Molecular Basis of *Casuarina/Frankia* Symbiosis

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Abstract Our group has concentrated on the molecular study of the plant genes involved in the interaction between *Frankia* and *Casuarina glauca*. Tools for the functional analysis of candidate genes have been developed; they include genetic transformation procedures based on *Agrobacterium tumefaciens* and *A. rhizogenes*, gene silencing by RNA interference, and microarrays. ESTs and expression analyses from roots and nodules have been compared, providing insights in the genes expressed in the actinorhizal nodules. We recently showed that the casuarina receptor-like kinase gene *SymRK* is a vital component of the genetic basis for both plant-fungal and plant-bacterial endosymbioses and is conserved between legumes and actinorhiza-forming fagales. All together, with the new tools now available, our understanding of the molecular mechanisms of *C. glauca* has considerably increased in the past few years.

1 Introduction

Actinorhizal root nodules result from the interaction between a nitrogen-fixing actinomycete called *Frankia* and roots of dicotyledonous plants belonging to eight plant families and 25 genera (Benson and Silvester, 1993). Actinorhizal plants share common features; with the exception of *Datisca*, which has herbaceous shoots, they are perennial dicots and include woody shrubs and trees such as *Alnus* (alder), *Elaeagnus* (autumn olive), *Hippophae* (sea buckthorn) and *Casuarina* (beef wood). Most actinorhizal plants are capable of high rates of nitrogen fixation comparable to those found in legumes. As a consequence, these plants are able to grow in poor and disturbed soils and are important elements in plant communities worldwide. In addition, some actinorhizal species can grow well under a range of environmental stresses such as high salinity, heavy metal and extreme pH. This facility for adaptation has drawn great interest to actinorhizal plants, particularly to several species of Casuarinaceae such as *Casuarina glauca*, which can be used for fuelwood, agroforestry, and land reclamation in the tropics and subtropics.

The basic knowledge of the symbiotic association between *Frankia* and actinorhizal plants is still poorly understood, although it offers striking differences with the *Rhizobium*-legume symbiosis (Obertello *et al*., 2003; Vessey *et al*., 2005). *Frankia* is a filamentous, branching, Gram-positive actinomycete, whereas rhizobia are Gram-negative unicellular bacteria. *Frankia* can interact with a

diverse group of dicotyledonous plants; whereas rhizobia only enter symbiosis with plants from the legume family and with one non-legume *Parasponia*. In actinorhizal plants, the formation of the nodule primordia takes place in the root pericycle and the nodule consists of multiple lobes, each representing a modified lateral root without a root cap and with infected cells present in the cortex. In indeterminate nodules formed on roots of temperate legumes, the nodule primordium starts in the root inner cortex and determinate nodule primordia are formed in the root outer cortex of tropical and subtropical legumes. Legume root nodules represent stem-like structures with peripheral vascular bundles and infected cells in the central tissue, whereas actinorhizal nodules conserve the structure of a lateral root with a central vascular bundle and peripheral infected cortical tissue.

The molecular understanding of regulatory events in actinorhizal nodulation is mainly limited by the microsymbiont *Frankia*; this actinomycete is characterized by slow growth rate, high G + C DNA content and the lack of a genetic transformation system (Lavire and Cournoyer, 2003). So far, investigations to detect any DNA sequences homologous to the *nod* genes in the *Frankia* genome have failed. However, in the past decade, some progress has been made in the knowledge of the plant genes that are expressed at different stages of actinorhizal nodule differentiation. Differential screening of nodule cDNA libraries with root and nodule cDNA has resulted in the isolation of a number of nodule-specific or nodule-enhanced plant genes in several actinorhizal plants including *Alnus*, *Datisca*, *Eleagnus* and *Casuarina* (for reviews see Obertello *et al*., 2003; Vessey *et al*., 2005).

In this short review we summarize recent advances on molecular biology of *Casuarina/Frankia* symbioses.

2 Tools for Functional Analysis of Symbiotic Genes: Gene Transfer Based on *Agrobacterium rhizogenes* and *A. tumefaciens*

The natural susceptibility of members of the Casuarinaceae family to *A. tumefaciens* was used to develop a gene transfer procedure for *C. glauca* (Smouni *et al*., 2002). Epicotyls fragments of 2 cm in length were excised from 45-day-old plantlets and cocultivated with the disarmed strain C58C1 (pGV2260; pBIN 19). After selection on kanamycin, one to three transgenic calli were observed on 26% of the epicotyls. Integration of the transgenes was further confirmed by PCR and Southern blot analyses. Transgenic plants were regenerated in approximately nine to ten months for *C. glauca*. The nodulation efficiency was found to be similar in transgenic Casuarinaceae and in non-transformed control plants inoculated by *Frankia*, and the transgenic nodules fixed nitrogen at the same rate as those of the non transformed control nodules. This transgenic approach has contributed to the characterization of two symbiotic genes from *C. glauca*, *Cgenod* 40, a homolog of early nodulin gene *enod*40 from legumes (Santi *et al*., 2003), and *cg*12, an actinorhizal symbiotic gene encoding a subtilisin-like serine protease (subtilases) (Svistoonoff *et al*., 2003).

A rapid procedure for producing composite plants of *C. glauca* is also available. It relies on the induction of a hairy root system by *A. rhizogenes* while the aerial part of the plant remains untransformed. Young seedlings of *C. glauca* were wounded on the hypocotyl and inoculated with *A. rhizogenes* A4RS containing the chosen binary vector. After two weeks, highly branched roots exhibiting rapid growth were observed at the inoculation site. The normal root system was removed at the stem base, and the composite plant decontaminated with cefotaxime. Cotransformation with the wild-type T-DNA and the T-DNA from the binary vector was observed in about 50% of *C. glauca* hairy roots. After inoculation with *Frankia*, nodulation was observed on 40% of the

transformed roots. Using this "composite plant" approach, the pattern of expression conferred by the promoter region of a symbiotic gene can be studied in both roots and nodules of *C. glauca* within about 4 months (Diouf *et al*., 1998).

These composite plants are currently used to dissect symbiotic gene function by RNAi gene silencing. To demonstrate the potential of this approach for actinorhizal plants, two RNAi silencing vectors directed against the β-glucuronidase (GUS) gene were introduced into transgenic plants of *Casuarina* stably transformed by the GUS gene under the control of the 35S promoter. Our data established that the reporter gene was efficiently silenced in both roots and actinorhizal nodules of composite plants (Gherbi *et al*., 2008b).

3 Genomics of *Casuarina-Frankia* Symbiosis

Our group has developed the first genomic platform to identify new genes involved in the symbiotic process between *Frankia* and *C. glauca*. A total of 15,000 unigenes were obtained from cDNA libraries corresponding to mRNA extracted from (1) young nodules induced by *Frankia* and (2) non-infected roots. Unigenes were classified into functional categories. As expected, several nodule cluster sequences corresponded to proteins previously described as actinorhizal nodulins (*i. e.* hemoglobin, metallothioneins, subtilisin, rubisco activase, saccharose synthase, glycine and histidine rich proteins). In order to explore the early events of *C. glauca* - *Frankia* symbiosis, a substractive hybridisation library (SSH) was also constructed with roots sampled 4 days after infection. SSH sequences were validated and annotated revealing a large proportion of ESTs implicated in defence, cell wall structure and gene expression. More recently, unigenes were used to design microarrays. Expression analysis has led to the identification of few up regulated genes during the early steps of nodule development (Hocher *et al*., in preparation).

In order to test the usefulness of transciptomics data to identify symbiotic genes, genes were selected to verify nodule-specific and /or nodule-enhanced expression by Quantitative Real-Time RT-PCR (qRT-PCR) on the basis of their putative involvement in nodule development and/or functioning. Differential expression was observed between roots and nodules for genes coding for flavonoid biosynthesis enzymes, thus suggesting a possible role of flavonoids in actinorhizal nodule development (Hocher *et al*., 2006). Experiments are now in progress to characterize more precisely the expression of these genes at different stages of *C. glauca* nodule differentiation.

4 *SymRK* Defines a Common Genetic Basis for Plant Root Endosymbioses

Although several genetic components of the host-symbiont interaction have been identified in legumes; the genetic basis of actinorhizal symbiosis is still unknown. We demonstrated that the receptor like-kinase gene *SymRK*, which is required for nodulation in legumes, is also necessary for actinorhizae formation in *C. glauca*. This indicated that both types of nodulation symbiosis share genetic components. We have also shown that *SymRK* is involved in AM formation in *C. glauca* and can restore both nodulation and AM symbiosis in *Lotus japonicus SymRK* mutants. Taken together, our results demonstrate that *SymRK* has a key role for both plant-fungal and plant-bacterial endosymbioses and is conserved between legumes and actinorhizal plants (Gherbi *et al*., 2008a).

5 Conclusions

In the past decade, considerable advances have been made in the identification and characterization

of the plant genes involved in the development and functioning of actinorhizal nodules. However, in comparison with the progress achieved in the molecular dissectioning of the communication between *Rhizobium* bacteria and legumes our understanding of the early steps of the interaction between *Frankia* and the actinorhizal plants lags well behind.

The genetic transformation procedures and RNA interference technology developed in the Casuarinaceae family now make it possible to perform functional analysis of the actinorhizal symbiotic genes. Moreover, in the next years, emerging genomics tools (454 and solexa technology) may help investigate the early communication between the actinomycete and the host plant.

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