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By

Hamid Kheyrodin

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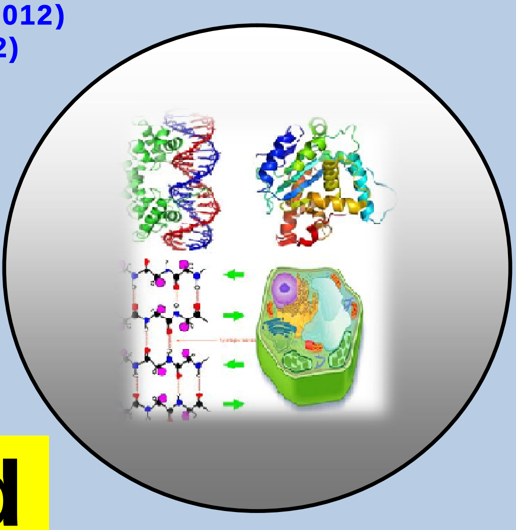

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Dr. Hamid Kheyrodin
http:// www.jbcr.in
jbiolchemres@gmail.com
info@jbcr.in

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Biological Nitrogen Fixation in Non-Leguminous Plants

Hamid Kheyrodin
Faculty of Desert Science in Semnan University, Iran

ABSTRACT

Nitrogen is an essential nutrient in plant growth. The ability of a plant to supply all or part of its requirements from biological nitrogen fixation (BNF) thanks to interactions with endosymbiotic, associative and endophytic symbionts, confers a great competitive advantage over non-nitrogen-fixing plants. Because BNF in legumes is well documented, this review focuses on BNF in non-legume plants. Despite the phylogenic and ecological diversity among diazotrophic bacteria and their hosts, tightly regulated communication is always necessary between the microorganisms and the host plant to achieve a successful interaction. Ongoing research efforts to improve knowledge of the molecular mechanisms underlying these original relationships and some common strategies leading to a successful relationship between the nitrogen-fixing microorganisms and their hosts are presented. Understanding the molecular mechanism of BNF outside the legume–Rhizobium symbiosis could have important agronomic implications and enable the use of N-fertilizers to be reduced or even avoided. Indeed, in the short term, improved understanding could lead to more sustainable exploitation of the biodiversity of nitrogen-fixing organisms and, in the longer term, to the transfer of endosymbiotic nitrogen fixation capacities to major non-legume crops.

Keywords: Non-Legumes, Microorganisms, Biodiversity, Fertilizers, and Biological Nitrogen Fixation.

INTRODUCTION

Nitrogen is an essential element in plant development and a limiting factor in plant growth. It represents about 2 % of the total plant dry matter that enters the food chain. Nevertheless, plants cannot directly access dinitrogen gas, which makes up about 80 % of the atmosphere. Plants absorb the available nitrogen in the soil through their roots in the form of ammonium and nitrates. The limited bio-availability of nitrogen and the dependence of crop growth on this element have spawned a massive N-based fertilizer industry worldwide.

About 60 % of synthetic nitrogen fertilizers are presently used for cereals, with irrigated rice production accounting for approx. 10 % of the use. Since >50 % of the fertilizer applied is actually used by plants, the inefficient use of nitrogen contributes to nitrate contamination of soils and ground water, leading to health hazards and compromising agricultural sustainability. Moreover, manufacturing N fertilizer requires six times more energy than that needed to produce either P or K fertilizers. Only some prokaryotes are able to use atmospheric nitrogen through a process known as biological nitrogen fixation (BNF), which is the conversion of atmospheric N_2 to NH_3, a form that can be used by plants. The bacteria responsible for nitrogen fixation are called diazotrophs; they encode nitrogenase, the enzyme complex that catalyses the conversion of N_2 gas to ammonia. The nitrogenase complex is highly conserved in free-living and symbiotic diazotrophs. Various types of associations/interactions occur between diazotrophs and their host plants. The highly specific and most efficient processes for nitrogen fixation involve the formation of root nodules on legumes and non-legumes. The diazotrophic bacteria involved in these endosymbiotic interactions include rhizobia (Gram negative) members of the alpha-subgroup of the phylum proteobacteria that associate with legumes (family Fabaceae) (not included in this review; see and the non-legume *Parasponia* species (family Cannabaceae), and *Frankia* sp. (Gram positive) members of the actinomycete family that associate with a broad spectrum of plants belonging to eight families collectively called actinorhizal plants. In addition, nitrogen-fixing cyanobacteria (mainly *Nostoc* sp.) have also been found to colonize different plant organs, either intracellularly in the family Gunneraceae or extracellularly in liverworts, hornworts, *Azolla* and Cycadaceae. In contrast with these symbioses, some diazotrophs, such as *Azospirillum* spp., *Azoarcus* spp. and *Herbaspirillum*, form associative and/or endophytic relationships with a wide variety of plant roots including those of cereals. In all these associations and symbioses, for the host plants the expected benefit of the interaction is the fixed nitrogen provided by the symbiotic partner, which, in return, receives reduced carbon and possibly all the other nutrients it requires. In addition, the symbiotic or endophytic plant structure colonized by the nitrogen-fixing microorganisms may provide the appropriate conditions for protecting the nitrogenase complex from oxygen exposure. Rhizosphere associations between nitrogen-fixing microorganisms and plants have been a major driving force in allowing organisms to spread across the biosphere, occupy new niches, and adapt to a variety of environmental stresses. This review presents an overview and recent advances in the understanding of the associations between a wide range of diazotrophs and non-legumes. Discoveries and breakthroughs in legume and non-legume nitrogen fixation provide new insight into ways of manipulating key steps in this process. Finally, new perspectives to engineer nitrogen-fixing ability in non-legume crops based on knowledge of endosymbiotic processes in non-legumes are discussed

Actinorrhizal plants and their major ecological role

Actinorrhizal plants have the ability to develop an endosymbiosis with the nitrogen-fixing soil actinomycete *Frankia*. The establishment of the symbiotic process results in the formation of root nodules in which *Frankia* provides fixed nitrogen to the host plant in exchange for reduced carbon. Actinorhizal plants represent a diverse group of about 220 species belonging to eight plant families distributed in the three orders, Fagales (Betulaceae, Casuarinaceae and Myricaceae), Rosales (Rosaceae, Eleagnaceae and Rhamnaceae) and Cucurbitales (Datiscaceae and Coriariaceae) (Wall, 2000; Pawlowski, 2009; Franche and Bogusz, 2011). All actinorhizal species belong to the Rosid I clade, thus sharing a common ancestor with legumes (Fabaceae), but differing from them in their wide distribution in numerous botanical families. It has been suggested that 100 million years ago (Mya), the common ancestor of Rosid I acquired a unique feature upon which a root nodule symbiosis (RNS) could evolve, and that this evolution occurred several times 50–60 Mya (Doyle, 2011).

Nitrogen-fixing actinobacteria *Frankia*

Frankia is a genus of soil actinomycetes in the family Frankiaceae that fix nitrogen, both under symbiotic and free-living aerobic conditions, while most rhizobia do notPhylogenetically, the filamentous gram-positive *Frankia* sp. and the unicellular gram-negative paraphyletic rhizobia are quite distant, suggesting that these two major groups of nitrogen-fixing symbionts have acquired mechanisms for nitrogen fixation from different evolutionary origins . The first successful isolation of *Frankia* was reported relatively recently from *Comptonia peregrina* root nodules . At present, over 200 strains of *Frankia* have been isolated from many, although not all, actinorhizal plant species. Phylogenetic analyses revealed that *Frankiae* form a coherent clade within actinobacteria, and that strains generally fall into three major groups or clusters.

Signals involved in plant–*Frankia* recognition and in the plant signalling pathway

It is assumed that, as observed in rhizobium–legume symbioses, the compatible interaction between *Frankia* and actinorhizal plants that leads to the development of nitrogen-fixing nodules is the result of a fine-tuned exchange of signals between the two partners (Franche and Bogusz, 2011). On the plant side, although the involvement of flavonoids in symbiosis is poorly understood, several studies indicate that they may play a significant role in the early stage of the interaction. These studies also suggest a role for *Frankia* in chemo-attraction and proliferation and in the enhancement of nodulation following the addition of seed washes from red alder *Alnus rubra*. Recently, a sensitive and reproducible bioassay based on early expression of symbiotic *C. glauca* genes was developed (Rhizogenesis group, Montpellier, France, unpubl. res.). This should help future work dealing with purification and complete characterization of *Frankia* signalling molecules involved in the early dialogue with the root system. Sugars and phytohormones may also be involved in the molecular dialogue. Interestingly, a study of whole-cell sugar contents showed that a monosaccharide, 2-*O*-methyl-D-mannose, was present in all *Frankia* strains tested. This sugar may thus play a role in interactions and communications between *Frankia* and its hosts (Kucho *et al.*, 2010).

Infection process and nodule development in actinorhizal plants

Two modes of root infection by *Frankia* have been described that depend on the host plant (Fig. 1). In the order Fagales, infection proceeds intracellularly via root hairs, whereas in Rosales, actinobacteria enter the root intercellularly. In Datiscaceae and Coriariaceae, the infection process is poorly known due to the difficulty involved in obtaining pure cultures of the symbionts and in studying the early stages of the infection process.

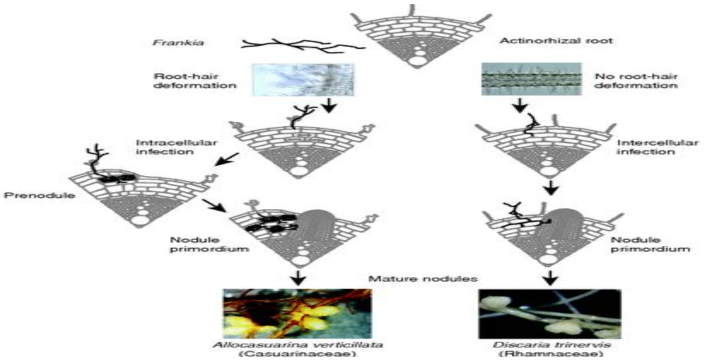

Fig 1. Schematic representation of actinorhizal root infection by *Frankia.*

Frankia penetrates via a root hair infection process in host plants from the families Betulaceae, Casuarinaceae and Myricaceae, and intercellularly in Eleagnaceae, Rosaceae and Rhamnaceae. Prenodule formation resulting from mitotic activity in the root cortical cells is observed only during the intracellular infection process. Nodule primordia arise from divisions in root pericycle cells, located opposite a protoxylem pole, and near the site of infection. *Frankia* hyphae progress either from cell to cell in the intracellular mode of infection, or apoplastically in a matrix secreted into the intercellular spaces. *Frankia* hyphae progress towards the nodule primordium where they will penetrate developing cortical cells intracellularly. Mature nodules consist of multiple lobes. Adapted from Franche and Bogusz (2011)

Molecular mechanisms underlying actinorrhizal infection and nodulation

Whereas no tools are yet available in *Frankia* to perform functional analysis of candidate genes, genetic transformation procedures based on *Agrobacterium tumefaciens* and *A. rhizogenes* are available for some actinorhizal plants, providing a tool for promoter studies and down-regulation of plant genes by RNAi. Functional and transcriptome analyses revealed that the common SYM pathway shared by rhizobium–legume and arbuscular mycorrhizal (AM) symbioses also controls nodulation by *Frankia*.

Table 1. Main features of plant cyanobacterial symbiotic associations.

Plant taxon	**Symbiotic host species**	**Symbiotic**	**Cyanobiont**	**Proposed time for plant origin**
Angiosperm	All known species of *Gunnera*	Stem gland	Intracellular *Nostoc*	80 Mya
Gymnosperm	All known *cydas* (150 species in 10 genera belonging to 3 families)	Root zone	Intercellular Nostoc or Calothrix	200-150 Mya
Pteridophyta	All species of the genus *Azolla*	Cavities in each dorsal leaf	Intercellular Nostocales obligatory synbiont	420Mya for the ferns, 120 Mya for Azolla fossils
Bryphyta	Only two of the 330 genera of liverwort, four of the six genera of hornwort	Cavities in the gametophyte	Intercellular Nostoc	400-500 Mya

This pathway includes a receptor-like kinase, nuclear pore proteins and potassium channels required for the induction of calcium oscillations. A putative calcium/calmodulin-dependent protein kinase (CCaMK) is also present and might thus recognize calcium ‘actinorhizal signatures’ (Singh and Parniske, 2012).

Bryophytes are small, non-vascular land plants including liverworts (Hepaticae), horworts (Anthocerotae) and mosses (Musci), a relatively small number of which are able to form epiphytic or endophytic associations with cyanobacteria. Epiphytic associations with mosses are not discussed in this review. Two liverwort species, *Blasia pusilla* and *Cavicularia densa* (Blasiales, Marchantiophyta), and all hornworts (Anthocerophyta) are able to form an endosymbiotic association with cyanobacteria that generally belong to the genus *Nostoc*. Endosymbiont filaments are hosted in specialized auricles on the ventral surface in Blasiales and in slime cavities within the thallus in Anthicerotophyta such as *Anthoceros* and *Phaeoceros*. During the development of the thallus, new auricles are continuously formed and infected by cyanobacteria. Cyanobacterial associations with pteridophytes are limited to the genus *Azolla* in the family Azollaceae. *Azolla* is a small floating aquatic fern with a worldwide distribution ranging from tropical to warm temperate regions. It has been exploited for many years as a source of nitrogen for agriculture and is extensively used as a green manure and biofertilizer for rice.

Diversity of cyanobacteria associated with plants

Cyanobionts are filamentous cyanobacteria and generally belong to the genus *Nostoc*, although a few other cyanobacteria such as *Calothrix* and *Chlorogloeopsis* have been reported. Cyanobacteria of the genus *Nostoc* belong to the order Nostocales and to Section IV of cyanobacteria. They are all characterized by their ability to differentiate some nitrogen-fixing cells called heterocysts, some resting spores called akinetes, and some motile filaments called hormogonia, which constitute the infective units during the establishment of the symbiotic process and contribute to short distance dispersal in free-living conditions

The role of hormogonia in the infection process

To establish a successful interaction, host plants must attract and internalize the cyanobacteria and then regulate their growth and differentiation. The cyanobacteria must avoid eliciting the plant defence response, and must adapt their metabolism to a new environment. Like in the previously described symbioses with actinorhizal plants and *Parasponia*, these events require sophisticated communication between the plant and the cyanobacteria (Fig. 2).

In nitrogen-free medium, *Nostoc* sp. filaments consist of vegetative cells (V) and regularly spaced heterocysts (H) that fix nitrogen. A hormogonium inducing factor (HIF) produced by the host under nitrogen starvation conditions leads to differentiation of motile small-celled hormogonial structures. Following the exchange of appropriate recognition signals, hormogonia penetrate the host symbiotic cavities and revert to vegetative filaments with a large number of heterocysts. The repression of hormogonia is linked to a hormogonia repressing factor (HRF).

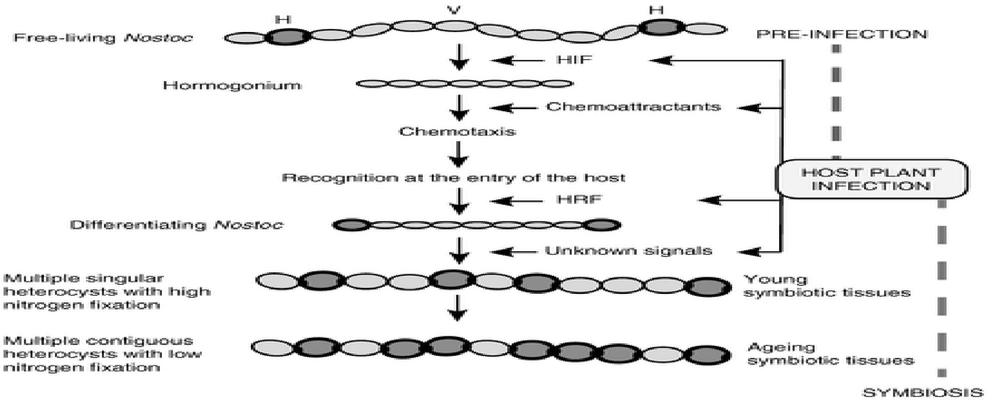

Fig 2. Schematic representation of the infection process in cyanobacteria–plant symbiosis.

In ageing symbiotic tissues, multiple contiguous heterocysts are observed that exhibit low nitrogen-fixation activity. Adapted from Rai *et al.* (2000) and Meeks 2005b. The conversion of vegetative filaments into motile and short-lived hormogonia is an essential step for the establishment of the symbiotic process and in laboratory conditions, and a cyanobacterial culture rich in hormogonia can increase the efficiency of plant infection.

Table 2. Association of cereals and nitrigen-fixing PGPR.

Cereals	**Diazotroph inoculants**	**Benefits % increase**	**References**
Rice	*Azoarcus*	16 (total dry weight)	Reinhold-Hurek and Hurek, 1997, Engelhard et al., 2014.
	Burkholderia	68 (shoot biomass) 19 (seed biomass)	Baldani et al., 2000
	B. vietnamiensis	13-22 (yield)	Tran Van et al., 2000
	Gluconacetobacter diazotrophicus	30 (total dry weight)	Muthukumarasamy et al., 2005
	Herbaspirllum seropedicae	37.6 (plant dry weight)	James et al., 2002.
	Serratia marcescens	30 (total dry weight)	Gyaneshwar et al., 2001
Maize	*Burkholderia sp.*	5.9-6.3 (yield)	Estrada et al., 2005
	Azospirllum brasilense	13-25 (yield)	Riggs et al., 2001
	Azotobacter	33 (grain yield)	Dobbelaere et al., 2001
	H. seropedicae	19.5 (yield)	Pandey et al., 1998
	Pseudomonas sp.	11.7 (total biomass)	Riggs et al., 2001
Wheat	*H. seropedicae*	49-82 (total biomass)	Riggs et al., 2001
	Azospirillum sp.		Boddey et al., 1986
	Azotobacter		Mrkovacki and Milic, 2001

***, †, Experiments in fields (*) or in controlled conditions (†).**

Adapted from Bhattacharjee *et al.* (2008) and Bhattacharyya and Jha (2012).

Hormogonia are short gliding filaments that lack heterocysts, with cells that are smaller than the cells in vegetative filaments. Although our knowledge on the molecular events involved in the differentiation of hormogonia is still limited, several genes affecting this process have been identified. In contrast to *Frankia* where no genetic tools are available to create mutations, procedures for genetic analysis and transposon mutagenesis have been developed in the large host range *Nostoc* ATCC 29133, thus providing a valuable tool for investigating the function of putative symbiotic genes.

Some of the main bacteria that can live in association with maize, rice and wheat and contribute to improved plant growth are presented in Table 2.

The conversion of vegetative filaments into motile and short-lived hormogonia is an essential step for the establishment of the symbiotic process and in laboratory conditions, and a cyanobacterial culture rich in hormogonia can increase the efficiency of plant infection. Hormogonia are short gliding filaments that lack heterocysts, with cells that are smaller than the cells in vegetative filaments. The reduced cell size results from cell divisions that are not accompanied by an increase in cell biomass and a significant synthesis of DNA. Different environmental stimuli and/or plant factors released during nitrogen starvation can stimulate the induction of hormogonia.

CONCLUSIONS

A number of non-legume plants have evolved multiple strategies in association with diazotrophs to deal with N deficiency. The most sophisticated associations are root nodule endosymbioses between *Frankia* and actinorhizal plants, rhizobium and *Parasponia* sp., and cyanobacteria that associate with *Gunnera* sp. in cells of the specialized stem gland. In recent years, a major breakthrough has been the demonstration of a common genetic basis for plant root endosymbioses with AM fungi, rhizobia and *Frankia* bacteria in both legumes and non-legumes. This finding strengthens the hypothesis of a single origin for all nitrogen-fixing root nodule endosymbioses, and that RNS could have been partially recruited from the more ancient AM. Compared with legumes, important questions remain to be answered including whether *Frankia* signals are structurally similar to Nod and Myc-factors, whether signals from actinorhizal plant and plant-hosting cyanobacteria are flavonoids – as in legumes and probably in *Parasponia* – and whether the conservation of the signalling pathway in *Parasponia* and actinorhizal plants goes beyond the common legume/rhizobium AM pathway. Progress in the knowledge of the basic mechanisms underlying symbiotic and endophytic associations in non-legumes has been generally slow, mainly due to the difficulties encountered in designing tools for the identification of candidate genes and their functional analysis. The value of comparative genomic approaches to help identifying, in addition to *nif* genes, common conserved gene functions specific to endosymbiotic and/or endophytic bacteria has been demonstrated. On the plant side, tools for 'omics' approaches and high-throughput sequencing technologies to finely explore transcriptomes are expected to provide new opportunities to decipher the molecular mechanisms underlying successful associations with diazotrophs.

The creation of artificial symbioses or associations between nitrogen-fixing microorganisms and plants of great agricultural importance is a primary goal in agriculture to reduce the demand for chemical nitrogen fertilizers. Since much of the basic work and major breakthroughs have been done on model legumes, strategies to expand the genetic capacity to fix nitrogen in symbiosis are currently based on that knowledge

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REFERENCES

Doyle, J.J. 2011. Phylogenetic perspectives on the origins of nodulation. *Molecular Plant–Microbe Interactions*, 24: 1289-1295.

Franche, C., Diouf, D. and Laplaze, L. 1998. The soybean (lbc3), Parasponia and Trema hemoglobin gene promoters retain symbiotic and nonsymbiotic specificity in transgenic Casuarinaceae: implications for hemoglobin genes evolution and root nodule symbioses. *Molecular Plant Microbe Interactions* 11: 887-894.

Franche, C., Lindström, K. and Elmerich, C. 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant and Soil* 321: 35-59.

Kucho, K., Hay, A.E. and Normand, P. 2010. The determinants of the actinorhizal symbiosis. *Microbes and Environments* 25: 241-252.

Pawlowski, K. and Pawlowski, K. 2009. Induction of actinorhizal nodules by Frankia. In: Pawlowski K, editor. Prokaryotic symbionts in plants. Berlin: *Springer*; p. 127-154.

Singh, S. and Parniske, M. 2012. Activation of calcium- and calmodulin-dependent protein kinase (CCaMK), the central regulator of plant root endosymbiosis. *Current Opinion in Plant Biology*, 15: 444-453.

Wall, L.G. 2000. The actinorhizal symbiosis. *Journal of Plant Growth Regulation* 19: 167-182.

Pawlowski, K., Newton, W.E., Wall, L.G. and Berry, A.M. 2008. Early interactions, infection and nodulation in actinorrhizal symbiosis. In: Pawlowski K, Newton WE, editors. Nitrogen-fixing actinorrhizal symbioses. Dordrecht: *Springer*; Vol. 6. pp. 147-166. Nitrogen fixation: origins, applications, and research progress.

Corresponding author: Dr. Hamid Kheyrodin, Faculty of Desert Science, Semnan University, Iran
Email: hkhyrodin@yahoo.com