia* sp. NRRL B-16219 (Ktari et al. 2017b) and two uncultured *Frankia* strains: *Candidatus Frankia datisc  e* Dg1 and *Candidatus Frankia californiensis* Dg2 (Persson et al. 2015; Nguyen et al. 2016). As in rhizobia, *nodABC* genes from *Candidatus F. datisc  e* Dg1 are

arranged in operons and are expressed in *Datisca glomerata* nodules (Persson et al. 2015). Unfortunately the presence of lipo-chito-oligosaccharides in *Frankia* exudates or in *D. glomerata* nodules has not been yet demonstrated and questions still remain regarding the hypothetical role of *Frankia nodABC* genes in actinorhizal symbiotic signalling.

Molecules potentially involved in symbiotic signalling have however been described in several *Frankia* strains. Root Hair Deformation Factors (RHDF) have been characterized in *F. alni* and *Frankia casuarinae* using the characteristic symbiotic deformation of plant root hairs observed few hours after the inoculation of *Alnus sp.* or *Casuarina sp.* respectively (Prin and Rougier 1987; Ghelue et al. 1997; C er emonie et al. 1999; Gabbarini and Wall 2011; Cissoko et al. 2018). RHDF is a relatively small (< 3 kDa), hydrophilic molecule that is resistant to heat and to a chitinase treatment, but its precise chemical structure remains unknown (C er emonie et al. 1999; Cissoko et al. 2018). Diffusible factors potentially involved in the interaction between *Frankia discariae* and *Ochetophila trinervis* (= *Discaria trinervis*) were also partially purified and interestingly these factors also act as RHDF on the non-host plant *Alnus acuminata* (Gabbarini and Wall 2008; Gabbarini and Wall 2011). In *C. glauca*, factors able to induce the expression of the symbiotic gene *NIN* (=NINA factors) were recently described. *NIN* encodes a transcription factor playing a crucial role in nodulation and importantly, it is expressed at pre-infection stages in legumes and actinorhizal plants in response to bacterial signalling molecules (Schauer et al. 1999; Radutoiu et al. 2003; Hocher et al. 2011; Clavijo et al. 2015). While rhizobial NFs are amphiphilic chitin-based molecules, NINA, like RHDF, is hydrophilic and resistant to chitinase (Chabaud et al. 2016). NINA shares many properties with RHDF but has apparently a different size (Cissoko et al. 2018).

Signalling related to infection and organogenesis

In actinorhizal plants two modes of infection have been described: intracellular (root hair) infection and intercellular infection. Intracellular infection is preceded by root hair deformation induced by RHDF secreted by *Frankia* and triggers limited cell-division

in the cortex leading to the formation of the so-called prenodules. At this step nitrogen fixation already occurs but prenodules are not apparent anymore once the mature nodule develops (Laplaze et al. 2000a). Intercellular infection does not involve root hair deformation or prenodules and *Frankia* filaments penetrate inside the root intercellularly via the apoplastic interface between epidermal cells. *Frankia* filaments are internalized at later stages inside nodule cortical cells. Intracellular infection is found in actinorhizal Fagales and intercellular infection in actinorhizal Rosales and Cucurbitales (Wall 2000; Svistoonoff et al. 2014). Infection is a crucial step in symbiotic interactions and genes expressed during infection are regulated by mechanisms conserved between actinorhizal and rhizobial symbioses. This is the case for example for *Cg12*, a symbiotic gene encoding a subtilase specifically expressed during the intracellular infection of *C. glauca* by *Frankia* (Laplaze et al. 2000b; Svistoonoff et al. 2003). Specific activation of the *Cg12* promoter (=Pro*Cg12*) was detected during the intracellular infection of *Medicago truncatula* by *Sinorhizobium meliloti* (Svistoonoff et al. 2004) and the intercellular infection of *O. trinervis* by *Frankia* (Fournier et al. 2018). Similar results were obtained with *MtEnod11*, a gene from *M. truncatula* expressed at pre-infection stages during rhizobial infection (Journet et al. 2001): activation of Pro*MtEnod11* was detected in *C. glauca* and *O. trinervis* during infection but interestingly no activation occurred at pre-infection stages (Svistoonoff et al. 2010; Imanishi et al. 2011).

As for other developmental processes plant hormones play an important role during actinorhizal infection and nodule organogenesis. The inhibition of auxin influx was shown to delay nodule formation in *C. glauca* and *O. trinervis* and auxins were detected in *Frankia*-infected cells in both species (P eret et al. 2007; Perrine-Walker et al. 2010; Imanishi et al. 2014). Treatments with exogenous auxins lead to the formation of thick lateral roots resembling nodules in actinorhizal Fagales (Hammad et al. 2003; Svistoonoff et al. 2003) and several *Frankia* strains are able to synthesize auxin (Wheeler et al. 1984; Berry et al. 1989; Hammad et al. 2003; Perrine-Walker et al. 2010; Solans et al. 2011). In *C. glauca*, an auxin influx carrier (*CgAUX1*) is expressed in infected cells whereas a PIN1-like auxin efflux carrier is present in surrounding uninfected cells probably leading to auxin

accumulation in infected cells, where auxins could induce changes in gene expression, and cell wall properties (Péret et al. 2007; Perrine-Walker et al. 2010). Specific inhibition of auxin signalling in infected cells using a dominant-negative version of the endogenous auxin-signalling regulator *IAA7* leads to increased nodulation suggesting that in *C. glauca* auxin is a negative regulator of symbiosis establishment (Champion et al. 2015). Pro*CgAux1* is not activated during AM formation (Péret et al. 2008) and *DtAUX1*, the putative orthologue of *CgAUX1* in *O. trinervis*, showed a different activation pattern in *O. trinervis* nodules compared to *CgAUX1* in *C. glauca* suggesting divergent roles of auxin and its transporters in those symbioses (Imanishi et al. 2014).

In addition to auxins other plant hormones might also be involved in actinorhizal nodulation. Ethylene and jasmonate are small organic molecules that are involved in plant response to a wide range of biotic and abiotic stresses (Bari and Jones 2009; Dar et al. 2015). In legumes, ethylene and jasmonate have been shown to inhibit plant nodulation, symbiotic calcium spiking in root hairs spiking and the expression of two early symbiotic genes *RIP1* and *MtEnod11* (Oldroyd et al. 2001; Sun et al. 2006). Little information is available regarding the potential involvement of plant stress hormones in actinorhizal nodulation. Ethylene is involved in modulating the susceptibility for nodulation of the basal portion of *O. trinervis* seedling roots (Valverde and Wall 2005). In *C. glauca* and *Datisca glomerata* higher levels of jasmonate were detected in nodules compared to roots. Immunolocalization experiments showed that the allene oxide cyclase enzyme which catalyses a committed step in JA biosynthesis is present in nodules but only in uninfected cells (Zdyb et al. 2011). We recently analyzed the combined effect of jasmonate and ethylene in *C. glauca* and found that both hormones negatively affect nodulation and deformation of root hairs (Ngom M., Cissoko M., Gray K. Hocher V., Svistoonoff S. and Champion A. unpublished results).

Symbiotic signal transduction pathways

In the past 20 years, much knowledge regarding the signalling pathways activated during RNS has been gathered in model legumes and many crucial components are now identified, particularly those involved in

NF signalling (Oldroyd 2013). Many components of the NF signalling pathway are also needed to form arbuscular mycorrhizae (AM) and constitute a Common Symbiotic Signalling Pathway (CSSP). Most actinorhizal plants are able to form AM and genes involved in the CSSP are also present in actinorhizal plants and are expressed during nodulation (Hocher et al. 2006, 2011; Demina et al. 2013; Griesmann et al. 2018). For two genes, *SymRK* and *CCaMK* functional studies using RNAi knockdown and gain of function approaches combined with complementation of legume mutants have proven their crucial role in actinorhizal nodulation (Gherbi et al. 2008; Markmann et al. 2008; Svistoonoff et al. 2013). High frequency calcium oscillations which are a central feature of the CSSP have also recently been described in actinorhizal Fagales in response to *Frankia* signals (Granqvist et al. 2015; Chabaud et al. 2016). Components of the NF signalling pathway that are not involved in AM are less well characterized in actinorhizal plants with the exception of *NIN*, a transcription factor crucial for Legume nodulation (Schäuser et al. 1999; Marsh et al. 2007) which is also essential for actinorhizal nodulation in *C. glauca* (Clavijo et al. 2015). Recently genome-wide comparative analysis of 37 plant genomes including legumes, several actinorhizal plants and related species unable to nodulate revealed that most genes involved in legume nodulation are present in all the analysed species regardless of their ability to form nodules with the exception of two genes: *NIN* and *RPG* (Griesmann et al. 2018). Loss or fragmentation of *NIN* and *RPG* correlates with the absence of nodules in several lineages of the nitrogen fixing clade pointing to a central role for those two genes in all root nodule symbioses (van Velzen et al. 2018; Griesmann et al. 2018). Importantly, losses of *NIN* and *RPG* occurred independently suggesting that the most recent common ancestor of the nitrogen fixing clade was able to form nitrogen fixing nodules but this ability was lost in several lineages probably because natural selection often counter selects nodulation (van Velzen et al. 2018; Griesmann et al. 2018).

Concluding remarks

Compared to model legumes, our knowledge of actinorhizal plants is still sparse and limited to a few

species. However, thanks to progress in molecular research on actinorhizal, many obstacles have fallen in the past 20 years and knowledge on this original nitrogen fixing symbiosis has taken a leap forward.

Actinorhizal nodulation shares many features with rhizobial nodulation. Until recently it was believed that the common ancestor of all nodulators evolved a predisposition for nodulation but was not a nodulator itself; nodulation would have evolved independently at least 11 times and conserved mechanisms would be the result deep homology (Doyle 2011). However recent broad scale phylogenomics favours an alternative hypothesis where the ancestor was a nodulator and nodulation was lost multiple times, probably because of a strong selection pressure against nodulation in certain environmental conditions (Streeter and Wong 1988; Kiers et al. 2003; Griesmann et al. 2018). One intriguing question then arises: if there is one single ancestor how can nodulation functional diversity be explained? One hypothesis is convergent evolution at different rates. The ancestor probably used the simplest mechanism i.e. intercellular infection together with a poor selection of bacterial partners which is still observed in actinorhizal Rosales, some legumes (*Arachis/Aeschynomene*; (Sprenst 2007) or in lotus mutants (Madsen et al. 2010). The availability of full-genome sequences for several actinorhizal trees also offers the possibility to develop deeper phylogenomic analyses and thus to confirm or deny these evolution hypotheses.

Another intriguing question related to actinorhizal symbioses concerns signalling molecules. Recognition of RHDF by non-compatible host actinorhizal plants also points to a common origin suggesting that a common molecular backbone is recognized by all actinorhizal species but additional decorations or co-factors are needed to achieve full compatibility (Wall 2000; Gabbarini and Wall 2011; Cissoko et al. 2018). It is surprising not to find LCOs or molecules related to chitin at pre-infection stages as these are not only involved in rhizobial nodulation with legumes and *Parasponia* but also in the more ancient and widespread AM symbiosis (Oldroyd 2013). Like most components of CSSP, orthologs of legume genes involved in NFs recognition are also present in the genomes of actinorhizal plants (Griesmann et al. 2018) but functional evidence regarding their role in the perception of actinorhizal signalling molecules has to be provided. Nonetheless these observations will

remain inconclusive until the molecular nature of actinorhizal signalling molecules like NINA or RHDF is determined. The availability of several bioassays developed to purify and characterize these signalling molecules (Chabaud et al. 2016; Cissoko et al. 2018) combined with promising results regarding the genetic transformation of *Frankia* reported by the UNH group (L. Tisa) during the 19th International Meeting on *Frankia* and Actinorhizal Plants will hopefully provide answers to these questions in the near future.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interest exists.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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