Genetic Transformation of Casuarinaceae Trees

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Abstract Actinorhizal species are non-leguminous perennial plants belonging to eight angiosperm families. They are able to form root nodules as a result of the infection by a nitrogen-fixing actinomycete called *Frankia*. Using the biological vectors *Agrobacterium rhizogenes* and *A. tumefaciens*, gene transfer has been successful in *Casuarina glauca*. Transgenic plants proved to be valuable tools for exploring the molecular mechanisms resulting from the infection process of actinorhizal plants by *Frankia* and should contribute in the future to the goal of engineering Casuarinaceae for enhanced performance and better adaptation to biotic and abiotic stress.

1 Introduction

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. 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*.

2 Gene Ttransfer Based on *Agrobacterium tumefaciens*

In order to successfully regenerate transgenic plants using the natural *A. tumefaciens* gene transfer system, a number of parameters has to be fulfilled (Gelvin, 2000): (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; and (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 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; pBIN19). 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 transformation procedure has two major advantages: the kanamycin selection is efficient and there are very few escapes; and only one medium is required for both bud differentiation and shoot elongation. The same approach is currently in progress to achieve the genetic transformation of *C. equisetifolia* and *C. cunninghamiana* (RITF and IFGTB, unpublished data).

This transgenic approach has contributed to the characterization of several symbiotic genes from *C. glauca*: *Cgenod*40, a homolog of early nodilin gene *enod*40 from legumes (Santi *et al*., 2002), *CgMT*1, a metallothionein-like gene of type 1 (Laplaze *et al*., 2002), *Cg*12, an actinorhizal symbiotic gene encoding a subtilisin-like serine protease (subtilases) (Svistoonoff *et al*., 2003), the auxin influx transporter gene *CgAUX*1 (Péret *et al*., 2007) and *CgSYMRK*, a leucine-rich-repeat receptor kinase gene required for nodulation and mycorrhization (Gherbi *et al*., 2008a).

3 Fast *Agrobacterium rhizogenes*-based Transformation System of *Casuarina glauca*

A rapid procedure for producing composite plants of *C. glauca* is also available (Diouf *et al*., 1995; Gherbi *et al*., 2008b). 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 (Franche *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).

4 Gene Transfer for the Improvement of Casuarinaceae

Gene transfer procedures based on *A. tumefaciens* and knowledge of gene expression conferred by

heterologous promoters such as the 35S in Casuarinaceae (Smouni *et al*., 2002; Obertello *et al*., 2005) pave the way for genetic engineering these tropical trees. Strategies can be developed to engineer *Casuarina* to resist major pathogens such as *Rhizoctonia solani*, and insect pests such as *Lymantria xylina* (Diouf *et al*., 2008). Transgenic trees that are more tolerant to adverse edaphic conditions such as salt and drought would also be very valuable in tropical regions. Other aspects may include modification of lignin content and/or composition to obtain trees that are more suitable for industrial uses. Paper production has more than tripled in the last 35 years and the paper industry suffers from the high cost of removing lignin from cellulose, which also has a negative environmental impact. Another goal linked to lignin modification could be to prevent casuarina wood from splitting when it dries. This is currently a major drawback for the use of casuarina wood for the manufacture of furniture.

No transgenic *Casuarina* trees have been planted in the field. Additional information on the stability of transgene expression in field-grown Casuarinaceae exposed to changing environments is thus needed to determine the real potential of genetic engineering for the introduction of valuable new traits in this tropical tree family. Furthermore, to prevent an uncontrolled escape into the environment, efforts should be made to obtain sterile transgenic Casuarinaceae trees that do not form fertile pollen or seeds.

5 Conclusions and Future Challenges

Actinorhizal plants input of fixed nitrogen on a global scale is enormous; they contribute to 15% of symbiotic nitrogen fixation (Franche *et al*., 2009). Casuarinaceae species are largely distributed and contribute to maintain/rehabilitate marginal lands, as well as to provide incomes to smallholders of various tropical and sub tropical countries. Understanding the development and functioning of the actinorhizal nodules is thus an important challenge. In the past decade, molecular tools have been developed and considerable advances have been made in the identification and characterization of genes implicated in actinorhizal symbiosis (Laplaze *et al*., 2008). The genetic transformation procedures developed for Casuarinaceae make it possible to perform functional analysis of the isolated symbiotic genes and offers numerous prospects for generating novel forest tree genotypes at an accelerated rate.

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