Review

Casuarina glauca: A model tree for basic research in actinorhizal symbiosis

CHONGLU ZHONG[1], SAMIRA MANSOUR[2], MATHISH NAMBIAR-VEETIL[3], DIDIER BOGUSZ[4] and CLAUDINE FRANCHE[4,*]

[1]*Research Institute of Tropical Forestry, Chinese Academy of Forestry, No.682 Guangshan Yilu, Longdong, Guangzhou 510520, People's Republic of China*

[2]*Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt*

[3]*Institute of Forest Genetics and Tree Breeding, Forest Campus, R. S. Puram, Coimbatore 641 002, India*

[4]*Equipe Rhizogenèse, UMR DIADE, IRD (Institut de Recherche pour le Développement), 911 avenue Agropolis, BP 64501, 34394 Montpellier, Cedex 5, France*

**Corresponding author (Email, claudine.franche@ird.fr)*

Casuarina glauca is a fast-growing multipurpose tree belonging to the *Casuarinaceae* family and native to Australia. It requires limited use of chemical fertilizers due to the symbiotic association with the nitrogen-fixing actinomycete *Frankia* and with mycorrhizal fungi, which help improve phosphorous and water uptake by the root system. *C. glauca* can grow in difficult sites, colonize eroded lands and improve their fertility, thereby enabling the subsequent growth of more demanding plant species. As a result, this tree is increasingly used for reforestation and reclamation of degraded lands in tropical and subtropical areas such as China and Egypt. Many tools have been developed in recent years to explore the molecular basis of the interaction between *Frankia* and *C. glauca*. These tools include *in vitro* culture of the host and genetic transformation with *Agrobacterium*, genome sequencing of *Frankia* and related studies, isolation of plant symbiotic genes combined with functional analyses (including knock-down expression based on RNA interference), and transcriptome analyses of roots inoculated with *Frankia* or *Rhizophagus irregularis*. These efforts have been fruitful since recent results established that many common molecular mechanisms regulate the nodulation process in actinorhizal plants and legumes, thus providing new insights into the evolution of nitrogen-fixing symbioses.

[Zhong C, Mansour S, Nambiar-Veetil M, Bogusz D and Franche C 2013 *Casuarina glauca*: A model tree for basic research in actinorhizal symbiosis. *J. Biosci.* **38** 815–823] **DOI**10.1007/s12038-013-9370-3

1. Introduction

Casuarina glauca Sieb. Ex Spreng., commonly known as swamp-she oak, swamp oak, or river oak, belongs to the family *Casuarinaceae* in the order Fagales. This medium-sized deciduous tree (8–20 m high) is native to the East coast of Australia (National Research Council 1984). Members of the family *Casuarinaceae* are angiosperms with a morphologically distinctive conifer-like appearance (Diouf *et al.* 2008). The leaves are reduced to lanceolate scales about 1 mm long, thereby reducing evapo-transpiration and contributing to the adaptation of *Casuarina* trees to arid and semi-arid climates. *C. glauca* is one of the most widely planted *Casuarina* species since it has proved to be the best under Mediterranean climates and can adapt to difficult sites. It can tolerate a wide range of conditions such as drought, frost (in the range of 0°C to −5°C), sea spray, acidity, alkaline or highly saline soils, and waterlogging. It has been successfully introduced in China, India, Cyprus, Egypt, Israel, Kenya, Singapore, South Africa, Thailand, Uganda and the United States of America.

Keywords. *Casuarina glauca*; actinorhizal symbiosis; nitrogen fixation; *Frankia*; symbiotic gene

Published online: 1 October 2013

In soils deprived of nitrogen, woody nodules consisting of multilobed structures reaching up to 10 cm in diameter are observed on the root system of *C. glauca*. These so-called actinorhizal nodules provide a niche for the actinobacteria *Frankia* which fixes atmospheric nitrogen and converts it into ammonia, which is then transferred to the plant and assimilated into amino acids (for a review, see Franche and Bogusz 2012; Pawlowski and Demchenko 2012). In addition, *C. glauca* can develop a symbiotic association with endomycorrhizae, which helps increase the uptake of plant nutrients such as phosphorous (He and Critchley 2008). These endosymbiotic root associations allow *Casuarina* trees to thrive in areas with nutrient-poor soils. Because of their wide adaptability, Casuarina trees have been introduced in Africa, Asia and in Central, South and North America for the reforestation of coastal areas and degraded lands (Diem and Dommergues 1990; Zhong *et al.* 2010). In areas where the tree is not native, the roots must be inoculated with the appropriate *Frankia* strains and mycorrhizal fungi to enhance growth in the field.

With growing environment-related concerns, there is renewed interest in biological nitrogen fixation as a way of limiting chemical fertilizers whose excessive use in past decades has contributed to greenhouse gas emission and underground water leaching (Olivares *et al.* 2013; Santi *et al.* 2013). Whereas association with legumes is the most important symbiosis in agriculture and is well-documented, actinorhizal symbiosis is still poorly understood even though its contribution to nitrogen soil fertility is of great environmental value in many areas. Basic research on the tree species *C. glauca* aims to improve our understanding of the root system and its relationship with root symbionts. Manipulation of these will indeed improve the growth of *Casuarina* trees and the efficient functioning of the *Casuarina-Frankia* symbiosis.

This article first describes the different uses of *C. glauca*, exemplified in China and Egypt, and then reviews the main achievements that have attracted the attention of the scientific community to this actinorhizal tree species. Progress in the knowledge of symbiotic genes expressed during the interaction of *Frankia* is highlighted.

2. *Casuarina glauca*, a valuable tree in semi-arid regions and coastal areas

In 1895, casuarinas were first introduced in the tropical parts of China. Thanks to past efforts by the Chinese government to stimulate forestry development, *Casuarina* trees currently cover about 300,000 hectares, mostly in the coastal areas (National Research Council 1984; Zhong and Zhang 2003; Zhong *et al.* 2010). Among the large number of species introduced, *Casuarina equisetifolia* L. Johnson, *C. cunninghamiana* Miq., *C. glauca* Sieber ex Sprengel and *C. junghuhniana* Miq., are the most successful, and are the focus of ongoing research and development in China. Casuarinas are economically and ecologically important as they provide a wide range of goods and services. Their wood is a major source of fuelwood and charcoal, and is also used for construction and other wood-based industries, e.g. woodchips for paper pulp and veneer for chipboard. Along the southern coast of China, casuarinas are commonly planted as windbreaks to stabilize drifting sand, in agroforestry to improve soil fertility for various crops, and in general soil rehabilitation programmes, such as former mining sites. Very few species can replace casuarinas at the foreshores. Along the southern coast of China (Provinces of Guangdong, Hainan and Fujian), huge numbers of *Casuarina* trees have been planted in an area 7000 km long and 0.5 to 5 km in width to stabilize sand dunes and protect the coastal area from strong winds and typhoons. In addition, each hectare of plantation annually provide up to 4 tons of litter and twigs that can be used for domestic fuel. Interestingly, in fuelwood plantations, cut trees rapidly regenerate from sprouts and consequently do not have to be replanted. However, this property has a drawback since *C. glauca* can be considered as a weed, as reported in Florida and Hawaii.

Casuarina trees were introduced in Egypt on a large scale at the end of the 18th century and *Casuarina* is currently the most widespread genus of trees used in Egyptian forestry (El-Lakany 1983; Mansour and Megahed 2002). Three species are grown, C. *equisetifolia*, *C. cunninghamiana* and *C. glauca*, and a natural hybrid between the last two species has also been described (Badran *et al.* 1979). *C. equisetifolia* is mainly grown on the Mediterranean coast, while the other taxa are grown inland with no preference for particular site conditions. Irrigation is needed to establish *C. glauca* in desert areas and the inoculation by *Frankia* is highly recommended when the tree is planted in new areas (Mansour 2003). Crushed nodules or sand collected beneath mature trees can be used to inoculate nursery seedlings. Currently, *Casuarina* trees are planted primarily as windbreaks around cultivated fields, along irrigation and drainage canals as well as along roads and highways, and as ornamentals in parks. Erosion control is another reason for planting *Casuarina* trees.

3. The early years: *in vitro* culture

A valuable feature of *C. glauca* is the large numbers of seeds the trees produce (Turnbull and Martensz 1982; El-Lakany *et al.* 1989). They flower at 3–4 years of age, and the trees then begin to shed about 4 tons of cones/year, with each cone producing about 70 seeds that have a good germination potential (up to 80%). At a temperature of 26°C, germination takes place 7 days later. This feature is particularly useful for scientific experiments that require a large number of plants, such as functional analyses of plant genes based on RNA interference (RNAi) (400 plants are needed to obtain significant data with RNAi).

In China, easy asexual propagation techniques have been developed, enabling the massive clonal propagation of elite trees for afforestation programmes: 5–8 cm shoots of *C. glauca* are incubated for 1 day with 10 μM of the hormone indol-3-butyric acid, and then transferred in water, which is renewed every day until rooting. Using this auxin treatment, around 90% of the shoots root in 3 weeks (Liang and Chen 1982; Zhong *et al.* 2010). This method is currently used to maintain and propagate the most valuable transgenic lines of *C. glauca* isolated in laboratories.

Shoot regeneration from the callus is often a critical bottleneck in the development of a genetic transformation procedure. It requires an appropriate choice of explant and the right hormonal balance to achieve plant regeneration. After testing cotyledons, hypocotyls and epicotyls, 2 cm fragments of epicotyls excised from 45-day-old seedlings of *C. glauca* appeared to be the best choice for these experiments of shoot regeneration from callus (Duhoux *et al.* 1996). When grown on MS gelled medium containing 2% sucrose, supplemented with Nitsch and Nitsch vitamins, 2.5 μM of BAP and 0.5 μM of NAA, calli appeared at the wound surfaces of most explants after 15 days of incubation at 26°C in a growth chamber. Bud organogenesis was observed after about 4 months and the first shoots could be rooted from 50% of the calli after 6 months. Due to the high accumulation of phenolic compounds, the calli had to be transferred to a fresh nutrient medium every 3 weeks, and patches of browning had to be carefully removed with a scalpel.

4. Symbiotic *Frankia*

In 1983, Diem *et al.* isolated the first effective strain of *Frankia* from nodules of Casuarinaceae, and since then, many strains have been isolated. They belong to Cluster I consisting in *Frankia* strains isolated from actinorhizal plants belonging to the order Fagales (Normand *et al.* 1996; Benson and Clawson 2000; Hahn 2008); they have the most specific host range and are only able to interact with plants belonging to this clade.

In recent years, several *Frankia* genomes have been sequenced, including the genome of *strain* CcI3, which was isolated from nodules collected on *Casuarina cunninghamiana,* and can nodulate *C. glauca* (Normand *et al.* 2007a; Rawnsley and Tisa 2007). The genome is circular and does not contain any replicating plasmids. Like other *Frankia* strains, CcI3 is characterized by a high G+C content (70.07%), but a striking feature is that it has one of the smallest *Frankia* genomes with 5.43 Mbp and 4499 protein coding. The *Frankia* infective strain with the largest genome sequenced so far is EAN1pec, with 9.04 Mbp and 7976 coding sequences. Isolated from *Eleagnus angustifolia,* this *Frankia* strain is characterized by a broad host range and wide geographical distribution. One study focused on the predicted highly expressed genes also revealed that CcI3 has a specific profile characterized by a restricted number of highly expressed genes in functional categories such as the transport and metabolism of lipids, biosynthesis of secondary metabolites, and the transport and metabolism of inorganic ions (Sen *et al.* 2008). These characteristics may be related to *Frankia* CcI3's narrow host range and the restricted Australian area of origin of the host plant. Unlike *Alnus* and *Elaeagnus* symbiotic strains, casuarina strains are limited to soils where the host plants are native, and are very close to one another, with DNA homologies greater than 70% (Fernandez *et al.* 1989; Normand and Fernandez 2009). These data suggest that CcI3 is becoming a symbiotic specialist and that during their evolution, Casuarinaceae and their symbiont *Frankia* co-adapted to the hot, dry climate of the Australian interior.

Analysis of the *Frankia* genome revealed the absence of canonical *nod* genes in CcI3. Only a few, low similarity *nodB* and *nodC* homologues were detected. Moreover genes known to be involved in symbiosis such as *nif* (nitrogenase), *shc* (squalene hopene cyclase), *hup* (hydrogenase uptake) and *suf* (sulfur-iron cofactor synthesis) are scattered over the genome away from the distant putative *nod* homologues (Normand *et al.* 2007b). This lack of a symbiotic island is in sharp contrast to the situation of rhizobia, where *nif* genes are clustered with *nod* and ancillary genes, and is a likely sequel of recent lateral transfers (Pappas and Cevallos 2011).

Transcriptome and proteome analyses led to the identification of genes potentially involved in the symbiotic process, but to date, no direct functional evidence that these genes are involved in symbiotic nitrogen fixation has been found. This is the due to the difficulty involved in developing tools for genetic analysis in *Frankia*. So far, there is no way to knock down or over-express targeted genes in any *Frankia* strain. Gene transfer can be transiently achieved by electroporation, including in CcI3, but no true transformants have been obtained (Kucho *et al.* 2009).

5. The intracellular infection process

Nodulation in *C. glauca* occurs through intracellular penetration of *Frankia* hyphae via root hair infection (Berry and Sunnel 1990; Wall and Berry 2008; Franche and Bogusz 2012). Four major stages can be distinguished during this process: (1) the presymbiotic stage, which involves perception and recognition of specific and as yet unknown plant and *Frankia* signalling molecules; within 24 h, this plant–bacteria signalling dialogue results in the chemoattraction of actinobacteria hyphae by root hairs and, after contact with *Frankia*, root hair branching or curling is observed; (2) root hair infection by *Frankia* hyphae; some hyphae captured

within a curled root hair partly dissolve the plant cell wall and enter the root hair by invagination of the plant plasma membrane. When the bacterium penetrates the cell wall, it becomes an intracellular endophyte; (3) the induction and infection of a prenodule resulting from the division of root cortical cells where *Frankia* will proliferate, leading to the hypertrophy of the infected cells; this stage is usually observed seven to 10 days after inoculation by *Frankia*; (4) the induction and invasion of a nodule primordium initiated from divisions in the root pericycle. The resulting actinorhizal nodule is a coralloid cluster of small lobes, each of which possesses a central vascular system and *Frankia* infected cells in the expanded cortex. No differentiation of vesicles is observed in the endophytic *Frankia*. Behind the nodule meristem, infection occurs progressively, with the youngest stages being closest to the apex. Within the cortex, infected cells occur in layers surrounded by smaller non-infected cell layers that contain heavy deposits of phenolic compounds. A specific feature observed in *Casuarina* nodules is the lignification of *Frankia* infected cells (Berg and McDowell 1987). In addition, the bordering wall of adjacent non-infected cells undergoes secondary thickening during maturation of infected cells. This lignification process could be considered a host defense against actinobacteria invasion and to contribute to oxygen protection of the nitrogenase complex. In plantations, nodules are found near the base of the trunk and as deep as 10 m and can reach 20 cm in diameter. The largest number of nodules in *C. glauca* plantations is found in soils with pH ranging from 6 to 8.

Several procedures have been developed to easily monitor the nodulation process of *C. glauca* by pure cultures of *Frankia* in laboratory conditions. The first procedure includes the transfer of 30-day-old seedlings of *C. glauca* grown in aseptic conditions in Gibson tubes; this allows continuous and non-destructive observation of the root system (Duhoux *et al.* 1996). Plants are kept in a growth chamber at 26°C with a 16 h/day photoperiod, and inoculated with *Frankia* after about 1 month, when the root system has developed numerous lateral roots. Nodulation in hydroponic conditions is an alternative to this method. Plantlets with a root system of about 4 cm are transferred in pots (4 plants/pot) containing some Hoagland and Arnon liquid medium. When the aerial system has developed several ramifications and reaches about 5 cm in height, plants are deprived of nitrogen for 1 week and then inoculated with *Frankia*. With this system, plants can be grown in a phytotron for at least 6 months, whereas they can only be kept for 3 months in Gibson tubes.

6. Genetic transformation of *Casuarina glauca*

Whereas the genetic transformation by a disarmed strain of *Agrobacterium tumefaciens* of the species *Allocasuarina verticillata* was first achieved in 1997, it took almost five additional years for the development of a suitable protocol for the regeneration of transformed *C. glauca* plants (Smouni *et al.* 2002). The significant accumulation of phenolic compounds in this species, together with the negative impact of kanamycin selection on the organogenic potential of the transgenic calli, were major limiting factors during the regeneration process (reviewed in Svistoonoff *et al.* 2010a). Transgenic calli obtained after genetic transformation of epicotyl fragments excised from young plantlets, had to be transferred to fresh selection medium every 3 weeks to prevent plant cell necrosis, and kanamycin (50 mg L^{-1}) had to be removed 4 months after contact with *A. tumefaciens* to improve bud differentiation and shoot elongation. Among several disarmed strains of *A. tumefaciens* tested in preliminary experiments, C58C1(pGV2260) was found to achieve the best transformation efficiency (Le *et al.* 1996). Transgenic rooted plants of *C. glauca* were regenerated in 6 to 9 months and successfully transferred to the greenhouse. Detailed analysis showed that there were no differences in growth parameters between transgenic and non-transgenic plants, and that *Frankia*'s nodulation ability was not affected (Smouni *et al.* 2002).

The first transgenic *C. glauca* plants expressed the selection marker *nptII* conferring resistance to kanamycin, and the β-glucuronidase reporter gene (*GUS)* (Jefferson *et al.* 1987) under the control of the constitutive *35S* promoter. With the discovery of the green fluorescent protein gene as an alternative to the GUS gene, transgenic *C. glauca* plants expressing constitutively the GFP5ER gene were then obtained and proved to be valuable tools to study the spatiotemporal expression conferred by promoters of symbiotic genes during the different stages of the nodulation process (Santi *et al.* 2002; Svistoonoff *et al.* 2003).

A. rhizogenes transformation of *C. glauca* was reported in 1995 by Diouf *et al. C. glauca* sensitivity to *A. rhizogenes* was exploited to develop a rapid method to generate composite plants of *C. glauca* consisting of a transformed root system growing on a non-transgenic aerial shoot. Three-week-old *C. glauca* seedlings were wounded in the hypocotyls with a needle dipped into a fresh colony of A4RS. After 6 days of cocultivation, the inoculated plants were further grown in presence of 300 mg L^{-1} cefotaxim to eliminate excess agrobacteria. Within 3 weeks, rapidly growing roots were observed on 75–90% of the inoculated plants and the non-transformed slow-growing root system was removed. Whereas about 9 months are necessary to obtain a rooted transformed *C. glauca* plant with *A. tumefaciens*, the expression of chimeric genes in composite plants can be studied in less than 4 months. Moreover, with this approach, it is possible – using *A. rhizogenes* – to retransform a transgenic plant obtained after genetic transformation with *A. tumefaciens*. The resulting transformed plant has a root system with

two different T-DNA (Gherbi *et al.* 2008b; Benabdoun *et al.* 2011). Compared to the transgenic *C. glauca* plants obtained with *A. tumefaciens*, the drawback of the method based on *A. rhizogenes* is that the aerial part of the composite plants is not transformed, thus preventing any functional analyses in the shoots. In addition, a decrease in the nodulation ability is observed: whereas more than 90% of *C. glauca* plants transformed by *A. tumefaciens* are nodulated by *Frankia* CcI3, only 30 to 50% of the composite plants develop some nitrogen-fixing nodules (Gherbi *et al.* 2008b).

7. Isolation and characterization of *Casuarina glauca* genes expressed in nodules

Two strategies were developed to identify plant genes involved in the actinorhizal symbiosis. First, the reverse transcription polymerase chain reaction was used to clone homologues of plant genes isolated from legumes or other actinorhizal species; and second, *C. glauca* nodule and root cDNA libraries were screened to isolate specific and nodule-enhanced cDNA (Hocher *et al.* 2006). More recently, *C. glauca* large-scale expressed sequence (EST) databases were created with the aim of identifying new symbiotic genes; these EST are also a resource for comparative genomics with legume-rhizobium and mycorrhizal symbioses (Hocher *et al.* 2011; Tromas *et al.* 2012).

The first genes isolated in the Casuarinaceae family were *C. glauca* haemoglobin DNA sequences. Using a promoter-reporter gene approach, the spatiotemporal expression conferred by their promoters was studied in 1995 in a transgenic legume (Jacobsen-Lyon *et al.* 1995). These authors demonstrated that *C. glauca* harbours two haemoglobin genes: one is only expressed at high levels in the nodule and bears striking similarities with symbiotic leghaemoglobin genes, and the other one, expressed in non-symbiotic tissues, displays a deduced amino acid sequence closely related to the haemoglobin gene of the non-legume *Parasponia*. Furthermore, by using *in situ* hybridization, *C. glauca* symbiotic haemoglobin transcripts were localized in young *Frankia* infected cells prior to the detection of *Frankia nif* gene mRNA. This pattern of expression suggests a role in reducing free oxygen in nitrogen-fixing cells (Gherbi *et al.* 1997).

Since then, several *C. glauca* genes have been isolated, and, thanks to the development of molecular tools and genetic transformation of *A. verticillata* and *C. glauca* (Diouf *et al.* 1995; Franche *et al.* 1997), functional analyses of some of these genes were achieved. In 1999, in order to investigate the function of polyphenols in *C. glauca* nodules, a chalcone synthase cDNA (*CgCHS1*) was isolated and used as a marker of flavan synthesis (Laplaze *et al*, 1999). *In situ* hybridization of *CgCHS1* cDNA, together with histochemical characterization and localization of polyphenols, demonstrated that phenolic compounds belonging to the flavan class delimit *Frankia*-infected areas in the nodule cortex. More recently, eight *C. glauca* genes involved in flavonoid biosynthesis were identified and their expression was monitored during the nodulation time course. Results suggest that isoflavonoids are involved in actinorhizal nodulation (Auguy *et al.* 2011). Further evidence for the critical role of flavonoids during actinorhizal nodulation was provided by knocking down *CgCHS1* expression. This was found to reduce the level of specific flavonoids and resulted in severely impaired root hair infection (Abdel-Lateif *et al.* 2013).

As part of the study of the intracellular infection process in *C. glauca, Cg12*, a homologue of *Alnus glutinosa* nodule-specific subtilisine-like protease gene, was isolated (Laplaze *et al.* 2000). Using *in situ* hybridizations, it was found that *Cg12* transcripts accumulated in the cortical cells of the nodule, in the infection zone, suggesting that this subtilase could be involved in cell-wall degradation during progression of the infection thread into the cortex of the root (Svistonoff *et al.* 2003). Another gene found to be expressed in *Frankia*-infected cells was *CgMT1,* whose sequence shared homology with the class 1 type 1 metallothionein gene (Laplaze *et al.* 2002). Transcripts were shown to be more abundant in *C. glauca* roots and nodules than in aerial parts and transcript accumulation in nitrogen fixing cells infected by *Frankia* suggest a role for *CgMT1* in the nitrogen fixation process. Further analyses included the study of factors regulating the expression of the promoter region and over expression of *CgMT1* in transgenic *Arabidopsis thaliana* (Obertello *et al.* 2007). Taken together, these results show that *CgMT1* is part of the antioxidant system that prevents accumulation of reactive oxygen species in the nitrogen-fixing cells of *C. glauca* nodules.

Actinorhizal nodule lobes resemble modified lateral roots because they originate from primordia which arise from pericycle cells located opposite xylem poles and conserve a lateral root structure with a central vascular bundle. Since regulation of root formation is tightly controlled by plant hormones like auxin (Péret *et al.* 2009), the involvement of this hormone was explored in the *C. glauca-Frankia* interaction. *CgAux1*, encoding an auxin influx carrier, was cloned and shown to be expressed in *Frankia* infected cells, including infected root hairs (Péret *et al.* 2007). In addition, using a combination of computer modeling and cell biology approaches, it was established that expression of *CgAux1* leads to specific accumulation of auxin in *Frankia*-infected cells. Many hypotheses have been proposed to explain the role of auxin in infected cells, including cell wall remodeling during infection, limitation of plant defense mechanisms and a role in the mechanism which increases the size of infected cells (Perrine-Walker *et al.* 2010).

8. RNAi for the functional analyses of genes from the symbiotic signalling pathway

In addition to the development of methods for gene transfer, the possibility to modulate gene expression using antisense and sense strategies was explored several years ago. However, due to the difficulty of obtaining enough transgenic lines (the analysis of 50 antisense lines was necessary to obtain significant data), no significant results were obtained. In 2008, a more efficient approach based on RNA interference (RNAi) was investigated in Casuarinaceae. Down regulation of gene expression by RNAi is obtained when plant transformation is achieved with a hairpin RNA (hpRNA) construct composed of inverted repeats of the targeted gene with an intron between the inverted repeat elements. GUS silencing constructs were introduced by *A. rhizogenes* in transgenic plants of *A. verticillata* containing a *35S-GUS* construct, and the resulting composite plants were analysed (Gherbi *et al.* 2008b). GUS histochemical analyses and qRT-PCR for the measurement of the accumulation of GUS transcripts revealed highly effective silencing of the ß-glucuronidase gene in the *35S-GUS*/35S-hpRNAiGUS root system. Fluorometric data further established that the level of GUS silencing was greater than 90% in hairy roots containing the 35S-hpRNAiGUS construct. The analysis of the aerial parts of the composite plants indicated that the silencing did not spread from the *35S-GUS*/35S-hpRNAiGUS root to the *35S-GUS* shoots. This methodological approach proved to be very valuable to explore the function of candidate symbiotic genes in *C. glauca*.

Genetic analyses of the model legumes *Medicago truncatula* and *Lotus japonicus* led to the identification of molecular components of the symbiotic signaling pathway which controls nodule development (for recent reviews see Venkateshwaran *et al.* 2012; Oldroyd 2013). Interestingly, some of these genes are also required for the formation of arbuscular mycorrhizae (AM) suggesting that the more recent rhizobial association has recycled part of the ancestral programme used by most plants to interact with AM fungi (Parnishke 2008; Delaux *et al.* 2013). To investigate if the common AM and rhizobial signaling pathway is shared with actinorhizal symbiosis, the *C. glauca* receptor-like kinase gene *SymRK* required for nodulation and AM in legumes, was identified and characterized (Gherbi *et al.* 2008a). It was demonstrated that *CgSymRK* also controls *Frankia* nodulation and arbuscular mycorrhization. To determine the extent of the conservation of the actinorhizal symbiotic signaling pathway in *C. glauca*, a homologue of the *M. truncatula* calcium/calmodulin-dependent protein kinase (*CCaMK*) was cloned and analysed (Svistoonoff *et al.* 2013). In legumes, CCaMK is presumed to act as a decoder of the Nod factor-induced calcium signal during nodulation and arbuscular mycorrhization and is a key regulator of both rhizobial infection and nodule organogenesis. Similarly, in *C. glauca*, *CgCCaMK* was shown to play a central role in *Frankia* infection and nodule organogenesis (Svistoonoff *et al.* 2013). In addition, comparative transcriptomics of *C. glauca* and *Alnus glutinosa* revealed gene homologues of the common and nodule-specific signaling pathway known in legumes. This result reinforces the hypothesis of a common plant ancestor of the Fabid (Eurosid 1) nodulating clade with a genetic predisposition for nodulation (Hocher *et al.* 2011).

9. The importance of promoter studies

Transgenic *Casuarinaceae* trees are valuable tools to investigate the conservation of the mechanisms behind nodule-specific expression between legumes and actinorhizal plants. Using the *gus* or *gfp* reporter gene, chimeric constructs containing promoters from early and late nodulin genes from legumes were introduced in transgenic *Casuarina*. Regulation of reporter gene expression during the ontogenesis of actinorhizal nodules was investigated and the results were compared to those reported in legumes. Using this approach, the conservation of the spatiotemporal expression of the Hb promoters from soybean (Franche *et al.* 1998), the *enod12* promoter from *Pisum sativum* (Sy *et al.* 2007) and the *enod11* promoter from *M. truncatula* (Svistoonoff *et al.* 2010b) was established in transgenic nodules of *C. glauca* and *A. verticillata.* Similarly, the promoter from the subtilase gene *cg12* from *C. glauca* was found to confer the same spatiotemporal expression in Casuarinaceae and in *M. truncatula* (Svistoonoff *et al.* 2004).

One exception to these observations is the gene *ENOD40* (Santi *et al.* 2003). This gene was shown to be induced very early in legume nodule induction, and expressed in the nodule vascular system throughout nodule development. In RNAi experiments in *L. japonicus* demonstrated that ENOD40 was required for nodule initiation and subsequent organogenesis (Kumagai *et al.* 2006). Legumes and *C. glauca ENOD40* promoter–reporter gene analyses demonstrated that only legume, not actinorhizal, *ENOD40* genes are active during nodule development, suggesting that the induction of actinorhizal nodules does not involve *ENOD40* (Santi *et al.* 2003).

When candidate symbiotic genes from *C. glauca* became available, the study of the promoter regions provided valuable data for hypotheses concerning the putative function of the corresponding coding sequence during the symbiotic process. This can be illustrated by the characterization of the promoters *Cg12* and *CgAUX1*. Both sequences drive some expression in *Frankia*-infected cells, linking the role of the corresponding genes to infection by the symbiont. In addition, *Cg12* is not expressed during the formation of mycorrhizae, suggesting that this gene is highly specific to actinorhizal symbiosis (Svistoonoff *et al.* 2003). Whereas *CgAUX1* expression was observed in the root primordia, no expression was observed in the nodule primordium

(Péret *et al.* 2007). This result suggests that specific molecular mechanisms are involved in the differentiation of the nodule primordium.

10. Conclusions and future prospects

One of the main advantages of the multipurpose actinorhizal tree *C. glauca* for basic research on symbiosis is that it can be manipulated through genetic engineering, thereby allowing functional analyses of candidate genes by RNAi and promoter studies (Svistoonoff *et al.* 2010a). It also has a relatively small genome, which could consequently be relatively easy to sequence in the future. In addition, the symbiotic *Frankia* can be grown in pure culture and a major effort is currently underway to develop genetic tools in the strain CcI3, which is symbiotic with *C. glauca* (Kucho *et al.* 2009). Ongoing research by the actinorhizal community has led to major advances in our knowledge of symbiotic genes (Franche and Bogusz 2012; Hocher *et al.* 2011). The major challenge in the coming years will be the isolation of the receptor for the signal molecules emitted by *Frankia* in response to contact with the root system of the host, and the biochemical characterization of these molecules. For the latter, transgenic *C. glauca* plants with promoters from symbiotic genes responding to *Frankia* signals during the perception stage will provide the basis for a valuable biological test of the purification of the symbiotic molecules. Expected data will highlight the diversity of molecules and molecular mechanisms underlying endosymbiotic root symbioses.

Gene transfer procedures also pave the way for genetic engineering of *C. glauca*. Strategies can be developed to confer resistance to major pathogens and insects such as *Ralstonia solanacearum*, *Subramanianospora vesiculosa* and *Lymantria xylina* (Narayanan *et al.* 1996, 2003). Transgenic actinorhizal trees that are more tolerant to abiotic stress such as salt and drought could also be valuable in tropical regions (Diouf *et al.* 2008; Nambiar-Veetil *et al.* 2011). Safe development of transgenic Casuarina plantations will require environmental impact assessment and strategies to avoid or minimize gene flow.

Acknowledgements

Research on actinorhizal plants was supported by the Research Institute of Tropical Forestry, the Chinese Academy of Forestry, the Suez Canal University, the Institut of Forest Genetics and Tree Breeding, the Institute de Recherche pour le Développement (IRD), the Montpellier University 2, the Agence Nationale de la Recherche (ANR) Blanc project NewNod (ANR-06-BLAN-0095) and SESAM (BLAN-1708-01), and the PHC-IMHOTEP 27467SA. We thank Jocelyne Bonneau (IRD) for help with the manuscript.

References

Abdel-Lateif K, Vaissayre V, Gherbi H, Verries C, Meudec E, Perrine-Walker F, Cheynier V, Svistoonoff S, Franche C, Bogusz D and Hocher V 2013 The silencing of the Chalcone Synthase (*CHS*) gene in *Casuarina glauca* highlights the important role of flavonoids during nodulation. *New Phytol.* doi: 10.1111/nph.12326

Auguy F, Abdel-Lateif K, Doumas P, Badin P, Guerin V, Bogusz D and Hocher V 2011 Isoflavonoids pathway activation in actinorhizal symbioses. *Funct. Plant Biol.* **38** 690–696

Badran DA, El-Lakany MH, El-Osta ML and Abu Gazia HA 1979 Breeding and improving casuarina trees. 1. Taxonomy and morphological characteristics of *Casuarina* spp. growing in Egypt. *Alex. J. Agric. Res.* **24** 603–684

Benabdoun M, Nambiar-Veetil M, Imanishi L, Svistoonoff S, Ykhlef N, Gherbi H and Franche C 2011 Composite Actinorhizal Plants with Transgenic Roots for the Study of Symbiotic Associations with *Frankia*. *J. Bot.* doi:10.1155/2011/702947

Benson DR and Clawson ML 2000 Evolution of the actinorhizal plant symbioses ; in *Prokaryotic nitrogen fixation: A model system for analysis of biological process* (ed) EW Triplett (Wymondham: Horizon Scientific Press) pp 207–224

Berg RH and McDowell L 1987 Endophyte differentiation in *Casuarina* actinorhizae. *Protoplasma* **136** 104–117

Berry AM and Sunnel LA 1990 The infection process and nodule development ; in *The biology of Frankia and Actinorhizal plants* (eds) CR Schwintzer and JD Tjepkema (New York Academic Press) pp 61–81

Delaux P-M, Sejalon-Delmas N, Bécard G and Ané J-M 2013 Evolution of the plant-microbe symbiotic tool-kit. *Trends Plant Sci.* **18** 298–304

Diem HG, Gauthier D and Dommergues Y 1983 An effective strain of *Frankia* from *Casuarina*. *Can. J. Bot.* **61** 2815–2821.

Diem HG and Dommergues YR 1990 Current and potential uses and management of Casuarinaceae in the tropics and subtropics ; in *The biology of Frankia and Actinorhizal plants* (eds) CR Schwintzer and JD Tjepkema (New York: Academic Press) pp 317–342

Diouf D, Gherbi H, Franche C, Duhoux E and Bogusz D 1995 Hairy root nodulation of *Casuarina glauca*: a system for the study of symbiotic gene expression in an actinorhizal tree. *Mol. Plant-Microbe Interact.* **8** 532–537

Diouf D, Sy M-O, Gherbi H, Bogusz D and Franche C 2008 *Casuarinaceae* ; in *Compendium of transgenic crop plants: transgenic forest tree species* vol 9 (eds) CR Kole, RScorza and TC Hall (Oxford: Blackwell Publishing) pp 279–292

Duhoux E, Franche C, Bogusz D, Diouf D, Le VQ, Gherbi H, Sougoufara B, Le Roux C and Dommergues Y 1996 *Casuarina* and *Allocasuarina* ; in *Biotechnology in Agriculture and Forestry* vol 35 Tree V (ed) YPS Bajaj (Berlin: Springer-Verlag) pp 76–94

El-Lakany MH 1983 Breeding and improving of casuarina: a promising multipurpose tree for arid region in Egypt ; in *Casuarina ecology, management and utilization* (eds) SJ Midgley, JW Turnbull and RD Johnston (Melbourne: CSIRO) pp 58–65

El-Lakany MH, Omran TA and Shehata MS 1989 Variation in seed characteristics of Casuarina as affected by species, season of

collection and position on tree crown. *Intern. Tree Crops J.* **5** 237–245

Fernandez MP, Meugnier H, Grimont PAD and Bardin R 1989 Deoxyribonucleic acid relatedness among members of the genus *Frankia*. *Int. J. Syst. Bacteriol.* **39** 424–429

Franche C, Diouf D, Le QV, N'Diaye A, Gherbi H, Bogusz D, Gobé C and Duhoux E 1997 Genetic transformation of the actinorhizal tree *Allocasuarina verticillata* by *Agrobacterium tumefaciens*. *Plant J.* **11** 897–904

Franche C, Diouf D, Laplaze L, Auguy F, Rio M, Frutz T, Duhoux E and Bogusz D 1998 The soybean (*lbc3*), *Parasponia* and *Trema* hemoglobin gene promoters retain their symbiotic and nonsymbiotic specificity in transgenic *Casuarinaceae*. Implications for the evolution of hemoglobin genes and root nodule symbioses. *Mol. Plant Microbe Interact.* **11** 887–894

Franche C and Bogusz D 2012 Signaling and communication in the actinorhizal symbiosis; in *Signaling and communication in plant symbiosis* (eds) S Perotto and F Baluska (Berlin: Springer) pp 73–92

Gherbi H, Franche C, Duhoux E and Bogusz D 1997 Cloning of a full-lengh symbiotic hemoglobin cDNA and *in-situ* localization of hemoglobin mRNA in *Casuarina glauca* and *Allocasuarina verticillata* root nodule. *Physiol. Plant.* **99** 608–616

Gherbi H, Markmann K, Svistoonoff S, Estevan J, Autran D, Giczey G, Auguy F, Péret B, *et al.* 2008a *SymRK* defines a common genetic basis for plant root endosymbioses with AM fungi, rhizobia and *Frankia* bacteria. *Proc. Natl. Acad. Sci. USA* **105** 4928–32

Gherbi H, Nambiar-Veetil M, Zhong C, Félix J, Autran D, Girardin R, Vaissayre V, Auguy F, Bogusz D and Franche C 2008b Post-transcriptional gene silencing in the root system of the actinorhizal tree *Allocasuarina verticillata*. *Mol. Plant-Microbe Interact.* **21** 518–524

Hahn D 2008 Polyphasic taxonomy of the genus *Frankia* ; in *Nitrogen-fixing actinorhizal symbioses. Nitrogen fixation: Origins, applications, and research progress*, vol. 6 (eds) K Pawlowski, WE Newton (Dordrecht: Springer) pp 25–47

He X H and Critchley C 2008 *Frankia* nodulation, mycorrhization and interactions between *Frankia* and mycorrhizal fungi in *Casuarina* plants. in *Mycorrhiza* (ed) A Varma (Berlin Heidelberg: Springer-Verlag) pp 767–781

Hocher V, Auguy F, Argout X, Laplaze L, Franche C and Bogusz D 2006 Expressed sequence tag analysis in *Casuarina glauca* actinorhizal nodule and root. *New Phytol.* **169** 681–688

Hocher V, Alloisio N, Auguy F, Fournier P, Doumas P, Pujic P, Gherbi H, Queiroux C, Da Silva C, Wincker P, Normand P and Bogusz D 2011 Transcriptomics of actinorhizal symbioses reveals homologs of the whole common symbiotic signaling cascade. *Plant Physiol.* **156** 700–711

Jacobsen-Lyon K, Jensen EO, Jorgensen J-E, Marcker KA, Peacock WJ and Dennis ES 1995 Symbiotic and non-symbiotic hemoglobin genes of *Casuarina glauca*. *Plant Cell* **7** 213–222

Jefferson RA, Kavanagh TA and Bevan MW 1987 GUS fusion: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6** 3901–3907

Kucho K, Kakoi K, Yamaura M, Higashi S, Uchiumi T and Abe M 2009 Transient transformation of *Frankia* by fusion marker genes in liquid culture. *Microbes Environ* **24** 231–240

Kumagai H, Kinoshita E, Ridge RW and Kouchi H 2006 RNAi knock-down of *ENOD40s* leads to significant suppression of nodule formation in *Lotus japonicus*. *Plant Cell Physiol.* **47** 1102–1111

Laplaze L, Gherbi H, Frutz T, Pawlowski K, Franche C, Macheix JJ, Auguy F, Bogusz D and Duhoux E 1999 Flavan-containing cells delimit *Frankia*-infected compartments in *Casuarina glauca* nodules. *Plant Physiol.* **121** 113–122

Laplaze L, Ribeiro A, Franche C, Duhoux E, Auguy F, Bogusz D and Pawlowski K 2000 Characterization of a *Casuarina glauca* nodule-specific subtilisin-like protease gene, a homologue of *Alnus glutinosa ag12*. *Mol. Plant-Microbe Interact.* **13** 113–117

Laplaze L, Gherbi H, Duhoux E, Pawlowski K, Auguy F, Guermache F, Franche C and Bogusz D 2002 Symbiotic and nonsymbiotic expression of *cgMT1*, a metallothionein-like gene from the actinorhizal tree *Casuarina glauca. Plant Mol. Biol.* **49** 81–92

Le QV, Bogusz D, Gherbi H, Lappartient A, Duhoux E and Franche C 1996 *Agrobacterium tumefaciens* gene transfer to *Casuarina glauca*, a tropical nitrogen-fixing tree. *Plant Science* **118** 57–69

Liang Z and Chen B 1982 Vegetative propagation method on *Pseudomonas solanacearum* resistant clones of casuarina plants. *Sci. Silvae Sinica* **18** 199–202

Nambiar-Veetil M, Nair DN, Selvakesavan RK, Jayaraj RSC, Roopesh M, Prabhu SJ, Balasubramanian A, Venkatachalam R, *et al.* 2011 Development of an *in silico* gene bank for plant abiotic stresses: towards its utilization for molecular analysis of salt tolerant and susceptible *Casuarina equisetifolia* clones ; in *Improving smallholder livelihoods through improved casuarina productivity* (eds) C Zhong, K Pinyopusarerk, A Kalinganire and C Franche (China Forestry Publishing House, Beijing) pp144–151

Mansour SR 2003 Survival of *Frankia* strains under different soil condition. *Online J. Biol. Sci.* **3** 618–626

Mansour SR and Megahed M 2002 Interaction of soil and different *Frankia* strains on nodulation and mass production of three *Casuarina* species. *Eg. J. Microbiol.* **37** 323–342

Narayanan C, Dudzinski M, Sharma JK and Mohanan C 1996 The Extent, recognition and management of blister bark disease; in *Recent Casuarina research and development* (Eds) K Pinyopusarerk, JW Turnbull and SJ Midgley (Canberra, Australia: CSIRO Forestry and Forest Products) pp 74–79

Narayanan C, Sharma JK and Minter DW 2003 *Subramanianospora vesiculosa*: a hyphomycete causing wilt disease of *Casuarina equisetifolia. Indian Phytopathology* **56** 159–163

National Research Council 1984 Casuarinas: nitrogen-fixing trees for adverse sites (Washington: National Academic Press)

Normand P and Fernandez MP 2009 Evolution and diversity of *Frankia*. *Mol. Monogr*. **8** 103–125

Normand P, Orso S, Cournoyer B, Jeannin P, Chapelon C, Dawson J, Evtushenko L and Misra AK 1996 Molecular phylogeny of the genus *Frankia* and related genera and emendation of the family *Frankiaceae*. *Int. J. Syst. Bacteriol.* **46** 1–9

Normand P, Lapierre P, Tisa LS, Gogarten JP, Alloisio N, Bagnarol E, Bassi CA, Berry AM, *et al.* 2007a Genome characteristics of facultatively symbiotic *Frankia* sp. strains reflect host range and host plant biogeography. *Genome Res*. **17** 7–15

Normand P, Queiroux C, Tisa LS, Benson DR, Rouy Z, Cruveiller S and Medigue C 2007b Exploring the genomes of *Frankia*. *Physiol. Plant*. **130** 331–343

Obertello M, Wall L, Laplaze L, Nicole M. Auguy F, Gherbi H, Bogusz D and Franche C 2007 Functional analysis of the metallothionein gene *CgMT1* isolated from the actinorhizal tree *Casuarina glauca*. *Mol. Plant-Microbe Interact.* **20** 1231–1240

Oldroyd ED 2013 Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Com.* **11** 252–263

Olivares J, Bedmar EJ and Sanjuan J 2013 Biological nitrogen fixation in the context of global change. *Mol. Plant-Microbe Interact.* **26** 486–494

Pappas KM and Cevallos MA 2011 Plasmids of the *Rhizobiaceae* and Their Role in Interbacterial and Transkingdom Interactions. *Soil Biol.* 23 295–337

Parnishke M 2008 Arbuscular mycorrhiza : the mother of plant root endosymbioses. *Nat. Rev. Microbiol.* **6** 763–775

Pawlowski K and Demchenko KN 2012 The diversity of actinorhizal symbiosis. *Protoplasma* doi: 10.1007/s00709–012–0388–4.

Péret B, Swarup R, Jansen L, Devos G, Auguy F, Collin M, Santi C, Hocher V, *et al.* 2007 Auxin influx activity is associated with *Frankia* infection during actinorhizal nodule formation in *Casuarina glauca*. *Plant Physiol.* **144** 1852–1862

Péret B, De Rybel B, Casimiro I, Benková E, Swarup R, Laplaze L, Beeckman T and Bennett MJ 2009 *Arabidopsis* lateral root development: an emerging story. *Trends Plant Sci.* **14** 399–408

Perrine-Walker F, Doumas F, Lucas M, Vaissayre V, Beauchemin NJ, Band LR, Chopard J, Crabos A, *et al.* 2010 Auxin carriers localization drives auxin accumulation in plant cells infected by *Frankia* in *Casuarina glauca* actinorhizal nodules. *Plant Physiol.* **154** 1372–1380

Rawnsley T and Tisa LS 2007 Development of a physical map for three *Frankia* strains and a partial genetic map for *Frankia* EuI1c. *Physiol. Plant*. **130** 427–439

Sen A, Sur S, Bothra AK, Benson DR, Normand P and Tisa LS 2008 The implication of life style on codon usage patterns and predicted highly expressed genes for three *Frankia* genomes. *Anton. Van Leeuw.* 93 335–46

Santi C, Swistoonoff S, Constans L, Auguy F, Duhoux E, Bogusz D and Franche C 2002 Choosing a reporter for gene expression studies in transgenic actinorhizal plants of the *Casuarinaceae* family. *Plant Soil* 254 229–237

Santi C, von Groll U, Chiurazzi M, Auguy F, Bogusz D, Franche C and Pawlowski K 2003 Comparison of nodule induction in legume and actinorhizal symbiosis : the induction of actinorhizal nodules does not involve *ENOD40*. *Mol. Plant-Microbe Interact.* **16** 808–816

Santi C, Bogusz D and Franche C 2013 Nitrogen fixation in non legumes. *Ann. Bot.* **111** 743–767

Smouni A, Laplaze L, Bogusz D, Auguy F, Duhoux E and Franche C 2002 The *35S* promoter is not constitutively expressed in the transgenic tropical actinorhizal tree, *Casuarina glauca*. *Funct. Plant Biol.* **29** 649–656

Svistoonoff S, Laplaze L, Auguy F, Runions CJ, Duponnois R, Haseloff J, Franche C and Bogusz D 2003 *cg12* Expression is specifically linked to infection of root hairs and cortical cells during *Casuarina glauca* and *Allocasuarina verticillata* actinorhizal nodule development. *Mol. Plant-Microbe Interact.* **16** 600–607

Svistoonoff S, Laplaze L, Liang J, Ribeiro A, Gouveia MC, Auguy F, Fevereiro P, Franche C and Bogusz D 2004 Infection-related activation of the *cg12* promoter is conserved between actinorhizal and legume-rhizobia root nodule symbioses. *Plant Physiol.* **136** 3191–3197

Svistoonoff S, Gherbi H, Nambiar-Veetil M, Zhong C, Michalak Z, Laplaze L, Vaissayre V, Auguy F, *et al.* 2010a Contribution of transgenic *Casuarinaceae* to our knowledge of the actinorhizal symbioses. *Symbiosis* **50** 3–11

Svistoonoff S, Sy M-O, Diagne N, Barker D, Bogusz D and Franche C 2010b Infection-specific activation of the *Medicago truncatula Enod11* early nodulin gene during actinorhizal root nodulation. *Mol. Plant-Microbe Interact.* **23** 740–747

Svistoonoff S, Benabdoun F-M, Nambiar-Veetil M, Imanishi L, Vaissayre V, Cesari S, Diagne N, Hocher V, *et al.* 2013 The independent acquisition of root nitrogen-fixing symbiosis in Fabids recruited the same genetic pathway for nodule organogenesis. *PLoS ONE* **8** e64515

Sy M-O, Constans L, Obertello M, Geney C, Laplaze L, Auguy F, Bogusz D and Franche C 2007 The *PsEnod12B* promoter from the early nodulin gene of *Pisum sativum* does not drive gene expression during the early stages of actinorhizal nodule development in transgenic *Casuarinaceae*. *Plant Soil* **281** 279–287

Tromas A, Parizot B, Diagne N, Champion C, Hocher V, Cissoko M, Crabos A, Lahouse B, Bogusz D, Laplaze L and Svistoonoff S 2012 Heart of endosymbioses: transcriptomics reveals a conserved genetic programme among arbuscular mycorrhizal, actinorhizal, and legume-*Rhizobium* symbioses. *PLoS ONE* **7** e44742

Turnbull JW and Martensz PN 1982 Seed production, collection and germination of *Casuarinaceae*. *Austr. For. Res.* **12** 281–294

Venkateshwaran M, Volkening JD, Sussman MR and Ané J-M 2012 Symbiosis and the social network of higher plants. *Curr. Opin. Plant Biol.* **16** 1–10

Wall LG and Berry AM 2008 Early interactions, infection and nodulation in actinorhizal symbiosis. in *Nitrogen-fixing actinorhizal symbioses* (eds) K Pawlowski and WE (Newton Dordrecht: Springer) pp 147–166

Zhong C and Zhang Y 2003 Introduction, cultivation and management of casuarinas in China. *China Forest. Sci. Technol.* **17** 3–5

Zhong C, Zhang Y, Chen Y, Jiang Q, Chen Z, Liang J, Pinyopusarerk K, Franche C and Bogusz D 2010 Casuarina research in China. *Symbiosis* **1** 107–114