TECHNICAL ADVANCE

The Nod Factor–Independent Symbiotic Signaling Pathway: Development of *Agrobacterium rhizogenes*–Mediated Transformation for the Legume *Aeschynomene indica*

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The nitrogen-fixing symbiosis between *Aeschynomene indica* and photosynthetic bradyrhizobia is the only legume-rhizobium association described to date that does not require lipochito-oligosaccharide Nod factors (NF). To assist in deciphering the molecular basis of this NF-independent interaction, we have developed a protocol for *Agrobacterium rhizogenes*-mediated transformation of *A. indica*. The co-transformation frequency (79%), the nodulation efficiency of transgenic roots (90%), and the expression pattern of the 35S *Cauliflower mosaic virus* promoter in transgenic nodules were all comparable to those obtained for model legumes. We have made use of this tool to monitor the heterologous spatio-temporal expression of the *pMiENOD11*- β -glucuronidase fusion, a widely used molecular reporter for rhizobial infection and nodulation in both legumes and actinorhizal plants. While *MiENOD11* promoter activation was not observed in *A. indica* roots prior to nodulation, strong reporter-gene expression was observed in the invaded cells of young nodules and in the cell layers bordering the central zone of older nodules. We conclude that *pMiENOD11* expression can be used as an infection-related marker in *A. indica* and that *Agrobacterium rhizogenes*-mediated root transformation of *Aeschynomene* spp. will be an invaluable tool for determining the molecular basis of the NF-independent symbiosis.

The establishment of the symbiosis between the majority of legumes and nitrogen-fixing rhizobial bacteria starts with a chemical signal exchange between both partners. In a first step, legumes exude flavonoids that trigger the activation of bacterial *nod* gene expression. These *nod* genes encode proteins involved in the biosynthesis and secretion of lipochito-oligosaccharides (Nod factors [NF]) (Perret et al. 2000), which are in turn specifically recognized by plant receptors. Recognition of the

appropriate NF leads to successful rhizobial entry and triggers long-distance responses in the cortex leading to cell divisions and nodule organogenesis. The best-studied mode of rhizobial invasion involves root-hair curling and subsequent infection thread (IT) formation, both requiring NF perception. However, an alternative infection process, so-called “crack entry” infection, has been reported in tropical legumes belonging to the dalbergioid and genistoid groups (Capoen et al. 2005; Sinharoy and DasGupta 2009; Sprent 2007). Crack entry is initiated at the emergence sites of lateral roots under the control of NF and, in the case of legumes belonging to the dalbergioid clade infection, does not involve IT formation (Goormachtig et al. 2004; Sprent 2007; Wilson et al. 1987). In addition to this nonclassical mode of root invasion, the dalbergioid clade (of which *Aeschynomene indica* is a member) is typified by the formation of aeschynomenoid nodules, which are characterized by the absence of uninfected cells (Arora 1954).

Genetic screens in model legumes *Medicago truncatula* and *Lotus japonicus* have led to the identification of numerous genes involved in the plant symbiotic signaling pathway, which is activated by NF. In particular, at least seven *L. japonicus* genes (*CASTOR*, *POLLUX*, *SYMRK*, *CCaMK*, *CYCLOPS*, *NUP133*, and *NUP85*) (Markmann and Parniske 2009) are required for establishing both rhizobial nodulation and arbuscular mycorrhizal (AM) symbioses. Interestingly, it was recently demonstrated that *SYMRK* is also necessary for symbiosis between actinorhizal plants and the *Frankia* actinomycete (Gherbi et al. 2008a; Markmann et al. 2008). Similarly, it has been shown that the expression pattern of the *M. truncatula* early nodulin *ENOD11* gene, used as a marker for studying the initial developmental stages of both AM and nitrogen-fixing symbioses (Chabaud et al. 2002; Journet et al. 2001), is partly conserved in the actinorhizal plant *Casuarina glauca* (Svistoonoff et al. 2010). Together, this has led to the proposition that plants developing endosymbioses share common signal transduction mechanisms (Markmann and Parniske 2009).

Since its discovery 20 years ago, the molecular dialogue described above was thought to be a universal paradigm for all legume-*Rhizobium* interactions. However, the finding that two photosynthetic *Bradyrhizobium* strains (ORS278 and BTAi1) lack the canonical *nodABC* genes (Giraud et al. 2007) oblige

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us to reconsider the “universal” NF signaling model. Despite the absence of *nodABC* genes, these bradyrhizobia elicit nitrogen-fixing nodules on roots and stems of certain aquatic tropical legumes belonging to the *Aeschynomene* genus. This finding was particularly surprising, since it had previously been shown that several *Aeschynomene* photosynthetic *Bradyrhizobium* strains, including the strain ORS285, possess the canonical *nodABC* genes (Chaintreuil et al. 2001). In fact, two groups can be distinguished among photosynthetic bradyrhizobia. The *nodABC*-containing group including ORS285 possesses a wide host range including all stem-nodulated *Aeschynomene* species, such as *A. afraspera* and *A. indica*. In contrast, the *nodABC*-lacking group including ORS278 and BTAi1 are only able to nodulate a subset of *Aeschynomene* spp., such as *A. indica* but not *A. afraspera* (Giraud and Fleischman 2004). Interestingly, a *nodB* deletion mutant of the ORS285 strain loses its broad host range specificity but is still able to nodulate the *Aeschynomene* species nodulated by the *nodABC*-lacking strains (Giraud et al. 2007). These observations imply that two types of symbiotic associations coexist in certain *Aeschynomene* species, the first involving classical NF perception and the activation of the common endosymbiotic signaling pathway and the second an atypical association that is NF-independent.

Investigation of the mechanisms of infection and nodulation in *A. indica* can thus provide insights into the establishment of a nitrogen-fixing symbiosis that not merely involves crack-entry infection but that is also the only NF-independent legume-rhizobium association described to date. Efficient genetic transformation of the host plant is an essential prerequisite for functional studies of *Aeschynomene* symbiotic genes (e.g., gene silencing using RNAi, overexpression, spatio-temporal expression). The *Agrobacterium rhizogenes*-mediated transformation procedure generates so-called composite transgenic plants comprising wild-type shoots with transgenic roots. This rapid and convenient transformation system has been widely used in model legumes and has greatly facilitated the deciphering of both AM and nitrogen-fixing symbioses (Boisson-Dernier et al. 2001; Kumagai and Kouchi 2003; Kumagai et al. 2006; Limpens et al. 2005).

In this article, we describe an *Agrobacterium rhizogenes*-based transformation system for *A. indica*, and we have assessed the influence of both *Agrobacterium rhizogenes* strains and different inoculation procedures on cotransformation and nodulation efficiencies. In addition, the utility of the *Cauliflower mosaic virus* (CaMV)35S promoter for downregulating gene expression via silencing techniques has been analyzed in *A. indica*. Finally, we introduced the *MtENOD11* promoter into *A. indica*, both to validate our genetic transformation procedure and to investigate the symbiotic expression pattern of this early nodulin gene throughout the *Aeschynomene-Bradyrhizobium* symbiotic association.

RESULTS

Agrobacterium rhizogenes-mediated transformation of *A. indica*.

Two different wild-type *Agrobacterium rhizogenes* strains, A4RS (Jouanin et al. 1986) and ARqual (Quandt et al. 1993),

were tested for their capacity to induce transgenic roots on *A. indica*. In addition, we tested two infection procedures: i) the needle-inoculation method (also referred to as stab inoculation), which is the technique most commonly used to generate composite actinorhizal or legume plants (Cheon et al. 1993; Díaz et al. 1989; Diouf et al. 1995; Lee et al. 1993; Quandt et al. 1993; Van de Velde et al. 2003), and ii) the direct application of *Agrobacterium rhizogenes* on freshly sectioned radicles, which has given excellent results in the case of *M. truncatula* and *Arachis hypogaea* (Boisson-Dernier et al. 2001; Sinharoy et al. 2009). With this latter method, nontransformed (NT) adventitious laterals appear above the radicle section. This is independent of *Agrobacterium rhizogenes* inoculation and represents the normal response of *A. indica* seedlings to removal of the root apical meristem. Since no selection was applied, chimeric root systems obtained after direct application of *Agrobacterium rhizogenes* on freshly sectioned radicles comprised both NT and transformed roots. We therefore used the pHKN29 binary vector carrying the pCaMV35S::gfp construct to assess the frequency of cotransformation (Kumagai and Kouchi 2003).

Whatever the strain or the inoculation method used, approximately 80% of composite plants possessed at least one green fluorescent protein-positive root 21 days after inoculation (Table 1; Fig. 1A through d). However, the percentage of cotransformed roots per composite plant was higher for ARqual-mediated transformed plants (Table 1) and the root phenotype depended upon the *Agrobacterium rhizogenes* strain. As shown in Figure 1 (E through G), transformation with A4RS gives rise to a “bushy” root system with an increased number of adventitious laterals, while plantlets inoculated with ARqual have a root system comparable to the NT control. Taking these factors into account, direct application with ARqual on sectioned radicles was retained for further transformation tests. We then examined the effect of ARqual transformation on rootlet inception. As shown in Figure 2A, emergence of adventitious roots on both NT and transformed plants could be observed as early as 6 days following sectioning. At 11 dpi, 100% of control NT plants possessed at least one adventitious root compared with 85% for plants inoculated with ARqual, thus showing that the transformation procedure resulted in a slight delay in root development. However at 15 dpi, 100% of inoculated plants developed at least one root, and among these, almost three-quarters possessed a cotransformed root expressing green fluorescent protein (GFP) (Fig. 2A and B).

Nodulation of *A. indica* transgenic roots.

ARqual-transformed root systems were inoculated with *Bradyrhizobium* sp. strain ORS278. As shown in Figure 3A and B, nodules were observed on composite root systems on both transformed and NT roots, suggesting that the transformation did not perturb the symbiotic process. To evaluate nodulation on ARqual-transformed plants, we used plantlets with only a single GFP-positive root, in order to avoid potential systemic effects between NT and transformed roots. The first nodules were observed at 7 days postinoculation (dpi), irrespective of whether the root was transformed or not, and 90% of cotransformed roots were nodulated by 21 dpi (Fig. 3E). The average number of nodules on GFP-positive roots was comparable to

Table 1. *Agrobacterium rhizogenes*-mediated transformation of *Aeschynomene* spp.

<i>Aeschynomene</i> spp.	<i>Agrobacterium</i> strains	Hypocotyls inoculated	Cotransformation frequency ^a	GFP roots per composite plants
<i>A. indica</i>	A4RS	72	80%	47%
	ARqual	87	79%	62%
<i>A. afraspera</i>	ARqual	40	52%	ND

^a Percentage of plants that generated at least one green fluorescent protein (GFP)-positive root 21 days after infection with *Agrobacterium rhizogenes*, irrespective of the infection procedure.

that for control roots (9 and 10 nodules, respectively; $n_1 = n_2 = 28$, $P < 0.01$). Microscopic observations did not reveal any phenotypic differences, whatever the stage of development. In particular, the central tissue of mature nodules on transformed roots displayed the characteristic pink color of leghemoglobin, suggesting that the nodules are functional (Fig. 3C and D). Indeed, we have confirmed that the nitrogen-fixing activity of nodules on transformed roots is similar to that for control nodules (62 to 74 nmol of C_2H_4 nodule $^{-1}$ min $^{-1}$). Together, these data demonstrate that *Agrobacterium rhizogenes* ARqual-transformed roots of *A. indica* can be nodulated as efficiently as control NT roots.

With the goal of performing a comparative functional analysis between NF-dependent and -independent symbioses, we also examined the possibility of transforming *A. afraspera*, the host that interacts with the *nod*-containing group of *Bradyrhizobium* (ORS285). We found that the identical transformation

protocol using *Agrobacterium rhizogenes* ARqual yielded approximately 50% of composite plants with at least one GFP-positive root (Table 1), and the cotransformed roots were successfully nodulated by the *nodABC*-containing *Bradyrhizobium* ORS285.

Expression of the 35S promoter during root and nodule development in *A. indica*.

To investigate the expression pattern of the *CaMV35S* promoter in transgenic roots and nodules a p*CaMV35S::gusA-int* reporter construct was introduced into *A. indica*. The p*CaMV35S::gusA-int* was used because the construct contains a plant intron that prevents β -glucuronidase gene (*GUS*) expression from residual free-living agrobacteria (Vancanneyt et al. 1990). 35S-driven *GUS* expression in *A. indica* roots was observed in vascular tissues, the root tip, and to a lesser extent, in the root cortex (Fig. 4A and B). At 10 dpi with the *Bradyrhizobium* sp.

Fig. 1. *Agrobacterium rhizogenes* transformation of *Aeschynomene indica*. **A** and **B**, A4RS-transformed composite root observed under either white (**A**) or blue (**B**) light. **C** and **D**, ARqual-transformed composite root observed under either white (**C**) or blue light (**D**). **E**, Nontransformed plant. **F** and **G**, Hairy root phenotype of plants transformed with either A4RS (**F**) or ARqual (**G**). Scale bar = 1 cm.

Fig. 2. Kinetics of ARqual-transformed root emergence for *Aeschynomene indica*. **A**, Appearance of roots on ARqual-transformed plants versus nontransformed (NT) plants ($n = 36$). **B**, Appearance of green fluorescent protein (GFP)-positive roots on composite plants (cotransformation frequency) ($n = 153$). Hatched columns represent transformed plants that have not yet developed roots, gray columns represent transformed plants with only NT roots, and dark columns represent transformed plants with at least one GFP-positive root. dpi = days postinfection with ARqual.

strain ORS278, GUS activity was seen in the infected cells of the central tissue, in the vascular bundles of nodules, and in a few cells within the cortical tissue (Fig. 4C).

***MtENOD11* promoter activation during the NF-independent symbiosis.**

We took advantage of the *Agrobacterium rhizogenes* transformation protocol to introduce the *pMtENOD11-gusA* reporter into *A. indica* in order to study the expression pattern of the *MtENOD11* promoter during the NF-independent symbiosis between *A. indica* and photosynthetic *Bradyrhizobium* strains.

Prior to inoculation of *A. indica* transformed roots expressing *pMtENOD11-gusA*, we observed GUS staining at the root apices, in the root vasculature, and associated with crowns of root hairs located at the base of young lateral roots (Fig. 5A through C). This nonsymbiotic expression in the root apices and vasculature is reminiscent of that described for *pMtENOD11-gusA* expression in *M. truncatula* (Journet et al. 2001). In contrast, whereas *pMtENOD11* was activated in *M. truncatula* in the first 3 to 6 h following addition of *Sinorhizobium meliloti*, no specific activation of the reporter was observed during the four days following inoculation with the *nodABC*-lacking ORS278 strain. Since preinfection induction of *pMtENOD11* has been strictly correlated with NF perception in *M. truncatula*, we then examined whether the NF-producing ORS285 strain was able to acti-

vate early *pMtENOD11-gusA* expression. Similarly, no GUS expression was observed over the four-day period following inoculation with ORS285. To ascertain whether this lack of response might be due to a failure to induce the *nod* genes, we added purified NF from the ORS285 strain. Once again this failed to activate the *MtENOD11* promoter (data not shown). Therefore, we conclude that *pMtENOD11* is not activated during the early stages of the interaction of *A. indica* with photosynthetic bradyrhizobia strains, whether or not they contain the *nodABC* genes.

We also examined the expression pattern of *pMtENOD11-gusA* at later stages of the symbiotic association. Whatever the strain used (ORS278, ORS285, or ORS285 Δ *nodB*) the *pMtENOD11* expression pattern appeared to be the same. An intense blue staining was observed as soon as the first developing nodules could be detected, i.e., 5 dpi. This GUS activity colocalized with the presence of bacteria, as revealed by the GFP fluorescence used to tag ORS285 or ORS285 Δ *nodB* strains (Fig. 5D through F). Surprisingly, in more developed nodules (7 to 12 dpi), the *pMtENOD11* expression pattern drastically changed, as GUS activity was now only detectable in one or a few cortical cell layers bordering the infection zone (Fig. 5G through I). At this later stage, GUS staining (Fig. 5G) and GFP fluorescence (Fig. 5H) did not colocalize, demonstrating that *pMtENOD11* expression was restricted to noninvaded cells surrounding the infection zone (Fig. 5I). Mature nodules (from 12 dpi onwards) showed a *pMtENOD11::GUS* expression pattern restricted to vascular bundles (Fig. 5J through L).

Fig. 3. Nodulation of *Aeschynomene indica* ARqual1-transformed roots inoculated with *Bradyrhizobium* sp. strain ORS278. **A** and **B**, ARqual1-transformed nodulated composite root system observed under either white (**A**) or blue (**B**) light. Scale bar = 5 mm. **C**, Longitudinal section of a nodule formed on nontransformed (NT) roots observed under Bright-field microscopy. Scale bar = 100 μ m. **D**, Longitudinal section of a nodule formed on ARqual1-transformed roots expressing the *pCaMV35S::GFP* construct and observed under Bright-field microscopy. Scale bar = 100 μ m. **E**, Percentage of ARqual1-transformed or NT plants with at least one nodule ($n = 28$); dpi = days postinoculation with *Bradyrhizobium* sp. strain ORS278.

Fig. 4. Cauliflower mosaic virus (*CaMV*) 35S promoter expression in transformed roots and nodules. **A**, Transgenic roots expressing the *pCaMV35S::gusA-int* construct. Scale bars = 500 and 200 μ m in the magnification. **B**, Longitudinal section of a transgenic root expressing the *pCaMV35S::gusA-int* construct at the site of lateral root emergence. Scale bar = 200 μ m. **C**, Longitudinal sections of a nodule expressing the *pCaMV35S::gusA-int* construct. Scale bar = 100 μ m.

DISCUSSION

Genetic tools for functional studies of the NF-independent symbiosis.

The recent discovery that NF is not required during the symbiotic interaction between certain *Aeschynomene* species and photosynthetic bradyrhizobia requires that we reconsider from a new angle the well-established microbial-plant signaling mechanisms based on the rhizobium-legume model systems (Giraud et al. 2007). For this reason, it became a priority to develop an efficient root-transformation protocol for *A. indica* in order to gain insights into the host genetic program recruited during the NF-independent symbiotic association.

Based on the efficiency of generating cotransformed roots, we found that the A4RS and ARqua1 strains were equivalent (79%), with a cotransformation frequency similar to that for other *Agrobacterium rhizogenes*-transformed legumes (Boisson-Dernier et al. 2001; Quandt et al. 1993; Sinharoy et al. 2009; Stiller et al. 1997; Van de Velde et al. 2003). However, A4RS-transformed

roots had a “bushy” phenotype that is probably related to a higher level of phytohormone synthesis compared with ARqua1 (Fig. 1A through C). A similar difference in the “hairy root” phenotype induced by distinct *Agrobacterium rhizogenes* strains was also reported in *Vicia hirsuta* and led to a preference for the less-perturbing ARqua1 strain, based on the principle that phytohormone imbalance might interfere with nodule formation (Crespi and Frugier 2008; Mathesius 2008; Quandt et al. 1993). Since nodule number, size, shape, and nitrogen-fixation capacity were all identical on ARqua1-transformed roots compared with control roots, we also selected this *Agrobacterium rhizogenes* strain for further studies to evaluate the usefulness of *A. indica* composite plants for functional studies.

The *CaMV35S* promoter is commonly used to overexpress or silence target genes, since it is generally constitutively expressed in plant tissues (Estrada-Navarrete et al. 2006; Sinharoy et al. 2009; Van de Velde et al. 2003). In *A. indica* transgenic nodules, the *CaMV35S* promoter is expressed in all cell types, including the infected cells of the central region. Similar ex-

Fig. 5. *MtENOD11* promoter expression in *Aeschynomene indica*. Nonsymbiotic expression of *pMtENOD11* in **A**, apices, **B**, crowns of axillary hairs, and **C**, vascular bundles of lateral transgenic roots (longitudinal section of lateral root emergence). **D** through **L**, Symbiotic expression of *pMtENOD11* in transgenic nodules 8 to 10 days (**D** through **F**), 10 to 15 days (**G** through **I**), and after 15 days (**J** through **L**) following inoculation with *Bradyrhizobium* sp. strain ORS28-*GFP*. Longitudinal (**D** and **G**) or transversal (**J**) sections of young and mature nodules observed under Bright-field microscopy. Magnification of longitudinal sections of young and mature nodules observed under Bright-field (**E**, **H**, and **K**) or fluorescence (**F**, **I**, and **L**) microscopy. Scale bar = 100 μ m.

pression of *pCaMV35S::gus* in the infected cells of the nodule has been shown for *V. hirsuta*, *Phaseolus* spp., and *Arachis hypogaea* (Estrada-Navarrete et al. 2006; Quandt et al. 1993; Sinharoy et al. 2009). However, this is not always the case, as reported for *M. truncatula*, *C. glauca*, and *Allocauarina verticillata* (Auriac and Timmers 2007; Obertello et al. 2005; Smouni et al. 2002). Nevertheless, the *CaMV35S* promoter has been successfully used in these species to shut down the expression of various symbiotic genes via RNAi silencing (Gherbi et al. 2008b; Kumagai et al. 2006; Sinharoy et al. 2009). We can, therefore, conclude that *pCaMV35S* should be a useful tool for functional studies in *Aeschynomene* spp.

pMtENOD11 as an infection marker during the NF-independent symbiosis.

Two observations motivated us to examine to what extent the symbiotic expression pattern of the *MtENOD11* promoter was conserved in *A. indica*. First, this early nodulin gene is induced in *M. truncatula* root tissues during several stages of the endosymbiotic associations with both rhizobia and AM fungi (Chabaud et al. 2002; Journet et al. 2001). Second, it has recently been shown that the expression pattern of *MtENOD11* is partly conserved in the actinorhizal plant *C. glauca* (Svistonoff et al. 2010). As reported for *M. truncatula* and *C. glauca* (Journet et al. 2001; Svistonoff et al. 2010), we observed nonsymbiotic p*MtENOD11::gusA* expression in lateral roots apices and in the vasculature of primary and secondary roots, thus suggesting a similar regulation of this promoter in *A. indica* roots. In *M. truncatula*, *ENOD11* is also expressed in specialized tissues, such as glandular trichomes or leaf vascular strands that share high-level secretory capacity (Esau 1977; Wagner 1991). *MtENOD11* encodes a putative cell-wall repetitive proline-rich protein with a low potential for the formation of Tyr-dependent intermolecular cross-links. Consequently, the presence of *ENOD11* proteins in the cell wall might result in higher matrix porosity. It is, therefore, noteworthy in this context that the p*MtENOD11::gusA* reporter is highly expressed in rosettes of root hairs located in the axil of *A. indica* lateral roots and that it has been reported in *Stylosanthes* spp. that Golgi vesicles are particularly numerous in this cell type (Chandler et al. 1982).

In *M. truncatula*, *MtENOD11* is expressed during both the early and later stages of nodulation. In *A. indica*, no activation of the *MtENOD11* promoter was observed during the four days following inoculation with photosynthetic *Bradyrhizobium* strains, whether or not they possessed the *nodABC* genes. This suggests that the signaling pathway leading to p*MtENOD11* activation during the early steps of the *M. truncatula*–*S. meliloti* association is not conserved for the NF-independent interaction. Nevertheless, as observed in *M. truncatula*, infected cells of young *A. indica* nodules display strong GUS staining. This suggests that infection-related p*MtENOD11* activation is conserved during the NF-independent symbiosis. Two distinct regulatory regions have been identified in the *MtENOD11* promoter, a distal region (–391 to –358 from the start codon) that triggers preinfection *ENOD11* expression following rhizobial NF perception and a proximal region (the 257-bp region upstream of the start codon) that is responsible for *ENOD11* expression during bacterial or mycorrhizal infection (Andrianakaja et al. 2007). The strong expression of the p*MtENOD11::gusA* reporter in the infected cells of young *A. indica* nodules indicates that the promoter regulatory elements related to infection are functional in *Aeschynomene* spp.

We observed a major switch in p*MtENOD11::gusA* expression during *A. indica* nodule development. Between 7 to 12 dpi, reporter expression is restricted to noninfected cells bordering the central infection zone. However, this region does

not show any particular microscopic features that could account for this expression pattern (data not shown). Moreover, these cell layers are unlikely to be invaded during later nodule development, since the infected central tissue results from serial divisions of a single original invaded cortical cell. Thus, the activation of the *MtENOD11* promoter observed in these cell layers cannot be interpreted as preinfection-related gene expression. In mature indeterminate nodules of *M. truncatula*, p*MtENOD11* expression is restricted to the submeristematic preinfection zone and no signal is observed in the fixation zone (Journet et al. 2001). Since meristematic tissues are not present in fully mature determinate *A. indica* nodules, the expression of the p*MtENOD11::GUS* reporter is restricted to vascular tissues and can therefore be considered as nonsymbiotic expression.

Towards the characterization of the NF-independent signaling pathway.

Now that it is possible to genetically transform *Aeschynomene* spp., targeting approaches for key genes identified for the model legumes are conceivable. But the real challenge will be to characterize host symbiotic genes specific to the NF-independent pathway. In addition, the *Aeschynomene* genus offers the unique possibility of comparing NF-dependent (*A. afraspera*–ORS285) and NF-independent (*A. indica*–ORS278) symbiotic associations in related genetic backgrounds.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bradyrhizobium sp. strains ORS278, ORS285, and ORS285 Δ *nodB* (Giraud et al. 2007) were grown at 37°C in modified YM medium (YMm) (Giraud et al. 2000). *Agrobacterium rhizogenes* strains A4RS (Jouanin et al. 1986) and ARqual (Quandt et al. 1993) were cultivated at 28°C in *Agrobacterium* growth medium (in grams per liter: KH₂PO₄, 0.5; MgSO₄ 7 H₂O, 0.2; NaCl, 0.1; mannitol, 5; yeast extract, 1; casamino acid, 1). When required, the media were supplemented with kanamycin (50 µg ml^{–1}). As reporter-gene constructs, we used p*CaMV35S::GFP* and p*CaMV35S::gusA-int* cloned in pHKN29 binary vector (Kumagai and Kouchi 2003; Vancanneyt et al. 1990) and p*MtENOD11::gusA* cloned in pLP100 (Charron et al. 2004). Binary vectors were introduced into A4RS and Arqual by the freeze-thaw method as described by Höfgen and Willmitzer (1988).

Labeling of photosynthetic *Bradyrhizobium* strains.

To label *Bradyrhizobium* sp. strain ORS285 and ORS285 Δ *nodB* with GFP, we constructed the pMG103-*nptII-GFP* plasmid, which harbors a *GFP* gene under the control of the constitutive *nptII* promoter (Xi et al. 1999). The plasmid pMG103 is derived from a cryptic plasmid from *Rhodopseudomonas palustris*, and it has been shown to replicate in some *Bradyrhizobium* species (Inui et al. 2000). The *GFP* gene was amplified by using pRU1701 plasmid as template (Hosie et al. 2002) and primers am.AFP.f, 5'-AAGGCGGCCGAGATCTAATCAATCACCGGATCTAGATATCG-3', and av.AFP.r, 5'-CCCGTAGCCCGTTGCTTCGCAACGTTCAAATCC-3'. The polymerase chain reaction (PCR) product was cloned in the pGEM-T easy vector (Promega, Charbonnières-les-Bains, France) to create an intermediate plasmid with appropriate cloning sites. The *SalI* fragment of this intermediate plasmid containing the *GFP* gene was then cloned in the *SalI* site of pMG103 plasmid. The *nptII* promoter was amplified by using the *mTn5GUSA-pGFP* transposon (Xi et al. 1999) as template and primers PnptII.f, 5'-GCATGCCACGCTGCCGCAAGC ACTCAGG-3', and PnptII.r, 5'-ACTAGTATCCTGTCTCTT

GATCAGATCTTG-3'. The PCR product was cloned into pGEM-T vector, was spliced out by *SphI/SpeI* double digestion, and was inserted in the corresponding sites upstream of the *GFP* gene to generate pMG103-*nptII-GFP*. Finally, pMG103-*nptII-GFP* was introduced into ORS285 and ORS285 Δ *nodB* strains by electroporation, using the method described by Elbeltagy and coworkers (2001). For a reason that is currently unclear, this plasmid was not stable in ORS278.

***Agrobacterium rhizogenes*–mediated root transformation.**

A. indica seeds were surface-sterilized for 30 min with concentrated (95%) sulfuric acid and were rinsed 10 times with sterile deionized water. Four-day-old seedlings were inoculated either by needle infection (Van de Velde et al. 2003) or by direct application of *Agrobacterium rhizogenes* on freshly sectioned radicles (Boisson-Dernier et al. 2001; Sinharoy et al. 2009). Plants were grown on half-strength MS medium (Murashige and Skoog basal salt mixture; Sigma-Aldrich, Saint-Quentin Fallavier, France) at 20°C in the dark.

Plant cultivation.

Seven days after transformation with *Agrobacterium rhizogenes*, plants were transferred to solid half-strength MS supplemented with a cefotaxime at 50 μ g ml⁻¹ and were cultivated at 25°C with a 16-h light and 8-h dark photoperiod. One week later, plants were transferred on the same fresh medium and were grown an additional week. Chimeric root systems obtained after *Agrobacterium rhizogenes* transformation using the pCaMV35S::GFP construct were dissected in sterile conditions, in order to select one cotransformed root expressing GFP. Dissected plants were transferred to Falcon tubes previously filled with liquid buffered nodulation media supplemented with 5 mM KNO₃ (Ehrhardt et al. 1992). In the case of transformation assays using either the pCaMV35S::gusA-int or pMtENOD11::gusA construct, a single root per chimeric root system was arbitrarily chosen before transferring plants to Falcon tubes. A fragment of each selected root was a posteriori tested for its GUS expression, and plants harboring GUS-positive roots were retained for further expression-pattern studies. Plants were grown in a 28°C growth chamber with a 16-h light and 8-h dark regime and 70% humidity.

Nodulation of transgenic roots and acetylene reduction assay.

One week after the transfer to hydroponic growth, composite plants were inoculated with 1 ml of a 5-day-old *Bradyrhizobium* ORS278 culture grown in YMm. Plants were regularly observed for nodule formation, and nodulation was quantified 21 days after inoculation. The significance of the difference between treatments was determined by the Mann-Whitney U test. Nitrogen fixation of NT and transformed roots was assayed using nitrogenase acetylene reduction as previously described (Bonaldi et al. 2010).

Histochemical analysis and microscopy.

Observations were made either with an optical microscope (AZ100; Nikon, Campigny-sur-Marne, France) or microscope (PROVIS; Olympus, Rungis, France) under Bright-field or blue-light illumination, when fluorescent GFP was used as marker gene. Microscopic observations were performed on 30- to 40- μ m-thick vibratome (VT1000S; Leica, Nanterre, France) sections from fresh root and nodule samples. When required, GUS activity was localized by incubating whole roots or sectioned nodules in a GUS assay buffer at 37°C in the dark for periods of between 2 and 12 h, depending on the intensity of the GUS expression. The substrate mixture (adapted from Mendel and associates [1989]) contained 0.075% (wt/vol) of

X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) in 0.1 M K/Na phosphate buffer (pH 7.0), 20 mM NaEDTA, 0.02% Triton X-100, 1 mM K-ferricyanide, and 1 mM K-ferrocyanide.

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