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Harnessing Actinorhizal Symbiosis: Biological Strategies for Nitrogen Fixation, Sustainable Growth and Soil Reclamation

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"Biological nitrogen fixation (BNF) is the enzymatic conversion of atmospheric nitrogen (N_2) to ammonia (NH_3), predominantly performed by prokaryotes like Frankia, a genus of actinobacteria. Frankia forms symbiotic root nodules with non-leguminous actinorhizal plants across diverse families. These symbioses enable plants to thrive in various environments, from cold regions (e.g., *Alnus*) to arid areas (e.g., *Casuarina*). Frankia's nitrogen-fixing vesicles, enveloped in lipid layers, protect the oxygen-sensitive nitrogenase enzyme, ensuring efficient nitrogen fixation under low oxygen conditions. The infection process varies among host plants, involving either intracellular or intercellular pathways. Genomic studies highlight the importance of nif genes in nitrogen fixation, while the absence of canonical nod genes suggests Frankia employs unique signaling mechanisms. Additional adaptations, such as truncated hemoglobins and hydrogenases, enhance oxygen regulation and energy efficiency during nitrogen fixation. Actinorhizal plants play crucial ecological roles, particularly in nutrient-deficient and degraded soils. They are pioneer species in disturbed areas, contributing to soil stabilization, revegetation, and sustainable agriculture. The tripartite symbiosis between actinorhizal plants, Frankia, and mycorrhizal fungi enhances plant resilience in extreme conditions, making them instrumental in desertification prevention and nitrogen enrichment of ecosystems. Their ability to fix significant quantities of nitrogen further underscores their ecological and agricultural importance."

Introduction

Biological Nitrogen Fixation

This is the process of converting atmospheric nitrogen (N_2) into ammonia (NH_3) through a nitrogenase enzyme system. Only prokaryotic nitrogen-fixing organisms, like bacteria and cyanobacteria, can perform this reaction: $N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16\ Pi$**.** Certain non-leguminous plants form root nodules for nitrogen fixation. Examples include: *Frankia* with *Alnus sp.*, *Casuarina equisetifolia*, *Myrica gale*, *Rhizobium* with *Parasponia*, Leaf nodules formed by *Klebsiella* in *Psychotria* and *Burkholderia* in *Pavetta zimermanniana*

Actinorhizal symbioses

Frankia is a genus of gram-positive, pleiomorphic, filamentous actinobacteria in the family Frankiaceae. Known for slow growth and high GC content. This genus was named after its discoverer Frank in 1880. *Frankia* has three cell types: vegetative hyphae, nitrogen-fixing vesicles, and sporangia with spores. It lives in soil and forms symbiotic root nodules on non-leguminous, nitrogen-fixing actinorhizal plants. Actinorhizal plants, mainly trees and shrubs in eight plant families across Fagales, Rosales, and Cucurbitales, form root nodules with *Frankia*. These plants (about 220 species) benefit from fixed nitrogen provided by *Frankia* in exchange for carbon, adapting from cold regions (e.g., *Alnus*) to warm climates (e.g., *Casuarina*).

General Features

Vesicles are surrounded by a laminated lipid envelope containing a mixture of hopanoid lipids which are pentacyclic bacteriohoppanes. The thickness of the vesicle envelope increases with an increase in O_2 and helps to maintain partial oxygen pressure at levels that are not labile for the dinitrogenase complex. Most strains develop spores in environmental stress conditions. Hopanoids are biosynthesized from isopentenyl pyrophosphate (IPP) which is synthesized either through mevalonate pathwayor methyl erythritol phosphate pathway. The spores are formed in sporangia at the mycelial tips and are thought to contribute to the dissemination and survival of the microorganism. Whereas some Frankia strains, referred to as «Sp-», differentiate spores only in pure culture, others, called «Sp 1 » strains, can also sporulate in actinorhizal nodules.

The isolation of Frankia was difficult because of the slow growth, high variability, and ill-defined growth condition of the symbiont. All cultivation protocols for Frankia employed nitrogen-deficient media to select against nondiazotrophs. Recently, strains of Frankia have been isolated from nodules of some plants using different methods, namely, (1) enzymatic digestion, e.g., *Comptonia peregrina*, (2) microdissection, e.g., *Alnus rubra* (3) sucrose-density fractionation, e.g., *Elaeagnus umbellata* and *Alnus crispa*, (4) serial dilution, e.g., *Alnus crispa* (5) sephadex fractionation, e.g., *Myrica gale* and *Elaeagnus umbellate*, and (6) direct isolation from surface sterilized pieces of nodules, e.g., *Alnus glutinosa*.

Taxonomy

Since the first report of the infective Frankia strain isolated from nodules of *C. peregrina*, hundreds of Frankia isolates have been described. Individual strains can nodulate actinorhizal plants from different orders, thus implying that the host origin is not always a determining characteristic for strain classification.

In specific conditions, aerial nodulation has also been reported to occur on the trunks of actinorhizal trees of two Casuarina species, *C. glauca* and *C. cunninghamiana*. This stem nodulation requires high atmospheric humidity. Field experiments have shown that these nodules can fix nitrogen.

Genes Involved in Symbiosis

Nitrogen fixation or the conversion of atmospheric N_2 to NH_3 is catlyzed by the enzyme nitrogenase. In general, Frankia strains from lineages I, II and III are able to fix nitrogen and possess nitrogenase, while members of lineage IV are unable to fix nitrogen. The Frankia genomes contain 11–12 nif genes that are clustered together as an operon on 11.6–14 kb region. For Frankia genomes from lineage I and II, all of the nif genes clustered together, while genomes from lineage III are organized slightly different having a nifV located at distance from the general nif cluster. Furthermore, nifZ was present only in lineage III. The nif operon consists of one copy of each nif gene. However for genomes from lineage II, two copies of nifU are present. A second nifU-like is also present in hup operon in lineage I, III and IV genomes. Six non-nif genes (orf1, orf2, hesA orf3, orf4 and fdx) are also present and located between nif genes. Interestingly, no nif genes were found in Frankia genomes from lineage IV.

O_2 Regulation and Haemoglobin in Actinorhizal Nodules

Vesicles act as specialized structures for the nitrogen fixation process and are formed terminally on short side branches of hyphae that have a septum near their base. The mature vesicle is surrounded by an envelope that extends down the stalk of the vesicle past the basal septum, which separates the vesicle from the hypha. The envelope surrounding the vesicle is composed of multilaminated lipid layers containing primarily bacteriohopanetetrol and its derivatives. It is believed that this lipid envelope acts as an oxygen diffusion barrier to protect the nitrogenase enzyme from oxygen

Table 1: Frankia Clusters Based on Cross-Infectivity, Genotypic, and Phylogenetic Approaches with Distinct Host Ranges

Cluster	Nodulating Host Families	Key Characteristics
Cluster I	Betulaceae, Casuarinaceae (not Gymnostoma), Myricaceae	Strains nodulate these families
Cluster II	Coriariaceae, Datiscaceae, Rosaceae, Ceanothus (Rhamnaceae)	Mostly non-isolated endophytes; only one cultured strain from *Coriaria myrtifolia*. Basal and ancient group.
Cluster III	Elaeagnaceae, Rhamnaceae (not Ceanothus), Gymnostoma (Casuarinaceae)	Poorly understood strains
Cluster IV	Not specified; isolated from actinorhizal nodules	Atypical strains; unable to fix nitrogen or re-induce nodules on their initial host plants.

inactivation. Unlike other actinorhizal plants, Frankia found within the root nodules of Casuarina and Allocasuarina plants are devoid of symbiotic vesicle structures. A positive correlation was observed between the differentiation of intracellular hyphae and the lignifications of the host-infected cell walls. In several actinorhizal nodules, a low oxygen tension was shown to be consistent with the high concentrations of hemoglobin. Frankia are known to produce truncated hemoglobins (TrHbO). Frankia TrHbO may function under hypoxic conditions to shuttle oxygen to the respiratory chain, similar to mycobacteria. Besides hemoglobins, Frankia possess hydrogenases that may act as oxygen-scavenging enzymes. In Frankia, [NiFe]-uptake hydrogenase (Hup) consists of two subunits: a large catalytic HupL with Ni and

Fe atoms bound via four cysteine thiolates and a small HupS subunit with Fe-S clusters for electron transfer. Frankia alni ACN14a expresses two Hup syntons—synton 1 under free-living conditions and synton 2 in symbiosis—critical for recycling hydrogen produced by nitrogenase for energy efficiency. These hydrogenases also contribute to oxygen tolerance, enhancing nitrogen-fixing efficiency.

Common Nod Genes

Analysis of the Frankia genomes for common canonical nodABC genes (NodA-acyl transferase, NodB-chitin deacetylase, NodC-chitin synthase) failed to reveal their presence. Some similarity to other Nod-like proteins, such as chitooligosaccharide deacetylase are present and found across non-symbiotic prokaryotes. These homologs are relatively conserved among actinobacteria. These results suggest that Frankia uses a novel signaling compounds during the infection process of actinorhizal plants.

Signal Exchange between Microsymbiont and Host Plant

Actinorhizal plant roots release flavonoids that trigger the production of Frankia signals. The nodulation signaling pathway is triggered when as yet unknown receptors sense Frankia signals. Activation of the receptors produces oscillations of calcium concentration (calcium spiking). Two members of the CSP, a leucine-rich repeat receptor kinase (SYMRK) and a calcium calmodulin-dependent protein kinase (CCaMK), are expressed following Frankia infection. Like in legumes, the NIN gene is expressed during preinfection stages in developing root hairs and during Frankia infection. Expression of Cg12 encoding a subtilisin-like serine protease and of CgAUX1 encoding an auxin influx carrier have been shown to be specifically linked to plant cell infection by Frankia. Expression in prenodules and in mature nodule lobes has also been demonstrated, suggesting that some of the genes involved in symbiotic signaling may have other

Differentiation of the two infection processes

Aspect	Intracellular Infection	Intercellular Infection
Plant Families/Genera	Hamamelidae: *Alnus, Myrica, Comptonia, Casuarina, Gymnostoma*	17 genera: *Elaeagnaceae, Rhamnaceae, Rosaceae, Datiscaceae, Coriariaceae*
Root Hair Involvement	Root hairs undergo curling and deformation.	Root hairs are neither deformed nor invaded by Frankia.
Entry Point	Frankia invades the curled root hairs via invagination of growing filaments.	Frankia grows through intercellular spaces, penetrating the middle lamella.
Entry Point	Frankia invades the curled root hairs via invagination of growing filaments.	Frankia grows through intercellular spaces, penetrating the middle lamella.
Progression of Infection	Intracellular infection in the root cortex; Frankia hyphae are surrounded by a plant-derived encapsulation layer enriched in polygalacturonans.	Apoplastic progression through cortical cells within an electron-dense matrix secreted into intercellular spaces.
Infection Thread	Encapsulation layer in intracellular infection resembles the infection thread wall in legume nodules.	No infection threads are observed in the early stages; threads form later in nodule lobe primordium.
Nodule Formation	Prenodule formation occurs, consisting of infected and uninfected cells.	No prenodule stage is observed.
Nodule Primordium	Develops from mitotic activity in pericycle cells near prenodule; infection is coordinated with post-meristematic cell expansion.	Develops from pericycle without prenodule; intracellular penetration begins acropetally in cortical cells.
Host Plant Role	Host provides encapsulation layer surrounding Frankia filaments.	Host modifies intercellular apoplast size and composition to accommodate Frankia.
Ancestral Mode	Not considered ancestral.	Suggested as the ancestral mode of infection.

roles in nodule function. Hemoglobin (CgHb) and metallothionein (CgMT1) genes have been shown to be highly expressed in cells filled with Frankia both in prenodules and in mature nodule lobes.

Major Ecological Role

Many actinorhizal plants are also capable of forming mycorrhizal associations, and this tripartite symbiosis (host plant–Frankia–mycorrhiza) gives them a propensity to grow in marginal and poor soils. Some species are very well adapted to flooded land, arid regions, contaminated soils, extreme pH and high salinity. Due to these properties, some actinorhizal trees are pioneer species that colonize disturbed areas; they play extremely important ecological roles and are intensively used in the revegetation of different landscapes or to prevent desertification. For example, in Africa, Casuarinaceae are planted to stabilize coastal and desert dunes, and for reclamation of salt-affected soils as well as in inter-cropping systems. In these arid soils, the species *Casuarina equisetifolia* fixes an average of 15 kg N ha^{-1} $year^{-1}$. But in temperate climates, nitrogen-fixation activity in actinorhizal plants could be similar to the rate of 300 kg ha^{-1} $year^{-1}$ measured in legumes. In addition, these perennial plants contribute to the N cycle through litter fall and soil decomposition.

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