



16th  
International meeting on Frankia  
and actinorhizal plants - 2010

& International Symposium on  
Frankineae

# Abstract Book

5-8 September 2010  
Porto-Portugal





# People involved

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# Welcome message

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Dear colleague,

The Instituto de Biologia Molecular and Celular, the Faculdade de Ciências from University of Porto and the Tropical Research Institute from Portugal kindly invite you to the 16th International Meeting on *Frankia* and Actinorhizal Plants - 2010 & International Symposium on Frankineae that will be held in the city of Oporto from 5 to 8 of September 2010.

The meeting follows an old tradition of our community, representing a unique opportunity to discuss the major advances, perspectives and constraints on actinorhizal research and to strengthen and build up new partnerships. This year we will concomitantly hold the International Symposium on Frankineae, hoping to enlarge the discussion forum. Three main topics will be addressed: Systems biology (omics); Ecology and Biodiversity; and Physiology and Biochemistry.

Under the context of global climate changes and on the International Year of Biodiversity it is particularly important to highlight the potential of actinorhizal symbiotic systems and to encourage new and young researchers to join us.

Be very welcome to Porto and enjoy the mild Portuguese late Summer,

The Organizing Committee



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# Conference program

<b>Session I</b>	<b>Physiology, Biochemistry and Genetics</b>
<b>Session II</b>	<b>Ecology and Biodiversity</b>
<b>Session III</b>	<b>Systems Biology (Omics)</b>

## Sunday, 5<sup>th</sup> September 2010

<b>6:00 pm – 9:00 pm</b>	<b>Registration / Welcome Cocktail</b>
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## Monday, 6<sup>th</sup> September 2010

<b>9:00 am - 9:30 am</b>	<b>Opening remarks</b>
<b>9:30 am - 10:00 am</b>	<b>Transformed Hairy Roots of <i>Discaria trinervis</i>: A tool for studying actinorhizal symbiosis in the context of intercellular infection (OC1)</b> L Imanishi, A Vayssières, C Franche, D Bogusz, S Svistoonoff and <a href="#">L G Wall</a>
<b>10:00 am - 10:30 am</b>	<b>Searching for signalling molecules involved in actinorhizal symbioses (OC2)</b> <a href="#">S Svistoonoff</a> , F Auguy, D Moukouanga, L Tisa, A Crabos, V Hocher, D Bogusz, C Franche
<b>10:30 am - 11:00 am</b>	<b>Coffee Break</b>
<b>11:00 am - 11:30 am</b>	<b><i>Frankia</i> BCU110501 diffusible factor(s) involved in <i>Discaria trinervis</i> nodulation, an intercellular root infected actinorhizal symbiosis (OC3)</b> <a href="#">L A Gabbarini</a> and L G Wall
<b>11:30 am - 12:00 pm</b>	<b>Hydrogenase in <i>Frankia</i>: expression and structure (OC4)</b> C Kosawang, P Pucic, N Kudahettige and <a href="#">A Sellstedt</a>
<b>12:00 pm - 1:00 pm</b>	<b>Visit to Porto Botanical Garden</b>
<b>1:00 pm - 2:30 pm</b>	<b>Lunch</b>
<b>2:30 pm - 3:30 pm</b>	<b>Poster session (P1-P9)</b>
<b>3:30 pm - 4:00 pm</b>	<b>Coffee Break</b>
<b>4:00 pm - 4:30 pm</b>	<b>Recent trials for <i>Frankia</i> transformation (OC5)</b> M Yamaura, K Kakoi, M Abe, T Uchiumi and <a href="#">K-i Kucho</a>
<b>4:30 pm - 5:00 pm</b>	<b><i>Alnus</i> and <i>Frankia</i> in Alaska: a case study in the intra-generic variability of an intimate symbiosis (OC6)</b> <a href="#">M Anderson</a> , R W Ruess, D L Taylor



## Tuesday, 7<sup>th</sup> September 2010

9:30 am - 10:00 am	<b>Amplicon restriction patterns, nucleotide sequence and secondary folding of 5.8S-rRNA in resolution of taxonomic dispute of <i>Myrica</i> sp. (OC7)</b> M Yanthan and <a href="#">A K Misra</a>
10:00 am - 10:30 am	<b><i>Ceanothus americanus</i> impacts soil nitrogen and plant composition in tallgrass prairies of Illinois (OC8)</b> John Taft, Kristin Pink and <a href="#">Jeffrey O. Dawson</a>
10:30 am - 11:00 am	<b>Coffee Break</b>
11:00 am - 11:30 pm	<b>Activity of nodule-specific promoters in heterologous nodules: implications for the evolution of infection pathways (OC9)</b> <a href="#">K Pawlowski</a> , B Rashidi, S Mehrabi, M Plaszczycza, T Persson, I Demina, L Jingsi, P Santos, S Svistoonoff, C Franche, M Plaszczycza and A Ribeiro
11:30 pm - 12:00 pm	<b>Engineering the gene predisposition to nitrogen fixation : a global approach of the symbiotic program in <i>Casuarina glauca</i> and <i>Alnus glutinosa</i> (OC 10)</b> <a href="#">V Hocher</a> , N Alloisio, P Doumas, F Auguy, P Fournier, P Normand and D Bogusz
12:00 pm - 12:30 pm	<b>Functional analysis of defense-related genes involved in <i>Casuarina glauca</i>-<i>Frankia</i> symbiosis (OC 11)</b> P Santos, I Graça, B Rashidi, J Liang, A Fortunato, A Melo, J C Ramalho, A Pereira, B Day, K Pawlowski and <a href="#">A Ribeiro</a>
12:30 pm - 2:00 pm	<b>Lunch</b>
2:00 pm - 3:30 pm	<b>Poster session (P11-P17)</b>
3:30 pm - 4:00 pm	<b>Coffee Break</b>
4:00 pm - 4:30 pm	<b>Characterization of TTA codon containing genes in <i>Frankia</i> and exploration of the role of tRNA in regulating these genes (OC 12)</b> <a href="#">A Sen</a> , S Thakur, A Bothra, S Sur and L S Tisa
4:30 pm - 5:00 pm	<b>Genome-based Perspectives on the <i>Datisca</i>-<i>Frankia</i> Root Nodule Symbiosis (OC 13)</b> A M Berry



## Wednesday, 8<sup>th</sup> September 2010

9:30 am - 10:00 am	<b>What are the stories that the <i>Frankia</i> genomes are telling us? (OC 14)</b> <u>L S Tisa</u> , N Beauchemin, D Bogusz, T Furnholm, M Gtari, M Rehan, A Sen, L G Wall, F Xu
10:00 am - 10:30 am	<b>A comparative genomic approach to the study of Geodermatophilaceae (OC 15)</b> M Gtari, I Essoussi, R Boujmil, A Hamza, E Accattino, E Crotti, D Daffonchio, V Barbe, C Médigue, P Pujic, J Gury and <u>P Normand</u>
10:30 am - 11:00 am	<b>Coffee Break</b>
11:00 am - 12:00 pm	<b>Round Table</b> <i>Frankia</i> , Frankineae, Actinorhizal plants: the future that lies ahead
12:00 am - 12:15 pm	<b>Next International Meeting on <i>Frankia</i> and Actinorhizal Plants &amp; International Symposium on Frankineae – location and possible dates</b>
12:15 pm - 12:30 pm	<b>Closing Remarks</b>
12:30 pm - 2:00 pm	<b>Lunch</b>
Afternoon & Evening	<b>Porto Sightseeing Tour, Visit to Port wine cellars &amp; Conference Dinner</b>



# Oral Communications

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## Transformed hairy roots of *Discaria trinervis*: A tool for studying actinorhizal symbiosis in the context of intercellular infection

Leandro Imanishi<sup>1</sup>, Alice Vayssières<sup>2</sup>, Claudine Franche<sup>2</sup>, Didier Bogusz<sup>2</sup>, Sergio Svistoonoff<sup>2</sup>, **Luis G Wall**<sup>1</sup>

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The mechanisms and genetics by which actinorhiza forming plants recognize *Frankia* symbionts, before and during infection, remain unknown. Actinorhizal plant species are distributed within the orders Fagales, Cucurbitales and Rosales. The most studied model systems, in terms of molecular biology and genetics, belong to Fagales (*Alnus spp* and *Casuarina spp*) which at the same time are examples of the root hair infection pathway. *Discaria trinervis*, a Patagonian Rhamnaceae shrub, is an example of actinorhizal plants belonging to Rosales which has been described to be infected via intercellular pathway. We have set up a genetic transformation of *Discaria trinervis* root system based on *Agrobacterium rhizogenes*, comparing *in vitro* transformation system and *ex-vitro* one. Composite plants with transgenic roots can be specifically and efficiently nodulated allowing for functional nitrogen fixing symbiosis by inoculation with *Frankia* BCU110501. We studied in *Discaria trinervis* the activation of promoters of symbiotic marker genes from legumes (MtEnod11) or other actinorhizal plants as *Casuarina glauca* (CgAux1 and Cg12), which have been previously characterized in root hair infected actinorhizal plants. Prior to *Frankia* inoculation no activation was detected in plants transformed with ProCg12::GUS whereas the promoters of CgAux1 and MtEnod11 were strongly active in the root vasculature and root tips of plants. Upon the inoculation by *Frankia* BCU110501, strong activation of ProCg12 and ProMtEnod11 was detected in the root cortex in places where small nodule primordia were starting to appear. The expression was localized in the apical zone of the developing nodule corresponding to the infection zone. Upon the inoculation by *Frankia* BCU110501, strong activation of ProCg12 and ProMtEnod11 was detected in the root cortex in places where small nodule primordia were starting to appear. The expression was localized in the apical zone of the developing nodule corresponding to the infection zone. ProCg12 appears to be a good reporter for the study of intercellular root invasion. In contrast to ProCg12 and ProMtEnod11, ProCgAux1 was not active during *Frankia* intercellular infection. GUS expression was observed in young and mature nodular lobes, related to infection zone and initially infected hyperplastic cells but no GUS crystals were detected in infected cells with vesicle differentiation. These promising results prompt us to look for the orthologous genes in *Discaria trinervis*. Dt12 has been detected and seem to be specifically induced in nodules and not in roots. The developed strategy would now enable the study of the intercellular infection pathway and the molecular mechanisms of the interaction of *Frankia* with actinorhizal plant belonging to the order Rosales.

## OC2

### Searching for signalling molecules involved in actinorhizal symbioses

**Sergio Svistoonoff**<sup>1</sup>, Florence Auguy<sup>1</sup>, Daniel Moukouanga<sup>1</sup>, Louis Tisa<sup>2</sup>, Amandine Crabos<sup>1</sup>, Valérie Hocher<sup>1</sup>, Didier Bogusz<sup>1</sup>, Claudine Franche<sup>1</sup>

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We are studying the interaction between the tropical tree *Casuarina glauca* and the actinomycete *Frankia* that leads to the formation of actinorhizal nodules. We recently reported the existence of a signalling pathway shared between fungal and bacterial root endosymbioses in the actinorhizal plant *Casuarina* and in legumes<sup>1</sup>. However, the nature of the chemical signals exchanged between the two partners of actinorhizal symbioses is still unknown due to the lack of genetic tools in *Frankia* and of specific molecular markers of the symbiotic interaction.

To identify signalling compounds we are using *C. glauca* genes expressed at the early stages of the symbiotic interaction. These genes include *Cg12*<sup>2</sup>, a subtilase specifically expressed in *Frankia*-infected cells, *CgAUX1*<sup>3</sup>, an auxin influx carrier, and *MtEnod11*, a legume gene widely used as a symbiotic marker. Recently we also used global approaches (EST libraries, microarrays) to identify new symbiotic markers. A promising candidate is *CgNIN*, the ortholog of legume *NIN* genes found to be expressed in nodules and also during the early stages of the interaction.

1. Gherbi, H. et al. SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and Frankiabacteria. *Proc Natl Acad Sci U S A* **105**, 4928-4932 (2008).
2. Svistoonoff, S. et al. Infection-Specific Activation of the Medicago truncatula Enod11 Early Nodulin Gene Promoter During Actinorhizal Root Nodulation. *Mol. Plant Microbe Interact* **23**, 740-747 (2010).
3. Péret, B. et al. Auxin influx activity is associated with Frankia infection during actinorhizal nodule formation in *Casuarina glauca*. *Plant Physiol* **144**, 1852-1862 (2007).
4. Schauser, L., Roussis, A., Stiller, J. & Stougaard, J. A plant regulator controlling development of symbiotic root nodules. *Nature* **402**, 191-195 (1999).

***Frankia* BCU110501 diffusible factor(s) involved in *Discaria trinervis* nodulation, an intercellular root infected actinorhizal symbiosis**

**Luciano A Gabbarini** and Luis G Wall

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*Frankia* BCU110501, belonging to phylogenetic clade 3, induces nitrogen fixing root nodules in the Rosales actinorhizal plant *Discaria trinervis* via intercellular colonization, without root hair deformation. *Frankia* BCU110501 produces Diffusible Factors (DFs) which might be involved in early interactions with the *Discaria trinervis* roots, playing a role in the nodulation process. The induction of root nodule development in actinorhizal symbiosis would depend on the concentration of factors produced by the bacteria and the plant. A detailed analysis of nodulation kinetic revealed that those DFs produce changes at the level of initial rate of nodulation and also in nodulation profile. Diluted *Frankia* BCU110501 inoculum could be activated in less than 96 hours by DFs produced by *Frankia* BCU110501 cells previously washed. An attempt to biochemical characterization was carried out and showed that *Frankia* BCU110501 DFs would be of MW < 12kDa, negative charged at pH 7.0 and would contain a peptide bond necessary for its activity. Although *Frankia* BCU110501, belonging to *Frankia* Clade 3, does not induce nodules in *Alnus acuminata*, because of lack of full symbiotic recognition, it is able to deform root hairs as infective *Frankia* belonging to Clade 1 does. The root hair deforming activity of *Frankia* BCU110501 DFs show the same biochemical characteristics as the DFs involved in nodulation of *Discaria trinervis*, suggesting a basic structure for *Frankia* factors regardless the infection pathway activated in the interaction with the actinorhizal host plant.

# OC4

## Hydrogenase in *Frankia*: expression and structure

C Kosawang<sup>1</sup>, P Puci<sup>2</sup>, N Kudahettige<sup>1</sup> and **A Sellstedt**<sup>1</sup>

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*Frankia* are actinomycetes capable of living in symbiosis with dicotyledonous plants worldwide. Two hydrogenase functions - uptake hydrogenase and hydrogen-evolving hydrogenase, have so far been reported in *Frankia* species. The blast of the three available *Frankia* genomes with the [NiFe]-hydrogenase L1 and L2 signatures derived from [NiFe]-hydrogenases showed that *Frankia* CcI3 has a bifunctional (NADP) hydrogenase, which might mediate the hydrogen production under anaerobic conditions. However, the blast of the three genomes with the conserved region of [Fe] hydrogenase, subunits of cyanobacterial bidirectional hydrogenase, *hyd* and *hyn* genes resulted in very low similarity grade. Structures of large subunit of *Frankia* hydrogenase in ACN14a and CcI3 are presented based on the genome sequence. An additional strain, *Frankia* R43, was found to have hydrogen production both under aerobic and anaerobic condition regardless of nickel availability. However, it was not possible to show the presence of bidirectional hydrogenase genes in the strain R43 since its genome is not yet sequenced.

## Recent trials for *Frankia* transformation

Masatoshi Yamaura, Kentaro Kakoi, Mikiko Abe, Toshiki Uchiumi and **Ken-ichi Kucho**

Graduate School of Science and Engineering, Kagoshima University

In spite of numerous trials for long time, transformation of *Frankia* is not feasible which has been a main obstacle to elucidate molecular basis of the symbiosis. I had presented at the last meeting that we had obtained potential transformants of *Frankia* strain CcI3 in selective liquid media by transforming a fusion marker gene consisting of the strain's *infC* gene promoter and antibiotic resistance gene showing similarity in codon usage with *Frankia*. Importance of codon usage similarity on foreign gene expression in *Frankia* cell was demonstrated by a codon-optimized synthetic marker gene. Detailed inspection of the transformant genome, however, revealed that most of the marker genes were not integrated in chromosome but they existed as degraded molecules in the cells. To enrich true transformants having marker gene in their chromosome, the antibiotic-resistant cell population was passaged in selective liquid media. But marker genes in the population were reduced during passage culture. We also picked up antibiotic resistant colonies on solid media, propagated them in liquid media and then analyzed their genomic DNA. But none of them contained a marker gene. These results suggest occurrence of spontaneous resistants by prolonged selective culture. We concluded that antibiotic resistance gene is not a good choice as a selection marker for transformation. So we tried to establish another selection system using an uracil-requiring *pyrF* mutant and wild type *pyrF* gene. We mutagenized *Frankia* strain CcI3 cells by ethyl methanesulfonate and screened for *pyrF* mutants using a positive selection with 5-fluoroorotic acid. A *pyrF* mutant strain (CcI3E21), which carries 1-bp deletion in the coding sequence, was used as a host strain of transformation. Coding sequence of the wild type *pyrF* gene was fused with a promoter of pyrimidine synthesis operon or translation initiation factor 3 gene. They were introduced into the strain CcI3E21 and transformants were selected on minimal media without uracil. Result of the selection will be presented.

## OC6

### ***Alnus* and *Frankia* in Alaska: a case study in the intra-generic variability of an intimate symbiosis**

**Mike Anderson**<sup>1, 2</sup>, Roger W Ruess<sup>1</sup>, D Lee Taylor<sup>1</sup>

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Alders (*Alnus* spp.), all species of which are actinorrhizal, are widespread and ecologically important in the northern hemisphere. Many *Alnus* species inhabit a broad range of habitats, and some habitats also support multiple sympatric species, making the *Alnus-Frankia* symbiosis an excellent system for field studies investigating natural variation in symbiotic interactions. In interior Alaska it is possible to study such habitat and host-related variation using spatially mixed replicate sites representing particular habitats, including habitats with multiple *Alnus* species. Over the past several years we have used these unique resources to study natural variation in *Frankia* symbiotic with two species of alder - thinleaf alder (*Alnus tenuifolia*) and green alder (*Alnus viridis*) - across a broad range of Alaskan habitats. Our studies have focused on two sources of variation: host specificity and environmental variation.

With regard to the former, we have found clear differences between the two host species in: 1) assemblage ('population') structure of symbiotic *Frankia*, even in sites supporting both hosts in close proximity ( $\leq 5$  m), and 2) phylogenetic relationships among *Frankia* associated with the two hosts, with *A. viridis*-associated *Frankia* closely related to a large and widespread group of previously described strains which include ACN14a (the model strain for *Alnus*-infective *Frankia*), and *A. tenuifolia*-associated *Frankia* forming a separate cluster distinct from previously described strains. With regard to environmental sources of *Frankia* variation, the two host species also differ, with *A. viridis* nodules occupied largely by two closely related genotypes across multiple habitats and broad (hundreds of km) geographic scales, while *A. tenuifolia* symbiont assemblages exhibit clear structure related to habitat at relatively small ( $\leq 15$  km) scales. Collectively, the differences between these two alder species demonstrate the wide variability possible within a host genus in patterns of host-symbiont association, and suggest very different host-symbiont co-evolutionary dynamics for the two systems. Speculations on the latter are provided.

## **Amplicon restriction patterns, nucleotide sequence and secondary folding of 5.8S-rRNA in resolution of taxonomic dispute of *Myrica* sp.**

Mhathung Yanthan and **Arvind K Misra**

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Three different morphological variants of genus *Myrica* L. (Myricaceae) are found in Meghalaya, India. There is a dispute regarding their classification. Some authors treat them as two separate species (*Myrica nagi* and *Myrica esculenta*) while others feel that *M. nagi* and *M. esculenta* are synonyms. In an effort to resolve this dispute, we investigated the nuclear ribosomal RNA genes segment of DNA of these variants utilizing the amplicon restriction patterns, nucleotide sequences and secondary folding of the 5.8S rRNA. It is proposed that *M. nagi* and *M. esculenta* be treated as two separate species.

## OC8

### ***Ceanothus americanus* alters soil nitrogen and plant composition in tallgrass prairies of Illinois**

John Taft, Kristin Pink and <sup>1</sup>**Jeffrey O. Dawson**

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The role of *Ceanothus americanus* in community organization was examined in three Illinois prairie remnants. Plots with and without *C. americanus* were compared for difference in floristic similarity, diversity, and C<sub>3</sub> vs. C<sub>4</sub> species abundance. We examined size-class distribution and reproductive condition of *C. americanus* for evidence of recruitment. Floristic similarity was greater for samples within sites than comparisons between sites. Means for diversity and evenness among plots associated with *C. americanus* were significantly lower than other plots, nearly so for species density, but not different for sum total cover or total species richness. Nitrogen-demanding, cool-season C<sub>3</sub> graminoid species were significantly more abundant among *C. americanus* than were C<sub>4</sub> species, which were more abundant in plots away from *C. americanus*. There was no statistically significant gradient in total soil nitrogen near individual *C. americanus* shrubs, but there was a difference in total amino sugar nitrogen, a form associated more-closely with soil nitrogen fertility. Non-flowering *C. americanus* plants (8% to 28% of individuals among sites) tended to be smaller than flowering plants and may represent recruitment. The evidence supports the hypothesis that *C. americanus* patches increase  $\beta$  diversity among plants in tallgrass prairies of central North America.

Key words: *Ceanothus americanus*, actinorhizal plants, tallgrass prairie, C<sub>3</sub> and C<sub>4</sub> species, plant diversity.

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## Activity of nodule-specific promoters in heterologous nodules: implications for the evolution of infection pathways

**Katharina Pawlowski**<sup>1</sup>, Behnoosh Rashidi<sup>1</sup>, Sara Mehrabi<sup>1</sup>, Malgorzata Plaszczyca<sup>1</sup>, Tomas Persson<sup>1</sup>, Irina Demina<sup>1</sup>, Liang Jingsi<sup>2</sup>, Patricia Santos<sup>1,2</sup>, Sergio Svistoonoff<sup>3</sup>, Claudine Franche<sup>3</sup>, Marian Plaszczyca<sup>1</sup> and Ana Ribeiro<sup>2</sup>

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Actinorhizal plant genera can be found in three orders, Fagales (Betulaceae, Casuarinaceae, Myricaceae; intracellular infection), Rosales (Rosaceae, Elaeagnaceae, Rhamnaceae; intercellular infection) and Cucurbitales (Datisceae, Coriariaceae; novel infection mechanism). Conservation of infected cell-specific transcription factors from different types of root nodules was examined by expressing promoter::GUS fusions from *Casuarina glauca* and legumes in transgenic hairy roots of *D. glomerata* and promoter::GUS fusions from *D. glomerata* nodule-specific genes in hairy roots of *C. glauca* or *Lotus japonicus* or in transgenic *Medicago truncatula*. Two cell type-specific promoters – *Vflb3* (specific for infected cells of *Vicia faba* nodules), *Cg12* (specific for infection thread-containing cells of *C. glauca* nodules) - and two nodule specific promoters expressed in different nodule cell types – *Cgchi3* from *C. glauca* and *Dg282* from *D. glomerata* – were examined. The infected cell-specific expression of *Vflb3* was conserved in *D. glomerata*, but the infection thread-containing cell-specific expression of *Cg12* was not. The nodule-specific expression pattern of *Cgchi3* was conserved in *D. glomerata*, but not in *L. japonicus*. The nodule-specific expression pattern of *Dg282*, however, was conserved in *M. truncatula*. The implications of these results will be discussed.

## OC10

### Engineering the gene predisposition to nitrogen fixation: a global approach of the symbiotic program in *Casuarina glauca* and *Alnus glutinosa*

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Two actinorhizal symbiotic systems *Frankia-Casuarina glauca* and *Frankia-Alnus glutinosa* were used as models to develop a comparative genomic approach to study the molecular mechanisms leading to actinorhizal nodule development and functioning. This approach will allow identifying new key plant genes that control nodulation and genes implicated in the early symbiotic signalling pathway will be specially targeted. Indeed, recent work developed on the legumes–*Rhizobium* symbiosis have led to the identification of plant genes involved in the Nod and Myc signaling transduction pathways and it was recently demonstrated that the first gene of this common signaling pathway (*CgSymRK*), encoding a LRR protein kinase, is also involved in regulating nodulation and arbuscular mycorrhization in *Casuarina*.

#### EST sequencing

Thanks to a Genoscope project, a collection of around 40 000 EST was obtained for both species from root and nodulated root at different stages of symbiosis. After analysis and assembly, two datasets of 15,000 unigenes were obtained. These datasets were annotated according to Gene Ontology classification using Blast2GO (<http://blast2go.bioinfo.cipf.es/home>). Similar functional categories were found in the same proportion for the two species: around 50% of the genes were blast and/or GO annotated while 50% remains unknown. Based on legumes sequences comparison, new genes like *Nin* or *CCaMK* were found and their function is now studied in *C. glauca*.

#### Microarray

*C. glauca* and *A. glutinosa* unigenes were further used for 15K custom microarrays design (1 per species). Microarray hybridizations were performed by Diagnogen-Imaxio (<http://www.diagnogene.fr/>) using Agilent technology. QPCR run on around 40 genes per species were performed subsequently for microarray validation. Transcriptomic data were analyzed to identify regulated genes implicated in actinorhizal symbiosis. Around 2000 genes were found regulated or induced in *C. glauca* during nodulation against 1500 in *A. glutinosa*. Among the top 10 regulated/induced genes, we found *Cg12* and *Ag12*, a subtilase involved early in actinorhizal symbiosis. We thus expect the identification of other marker genes that will help us to identify symbiotic signal molecules from *Frankia* and plant. Furthermore, a comparison of the data obtained with the one obtained from Legumes is now performed and should allow the identification of common and divergent symbiotic pathways. Results obtained will be presented and discussed.

## Functional analysis of defense-related genes involved in *Casuarina glauca*-*Frankia* symbiosis

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Root nodule formation is an intricate and complex process that must be tightly regulated. The picture emerging from recent research indicates that similar mechanisms are used by the plant to recognize and accommodate pathogens and symbiotic microorganisms. At the plant level, the induction of defense events has been associated with nodule development, microsymbiont infection control and defense against external pathogens. The research conducted by the Eco-Bio/IICT team focuses on the role of defense-related genes/proteins during the symbiosis between *Casuarina glauca* and *Frankia* as well as on the physiology and proteomics of plant adaptation to salt stress. During this conference we will present an overview of our research strategy. Emphasis will be given to the follow-up of the work regarding the identification and characterization of five defense-related genes, *CgChi1* and *CgChi3* (encoding class I and III chitinases), *CgHin1* (encoding a Hairpin-inducible protein), *CgPox4* (encoding a peroxidase) and *CgGST* (encoding a glutathione S-transferase). Particularly we will focus on the functional analysis of three genes/proteins, *CgChi3*, *CgHin1* and *CgGST* through: i) promotor - reporter gene analysis in *C. glauca*, *Datisca glomerata* and *Lotus japonicus*; ii) complementation of knockout mutants in *Arabidopsis thaliana*; and iii) the production and characterization of recombinant proteins. In general, the results show a certain degree of conservation between *C. glauca*, *Datisca glomerata* and *Lotus japonicus*, as judged by the partial retention of reporter gene expression patterns driven by *C. glauca* gene promoters. Preliminary results, point for the ability of at least *CgHin1* to complement *A. thaliana* mutants and for the successful physiological analysis of the recombinant proteins.

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## OC12

### Characterization of TTA codon containing genes in *Frankia* and exploration of the role of tRNA in regulating these genes

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The TTA codon, one of the six available codons for the amino acid leucine, is the rarest codon among the high GC genomes of actinobacteria including *Frankia*. This codon has been implicated in various regulatory mechanisms involving secondary metabolism and morphological development. Although TTA-mediated regulation is well documented in *Streptomyces coelicolor*, but that role has not been investigated in other actinobacteria including *Frankia*. Among the sequenced *Frankia* genomes, *Frankia* sp. EAN1pec, CcI3 and ACN14a have 625, 361, and 350 TTA-containing genes, respectively. These numbers corresponds to highest percentages (7.9 %, 8.7 % and 5.2 % of *Frankia* sp. EAN1pec, CcI3 and ACN14a genomes, respectively) amongst the Actinomycetes with genome GC content more than 70%. In comparison, TTA-bearing genes comprised 1.7, 3.4 and 4.1 % of the *S. coelicolor*, *Streptomyces avermitilis* and *Nocardia farcinia* genomes. The functional distribution analysis of TTA codon bearing genes reveals that these genes are well represented in COG functional group 3 (Metabolism) and group 4 (Poorly Characterized). The predicted gene expression level of TTA-containing genes was estimated by the use of the Codon adaption index (CAI) and was significantly lower in TTA-containing genes than those values for all of the protein coding genes or ribosomal genes. The CAI values of these genes also show a positive correlation with the GC3. The tAI (t-RNA adaptation index) values of the TTA bearing genes were also determined and used to predict how these genes are co-adapted to the tRNA gene pool. The tAI values provides predictive information on the translational efficiency of these genes. Analysis of the three *Frankia* nucleotide sequences for the TTA -t-RNA revealed that *Frankia* sp. CcI3 and ACN14a were very similar, while *Frankia* sp. EAN1pec was slightly different. A full atomic 3D model of the *Frankia* leucyl t-RNA (with TAA anticodon) generated by the use of the NAST tool kit showed a 3D structure quite consistent with a typical L-shaped framework which allows them to fit into the P and A sites of the ribosome.

## Genome-based Perspectives on the *Datisca-Frankia* Root Nodule Symbiosis

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The *Datisca-Frankia* root nodule symbiosis represents an outlier, both in evolutionary terms, and with respect to the physiology of nitrogen assimilation. We showed earlier that host plant cytoplasmic glutamine synthetase is absent from *Frankia*-infected tissue, a surprising finding that suggested a strong role for nitrogen assimilation in *Frankia*, rather than direct ammonium export. However, understanding of the underlying mechanisms has been lacking. The genome of *Datisca-Frankia* has now been sequenced to an advanced draft. Root nodule transcriptome data, based on the genome sequence, allow us to assemble a novel picture of nitrogen fixation and nitrogen assimilation in the root nodules of *Datisca glomerata*.

## OC14

### What are the stories that the *Frankia* genomes are telling us?

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*Frankia* are nitrogen-fixing actinobacteria that form root nodules with dicotyledonous plants in 8 families of angiosperms that are only distantly related to each other. Symbiotic interactions between *Frankia* and the host plant are not well understood. The nature of the chemical signals exchanged between the two partners of actinorhizal symbioses is still unknown due to the lack of genetic tools in *Frankia* and of specific molecular markers of the symbiotic interaction. While we have focused on resolving this situation by developing genetic tools, we have also pursued new genomic approaches toward studying these bacteria. Three *Frankia* genomes were sequenced completely and six more genomes are currently in the pipeline being sequenced. In the absence of genetic tools, the availability of these genome sequences also provides opportunities to use bioinformatic approaches and other new technologies. Analysis of the *Frankia* genomes has provided a myriad of information on these bacteria and several surprises including the extraordinary size discrepancy among the six *Frankia* genomes (5.4 Mb for strain CcI3 to 9.1 Mb for strain EAN1pec). The absence of obvious nodulation genes similar to those found in *Rhizobia* genomes suggests that the actinorhizal symbiosis uses novel signal compounds during the infection process. These genomes reveal potential new metabolic capabilities and tolerance mechanisms. An analysis of the draft genome sequence of *Frankia* EuI1c elucidated the absence of nitrogenase genes and may explain the Fix<sup>-</sup> nature of this infective strain. An overview of the comparative genomics of these *Frankia* genomes and other actinobacterial genomes including *Mycobacteria* will be presented.

## A comparative genomic approach to the study of Geodermatophilaceae

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Calcareous stones, used for the construction of buildings since the dawn of humanity, constitute a habitat for a variety of microbes, prominent among which are the Geodermatophilaceae comprising three genera: *Geodermatophilus*, *Modestobacter* and *Blastococcus*. These bacteria contribute to deterioration of stones, both on the surfaces and inside of them. These bacteria are also very resistant to ionizing radiation, surviving massive doses of gamma radiation comparable to those published for *Deinococcus*<sup>1</sup>. To understand which determinants permit the strains to thrive in this biotope without obvious sources of organic carbon, and how they can resist high radiation doses, the genetic basis of their contrasted pigments, the genome of one representative of each genus was sequenced.

One genome, that of *Geodermatophilus obscurus*, was sequenced by the JGI in the course of the GEBA project<sup>2</sup> and is now complete. The other two genome, *Modestobacter multiseptatus* and *Blastococcus saxobsidens*, were determined by the Genoscope and are not completely finished but will be soon. The sizes of the 3 genomes range between 4.6Mb and 5.6Mb, have G+C% between 72 and 74% and contain 3-5 copies of *cox*, the gene responsible for the synthesis of the carbon monoxide dehydrogenase, suggesting, besides an heterotrophic metabolism, a potential autotrophic capacity. Several determinants putatively involved in the resistance to different stress agents, including heavy metals, salinity and radiations have been found, confirming the phenotype properties. The capacity of these actinobacteria to thrive in harsh environments like calcarenite rocks is discussed in the light of the properties of their metabolism deduced from study of the genome.

1. Rainey, F. A., K. Ray, M. Ferreira, *et al.* 2005. Extensive diversity of ionizing-radiation-resistant bacteria recovered from Sonoran Desert soil and description of nine new species of the genus *Deinococcus* obtained from a single soil sample. *Appl Environ Microbiol* **71**: 5225-5235.

2. Wu, D., P. Hugenholtz, K. Mavromatis, *et al.* 2009. A phylogeny-driven genomic encyclopaedia of Bacteria and Archaea. *Nature* **462**: 1056-1060.



## Posters

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## Study of early events in actinorhizal symbioses: role of *Casuarina* CCaMK in the *Frankia* signal transduction and the regulation of nodulation process

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Nitrogen-fixing root nodule symbioses are confined to four plant orders, and encompasses two distinct types of associations: the interaction of legumes (Fabales) with rhizobia bacteria and actinorhizal symbioses, where the bacterial symbionts are actinobacteria of the genus *Frankia*.

Little is known about the genetic basis of the actinorhizal symbioses and the mechanisms by which actinorhiza forming plants recognize *Frankia* symbionts remain unknown. In contrast, in the legume-rhizobium symbiosis, several downstream components of the Nod factors signaling cascade, including SymRK/DMI2/NORK, DMII/Castor&Pollux, CCaMK/DMI3 genes have been identified. We have previously demonstrated that *C. glauca* Symbiosis Receptor Kinase (SymRK) is necessary for both nodulation and mycorrhization (AM) and can functionally replace the legume SymRK (Gherbi et al., 2008). Recently, we have cloned the CCaMK/DMI3 homologue from *C. glauca*. CCaMK encodes a calcium and calmodulin-dependant kinase dually required for rhizobial and mycorrhizal symbioses in legumes. CCaMK is supposed to decode and transduce Nod factor specific calcium spiking response. CCaMK protein contains a predicted CaM-binding/autoinhibition domain that is central to the regulation of its activity. Interestingly, a deregulated version of the protein, by specific removal or targeted mutagenesis of this domain, can trigger spontaneous nodulation in the absence of rhizobia. Functional characterization of CgCCaMK is currently elaborated. Moreover, deregulated CgCCaMK constructs are being prepared and introduced in *C. glauca* in order to induce spontaneous nodules.

Our functional study will allow us to gain insight into the genetic basis of signal transduction in the actinorhizal symbiosis and data will finally give us information relative to the degree of conservation of genetic mechanisms between actinorhizal and legume symbioses.

## P2

### Specific auxin carriers localization actuates auxin accumulation in plant cells infected by *Frankia* in *Casuarina glauca* actinorhizal nodules

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Actinorhizal symbioses are mutualistic interactions between plants and the soil bacteria *Frankia* that lead to the formation of nitrogen-fixing root nodules. Little is known about the signaling mechanisms controlling the different steps of the establishment of the symbiosis. The plant hormone auxin has been suggested to play a role. Here we report that auxin accumulates within *Frankia*-infected cells in actinorhizal nodules of *Casuarina glauca*. Using a combination of computational modeling and experimental approaches, we demonstrate that this localized auxin accumulation is driven by the cell-specific expression of auxin transporters and by *Frankia* auxin biosynthesis in planta. Our results indicate that the plant actively restricts auxin accumulation to *Frankia*-infected cells during the symbiotic interaction.

## The role of flavonoids in actinorhizal symbiosis

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In the symbiosis between legumes and *Rhizobium*, flavonoids are key molecules for nodulation. Recent studies using RNA interference (RNAi) mediated silencing of key flavonoid enzyme has provided direct genetic evidence for the essential roles that these compounds play in nodulation (1, 2). In actinorhizal symbiosis, different studies suggest a role of flavonoid during nodulation. In *Casuarina glauca* nodule, an accumulation of flavanols was described concomitant with an accumulation of chalcone synthase (CHS) transcripts (3). In *Elaeagnus umbellata* Thunb, a chalcone isomerase gene was isolated and its expression appears to be restricted to nodule infected cells of the nitrogen fixation zone (4). Moreover, expressed sequence-tag (EST) analysis in roots and nodules of *Casuarina glauca* revealed that several genes linked to flavonoid biosynthesis pathway were more expressed in nodule compare to not inoculated roots (5). To go deeper in the understanding of the role of flavonoids in actinorhizal symbiosis, we used RNA interference strategy recently developed in our group for *C. glauca* (6) to silence chalcone synthase, the enzyme that catalyzes the first committed step of the flavonoid pathway. Chalcone synthase silenced *Casuarina glauca* plants are now under analyses: CHS transcript level was measured by qPCR, flavonoids were identified and quantified by LC-MS. Evaluation of nodulation ability of CHS silenced plants is in progress. This poster will discuss the first results of the characterization of *Casuarina glauca* CHS silenced roots.

(1) Wasson *et al.*, 2006 *Plant Cell* 18: 1617-1629.

(2) Subramanian *et al.*, 2007 *Trends in Plant Science* 12: 282-285.

(3) Laplaze *et al.*, 1999 *Plant Physiology* 121: 113-122.

(4) Kim *et al.*, 2007 *Molecules and Cells* 23: 405-409.

(5) Hocher *et al.*, 2006 *New Phytologist* 169: 681-688.

(6) Gherbi *et al.*, 2008 *Molecular Plant Microbe Interaction* 21: 518-524.

## P4

### Functional changes in alder-EMF relationships affect mycorrhizally driven phosphorus mobilization across a boreal forest succession sequence

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Ecosystem processes within the Alaskan boreal forest depend heavily on inputs of biologically fixed nitrogen (N) from *A. tenuifolia*, which contributes the majority of N accumulated during forest succession. Because of the high phosphorus (P) demands of this plant, we hypothesize that N-fixation inputs are controlled by the ability of alder to access P through associations with ectomycorrhizal fungi (EMF) that produce enzymes which mobilize organic and recalcitrant P forms. Because the forms and availability of P are known to change throughout forest succession and between soil horizons, and because resource availability is frequently linked to enzyme activity, we expected to see parallel shifts in EMF communities and function at these same scales. Using fluorogenic substrates, we measured the activity of acid phosphatase, phosphodiesterase and phytase enzymes bound to the surfaces of individual mycorrhizally-infected alder root tips (n=420) collected from organic and mineral soils across early, mid and late successional stands along the Tanana River floodplain in interior Alaska. Activities of acid phosphatase and phosphodiesterase were positively correlated across stages and horizons (all  $P < 0.05$ ) with the strongest relationships found in early succession ( $R^2 = 0.41$ ) and in mineral soils ( $R^2 = 0.35$ ) where it is generally assumed a low proportion of nutrients exist in organic forms. Potential rates of acid phosphatase and phosphodiesterase were highest in late successional stands (both  $P < 0.001$ ) where on average these enzymes mobilized 2.50 and 0.18  $\mu\text{mol P mm}^2 \text{ root tip surface area}^{-1} \text{ hr}^{-1}$ , respectively. Horizon effects were only seen for acid phosphatase ( $P=0.018$ ), which overall, was higher in organic vs. mineral soils, though, strong stage by horizon interactions were found for phosphodiesterase ( $P=0.029$ ). These data suggest that the functional traits of individual EMF species vary between soil horizons and throughout succession. We used ARISA (automated ribosomal intergenic spacer analysis) to analyze fungal ITS rDNA sequences isolated from individual alder root tips to a) determine if differences in enzyme activities across succession and between horizons were due to shifts in EMF community structure, and b) assess if enzyme activities within a given EMF species varied across these spatial scales. ARISA ribotypes will be matched with sequences of known EMF. Paired enzyme and community data will be linked with soil phosphorus availability (total P, resin extractable P and organic phosphorus fractions) and indices of plant N:P balance to evaluate how changes in the forms of soil P influence the structure and function of alder's EMF community. This work is part of a larger project at Bonanza Creek LTER that is manipulating partner choice in alder/EMF and alder/*Frankia* mutualisms using N and P fertilization.

## Effects of elevated CO<sub>2</sub> and soil nutrients and water conditions on photosynthetic and growth responses of *Alnus hirsuta*

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The objective is to clear the effects of multiple environmental conditions, such as elevated CO<sub>2</sub> and soil conditions, on the physiological and morphological properties of *Alnus* species in order to predict the responses to environmental changes in the future. We examined the responses of photosynthetic properties (maximum carboxylation rate (V<sub>cmax</sub>), maximum electron transport rate (J<sub>max</sub>), and light saturated photosynthesis (P<sub>sat</sub>) at growth condition), leaf characters (morphological characters, N content, and total non-structural carbohydrate contents), and growth properties (total biomass, biomass allocation, and N allocation within tree) of *Alnus hirsuta* to elevated CO<sub>2</sub> and soil properties (soil nitrogen (N) availability, soil phosphorus (P) availability, and soil drought) from the results of following two experiments. In experiment I, potted seedlings were grown at either ambient or elevated CO<sub>2</sub> concentrations (36 Pa and 72 Pa CO<sub>2</sub>), with different levels of N supplied (52.5, 5.25 and 0 mgN pot<sup>-1</sup> week<sup>-1</sup> for High-N, Low-N and N-free, respectively) in a natural daylight phytotron. In experiment II, potted seedlings were also grown at 36 Pa and 72 Pa CO<sub>2</sub>, with different levels of P supply (7.7 and 0.77 mgP pot<sup>-1</sup> week<sup>-1</sup> for High-P and Low-P, respectively) and water supply (three times and once week<sup>-1</sup> for Wet and Dry, respectively) in the same natural daylight phytotron. In the experiment II, N supply was set at the same level of Low-N in the experiment I. Therefore, we used the seedlings grown under the same treatment (Low-N and 36 Pa CO<sub>2</sub> in experiment I, High-P and Wet and 36 Pa CO<sub>2</sub> in experiment II) as a control, and we compared the relative variation under each treatment from the control. In general, the effects of P availability was more marked than those of N availability and soil drought, though there were some parameters, such as stomatal conductance, which showed no effects by P availability. Especially, it was remarkable that the effects of soil drought for some parameters depended on P availability. These results will contribute to predict the physiological and growth responses of N<sub>2</sub>-fixer to multiple environmental conditions by using simulation model in the future.

## P6

### Use of high-activity promoters and a codon-optimized marker gene for *Frankia* transformation

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*Frankia* is a nitrogen-fixing actinomycete which establishes symbiosis with woody plants called actinorhizals. Molecular mechanism of *Frankia* symbiosis is poorly understood at least partially because transformation method of *Frankia* has not been established. Previously, we tried to transform *Frankia* sp. strain CcI3 using a fusion marker gene consisting of translation initiation factor 3 gene (*infC*) promoter of the strain and tetracycline resistance gene (*tet<sup>R</sup>*) whose codon usage is similar to *Frankia*'s. The fusion marker gene was introduced into CcI3 cells by electroporation and transformants were selected in a liquid medium. As the result, antibiotic resistant cells were grown in the selective medium, but marker genes in the population were decreased during passage culture. Probably, the selective liquid culture would have contained spontaneous mutants which obtained antibiotic resistance by natural mutations and they would be more tolerant to the antibiotic than transformants. So we supposed that more active promoter increases transformation efficiency. Based on the *Frankia alni* ACN14a microarray data we selected 3 promoters which appeared to be more active than *infC* promoter. They were fused with *tet<sup>R</sup>* coding sequence and these fusion genes were electroporated to CcI3 cells. But growth rate of those cells in selective media was not significantly improved compared to the cell electroporated with the fusion gene with *infC* promoter. As an alternative trial, we synthesized a modified gentamicin resistance gene *fgm<sup>R</sup>* whose codon usage frequency was optimized to CcI3. The *fgm<sup>R</sup>* was connected to *infC* promoter. CcI3 cells electroporated with the *fgm<sup>R</sup>* fusion gene grew more rapidly in selective media than those electroporated with native *gm<sup>R</sup>* fusion gene. This result suggests that optimization of codon usage frequency improved expression of exogenous gene in *Frankia* cells.

## Isolation of uracil auxotrophic mutants in *Frankia*

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*Frankia* is a symbiotic actinobacterium which induces nitrogen-fixing nodules on root of actinorhizal plants. Molecular basis of the symbiosis is unclear due to a lack of the transformation system in *Frankia*. In this study, we isolated uracil auxotrophic mutants of *Frankia* sp. strain CcI3 by positive screening using 5-fluoroorotic acid (5-FOA) to establish the transformation system using an endogenous orotidine-5-phosphate decarboxylase gene (*pyrF*) as a selection marker. Cells of CcI3 were mutagenized with 0, 1, 2, 4 and 8% ethyl methanesulfonate (EMS) and selected 5-FOA resistant (5-FOA<sup>R</sup>) colonies on a solid media supplemented with uracil. Seven 5-FOA<sup>R</sup> colonies were isolated; six of them (CcI3E21, CcI3E22, CcI3E23, CcI3E24, CcI3E25 and CcI3E26) were induced by 2% EMS and one of them (CcI3E41) was induced by 4% EMS. Occurrence of 5-FOA<sup>R</sup> mutants is  $3.7 \times 10^{-5}$  and  $1.5 \times 10^{-6}$  for 2% and 4% EMS treatments. We checked uracil auxotrophy of the mutants on a minimal media. Strains CcI3E21, CcI3E23, CcI3E25 CcI3E26 and CcI3E41 exhibited uracil auxotrophy. We found that strains CcI3E21, CcI3E23, CcI3E25 and CcI3E41 were carried a mutation in *pyrF* while strains CcI3E22 and CcI3E24 carried a mutation in *pyrE*. We tried transformation using a *pyrF* mutant (CcI3E21) as a host. To generate functional *pyrF* marker gene, we fused a coding sequence of *pyrF* with two different promoters. One of them is derived from translation initiation factor 3 gene of *Frankia* CcI3 whose promoter activity was confirmed previously. The other is an own *pyrF* promoter predicted by gene arrangement of pyrimidine synthesis operon. The fusion marker genes were flanked by 4-kbp genomic sequences of CcI3 with an expectation that are integrated into the chromosome by single- or double-crossover recombination. We introduced the constructs into the cells by electroporation and selected transformants on a minimal media. Results of the transformation will be presented.

### **Transformed hairy roots of *Discaria trinervis*: A tool for studying actinorhizal symbiosis in the context of intercellular infection**

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The mechanisms and genetics by which actinorhiza forming plants recognize *Frankia* symbionts, before and during infection, remain unknown. Actinorhizal plant species are distributed within the orders Fagales, Cucurbitales and Rosales. The most studied model systems, in terms of molecular biology and genetics, belong to Fagales (*Alnus spp* and *Casuarina spp*) which at the same time are examples of the root hair infection pathway. *Discaria trinervis*, a Patagonian Rhamnaceae shrub, is an example of actinorhizal plants belonging to Rosales which has been described to be infected via intercellular pathway. We have set up a genetic transformation of *Discaria trinervis* root system based on *Agrobacterium rhizogenes*, comparing *in vitro* transformation system and *ex-vitro* one. Composite plants with transgenic roots can be specifically and efficiently nodulated allowing for functional nitrogen fixing symbiosis by inoculation with *Frankia* BCU110501. We studied in *Discaria trinervis* the activation of promoters of symbiotic marker genes from legumes (MtEnod11) or other actinorhizal plants as *Casuarina glauca* (CgAux1 and Cg12), which have been previously characterized in root hair infected actinorhizal plants. Prior to *Frankia* inoculation no activation was detected in plants transformed with ProCg12::GUS whereas the promoters of CgAux1 and MtEnod11 were strongly active in the root vasculature and root tips of plants. Upon the inoculation by *Frankia* BCU110501, strong activation of ProCg12 and ProMtEnod11 was detected in the root cortex in places where small nodule primordia were starting to appear. The expression was localized in the apical zone of the developing nodule corresponding to the infection zone. Upon the inoculation by *Frankia* BCU110501, strong activation of ProCg12 and ProMtEnod11 was detected in the root cortex in places where small nodule primordia were starting to appear. The expression was localized in the apical zone of the developing nodule corresponding to the infection zone. ProCg12 appears to be a good reporter for the study of intercellular root invasion. In contrast to ProCg12 and ProMtEnod11, ProCgAux1 was not active during *Frankia* intercellular infection. GUS expression was observed in young and mature nodular lobes, related to infection zone and initially infected hyperplastic cells but no GUS crystals were detected in infected cells with vesicle differentiation. These promising results prompt us to look for the orthologous genes in *Discaria trinervis*. Dt12 has been detected and seem to be specifically induced in nodules and not in roots. The developed strategy would now enable the study of the intercellular infection pathway and the molecular mechanisms of the interaction of *Frankia* with actinorhizal plant belonging to the order Rosales.

## The Effect of *Casuarina cunninghamiana* Root Exudates on *Frankia* Cc13 Growth, Physiology, and Gene Expression

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Although the symbiosis between *Frankia* and its actinorhizal host plants have been studied widely, very little is known about the initial molecular interaction between *Frankia* and the host. One hypothesis is that the first host signaling molecules received by *Frankia*, which initiates the infection process, are present in host root exudates released into the rhizosphere. To address this issue, we measured differences in *Frankia* physiology after exposure to host root exudates. *Casuarina cunninghamiana* root exudates were collected from plants under nitrogen-sufficient and -deficient conditions and tested on *Frankia* Cc13. Root exudates from plants of different ages were also tested to assess age effects. Root exudates from 60-day-old plants increased the growth yield of *Frankia*, while younger plants had less of an effect. Exposure to root exudates caused *Frankia* hyphal "curling" suggesting a chemotrophic response. This "curling" effect was also observed with *Frankia* EAN1pec and its host root exudates. Surface property changes in response to root exudates exposure were investigated by FTIR spectroscopy. Hypahae exposed to root exudates for 5 days showed changes in their FTIR profile indicating a modification in the chemistry of these hyphal surfaces. The most significant changes occurred in the region of C-H (aliphatics), C-N, and C-O bonds. The *Frankia* genome was data-mined for genes of interests for host-microbe interactions and the gene expression pattern of these genes are being analyzed by qPCR. Several genes showed up-regulation in response to root exudates exposure. The results of this study will be discussed in host-micobe signaling events.

## An overview of actinorhizal plants in Africa

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Diversity and biogeography of actinorhizal symbionts is less well-known in Africa than in other parts of the world. For example, no detailed distribution maps exist for most African actinorhizal plants. This review provides a synthesis of current information on Africa's actinorhizal plants compiled from floristic studies, literature on current and past distribution, as well as plant databases. Plant taxa able to bear actinorhizal root nodules are widespread in Africa, with greatest diversity in the native component of the flora than in introduced plants, and may exist in most African countries. African actinorhizal plants are comprised of fifty species, in six families and ten genera: *Betulaceae* (*Alnus*), *Casuarinaceae* (*Casuarina*, *Allocasuarina*, *Gymnostoma*), *Coriariaceae* (*Coriaria*), *Myricaceae* (*Myrica*, *Morella*), *Rhamnaceae* (*Ceanothus*, *Colletia*) and *Elaeagnaceae* (*Elaeagnus*). Many past and present ecological conditions in Africa are suitable for actinorhizal plants, but native actinorhizal species are universally lacking in extreme desert and tropical rainforest environments where tree legumes occupy a similar niche. This review illustrates the current status and need for more-detailed taxonomic analysis, distribution information, biological studies of exotics, and fossil material if we are to understand and benefit from Africa's wealth of actinorhizal plants.

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## Antioxidant defenses and iron toxicity in *Casuarina* nodules

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Iron is an essential nutrient with limited bioavailability. When present in excess, iron poses a threat to cells and tissues, and therefore iron homeostasis has to be tightly controlled. The effect of iron toxicity was explored with *Casuarina cunninghamiana* seedlings in water culture jars containing Hoagland's solution prepared with different iron concentrations (20 to 40  $\mu$ M) in the form of Fe-EDTA. Results showed that Fe-overdose (80  $\mu$ M) induced significant reduction in nodulation percent and number of nodules developed. The uninoculated and non-nodulated *Casuarina* seedlings were highly affected and recorded significant reduction in plant biomass and chlorophyll content. Iron overdose also affected the activity of some antioxidant enzymes such as catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) in tissues of *Casuarina* seedlings. A non-enzymatic antioxidant (glutathione) also affected significantly with different Fe-concentrations. Augmentation of the lipid peroxidation level was highly affected and recorded a positive correlated with iron overdose. Detection of the presence of high levels of enzymatic and non-enzymatic antioxidants in nodule tissues and the shoot systems of inoculated seedlings may reflect the importance of inoculation as a defense mechanism under iron toxicity.

**Key words** Iron toxicity, nodulation, *Frankia* strain, *Casuarina*

## Physiological and compositional responses of *Frankia* to N and P fertilization of *Alnus tenuifolia* in interior Alaska

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Thin-leaf alder (*Alnus tenuifolia*) dominates early-successional floodplain stands throughout interior Alaska, where it can fix > 100 kg N ha<sup>-1</sup> yr<sup>-1</sup>. Because the species persists through a range of environmental conditions and soil environments across 200 years of forest development, we tested the hypothesis that *Frankia* partner choice and physiological function would respond to changing soil nutrient conditions that either up- (+P) or down-regulate (+N) N<sub>2</sub>-fixation rate. Nitrogen fixation (<sup>15</sup>N<sub>2</sub>-uptake), nodule respiration and *Frankia* genetic structure (*nifD*-K spacer sequence haplotypes) were measured on nodule lobes (n=10 nodules per plant) from thin-leaf alder (n=5 plants per site) growing in mid-successional stands (n=3 sites) dominated by balsam poplar (*Populus balsamifera*) that were unfertilized or fertilized with N (100 kg ha<sup>-1</sup> yr<sup>-1</sup>) or P (80 kg ha<sup>-1</sup> yr<sup>-1</sup>). Relative to control (CTL) plots, P fertilization increased N fixation and nodule respiration 91% and 54%, respectively (both P<0.001), while N fertilization decreased these same parameters by 56% and 30%, respectively (both P<0.05). The ratio of nodule respiration to N fixation (a measure of cost) was significantly less in P-fertilized relative to CTL and +N plots (both P<0.0001), which did not differ. Eight *Frankia* haplotypes were found but two dominants accounted for over 90% of nodule occupancy across all plants. While there were some significant shifts in *Frankia* genetic structure across treatments, the two dominants showed very similar (but dramatic) degrees of up- and down-regulation in response to +P and +N treatments, respectively. Interestingly, the next most-dominant haplotype, which constituted less than 5% of the total number of nodules sampled, failed to up-regulate N fixation in response to P fertilization. This suggests that thin-leaf alder most commonly forms associations with *Frankia* haplotypes with the physiological plasticity to respond to varying soil nutrient conditions that influence plant N-P balance.

## ***Frankia* phylogeny depiction and species definition by Amplified Fragment Length Polymorphism (AFLP)**

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*Frankia* spp. is the unique known genus of the Frankiaceae family and its members display very specific traits, allowing their clear distinction from other actinomycetes (slow growing; original morphological structures such as multilocular sporangia and vesicles; ability to fix atmospheric nitrogen; etc...) Conversely, an important morphological and physiological diversity exists within *Frankia* spp., suggesting a great genetic diversity and the occurrence of several species within the genus. However, in addition to in-vitro isolation and biomass production difficulties, *Frankia* genomospecies definition has been limited by the drawbacks of the DNA-DNA Hybridization (DDH) technique – the golden standard for species definition – and the lack of single-locus analysis, such as 16S rDNA sequencing. In this work, Fluorescent Amplified Fragment Length Polymorphism (F-AFLP) has been used as an alternative to study the phylogenetic structure of *Frankia* genus and for genomospecies definition reappraisal. Forty *Frankia* strains representative of the major phylogenetic groups and some of them belonging to seven known DDH described genomospecies were typed with four primer/enzyme combinations. Primarily, AFLP analyses yielded a dendrogram splitted in three of the major compatibility groups (*Alnus-Myrica*, *Casuarina*, and *Elaeagnaceae*). In addition, all the strains belonging to the same genomic species were grouped consistently in coherent clusters supported by high bootstrap values. A strongly significant correlation was found between the DNA reassociation values and AFLP results. Secondly, the applicability of this approach was tested for the endophytic *Frankia* strain characterization, directly from nodule composite DNA (including plant and bacteria). Greenhouse experimentally inoculated plants with known strains were used for this purpose. The results showed that root DNA might not influence the reliable identification of the nodulant strains (only less than 1.3% of the total AFLP fragments were proved to be generated by the plant DNA). The method was also applied to field *Alnus incana* and *Alnus viridis* nodules, harbouring non-isolated in-planta sporulating strains (Sp+ *Frankia* strains). An AFLP genotyping of uncultured microorganisms was then reported for the first time to our knowledge. In conclusion, the AFLP technique is a suitable and robust alternative for *Frankia* species delineation, reliable typing of novel isolates as well as for population structure studies.

## Assessment of $^{15}\text{N}$ natural abundance in leaves of *Coriaria ruscifolia* and other plant species occurring in humid forests of northwest Patagonia

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The actinorhizal species from Northwest Patagonia growing in the semiarid steppe, *Ochetophila trinervis* and *Discaria chacaye* (Rhamnaceae), have a foliar natural abundance of  $^{15}\text{N}$  ( $\delta^{15}\text{N}$ ) different than that of reference plants and fix considerable amounts of N (Chaia & Myrold, 2010). Corresponding information about actinorhizal plants growing in the humid forests of the region is lacking. To better understand the role of native actinorhizal plants in the N economy of northwest Patagonian ecosystems we assessed the foliar  $\delta^{15}\text{N}$  and N concentrations of *Coriaria ruscifolia* (Coriariaceae), the rhamnaceous species *D. chacaye* and *Colletia hystrix*, and some non-actinorhizal species growing adjacent to or distant from the actinorhizal plants at four sites. In addition, concentrations of N and  $\delta^{15}\text{N}$  of soils under sampled plants were measured. Foliar N concentrations in *C. ruscifolia*, *D. chacaye* and *C. hystrix* were significantly higher than in non-actinorhizal shrubs growing at the same sites, even though soils under the studied plants had very low and similar N concentrations. Foliar N concentration in reference plants growing either adjacent to or distant from the actinorhizal plants was similar. Foliar  $\delta^{15}\text{N}$  of *C. ruscifolia*, *D. chacaye* and *C. hystrix* was close to 0 and was not correlated with soil  $\delta^{15}\text{N}$  values across the sites. A tendency towards higher foliar  $\delta^{15}\text{N}$  mean values in actinorhizal plants than in most non-actinorhizal plants was observed in three out of four studied sites. Preliminary evaluation of the data indicates that all studied actinorhizal species fix atmospheric nitrogen in the humid Patagonian forests.

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## Actinorhizal Non-*Frankia* isolates provide an alternative to study the symbiotic infection process

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Molecular events occurring during the infection process of actinorhizal plant by *Frankia* remain an enigma mainly due to the lack of genetic transformation protocols for this filamentous actinobacterium. Analysis of three *Frankia* genomes revealed the absence of a symbiotic island or canonical *nod*-gene cluster suggesting novel mechanisms for the infection process. The aim of our research is the detection of symbiotic-related genes horizontally transferred from *Frankia* to closely related and sympatric actinobacteria amenable to genetic manipulation.

Several actinobacteria have been isolated from actinorhizal root nodules isolated from *Casuarina* sp. plants in Tunisia. They have been assigned to *Streptomyces*, *Nocardia* and *Micromonospora* based on 16S RNA sequences. The isolates have been analysed for plant infectivity, the presence of symbiotic genes (*nif*, *shcI*, *hup1*, *glbO*, *hup2*), their effect on plant growth, and beneficial physiological traits including hydrolytic activities, indol-3-acetic acid (IAA) production, and antagonistic activities.

The genomes for two of selected isolates are currently being sequenced. The availability of these genomes will aid efforts in the identification and manipulation of common core genes involved in actinorhizal infection process.

## Casuarinaceae for soil rehabilitation in Algeria

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*Casuarinaceae* trees are fast-growing multipurpose species which do not require chemical fertilizers due to their symbiotic association with the nitrogen-fixing actinomycete *Frankia* and with mycorrhizal fungi that will contribute to improve phosphorous and water acquisition by the root system. *Casuarinaceae* can grow in difficult sites, colonize eroded lands and improve their fertility, allowing the subsequent growth of more demanding plant species. Therefore, these trees have been increasingly used for reforestation and reclamation of degraded lands in tropical and subtropical areas.

In Algeria, sand mining activities are developed to fulfill the need for building. These activities have indeed a negative impact on the environment due to the destruction of natural ecosystems through removal of soil and vegetation. The restoration of mined land includes ecosystem reconstruction via the reestablishment of the capability of the land to capture and retain fundamental resources.

The objective of the project that is currently developed between Montpellier and Oran is to evaluate the potential of *Casuarinaceae* trees for the rehabilitation of areas degraded due to intensive sand extraction in the region of Mostaganem (Société des Carrières de l'Ouest). Some *Casuarinaceae* species such as *C. equisetifolia* have already been introduced in Algeria. So far, to our knowledge, no data are available concerning the identification of symbionts associated with *Casuarina* in Algeria.

The *Casuarinaceae* species that will be the most appropriate for these degraded sandy and salty areas will be identified. The production of *Casuarina* trees for land reclamation will imply the inoculation with suitable *Frankia* and mycorrhizal strains. Their effectiveness for nitrogen and phosphorous acquisition will be evaluated together with their ability to persist in the soil after planting. Contribution of *Casuarina* to soil fertilisation will be determined.

## Gene transfer for the improvement of Casuarinaceae

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Long breeding cycles, large size, high levels of heterozygosity, and the economics of producing and evaluating large segregating populations of trees are some of the difficulties encountered in breeding forest trees. Genetic engineering offers prospects for generating novel forest tree genotypes at an accelerated rate. One major advantage of this approach over conventional breeding is that only the characteristics of interest are inserted into the recipient plant while the original genetic framework remains unchanged. Although genetic engineering in trees is still in its infancy, several studies have clearly established its potential for introducing novel genetic characters, such as herbicide tolerance, insect resistance, or modifying lignin content.

In order to successfully regenerate transgenic plants using the natural *Agrobacterium tumefaciens* gene transfer system, a number of parameters has to be fulfilled: 1) the virulence of the *Agrobacterium* strain should permit the transfer of the T-DNA into the wounded plant cells 2) the transformed cells should be efficiently selected among the population of non-transformed cells 3) the transformed cells should be regenerated into plants. The natural susceptibility of members of the *Casuarinaceae* family to *A. tumefaciens* was used to develop gene transfer procedures for *Allocasuarina verticillata*, *C. glauca* and *C. cunninghamiana*. Experiments are also in progress for *C. equisetifolia*.

*Casuarina* plantations are faced with a number of problems including diseases and pests. Developing gene transfer techniques in *Casuarinaceae* trees is therefore a major issue to contribute to the genetic improvement of these valuable tropical tree species. Besides, it is an important tool for the basic molecular knowledge of the symbiosis established between *Frankia* and *Casuarina*.

## Defense-related events in the early stages of *Casuarina glauca* – *Frankia* symbiosis

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The soil actinomycete *Frankia* has the ability to establish a nitrogen-fixing symbiosis on the roots of actinorhizal plants. The symbiotic process involves a complex interplay between the host and its symbiont, and results in a drastic alteration of the expression of the host actinorhizal genes. Within the framework of a bilateral project PESSOA (Portugal/France), some defence related events were investigated during the symbiotic interaction between the tropical tree *Casuarina glauca* and the *Frankia* strain CcI3.

Transcriptomic analyses are in progress to reveal the molecular mechanisms of the plant response upon *Frankia* infection. Gene expression during the early stages the symbiotic process was analysed by means of a cDNA array including 15 000 non-redundant unigenes. A hundred of genes potentially involved in biotic or abiotic stress were found, among them the majority is regulated in nodules and/or in roots 7 days after the inoculation with *Frankia*. Three genes were selected for further analyses: *PR1* (Pathogenesis-related 1), *NPR1* (Non-expressor of Pr1) and *APX* (Ascorbate peroxidase). Their expression profile was assessed using Q-RT-PCR in roots and nodules. Their potential involvement in pathogen-related responses was assessed by analyzing their expression in roots incubated in salicylic acid, a plant hormone known to elicit the expression of several pathogen-responsive genes. Additionally, the presence of reactive oxygen species (ROS) was observed in young and mature nodules. Our results suggest that defence-like responses are activated in symbiotic tissues and may play a role during the early stages of the symbiotic interaction

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## Genome analysis of *Frankia* by next generation sequencer

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Recent advances in high-throughput next generation sequencer have opened a new era in microbial genome biology. We have made use of Applied Biosystems SOLiD sequencer for *Frankia* genome analysis. As a first trial, we resequenced two known genomes of *Frankia* strains CcI3 and ACN14a. We obtained about 2 Gbp sequence data consisting of more than 40 million of 50-bp reads for each strain. Those reads were mapped on 96.3% and 99.4% of CcI3 and ACN14a genomes. Relatively low mapping rate of CcI3 data suggests deletions of chromosomal segments in the strain maintained in our laboratory. CcI3 contained 503 unmapped regions with average length of 404 bp and maximum length of 5398 bp. Unmapped regions contain many transposons, suggesting that those mobile elements are still active in the strain.

Because individual reads obtained by SOLiD are very short, it is quite difficult to assemble them into a complete genome sequence consisting of a single scaffold when genomes of novel strains are sequenced. Therefore we tried to establish a strategy to detect homologous genes in a novel genome using known genome sequences as references. As a test case, we performed pilot analyses using short reads of strain CcI3 and reference genome sequence of strain EAN1pec. Two strategies were tested, in which individual short reads (strategy 1) or small contigs resulted from *de novo* assembly (strategy 2) were used as queries for homology search of the EAN1pec reference genome and EAN1pec genes with significant homology to CcI3 reads were assigned. Result of *de novo* assemble analysis was poor; mean and maximum length of contigs were only 464 bp and 3660 bp those which covered 76% of CcI3 genome. Using a threshold of >30% of similarity, we detected 43% for strategy 1 and 30% for strategy 2 of CcI3 orthologs in EAN1pec genome. 85% for strategy 1 and 82% for strategy 2 of positive genes were true orthologs. These results indicate that strategy 1 is more effective method to detect homologous genes.

## P20

### The Ins and Outs of *Frankia* Metal Resistance

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*Frankia* are actinobacteria that form a symbiotic nitrogen-fixing association with actinorhizal plants, and are suspected to play a significant role in actinorhizal plant colonization of metal-contaminated areas. From a bioremediation standpoint, it is important to understand mechanisms that allow the endosymbiont to survive and infect actinorhizal plants in metal-contaminated soils. Many *Frankia* strains are known to be resistant to several toxic metals including  $Pb^{+2}$ ,  $Al^{+3}$ ,  $SeO_2$ ,  $Cu^{+2}$ ,  $AsO_4$ , and  $Zn^{+2}$ . With the availability of seven *Frankia* genome databases, a comparative genomic approach was used to investigate *Frankia* metal resistance properties. Data mining of the *Frankia* genomes revealed several potential heavy metal resistance genes including multiple P-type ATPases, CBA efflux pumps, cation diffusion facilitators (CDF), and heavy metal detoxification proteins. A bioinformatics approach employing multiple algorithms is being used to explore potential pre- and post-transcriptional regulators of metal resistance and to possibly identify new metal resistance mechanisms. Two potential metal-specific regulatory consensus sequences have been identified so far, and further *in silico* analysis is underway to determine factors affecting expression levels including codon usage and shine-dalgarno sequence strength. To confirm the *in silico* findings, *Frankia* sp EAN1pec cells were exposed to paraquat,  $Pb^{+2}$ ,  $Al^{+3}$ , and  $Zn^{+2}$  and the relative expression of selected potential metal resistance genes were determined using qPCR. Preliminary results indicated that exposure times were critical for metal-specific responses. After  $Pb^{+2}$  and  $Al^{+3}$  exposure, a general stress response occurred at 1h for the CDF (franean1\_2440), Cu-ATPase 1 and 2 (franean1\_5747 and \_6538) genes. Zn-specific up-regulation of the CDF and Cu-ATPase1 gene expression occurred at 24 h, whereas a Pb-specific response required several days of exposure. CDF (franean1\_2440) gene expression showed a dose-dependent response to  $Pb^{+2}$  challenge which peaked at day 3. FTIR spectroscopy of  $Pb^{+2}$  challenged *Frankia* showed significant alteration to the surface properties compared to control cells suggesting a Pb-binding mechanism for resistance. A shotgun proteomics approach incorporating an isobaric labeling system (iTRAQ) is being used to explore global gene regulation in response to metal challenge. Cells were exposed to  $Pb^{+2}$  and  $Zn^{+2}$  challenges for 5 days, and protein samples of these cells are being analyzed by both iTRAQ and conventional MuDPIT proteomics methods. These data will be discussed in regard to heavy metal tolerance and potential role in the ability of these plants to colonize heavy-metal-contaminated soils.

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