

# The cell-cycle promoter *cdc2aAt* from *Arabidopsis thaliana* is induced in the lateral roots of the actinorhizal tree *Allocauarina verticillata* during the early stages of the symbiotic interaction with *Frankia*

Mame Ourèye Sy<sup>a,b</sup>, Valérie Hocher<sup>a</sup>, Hassen Gherbi<sup>a</sup>, Laurent Laplaze<sup>a</sup>, Florence Auguy<sup>a</sup>, Didier Bogusz<sup>a</sup> and Claudine Franche<sup>a,\*</sup>

<sup>a</sup>Groupe Rhizogenèse Symbiotique, UMR DIAPC, IRD (Institut de Recherche pour le Développement), 911 avenue Agropolis, BP 5045, 34394 Montpellier Cedex 5, France

<sup>b</sup>Laboratoire Campus de Biotechnologies Végétales, Département de Biologie Végétale, Faculté des Sciences et Techniques, Université Cheikh Anta Diop, BP 5005, Dakar-Fann, Sénégal

## Correspondence

\*Corresponding author,  
e-mail: franche@mpl.ird.fr

Received 2 October 2006; revised 8  
January 2007

doi: 10.1111/j.1399-3054.2007.00884.x

The symbiosis between the actinorhizal tree *Allocauarina verticillata* and the actinomycete *Frankia* leads to the formation of root nodules inside which bacteria fix atmospheric nitrogen. Actinorhizal nodule organogenesis starts with the induction of cell divisions in the root cortex and in the pericycle cells opposite protoxylem poles near *Frankia*-infected root hairs. To study the ability of *Frankia* to induce progression through the cell cycle, we monitored the expression of the  $\beta$ -glucuronidase (*gus*) gene driven by the promoter from *cdc2aAt*, an *Arabidopsis* cyclin-dependent kinase gene that displays competence for cell division, during plant growth and nodule ontogenesis. In non-symbiotic tissues, the *gus* gene was mainly expressed in primary and secondary meristems of roots and shoots. Auxins and cytokinins were found to induce reporter gene activity in the root system of whole plants, showing that the promoter *cdc2aAt* displayed the same regulation by hormones in *Allocauarina* as that reported in *Arabidopsis*. In transgenic nodules, *gus* expression was found to be restricted to the phellogen. During the early stages of the interaction between *Frankia* and the plant root system, *cdc2aAt* was strongly induced in the lateral roots surrounded by hyphae of the actinomycete. Histochemical analysis of  $\beta$ -glucuronidase activity revealed that cells from the pericycle opposite protoxylem poles were very deeply stained. These data indicate that upon *Frankia* infection, cells from the lateral roots, and notably pericycle cells that can give rise to a nodule or a root primordium, prepare to re-enter the cell cycle.

## Introduction

Actinorhizal plants have the ability to form a root symbiosis with the soil actinomycete *Frankia*, a nitrogen-fixing filamentous prokaryote (Benson and Silvester 1993, Pawlowski 2002). Actinorhizal hosts described to date are shrub and tree species from 25 genera of

angiosperm plants belonging to eight phylogenetically diverse families. The establishment of the actinorhizal symbiosis is a complex process where both the host plant and *Frankia* undergo biochemical, physiological and molecular changes (Franche et al. 1998, Vessey et al. 2005). It is likely that these modifications involve

**Abbreviations** – 2iP, 6- $\gamma$ - $\gamma$ -(dimethylamino)-purine; 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzylaminopurine; CDK, cyclin-dependent kinase; GUS,  $\beta$ -glucuronidase; NAA,  $\alpha$ -naphthalenacetic acid; X-Gluc, 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronic acid.

compounds that are synthesized by the two symbiotic partners. However, compared with the progress made in elucidating the first steps of the interaction between *Rhizobium* and legumes (Geurts et al. 2005), little is known about the early stages of the actinorhizal symbiosis.

Actinorhizal nodules are perennial multilobed structures that resemble modified lateral roots with a central vascular system, and which originate from the pericycle (Berry and Sunell 1990). Depending on the host, nodules are initiated either via root hair infection by *Frankia* or by intercellular invasion. In the *Casuarinaceae* family, the infection process occurs intracellularly and starts with curling and branching of the root hair induced by unknown *Frankia* signals (Callaham et al. 1979). *Frankia* hyphae associated with deformed root hairs penetrate at the site of folding of deformed root hairs and become sheathed by host plasmalemma and host-derived wall material containing pectin, cellulose and hemicellulose (Berg 1999). This interfacial matrix is the equivalent of the infection thread wall in legume nodules. Upon infection, cell divisions occur in the root cortex near the infected hairs, leading to a small external protuberance called pre-nodule (Berry and Sunell 1990). Most of the pre-nodule cells are invaded by *Frankia* filaments, but the pre-nodule does not evolve into a nodule. At the same time, mitotic activity occurs in pericycle cells opposite a protoxylem pole close to the pre-nodule, giving rise to a lobe primordium that will grow and become infected by encapsulated hyphae of *Frankia* (Callaham and Torrey 1977). Each nodule lobe has an indeterminate growth pattern and four different zones: a persistent apical meristem (zone I), an infection zone (zone II), a nitrogen fixation zone (zone III) and in older portions of nodules, a senescent zone (zone IV).

Following nitrogen starvation and inoculation by *Frankia*, fully differentiated root cells of actinorhizal plants re-enter the cell cycle. Cell division in the cortex near the infected root hair results in the formation of a pre-nodule, while the division of pericycle cells located near these activated cortical cells will generate a nodule primordium. In eukaryotes, the progression through the cell cycle is regulated by cyclin-dependent kinases (CDKs) in association with regulatory subunits, called cyclins (Pines 1995). In plants, the CDK/cyclin complexes are influenced by a combination of different gene products and factors which ensure that the progression of the cell cycle is initiated as an integral part of the growth and development program or in response to the environment (Joubès et al. 2000, Stals et al. 2000). In the model plant *Arabidopsis thaliana*, the family of CDK-related kinases is composed of 12 proteins grouped in six

types (Wang et al. 2004). The P34<sup>cdc2</sup> kinase, which is encoded by the gene *cdc2a*, belongs to the A-type CDKs and is a key component in the regulation of the G1 to S and G2 to M transitions (Ferreira et al. 1991, Hemerly et al. 1992). In *Arabidopsis*, *cdc2aAt* is highly expressed in all meristems and apparently throughout all phases of the cell cycle (Hemerly et al. 1992, 1993). It is also expressed in cells, which have not yet entered the mitotic cell cycle but have increased competence to do so.

When the promoter region of *cdc2aAt* was fused to the  $\beta$ -glucuronidase (GUS) gene and genetically transformed into *Arabidopsis*, the pattern of reporter gene activity was found to resemble the pattern of *cdc2aAt* mRNA accumulation observed by in situ hybridization (Hemerly et al. 1993). External signals such as light, wounding and hormones were found to regulate *cdc2aAt-gus* transcription. When introduced into the heterologous host tree *Populus*, *cdc2aAt-gus* was shown to retain the same specificity of expression as that observed in *Arabidopsis* (Rohde et al. 1997). It also appeared to be a useful indicator of growth activity in individual axillary buds of poplars subjected to different growth conditions and abiotic stress. *cdc2aAt-gus* expression can thus be used to monitor the cells that are progressing through the cell cycle or that acquire competence for cell division. However, more recent data established that *cdc2aAt* could also direct transcription during specific stages of cell morphogenesis. A deletion analysis of the promoter *cdc2aAt* showed that a 595-bp DNA fragment located upstream from the transcription start could direct transcription in a period of trichome development that did not include cell division (Imajuku et al. 2001).

In this paper, we obtained transgenic plants of the tropical actinorhizal tree *Allocauarina verticillata* expressing the *cdc2aAt-gus* construct. We first determined the GUS expression pattern conferred by *cdc2aAt* in roots and shoots and compared the data we obtained to those reported for *Arabidopsis* (Hemerly et al. 1993). We then checked if hormones such as auxins and cytokinins could regulate the transcription of *cdc2aAt* in the root system of *Allocauarina*. Finally, to study the ability of *Frankia* to induce the progression of root cells through the cell cycle, we performed a time course analysis of *cdc2aAt-gus* expression during actinorhizal nodule ontogenesis.

## Materials and methods

### Plasmid and bacterial strains

The chimeric fusion *Pcdc2aAt-gus* consisted of the 1.7-kb promoter and leader sequences of the *A. thaliana* (L.) Heynh. *cdc2aAt* gene (Hemerly et al. 1993) fused to the GUS (*gus* or *uidA* from *Escherichia coli*) reporter gene

(Jefferson et al. 1987). The binary vector pGSV4-*Pcdc2aAt-gus* (Hemerly et al. 1993) kindly provided by D. Inzé was electroporated into the *Agrobacterium tumefaciens* strain C58C1 (pGV2260) (Deblaere et al. 1985). The presence and the integrity of the chimeric gene fusion introduced into *Agrobacterium* were verified by Southern blot analysis of digested bacterial DNA (data not shown). Prior to genetic transformation of *A. verticillata*, the *A. tumefaciens* strain was grown at 28°C in Ag medium (Le et al. 1996) supplemented with appropriate antibiotics.

The *Frankia* strain Allo2 (Girgis and Schwencke 1993) used for inoculation and nodulation of *A. verticillata* plants was grown in a modified BAP-PCM (basic phosphate medium with phosphatidyl choline and MES) (Benoist et al. 1992).

### Plant material and culture media

*Allocasuarina verticillata* seeds were collected in Australia and provided by the Versepuy Company (le Puy-en Velay, France). Seeds were scarified with 95% sulfuric acid for 2 min and washed for 30 min under running tap water. They were then surface sterilized with 5% calcium hypochlorite for 35 min, and rinsed three times with sterile distilled water. Seeds were germinated under sterile conditions on a quarter-strength Hoagland medium (H medium) supplemented with 0.13 mM ammonium sulfate (Hoagland and Arnon 1938) at pH 5.6 and solidified with 8 g l<sup>-1</sup> Difco bacto-agar as described previously (Le et al. 1996). The plantlets were grown for 1–2 months at 28°C with a 16-h photoperiod and a light intensity of 50 μE m<sup>-2</sup> s<sup>-1</sup>.

### Genetic transformation and regeneration of transgenic plants

Transgenic *A. verticillata* plants containing the chimeric gene construct *Pcdc2aAt-gus* were generated by *A. tumefaciens* mediated transformation as previously described (Franche et al. 1997). Transgenic calli were selected on nutrient Murashige and Skoog modified for Casuarina medium (Franche et al. 1997) supplemented with 6-benzylaminopurine (BA; 2.5 μM), α-naphthalenacetic acid (NAA; 0.5 μM), kanamycin (100 mg l<sup>-1</sup>) and cefotaxime (300 mg l<sup>-1</sup>). Gene transfer was confirmed in regenerated rooted plants by polymerase chain reaction using *nptII* and *gus* primers (data not shown). Untransformed plants were regenerated from non-transgenic calli and used as negative controls.

### Plant inoculation by *Frankia*

Transgenic and non-transformed *A. verticillata* plants with a main root at least 3 cm in length were transferred to

tubes (Gibson 1963) containing H liquid medium with ammonium sulfate and placed in a controlled environment chamber as described above. When the root system reached at least 10 cm in length, plants were deprived of nitrogen and inoculated by *Frankia* Allo2 (Smouni et al. 2002). Nodule initiation was monitored weekly using a stereomicroscope.

### Plant growth regulator treatments

At least five plants of each transgenic callus line were grown for 3 weeks in sterile conditions in H liquid medium. All the hormones tested were applied at a concentration ranging from 1 to 10 μM. After 1–3 days of induction, GUS activity was analyzed histochemically in the root system as described below.

### Histochemical GUS assay

Excised shoots or roots were immersed overnight at 37°C into a staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-Gluc) substrate and incubated at 37°C as recommended by Jefferson et al. (1987). To confine the localization of the blue staining, 0.5 mM potassium ferrocyanide and 0.5 mM potassium ferrocyanide were added as catalysts. Stained samples were then fixed for 1 h in a solution containing 5% formaldehyde, 5% acetic acid and 50% ethanol. Soluble pigments were removed by immersing the plant material in several baths of 70% ethanol until cleared.

To allow the penetration of the substrate X-Gluc, actinorhizal nodules were embedded in 3% agarose and sliced into 45–50-μm thick longitudinal or transverse sections on a vibratome (VT1000E; Leica, Wetzlar, Germany). After incubation in the X-Gluc solution, samples were mounted on glass slides with a drop of 50% glycerine solution and examined under a stereomicroscope.

### Microscopy

Transgenic-stained shoots and roots were fixed and embedded in Histo-Technovit 7100 resin as described by Laplaze et al. (1999). Thin 4–8-μm sections were made with a microtome (Historange 2218; LKB, Bromma, Sweden), immersed for 10 min in 5% ruthenium red and mounted on glass slides with Clearium Mountant (Surgipath Medical Instruments, Richmond, IL). Micrographs were taken with a light microscope (model DMR13; Leica).

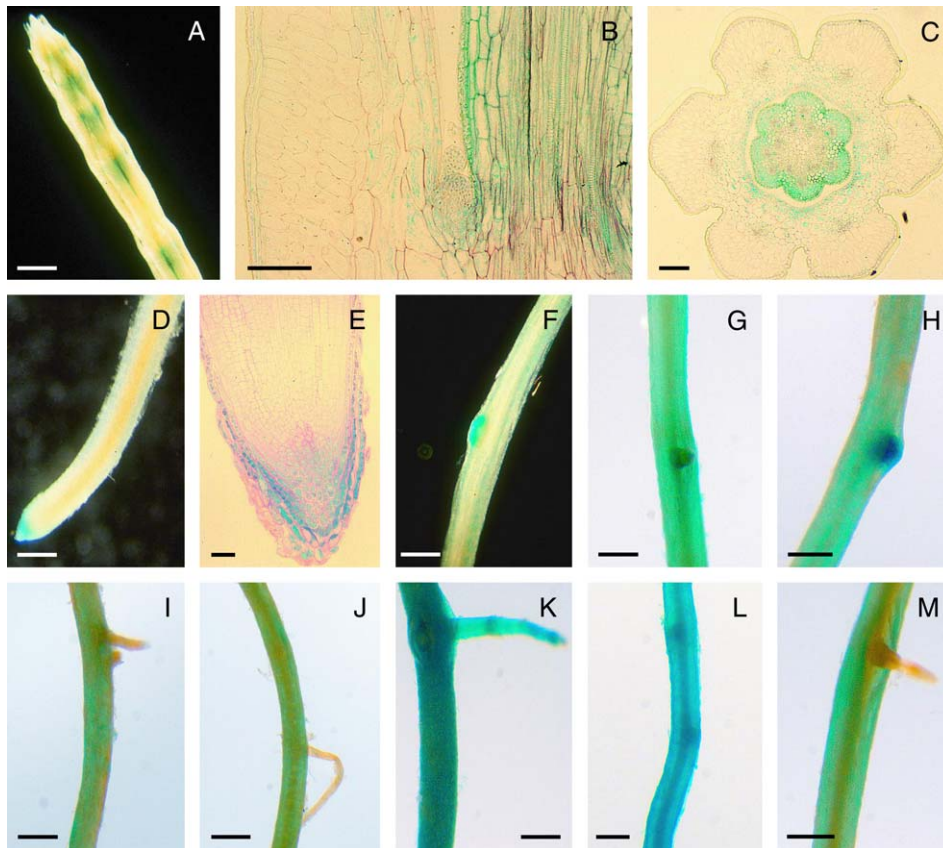
## Results

### Analysis of *cdc2aAt* promoter activity in shoots and roots of transgenic *A. verticillata* plants

From two separate experiments involving the genetic transformation of 200 epicotyls fragments of *A. verticillata*, 38 kanamycin-resistant calli were obtained and further analyzed. Gene transfer was monitored by the expression of the GUS gene and by the amplification of the *gus* and *nptII* genes (data not shown). Five independent transgenic callus lines exhibiting representative GUS patterns and strong reporter gene activity were chosen for detailed cytological analyses and induction experiments. For each callus line, an average of five to seven plants were incubated with X-Gluc and histochemically analyzed. Non-transformed control plants did not exhibit any endogenous GUS activity (data not shown).

First, we checked whether the expression of the chimeric *cdc2aAt-gus* construct could be used as an

indicator of cell division in *Allocasuarina*. All through the shoots, *cdc2aAt* was found to drive reporter gene activity at the node level under the scale-like leaves of *A. verticillata* (Fig. 1A). The intensity of blue staining was uniform in the nodes along the shoot and was observed in the apical region and near the base of the shoot. To further localize *cdc2aAt*-driven *gus* expression, longitudinal and transverse sections were made with a microtome in the *gus*-expressing region (Fig. 1B, C). In the longitudinal section (Fig. 1B), GUS activity was localized at the base of axillary buds and in proximal tissues of the stem and leaves. On the transgenic node cross section made just above the axillary buds (Fig. 1C), *cdc2aAt* gene expression was observed in the internal part of the foliar sheath and in the epidermis of the stem. Blue staining was also observed in the epidermis and in the subepidermal cell layers on the adaxial face of the scales. *cdc2aAt-gus* was also expressed in the cells of the foliar mesophyll surrounding the conductive tissues, but here blue staining



**Fig. 1.** Histochemical localization of GUS activity in transgenic *cdc2aAt-gus Allocasuarina verticillata* plants. (A) Shoot; (B) longitudinal section through a shoot at level of the node; (C) transverse section through a shoot just above a node; (D) apex of a primary root; (E) longitudinal section through the apex of a primary root; (F) meristematic dome of an emerging lateral root; (G) root treated with  $10^{-5}$  M 2iP for 3 days; (H) root treated with  $10^{-5}$  M BAP for 3 days; (I) root treated with  $10^{-5}$  M kinetin for 3 days; (J) root treated with  $10^{-5}$  M zeatin for 3 days; (K) root treated with  $10^{-5}$  M 2,4-D for 3 days; (L) root treated with  $10^{-5}$  M NAA for 3 days; (M) root treated with  $10^{-5}$  M IAA for 3 days. Scale bars: 1 mm (A, D, F–M); 100  $\mu$ m (B, C, E).

was less intense. There was no blue staining in the palisade parenchyma of the scales. In contrast, all the meristematic cells of the internal young axillary buds expressed the *cdc2aAt*-driven reporter gene. Positive GUS activity was confined to the phloem poles of the vascular bundles in the scales. In the internodes, no GUS activity was observed on the longitudinal and transverse sections (data not shown).

In the primary roots, *cdc2aAt-gus* expression was restricted to the apex (Fig. 1D). No expression was observed in either the elongation zone or in older parts of the root system. The root vascular system was completely devoid of indigo blue crystals. A longitudinal section made in a primary root tip showed blue staining in apical and basal meristem cells, in the quiescent center and in the root cap cells (Fig. 1E). In the lateral roots, strong reporter gene expression was observed in the meristematic domes of emerging lateral roots (Fig. 1F). GUS staining persisted in lateral root apical meristems throughout root development (data not shown).

### Control of *cdc2aAt-gus* expression by hormones

We investigated if regulation of *cdc2aAt* by hormones observed in transgenic *Arabidopsis* (Hemerly et al. 1993) also occurred in the tropical actinorhizal tree *A. verticillata*. Two different concentrations of hormones (1 or 10  $\mu\text{M}$ ) were tested on the plant root system of five plants regenerated from each of the callus lines previously selected. The plant root system was incubated in X-Gluc after 1, 2 or 3 days of hormone treatment.

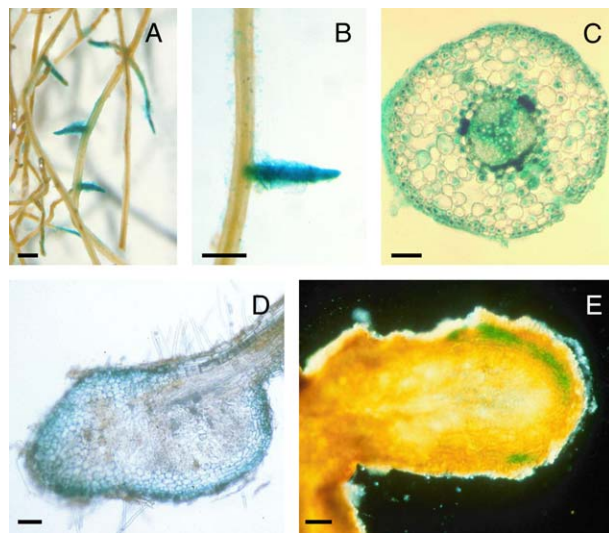
The cytokinins tested included 6-( $\gamma$ - $\gamma$ -dimethylamino)purine (2iP), BAP, kinetin and zeatin. Reporter gene induction was detected 24 h after incubation with 1  $\mu\text{M}$  of cytokinin (data not shown). After a 72-h treatment with 10  $\mu\text{M}$  of cytokinin, the roots showed increased GUS activity in the primary root whatever the hormone tested (Fig. 1G–J). 2iP and BAP provided the best induction of the *cdc2aAt* promoter in the primary root and along the young lateral roots including the emerging meristematic domes.

We then analyzed *cdc2aAt-gus* induction by 1 or 10  $\mu\text{M}$  of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D), NAA or IAA. After 72 h of hormone treatment, GUS staining was observed in the transgenic *A. verticillata* root system. Intense blue staining was obtained in the plant incubated with 2,4-D and NAA in both primary and lateral roots (Fig. 1K–L). With IAA, induction was mainly observed in the primary roots (Fig. 1M). These differences might be linked to the stability of the exogenously applied auxins, IAA being a less stable growth regulator than NAA and 2,4-D (Teale et al. 2006).

### *cdc2aAt* expression upon *Frankia* infection and nodule ontogenesis

Twenty transgenic *cdc2aAt-gus* plants from each callus line were inoculated with *Frankia* Allo2 and three plants were tested for GUS activity at different stages of the nodulation process.

In the first stages of the infection process in *A. verticillata*, *Frankia* hyphae are embedded within a mucilage layer in the root hairs and in the epidermal layer. This mucilage layer is secreted by root hairs (Berry and Sunell 1990, Bhuvaneswari and Solheim 2000). In transgenic *cdc2aAt-gus* plants, lateral roots surrounded by *Frankia* hyphae were found to express strong reporter gene activity (Fig. 2A, B). In the primary root, staining was restricted to root hairs. A control experiment was conducted to check that *Frankia* Allo2 did not express any endogenous GUS activity (data not shown). A cross section made in *gus*-expressing lateral roots revealed that all cell types stained blue, although the intensity of staining varied (Fig. 2C). The pericycle cells opposite the xylem poles exhibited a very strong *gus* expression; intense blue staining was also observed in the root epidermis. However, because cells in the epidermis and in the cortex are not of the same size, it is difficult to conclude that the varying intensity of staining is the result of different GUS activities.



**Fig. 2.** Histochemical localization of GUS activity in transgenic *cdc2aAt-gus Allocasuarina verticillata* plants inoculated by *Frankia*. (A) Whole root system inoculated for 7 days with *Frankia*; (B) close-up of a lateral root; (C) transverse section through a lateral root inoculated for 7 days with *Frankia*; (D): vibratome section through a young nodule collected 4 weeks after inoculation; (E) longitudinal section through a 2-month-old nodule. Scale bars: 2 mm (A, B); 100  $\mu\text{m}$  (C–E).

After about 3 weeks, the first nodules with one or two lobes were observed and GUS activity was analyzed on vibratome sections. Pale blue staining suggested that GUS activity was lower than in roots (Fig. 2D). *cdc2aAt-gus* expression was observed in a peripheral nodule layer corresponding to the phellogen. In more mature nodules exhibiting five to eight nodular lobes, a similar pattern of blue staining was observed (Fig. 2E). No *gus* expression was seen in the nodule vascular system or in the cortical region containing both *Frankia*-infected cells and uninfected cells.

## Discussion

We used GUS fusion and transgenic plants of the actinorhizal tree *A. verticillata* to monitor the expression of the cell-cycle marker *cdc2aAt* from *A. thaliana* during plant development and nodule ontogenesis. In *Arabidopsis*, the expression of *cdc2aAt* has been mainly associated with cell division and competence to divide (Hemerly et al. 1993). Additional roles during cell morphogenesis and cell elongation have been suggested more recently (Bursens et al. 2000; Imajuku et al. 2001).

In *Arabidopsis*, *cdc2aAt* is expressed without any specificity in all plant meristems and in dividing cells (Hemerly et al. 1993). The promoter displayed similar specificity of expression in *A. verticillata* and was found to be mainly expressed in both primary and secondary meristems of roots and shoots. However, in *Arabidopsis* roots, GUS staining was also observed throughout the pericycle and parenchyma of the vascular cylinder, suggesting that *cdc2aAt* expression reflects a state of competence for cell division (Hemerly et al. 1993). Whatever the transgenic line of *Allocauarina*, we observed no GUS expression in the root vascular cylinder, suggesting that the expression of *cdc2aAt* is strictly meristem specific in the root system during the development of actinorhizal trees. In transgenic nodules, whatever their developmental stage, the *gus* expression conferred by *cdc2aAt* was only observed in the phellogen. The phellogen is a cork cambium corresponding to a lateral ring of meristematic tissue which produces cork on the outside and phelloderm on the inside. *cdc2aAt* activity was not observed in *Frankia*-infected cells. In the same way as in the *Rhizobium*-legume symbiosis, the infected threads containing the *Frankia* hyphae do not penetrate the dividing cells. Penetration of the actinomycete hyphae in the nodule cortical cells is only followed by enlargement of host cells that become surrounded by a specialized cell layer with hydrophobic properties (Berry and Sunell 1990).

Hormones control cell division in plants (Francis and Sorrell 2001, del Pozo et al. 2005). In *Arabidopsis*,

expression of *cdc2aAt* is regulated by various hormone treatments (Hemerly et al. 1993) and the promoter contains both abscisic acid and auxin response elements (Chung and Parish 1995). In *A. thaliana*, after 72 h of treatment either with cytokinins including BA and kinetin, or auxins such as IAA, NAA and 2,4-D, the transgenic roots *cdc2aAt-gus* showed increased reporter gene activity in the pericycle and parenchyma cells of the vascular cylinder. Our data showed that *cdc2aAt* retains its inducibility by auxins and cytokinins in the root system of whole plants of *A. verticillata*. In plants, auxins play a central role in triggering lateral root formation (Casimiro et al. 2003, Teale et al. 2006). With a local increase in auxin, pericycle cells in regions immediately adjacent to protoxylem poles re-enter the cell cycle and divide to form a primordium that eventually forms a lateral root.

Under conditions of limited combined nitrogen and active photosynthesis, a few cells close to the infected root hair acquire the competence to enter the nodule development program in *Allocauarina*. In this zone, the cell cycle is activated in the cortical cells that subsequently give rise to the pre-nodule, and in pericycle cells opposite the protoxylem pole near the pre-nodule. We found that highest level of *cdc2aAt-gus* expression occurred during the initial phases of *Frankia* infection, before the nodules had developed. At the stage where *Frankia* is embedded in a mucilage layer excreted by the deformed root hairs (Bhuvanewari and Solheim 2000), the young lateral roots exhibited strong and uniform *cdc2aAt-gus* activity from the root tip to the zone of attachment to the primary root. Interestingly, intense GUS staining was observed in the pericycle cells opposite the protoxylem poles, which can give rise either to a nodule primordium or to a root primordium. These data suggest that in response to *Frankia*, the lateral root cells are able to recover mitotic competence.

Modifications in endogenous phytohormone gradients probably mediate the activation of cell cycle during actinorhizal ontogenesis. High levels of auxin conjugates, cytokinin derivatives and gibberellins have been measured in early active actinorhizal nodule tissue (Wheeler et al. 1979). In addition, IAA and cytokinins have been reported in *Frankia* culture medium (Berry et al. 1989, Stevens and Berry 1988, Wheeler et al. 1984), and two *Alnus*-infective *Frankia* strains and two *Elaeagnus*-infective strains were recently found to produce auxin phenyl acetic acid at a concentration of  $10^{-5}$  to  $10^{-6}$  M (Hammad et al. 2003). It should also be noted that in the actinorhizal plant *Alnus*, the number of lateral root primordia initiated on roots following inoculation by *Frankia* is higher than that on uninoculated control roots, indicating that the actinomycete can stimulate lateral root initiation (Wheeler et al. 1979). Auxins and cytokinins

secreted by the actinomycete may also be involved in the hypertrophy of *Frankia*-infected cells.

In legumes, it has been established that Nod factors are involved in the activation of the cell cycle during the early stages of the interaction. After the addition of Nod factors to a microcallus cell suspension of *Medicago sativa*, an increase in *cdc2* expression was observed within 2 h, indicating that Nod factors stimulate re-entry of quiescent cells into the cell cycle (Savouré et al. 1997). This induction was followed by the expression of the S-phase-specific histone H3 gene and the expression of a mitotic cyclin. Reactivation of the cell cycle by Nod factors was also shown *in planta* in alfalfa and pea roots (Yang et al. 1994) and in *Medicago truncatula* (Foucher and Kondorosi 2000). Several studies suggest that Nod factors affect local distribution and concentration of auxins and cytokinins, which then appear to play a major role in controlling the plant symbiotic programs (Mulder et al. 2005). Using the auxin-sensitive promoter *GH3* fused to the *gus* reporter gene, transient inhibition of auxin transport was observed in white clover, leading to the accumulation of auxin at the site of nodule initiation (Mathesius et al. 1998). With the cytokinin-sensitive promoter *ARR5*, upregulation of cytokinin levels was observed in root hairs undergoing deformation and in initially dividing cells in the root cortex (Lohar et al. 2004).

In actinorhizal plants, the deformation of root hairs is the first morphological indication of molecular signal exchange between the host plant and *Frankia*. These deformations can be induced by cell-free filtrates of *Frankia*, but the chemical nature of the deformation factors (DF) is still not known (Cérémonie et al. 1999). Attempts to purify the DF have failed because of technical difficulties associated with the slow rate of growth and biomass production in *Frankia* cultures and the absence of mutagenesis and transformation techniques. Recent sequencing of the genome of *Frankia* strains failed to locate any genes resembling rhizobial common *nod* genes, except for *NodB*, suggesting that rhizobia Nod factors and *Frankia* DF are structurally divergent (P. Normand, unpublished data). Postgenomic analyses of *Frankia* using DNA chips to monitor the modification of transcripts during the early stages of the interaction and proteomic analyses should provide valuable information on the signals produced by *Frankia* that induce plant root cells to progress through the cell cycle. Furthermore, the isolation and functional characterization of cyclin and CDK genes isolated from *Casuarinaceae* will be of great importance for a deep understanding of the cell cycle modifications and regulations linked to *Frankia* infection.

**Acknowledgements** – The authors are very grateful to Dr D Inzé for providing the *cdc2aAt-gus* construct. This work was supported by the Institut de Recherche pour le Développement (IRD). M. O. Sy. is Assistant Professor at the University Cheikh Anta Diop of Dakar in Senegal; she was financially supported by grants from IRD and by AUF (Agence Universitaire de la Francophonie).

## References

- Benoist P, Müller A, Diem HG, Schwencke J (1992) High-molecular-mass multicatalytic proteinases complexes produced by the nitrogen-fixing actinomycete *Frankia* strain BR. *J Bacteriol* 174: 1495–1504
- Benson DR, Silvester WB (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol Rev* 57: 293–319
- Berg RH (1999) *Frankia* forms infection threads. *Can J Bot* 77: 1351–1357
- Berry AL, Sunell LA (1990) The infection process and nodule development. In: Schintzer CR, Tjepkema JD (eds) *The Biology of Frankia and Actinorhizal Plants*. Academic Press, NY, pp 61–81
- Berry AM, Kahn RKS, Booth MC (1989) Identification of indole compounds secreted by *Frankia* HFPAr13 in defined culture medium. *Plant Soil* 118: 205–209
- Bhuvaneshwari TV, Solheim B (2000) Root-hair interactions in actinorhizal symbioses. In: Ridge RW, Emons AMC (eds) *Root Hairs – Cell and Molecular Biology*. Springer, Paris, pp 311–327
- Burssens S, Himanen K, van de Cotte B, Beeckman T, Van Montagu M, Inzé D, Verburggen N (2000) Expression of cell cycle regulatory genes and morphological alterations in response to salt stress in *Arabidopsis thaliana*. *Planta* 211: 632–640
- Callaham D, Torrey JG (1977) Prenodule formation and primary nodule development in roots of *Comptonia* (*Myricaceae*). *Can J Bot* 55: 2306–2318
- Callaham D, Newcomb W, Torrey JG, Peterson RL (1979) Root hair infection in *Casuarina*, *Myrica* and *Comptonia*. *Bot Gaz* 140: S1–S9
- Casimiro I, Beeckman T, Graham N, Bhalerao R, Zhang H, Casero P, Sandberg G, Bennett MJ (2003) Dissecting *Arabidopsis* lateral root development. *Trends Plant Sci* 8: 165–171
- Cérémonie H, Debelle F, Fernandez M (1999) Structural and functional comparison of *Frankia* root hair deforming factor and Rhizobia Nod factor. *Can J Bot* 77: 1293–1301
- Chung SK, Parish RW (1995) Studies on the promoter of the *Arabidopsis thaliana cdc2a* gene. *FEBS Lett* 362: 215–219
- Deblaere R, Byteblier B, De Greve H, Deboeck F, Schell J, Van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. *Nucleic Acids Res* 13: 4777–4788

- Ferreira PCG, Hemerly AS, Villarroel R, Van Montagu M, Inzé D (1991) The *Arabidopsis* functional homolog of the p34<sup>cdc2</sup> protein kinase. *Plant Cell* 3: 531–540
- Foucher F, Kondorosi E (2000) Cell cycle regulation in the course of nodule organogenesis in *Medicago*. *Plant Mol Biol* 43: 773–786
- Franche C, Diouf D, Le QV, N'Diaye A, Gherbi H, Bogusz D, Gobé C, Duhoux E (1997) Genetic transformation of the actinorhizal tree *Allocasuarina verticillata* by *Agrobacterium tumefaciens*. *Plant J* 11: 897–904
- Franche C, Laplaze L, Duhoux E, Bogusz D (1998) Actinorhizal symbioses: recent advances in plant molecular and genetic transformation studies. *Crit Rev Plant Sci* 17: 1–28
- Francis D, Sorrell DA (2001) The interface between the cell cycle and plant growth regulators: a mini review. *Plant Growth Regul* 33: 1–12
- Geurts R, Federova E, Bisseling T (2005) Nod factor signaling genes and their function in the early stages of *Rhizobium* infection. *Curr Opin Plant Biol* 8: 346–352
- Gibson AH (1963) Physical environment and symbiotic nitrogen fixation. I. The effect of root temperature on recently nodulated *Trifolium subterraneum* L. plants. *Aust J Biol Sci* 16: 28–42
- Girgis Z, Schwencke J (1993) Differentiation of *Frankia* strains by their electrophoretic pattern of intracellular esterases and aminopeptidases. *J Gen Microbiol* 139: 2225–2232
- Hammad Y, Nalin R, Marecahl J, Fiasson K, Pepin R, Berry AM, Normand P, Domenach A-M (2003) A possible role for phenyl acetic acid (PAA) on *Alnus glutinosa* nodulation by *Frankia*. *Plant Soil* 254: 193–205
- Hemerly AS, Bergounioux C, Van Montagu M, Inzé D, Ferreira P (1992) Gene regulating the plant cell cycle: isolation of a mitotic-like cyclin from *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 89: 3295–3299
- Hemerly AS, Ferreira PCG, de Almeida Engler J, van Montagu M (1993) *cdc2a* expression in *Arabidopsis thaliana* is linked with competence for cell division. *Plant Cell* 5: 1711–1723
- Hoagland R, Arnon I (1938) The water culture medium method for growing plants without soil. Circular 347. University of California, College of Agriculture, Agricultural Experiment Station, Berkeley, California
- Imajuku Y, Ohashi Y, Aoyama T, Goto K, Oka A (2001) An upstream region of the *Arabidopsis thaliana* *CDCKA;1* (*CDC2aAt*) gene directs transcription during trichome development. *Plant Mol Biol* 46: 205–213
- Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6: 3901–3907
- Joubès J, Chevalier C, Dudits D, Heberle-Bors E, Inzé D, Umeda M, Renaudin J-P (2000) CDK-related protein kinases in plants. *Plant Mol Biol* 43: 607–620
- Laplaze L, Gherbi H, Frutz T, Pawlowski K, Franche C, Macheix JJ, Auguy F, Bogusz D, Duhoux E (1999) Flavan-containing cells delimit *Frankia*-infected compartments in *Casuarina glauca* nodules. *Plant Physiol* 121: 113–122
- Le QV, Bogusz D, Gherbi H, Lappartient A, Duhoux E, Franche C (1996) *Agrobacterium tumefaciens* gene transfer to *Casuarina glauca*, a tropical nitrogen-fixing tree. *Plant Sci* 118: 57–69
- Lohar DP, Schaff JE, Laskey JG, Kieber JJ, Bilyeu KD, Bird DM (2004) Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. *Plant J* 38: 203–214
- Mathesius U, Schlaman HR, Spaink HP, Of Sautter C, Rolfe BG, Djordjevic MA (1998) Auxin transport inhibition precedes root nodule formation in white clover and is regulated by flavonoids and derivatives of chitin oligosaccharides. *Plant J* 14: 23–34
- Mulder L, Hogg B, Bersoult A, Cullimore J (2005) Integration of signaling pathways in the establishment of the legume-rhizobia symbiosis. *Physiol Plant* 123: 207–218
- Pawlowski K (2002) Actinorhizal symbioses. In: Leigh GJ (ed) *Nitrogen Fixation at the Millennium*. Elsevier Science, Paris, pp 167–189
- Pines J (1995) Cyclins and cyclin-dependent kinases: a biochemical view. *Biochem J* 308: 697–711
- del Pozo JC, Lopez-Matas MA, Ramirez-matas E, Gutierrez C (2005) Hormonal control of the plant cell cycle. *Physiol Plant* 123: 173–183
- Rohde A, Van Montagu M, Inzé D, Boerjan W (1997) Factors regulating the expression of cell cycle genes in individual buds of *Populus*. *Planta* 201: 43–52
- Savouré A, Sallaud C, El-Turk J, Zuanazzi J, Ratet P, Schultze M, Kondorosi A, Esnault R, Kondorosi E (1997) Distinct response of *Medicago* suspension cultures and roots to Nod factors and chitin oligomers in the elicitation of defense-related responses. *Plant J* 11: 277–287
- Smouni A, Laplaze L, Bogusz D, Guermache F, Auguy F, Duhoux E, Franche C (2002) The 35S promoter is not constitutively expressed in the transgenic tropical actinorhizal tree, *Casuarina glauca*. *Funct Plant Biol* 29: 649–656
- Stals H, Casteels P, Van Montagu M, Inzé D (2000) Regulation of cyclin dependent kinases in *Arabidopsis thaliana*. *Plant Mol Biol* 43: 583–593
- Stevens GA Jr, Berry AM (1988) Cytokinin secretion by *Frankia* sp. HFPAr13 in defined medium. *Plant Physiol* 87: 15–16
- Teale WD, Paponov IA, Palme K (2006) Auxin in action: signalling, transport and the control of plant growth and development. *Nat Rev Mol Cell Biol* 7: 847–859
- Vessey JK, Pawlowski K, Bergman B (2005) Root-based N<sub>2</sub>-fixing symbioses: legumes, actinorhizal plants, *Parasponia* sp. and cycads. *Plant Soil* 274: 51–78

- Wang G, Kong H, Sun Y, Zhang X, Zhang W, Altman N, De Pamphilis CW, Ma H (2004) Genomic wide analysis of the cyclin family in *Arabidopsis* and comparative phylogenetic analysis of plant cyclin-like proteins. *Plant Physiol* 135: 1084–1099
- Wheeler CT, Henson IE, McLaughlin ME (1979) Hormones in plants bearing actinomycete nodules. *Bot Gaz (Chicago)* 140(Suppl.): S52–S57
- Wheeler CT, Crozier A, Sandberg G (1984) The biosynthesis of indole-3 acetic acid by *Frankia*. *Plant Soil* 78: 99–104
- Yang WC, de Blank C, Meskiene I, Hirt H, Bakker J, van Kammen A, Franssen H, Bisseling T (1994) *Rhizobium* Nod factors reactivate the cell cycle during infection and nodule primordium formation. *Plant Cell* 6: 1415–1426