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TECHNICAL ADVANCE

Transformed Hairy Roots of *Arachis hypogea*: A Tool for Studying Root Nodule Symbiosis in a Non–Infection Thread Legume of the Aeschynomeneae Tribe

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***Arachis hypogea* is a non–"infection thread" (IT) legume where rhizobial entry or dissemination in the nodules never involves IT. Rhizobia invade through epidermal "cracks" and directly access the cortical cells to develop the characteristic aeschynomenoid nodules. For investigating these nonclassical nodulation features in *Arachis* spp., we developed an efficient procedure for *Agrobacterium rhizogenes* R1000-mediated transformation of this plant. In this study, we optimized the induction of hairy roots and nodulation of composite *Arachis hypogea* plants in the presence of *Bradyrhizobium* sp. (*Arachis*) strain NC92. 35S promoter-driven green fluorescent protein and β-glucuronidase expression indicated transformation frequency to be above 80%. The transformed roots had the characteristic rosette-type root hairs and had normal level of expression of symbiosis-related genes *SymRK* and *CCaMK*. The transgenic nodules resembled the wild-type nodules with an exception of 2 to 3%, where they structurally deviated from the wild-type nodules to form nodular roots. A 16S rRNA profile of an infected-zone metagenome indicated that identical populations of bradyrhizobia invaded both composite wild-type plants grown in natural soil. Our results demonstrate that Arachis hairy root is an attractive system for undertaking investigations of the nonclassical features associated with its nitrogen-fixing symbiotic interactions.**

Nitrogen-fixing root-nodule symbioses (RNS) in plants is restricted to eurosid angiosperms, of which the ability to form RNS with rhizobia is restricted to the legume family and a single nonlegume *Parasponia* sp. (Markmann et al. 2008). Comparative genome analysis indicates that putative orthologs of several genes known to be required for RNS are present in nonlegumes, too (Zhu et al. 2006). It is clear that preexisting genes functioning in different plant development pathways have been recruited to function during the evolution of nitrogen-fixing RNS, and the borderline between a nonlegume and a legume may not be that wide (Hirsch et al. 2001; Szczyglowski and Amyot 2003; Zhu et al. 2006). The golden question that has emerged is "What are the features that enabled legumes to be predisposed to nodulation?" (Szczyglowski and Amyot 2003). To answer this question, the prerequisite is to understand the molecular principles of functional nodule establishment in several members of the legume family.

The mode of rhizobial invasion and the nodule morphogenetic program are evidenced to be under the control of the host plant (Gleason et al. 2006; Tirichine et al. 2006). Investigation of nodulation on a molecular basis is restricted to the subfamily Papilionoideae, most members of which nodulate (Lavin et al. 2005; Sprent 2007; Wojciechowski et al. 2004). Four major groups of this subfamily are aeschynomenoid/dalbergoid, genistoid, hologalegina, and phaseoloid/millettoid (Doyle and Luckow 2003), which largely differ in their rhizobial invasion strategies and nodule developmental pattern (Sprent 2007). The most common invasion strategy is through epidermis by root hair curling and infection thread (IT) formation. Model legumes *Lotus japonicus* and *Medicago truncatula* of the hologalegina group use this epidermal infection strategy (Geurts and Bisseling 2002; Oldroyd and Downie 2004, 2006). Forward genetic approaches have unraveled important determining factors that establish RNS in these plants (Catoira et al. 2000). The alternate mode of rhizobial invasion is known as "crack invasion". This is a characteristic feature of legumes belonging to the aeschynomeneae tribe, where rhizobia directly access the cortical cells through epidermal "cracks" for development of their characteristic aeschynomenoid nodules. This strategy of developing RNS by direct cortical colonization through crack invasion is relatively less investigated. Investigation on crack invasion is undertaken with *Sesbania rostrata*, again a hologaleginoid legume, that switches to this mode of bacterial entry from the IT mode depending on environmental cues (Spent 2007). But even when the epidermal invasion is through cracks, the ultimate colonization in the cortical cells in *Sesbania* spp. is through formation of infection threads from localized infection pockets (Goormachtig et al. 2004a and b; Spent 2007). There are also major differences in the nodule primordia developmental pattern among the legumes; it can be indeterminate, with persistent meristems, or determinate, where meristematic activity is lost in mature nodules (Barker et al. 1990; Handberg and Stougaard 1992; Sprent 2007). Among the most studied legumes, nodulation in *Medicago* spp. is indeterminate compared with *Lotus, Sesbania*, and *Soybean* spp., where it is determinate in nature.

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The above model systems are valuable because they provide the tools for detailed analysis but they do not address all the fundamental questions regarding evolution of nodule organogenesis (Hirsch et al. 2001). The aeschynomenoid/dalbergioid and genistoid groups of Papilionoideae account for approximately 25% of all legume genera but have not been investigated in molecular terms as yet (Sprent 2007). The former group is typified by aeschynomenoid nodules (Lavin et al. 2001), which are determinate nodules with no uninfected cells in the infected zone. Aeschynomenoid legumes bypass the complex processes involved in epidermal infection through transcellular IT via root hairs; instead, they allow crack invasion through the openings generated during lateral root emergence (Goormachtig et al. 2004a and b; Spent 2007). Cortical or pericycle cells get infected and divide repeatedly to give the characteristic uniformly infected central tissue, with loss of meristematic activity (Alazar and Duhoux 1990; Fedorova et al. 2007; Lavin et al. 2001; Tajima et al. 2008). Recently, two plants of the tribe aeschynomenoid/dalbergioid, *Aeschynomene sensitiva* and *A. indica*, have been demonstrated to undergo successful nodulation in the absence of nod factor signaling, which is a primary requisite for nodulation in the IT legumes, highlighting basic differences in the developmental regulations among the IT and non-IT legumes (Doyle and Luckow 2003; Giraud et al. 2007).

A well-known member of the aeschynomeneae tribe is *Arachis hypogea,* which is an important oil seed crop (peanut or groundnut) and has hitherto been generally recalcitrant to transformation (Nag et al. 2006; Seal et al. 2001; Sharma and Anjaiah 2000). The infection of this plant by *Bradyrhizobium* spp. through epidermal cracks and the distinctive features of its aeschynomenoid nodules have been studied in depth (Allen and Allen 1940; Bal et al. 1989; Chandler 1978; Sen et al. 1986). In addition to these nonclassical nodulation features, *Arachis* roots are distinct from other root systems in lacking root hairs. However, tufted clusters or rosettes of root hairs are frequently found in young roots in the junction of the taproot and the lateral root and are thought to be important for bacterial invasion (Bhuvaneswari et al. 1981; Nambair et al. 1983; Uheda et al. 2001). Nod factor has been identified from *Arachis* spp.-specific *Bradyrhizobium* spp. (Taurian et al. 2008) but it is not yet known whether *Arachis* spp., like the other members of the aeschynomenoid/dalbergioid tribe, can undergo successful nodulation in the absence of nod factor (Giraud et al. 2007). However, root exudates of *Arachis* spp. induce NodA–β-glucuronidase (GUS) reporter expression in *Bradyrhizobium* sp. (*Arachis*) strain NC92, indicating that the host and the symbiont are capable of undertaking the familiar *Rhizobium*-legume dialogue (Gillette and Elkan 1996). Investigation of nodulation processes in *Arachis* spp. can provide insight into the molecular mechanism of i) intercellular invasion and direct cortical colonization of the cognate rhizobia and ii) formation of aeschynomenoid nodule and iii) may also shed light on the evolution of essentiality of nod-factor-dependent signaling in the modern legumes.

Composite plants (an untransformed plantlet with transformed roots) are rapid and convenient alternatives to generating stable transgenic lines and are being widely used in studies focused on root characteristics (Díaz et al. 1989; Jensen et al. 1986; Petit et al. 1987; Stougaard et al. 1987). Composite plants do not transmit the transgenic trait to their progeny and, thus, are of little use in crop improvement. However, it is a routine procedure for overexpressing or suppressing endogenous genes in transformed roots of model legumes for studying the molecular detail of nodulation, and the procedure has already been adapted for *L. corniculatus* (Jensen et al. 1986; Petit et al. 1987), *Trifolium repens* (Díaz et al. 1989), *Vigna aconitifolia* (Lee et al. 1993), *Glycine max* (Cheon et al. 1993), *Vicia hirsuta* (Quandt et al. 1993), *L. japonicus* (Stiller et al. 1997), *T. pratense* (Díaz et al. 2000), *M. truncatula* (Boisson-Dernier et al. 2001), *S. rostrata* (Van de Velde et al. 2003), and *Phaseolus* spp. (Estrada-Navarrete et al. 2006). Here, we have demonstrated successful transformation of *A. hypogea* by *Agrobacterium rhizogenes* R1000, where transgenic roots in the composite plants showed expression of the cotransferred marker green fluorescent protein (GFP) and GUS under a 35S promoter. The level of expression of the symbiotic marker genes *SymRK* (symbiotic receptor kinase) and *CCaMK* (calcium and calmodulin-dependent protein kinase) in the transgenic roots was found to be identical to what was observed with wild-type roots. We show that the transgenic roots can be efficiently nodulated by *Bradyrhizobium* sp. (*Arachis*) NC92 strain and the developed nodules are aeschynomenoid in nature. The developed strategy would now enable undertaking investigation on the molecular mechanism of root-microbe interaction in *Arachis hypogea.*

RESULTS

Agrobacterium rhizogenes-mediated transformation of *Arachis hypogea*.

We have used *Agrobacterium rhizogenes* R1000 harboring the pRiA4b plasmid for generating composite *Arachis hypogea* plants to study its rhizospheric interactions. This same plasmid is harbored by MSU440 or ARqua1 strains that are widely used for generating composite plants with the model legumes *L. japonicus* and *M. truncatula* (Quandt et al. 1993). A preliminary report on *Arachis* spp. transformation using an embryonic axis from dry seed as explant encouraged us to initiate our attempts using the same explant (Akasaka et al. 1998). For cocultivation with *Agrobacterium* spp., we used either the upper one-third or the upper two-thirds of the apical portion of the embryonic axis as explants. The relative efficiency of generation of composite plants using these two explants as embryonic axis is summarized in Table 1 and the induction of transformed hairy roots along with phenotypic characteristics of the generated composite plants is demonstrated in Figure 1. With the upper two-thirds of the embryonic axis, within two weeks after infection, a globular tumor developed at the cut site of the epicotyl and, at about the same time, roots were found to emerge from the tumor (Fig. 1A). Within three to four weeks, growth of hairy roots was observed in 95% of the explants (Fig. 1B and C). Akasaka and associates (1998) observed root growth from 90% of control untransformed explants. In contrast, even after four weeks, development of the root system was rarely noticed in the nontransformed mock-treated explant in the presence of kanamycin in our experiments (data not shown). This is consistent with earlier observations with *Medicago* spp. where growth of nontransformed roots was inhibited in the presence of kanamycin (Boisson-Dernier et al. 2001). Within four to five weeks, the development of the composite plants was complete (Fig. 1E), whereupon they were trans-

Table 1. *Agrobacterium rhizogenes* R1000-mediated transformation of *Arachis hypogea* with the binary vector pCAMBIA2301[a]

Choice of explant	Explants inoculated (composite plants)	Composite plants (%)	Roots per composite plant[b]
Upper 1/3 embryonic axis	80 (14)	17.5	14 ± 8*
Upper 2/3 embryonic axis	500 (447)	89.4	8 ± 2**

[a] Ten plants were tested.
[b] Number of cotransformed β-glucuronidase-positive hairy roots per composite plant; * and ** = 8 and 6 weeks after infection, respectively.

ferred to sterile vermiculite pots for nodulation assays (Fig. 1F). The shoot systems of the composite plantlets had no differences in their morphology when compared with the corresponding wild-type (nontransgenic) plants but the transformed roots exhibited characteristic active elongation and plageotropic growth (Fig. 1E) instead of the thickened taproots in nontransformed plants (not shown here) (Bianucci et al. 2008; Daimon and Mii 1995; Porter 1991). Wild-type roots of *Arachis* spp. lack root hairs on the surface of the tap root but rosette-type root hairs are present at the branch of the main root at the base of lateral roots (Meisner and Karnok 1991; Uheda et al. 1997; Yarbrough 1949). The transformed roots were also found to contain such rosette-type root hairs as shown in Figure 1D indicating that, morphologically, the transformed roots perfectly resembled the nontransformed roots. It needs to be noted that, under identical conditions when we used the upper one-third of the embryonic axis as explants, the growth of the transgenic roots was much slower (Fig. 1G and H). Roots started emerging only after two to three weeks (Fig. 1G) and, at approximately six weeks, there was vigorous growth of roots (Fig. 1H). Roots emerged from all over the explant (Fig. 1G and H) instead of showing restricted growth in the cut site as observed earlier with the upper two-thirds of the embryonic axis (Fig. 1A through C). In addition, transgenic roots emerged from only 30 to 40% of the explants compared with 95% when the upper two-thirds of the embryonic axis was used. Most importantly, we were unsuccessful in generating composite plants in a large number of cases when the upper one-third of the embryonic axis was used (Table 1), and the composite plants that could be generated occasionally showed transformed

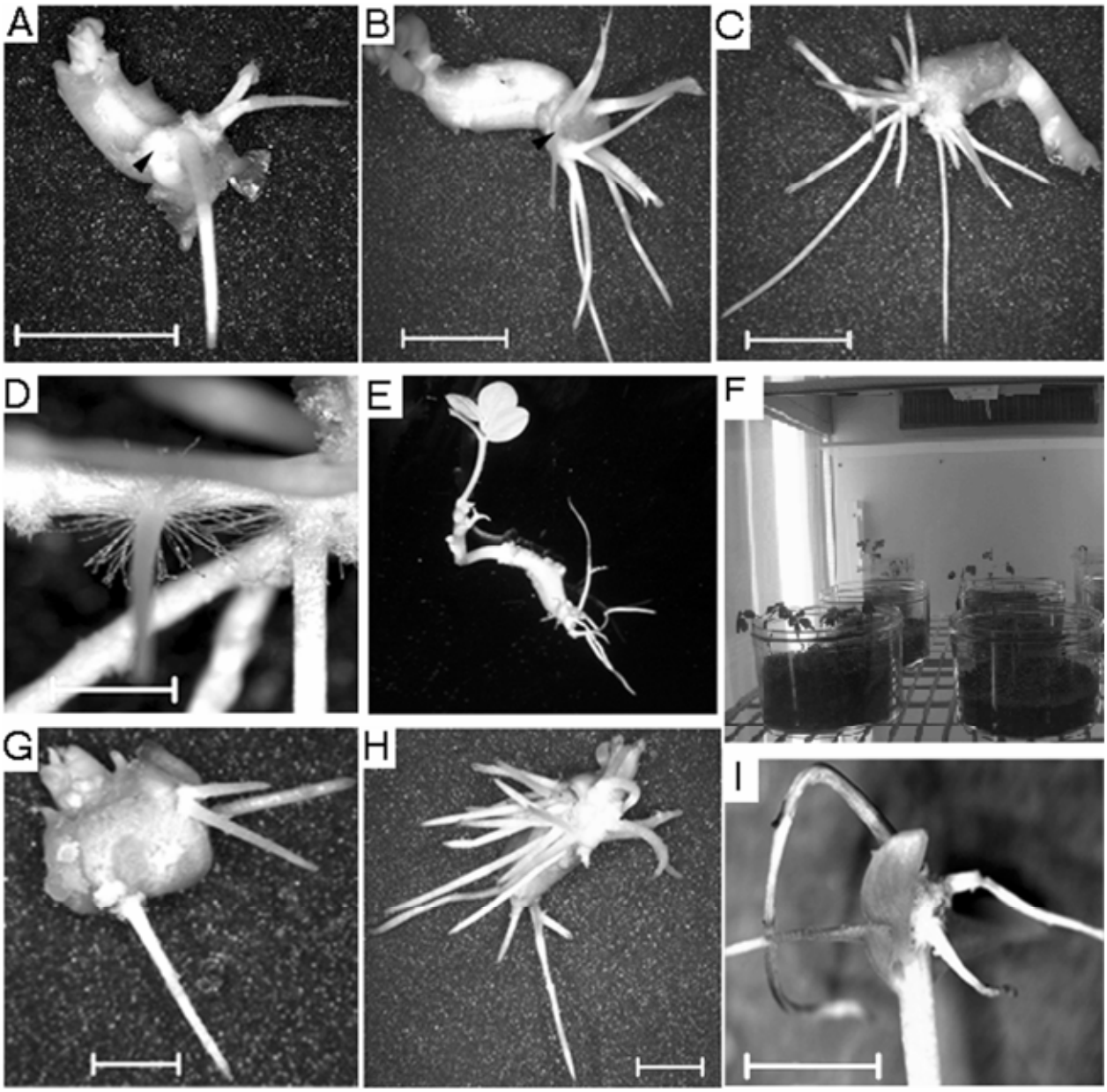

Fig. 1. *Agrobacterium rhizogenes* R1000 induced calli and hairy roots in *Arachis hypogea*. **A** through **F,** Upper two-thirds of the embryonic axis inoculated with *Agrobacterium rhizogenes*. **A,** Calli formation at the cut site and emergence of hairy roots within 2 weeks. **B** and **C,** Transformed root growth after 3 to 4 weeks. **D,** Rosette-type root hairs at the base of lateral root. **E,** Composite *Arachis hypogea* plant generated within 4 to 5 weeks and **F,** transferred to vermiculite pots for nodulation assay (described in Materials and Methods). **G** and **H,** Upper one-third of the embryonic axis inoculated with *Agrobacterium rhizogenes*. **G,** Emergence of hairy roots after 3 to 4 weeks. **H,** Vigorous root growth after 5 to 6 weeks. **I,** Ectopic root formation from shoot apical meristem. Tumor formation is indicated by arrowheads. Bars: A through C, 5 mm; D, 1 mm; G through I, 2 mm.

roots ectopically emerging from the shoot apical meristem. (Fig. 1I). Therefore, we generated transformed roots and composite plants of *A. hypogea* from the upper two-thirds of the embryonic axis as explants for investigating the nodulation features of this plant. A list of botanical types and cultivars of *A. hypogea* of different origin where our transformation procedure has been tested to be successful is shown in Table 2.

Expression of transgenes in the transformed roots of *A. hypogea.*

To verify root transformation via cotransfer and expression of the reporter genes, we have used both bacterial GUS and the jellyfish GFP. For GUS expression, we used *Agrobacterium rhizogenes* R1000 strain carrying the binary vector pCAMBIA 2301 with a 35S-GUS intron marker construct. Within two weeks, almost 70% of the explants with transgenic roots had one or more GUS-positive cotransformed root (Fig. 2A). Expression of glucuronidase activity ensured nuclear integration of the transgene in the plant cells because presence of an intron prevented GUS expression from residual free-living, coinhabiting *A. rhizogenes* cells. The 35S-driven GUS expression pattern in uninoculated *Arachis* spp. roots was primarily observed in vascular tissues and root tip and, to a lesser extent, in the root cortex (Fig. 2A and B, indicated by arrows).

We also examined the efficiency of the transformation procedure for *Arachis* spp. by screening for cotransformation events using *A. rhizogenes* R1000 derivatives with binary vector pK7WGF2, which expresses eGFP under 35S promoter. Two weeks after infection, more than 80% of roots were found to be GFP positive, and GFP expression was uniformly present in the roots (Fig. 2C). The rosette-type root hairs at the site of emergence of lateral roots also show strong GFP fluorescence (Fig. 2D). In addition, these cotransformation experiments were also successfully done using a similar binary vector pK7GWIWG2D(II). The binary vector pK7GWIWG2D(II) is used for silencing target genes and the vector backbone delivers a hairpin RNA for the *ccdb* gene, the corresponding sequence being absent in plant cells. Double-stranded (ds)RNAs are occasionally reported to elicit sequence-nonspecific PTGS; therefore, it was important to check the health of the transformed roots with this vector to eliminate such possibilities. Our results have shown that there was no phenotypic difference between the composite plants generated by transformation with pK7WGF2 and pK7GWIWG2D(II), clearly showing that the backbone dsRNA in the pK7GWIWG2D(II) vector had no deleterious effect on the health of the composite plants (data not shown).

Molecular characterization of the transformed roots.

To understand the molecular integrity of the transformed roots, we have chosen to check the expression of two genes that are required for both nodulation and mycorrhization, indicating their importance in root microbe symbiosis (Oldroyd and Downie 2006). These two genes code for SymRK and CCaMK, respectively. Quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis indicated the expression level of these two symbiotic marker genes (AhSymRK: accession number EU982408 and AhCCaMK: accession number EU395429) to be comparable in the nontransformed and the transformed roots, indicating molecular competence of these roots for nodulation (Fig. 3A). The data was normalized with the expression level of AhActin (accession number EU982407). The stable introduction and successful expression of the transformation marker *rolB* and the cotransformation marker GFP in the composite plants was confirmed by RT-PCR (Fig. 3B).

Table 2. *Agrobacterium rhizogenes*-mediated transformation of *Arachis hypogea* cultivars with the binary vector pCAMBIA2301[a]

Arachis spp. (ICRISAT no.)	Region	Total no. of explants inoculated (composite plants)	Composite plant (%)	Roots per plant[b]
A. fastigiata (ICG 332)	Brazil	25 (20)	81	8 ± 1
A. vulgaris (ICG 4729)	China	25 (22)	88	7 ± 4
A. peruviana (ICG 10036)	Peru	25 (18)	72	6 ± 2
A. hypogea				
Spanish bunch (ICG 3700)	Nigeria	25 (19)	78	8 ± 2
Valencia (ICG 2716)	United States	25 (20)	81	7 ± 3
Virginia bunch (ICG 6284)	Honduras	25 (19)	78	8 ± 2
Virginia runner (ICG 5262)	Zambia	25 (22)	90	6 ± 3

[a] Ten plants were tested.
[b] Number of cotransformed β-glucuronidase-positive hairy roots per composite plant 6 weeks after infection.

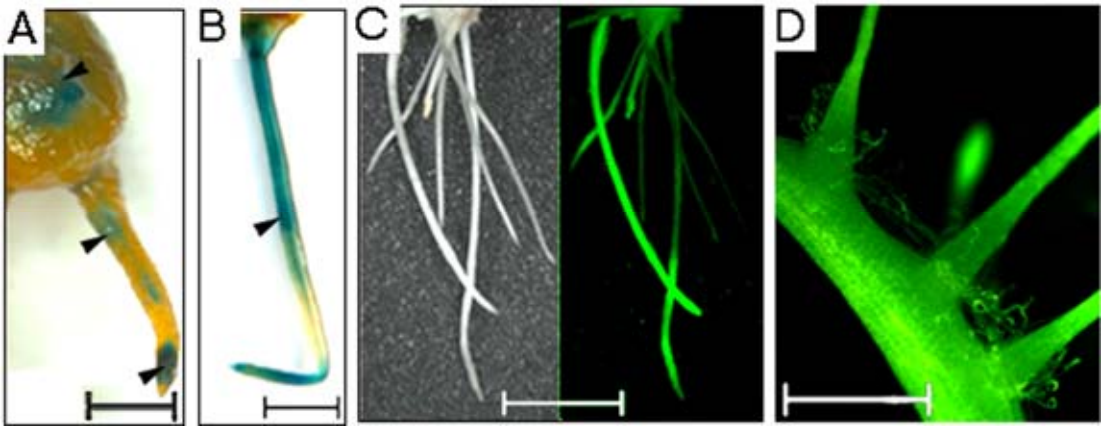

Fig. 2. Expression of cotransformation markers β-glucuronidase (GUS) and enhanced green fluorescent protein (eGFP) expression in 2- to 4-week-old transgenic roots of *Arachis hypogea*: **A** and **B,** GUS expression in transgenic roots. Arrowhead indicates high GUS expression in root tips, vascular tissues, and during root emergence from the globular calli. **C,** Transgenic roots showing GFP fluorescence. **D,** Rosette-type root hairs at lateral root base shows GFP fluorescence. Bars: A through C, 1 mm; D, 0.5 mm.

Transformed roots of *Arachis hypogea* nodulates efficiently.

Four to five weeks after transformation with *Agrobacterium rhizogenes* R1000 carrying the binary vectors pK7WGF2, pK7GWIWG2D(II), or pCAMBIA 2301, the hairy roots in the *Arachis* spp. composite plants were inoculated with *Bradyrhizobium* sp. (*Arachis*) strain NC92 (Gillette and Elkan 1996). Within three weeks after infection (WAI), the composite plants developed spherical nodules, the nodulation kinetics being slightly delayed (3 to 4 days) compared with what was observed for nontransformed wild-type plants. Almost 90% of the transformed roots expressing either GFP or GUS effectively nodulated (Fig. 4A through D). The number of nodules in the mature plant (8 WAI) was higher in transformed roots, ranging from 100 to 180 compared with 80 to 120 in the wild-type roots which are averaged from 25 plants of each kind. This is consistent with the fact that nodulation in *Arachis* spp. occurred on sites of lateral root emergence and such sites are numerous in transgenic roots for increased branching compared with control untransformed roots (Fig. 4E). Similar to the nontransgenic nodules, almost all the nodules in the transformed roots were spherical determinate type (Fig. 4F). However, in almost all the transgenic plants, 2 to 3% of the nodules contained nodular roots (Fig. 4G). Half-sectioned transformed nodules revealed the central infected zone with red pigmentation of leghemoglobin, which is characteristic for nitrogen-fixing nodules (Fig. 4H). Acetylene reduction assay also indicated the functional efficacy of the transformed nodules to be similar to the nontransformed nodules (data not shown). The distribution of 35S promoter-driven expression of cotransformation markers GFP and GUS was similar in the mature nodules. Both GFP fluorescence (Fig. 4I and L) and GUS staining (Fig. 4J and K) was positive in the central invaded zone and the vascular bundles but absent in the peripheral uninvaded cells. GFP fluorescence had very low intensity and could be detected only with high laser power in a confocal microscope (described below). Because 35S-driven GUS expression was strong in the invaded region of the nodule, low fluorescence of GFP was not due to silencing of the promoter. Rather, it indicates quenching of the GFP fluorescence in the invaded region, as reported earlier (Auriac and Timmers 2007; Estrada-Navarrete et al. 2006; Govindarajulu et al. 2008).

The anatomy of the nodules from transformed roots is shown by several cell-permeant nucleic-acid-staining fluorophores such as 4′,6-diamidino-2-phenylindole (DAPI), SYTO9, and propidium iodide (PI) (Fig. 4M through R). The tissue organization was aeschynomenoid where there were no uninfected cells in the infected nodule central zone (Fig. 4M and N). There was no endodermis layer separating the outer cortex and the inner parenchymal cortex (Fig. 4M). This common endodermis is recognized as an advanced feature, present only in certain species of the subfamily Papilionoideae (Boogerd and Rossum 1997). The peripheral vascular bundles in the nodule cortex were surrounded by vascular endodermis (Fig. 4M). In the infected zone, cells are tightly packed without intercellular air spaces (Fig. 4N). Fluorescence of SYTO9 has been reported to be quenched in the invaded region earlier (Auriac and Timmers 2007). In *Arachis* nodules, SYTO9 fluorescence was very strong and was detected using a very low-power laser that failed to detect any fluorescence from the co-transformation marker GFP (Fig. 4L and O). Infected cells in the transgenic nodules are filled with spherical symbiosomes as indicated by both confocal (DAPI, PI, and SYTO9 staining) and scanning electron microscopy (Fig. 4P through S). This is exactly similar to what has been observed with nontransformed standard nodules (data not shown). However, this is in contrast to *Medicago, Pisum*, and *Phaseolus* spp. (Estrada-Navarrete et al. 2006; Mergaert et al. 2006), where the symbiosomes were observed to be of elongated shape. Whereas DAPI and PI stained the nucleic acid material of the symbiosomes (Fig. 4P and Q), SYTO9 distinctively stained the symbiosome membranes (Fig. 4R). The reason for SYTO9 staining the symbiosome membranes remains unclear; however, it can be used as an effective tool for observing symbiosomes in *Arachis* nodules.

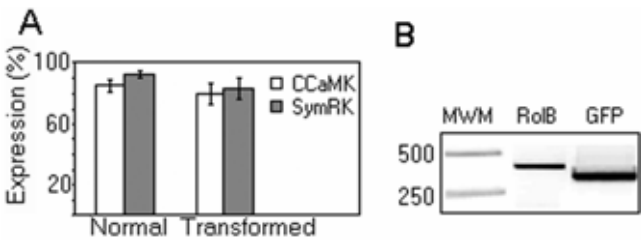

Fig. 3. Molecular characterization of the transformed roots: **A,** Quantification of symbiotic marker genes calcium and calmodulin-dependent protein kinase (CCaMK) and symbiotic receptor kinase (SymRK) mRNA levels in transformed (4-week-old) and nontransformed (1-week-old) roots. Relative transcript abundance was determined by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and normalized against *AhACTIN*, which is constitutively expressed in roots. **B,** Proper genomic integration and transcription of transformation markers is demonstrated by amplification of the transformation and co-transformatin markers *rolB* (438 bp) and green fluorescent protein (GFP) (350 bp), respectively, by RT-PCR; Molecular-weight marker sizes are indicated to the left.

Choice of symbionts by the composite plants was identical to the wild-type plants.

16S rDNA profiling was done for the infected zone metagenome in the nodules developed in composite plants grown in natural soil. In total, 75 rDNA clones were analyzed for generating a representative profile. From the metagenome of the infected zone of five randomly picked nodules collected from five composite plants, 25 clones were analyzed, and three such sets gave a total of 75 clones. Representation of rDNA from *Bradyrhizobia* spp. compared with mitochondrial rRNA (EU982414) and plastid rRNA (EU982406) were 82.6, 12, and 5.3%, respectively (Fig 5A). Five different *Bradyrhizobia* spp. named uncultured *Bradyrhizobia* sp. *Arachis* 1 to 5, with accession nos. EU982409, EU982410, EU982411, EU982412, and EU982413, had a representation of 25.8, 8, 35.4, 6.5, and 24%, respectively, which showed 91 to 98% identity with *Bradyrhizobia* spp. reported in the database. From our 16S rDNA-based phylogenetic analysis, it is apparent that the bradyrhizobial sequences identified by us form two distinct clusters belonging to group II bradyrhizobia that includes *Bradyrhizobium japonicum* and BTAi1 and are distant from *B. elkani*, a member of group I bradyrhizobia (Kwon et al. 2005) (Fig. 5B). The uncultured *Bradyrhizobia* sp. *Arachis* 1 to 5 identified in *Arachis* nodules appear to be relatively closer to the point of divergence of the genera *Bradyrhizobium* and *Rhizobium.* Similar analysis was performed with wild-type plants under identical conditions, where the representation of the bradyrhizobial sequences were identical to what has been obtained with the composite plants (data not shown). These data clearly reflect that the choice of the symbiont of the composite plants remain unchanged after being transformed.

DISCUSSION

Investigation of crack invasion has so far been undertaken with *S. rostrata*, which adapts to this mode of bacterial entry under submerged growth conditions but resorts to formation of IT for ultimate cortical colonization (Goormachtig et al. 2004b). In contrast, in *Arachis* spp., IT are not formed for epidermal invasion or for cortical invasion or nodular dissemination

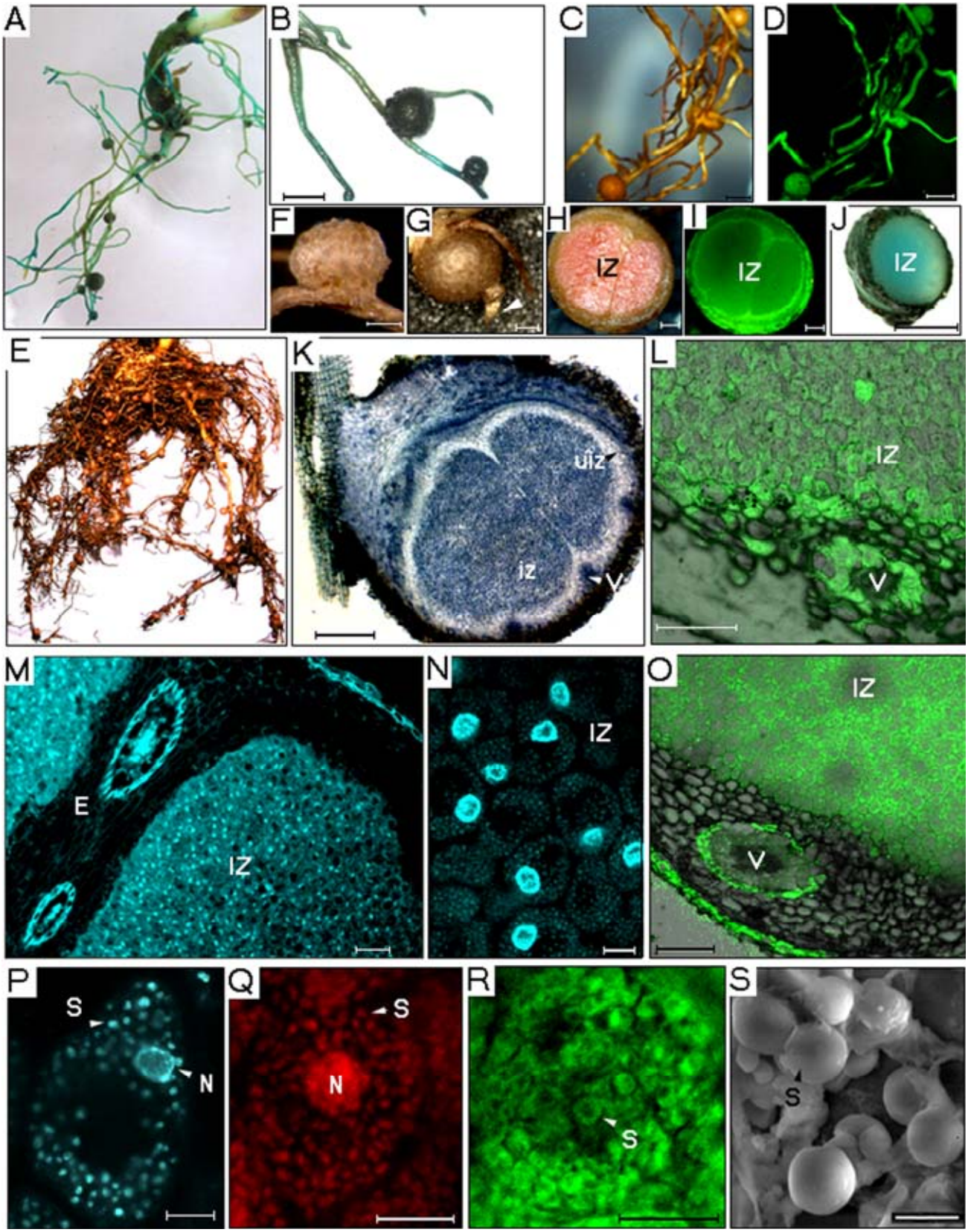

Fig. 4. Characterization of transformed nodules of *Arachis hypogea*: **A** and **B,** β-Glucuronidase (GUS) and **C** and **D,** enhanced green fluorescent protein (eGFP) cotransformed mature transgenic root nodules 3 and 6 weeks after infection (WAI), respectively, with *Bradyrhizobium* spp. (*Arachis*) strain NC92. **E,** Transgenic roots with mature root nodules at 8 WAI. **F,** Standard nodules. **G,** Nodules with apical nodular roots, indicated by an arrow. **H** through **J,** Half-sectioned transformed nodules showing red coloration of leghaemoglobin, eGFP, and GUS expression. **K** through **R,** Longitudinal section of nodules expressing **K,** GUS; **L,** eGFP; **M, N,** and **P,** stained with 4′,6-diamidino-2-phenylindole; **O** and **R,** stained with SYTO9; **Q,** stained with PI. **S,** Scanning electron micrograph of nodules showing spherical symbiosomes. Infected zone (iz), uninfected zones (uiz), endodermis (E), nucleus (N), and vascular bundle (v) are indicated. Bars: A through D, 1 mm; F, G, and J, 0.5 mm; H, I, and K, 0.2 mm; L, M, and O, 100 μm; N, P, Q, and R, 10 μm; and S, 5 μm.

(Boogerd and Rossum 1997; Chandler 1978). Apart from having a nonclassical rhizobial invasion strategy, *Arachis* spp. also have a distinguishing aeschynomenoid nodule morphogenesis program, where the core infected zone is not interspersed with uninfected cells (Boogerd and Rossum 1997). It is interesting to note that aeschynomenoid nodulation is always associated with crack invasion, though the reverse is not true (Goormachtig et al. 2004a). The fact that nod factor is indispensable for crack invasion in *S. rostrata* (D'Haeze et al. 1998) and dispensable in at least two aschynomene plants (Giraud et al. 2007) that are taxonomically related to *Arachis* spp. clearly indicates that there may be major differences in the molecular mechanisms of rhizobial invasion and nodule organogenesis, even among legumes that are adapted to crack invasion.

An efficient transformation procedure was crucial for undertaking investigation of nonclassical nodulation features of *Arachis hypogea*. Therefore, we have developed a fast, reproducible, and efficient transformation procedure for this plant using *Agrobacterium rhizogenes*-R1000 strain (agropine type) harboring the pRiA4b plasmid. Depending on whether we used the upper two-thirds or upper one-third of the embryonic axis as explant, there was significant difference in the efficiency of composite plant generation (Table 1; Fig. 1). In both cases, the efficiency to generate cotransformed roots (above 80%) was similar to cotransformation frequencies reported for other *A. rhizogenes*-transformed legumes (Boisson-Dernier et al. 2001; Estrada-Navarrete et al. 2006; Quandt et al. 1993; Stiller et al. 1997; Stougaard et al. 1987, Van de Velde et al. 2003). However, in the later case, with the upper one-third embryonic axis as explant, the shoot had slow growth kinetics giving inconsistent and poor generation of composite plants. In contrast, the upper two-thirds of the embryonic axis was highly consistent in generating transformed roots and composite plants and was used for our nodulation experiments (Fig. 1). The procedure has been tested for several *Arachis hypogea* cultivars where it was equally successful (Table 2).

Cotransformation was monitored by the expression of two marker genes, GUS and eGFP, under the constitutive 35S *Cauliflower mosaic virus* promoter. The pCAMBIA2301 binary vector contains the *Escherichia coli* gusA with an intron from the castor bean catalase gene inside the coding sequence to ensure that expression of glucuronidase activity is derived from eukaryotic cells. Therefore, GUS expression ensured proper integration of the transformed genes. In uninoculated *Arachis* roots, 35S-driven *gus* expression was primarily in vascular tissues and the root tip (Fig. 2A and B), which matched with the patterns published earlier for other systems (Martirani et al. 1999; Stiller et al. 1997; Van de Velde at al. 2003). RT-PCR-based amplification of *rol*B and GFP from transformed roots further confirmed successful integration of the T-DNA and the marker genes (Fig. 3B). Quantitative RT-PCR indicated that expression of CCaMK and SymRK, two symbiotic markers, were similar in the wild-type and the transformed roots, indicating similar symbiotic competence of the transformed roots (Fig. 3A). Distribution of 35S-driven expression of cotransformation markers GUS and GFP was similar in the nodule tissue. They were detected in the central infected region and in the vascular bundles in the peripheral uninvaded zone (Fig. 4I through L). The 35S promoter had been demonstrated to be active in the invaded cells of the infected zone in the case of *Sesbania* spp. (Van de Velde et al. 2003), soybean (Govindarajulu et al. 2008), and *Phaseolus* spp. (Estrada-Navarrete et al. 2006) but, in several other plants, the 35S expression was restricted only to the noninvaded cells of the nodule tissue; for example, *V. hirsuta* (Quandt et al. 1993), *M. sativa* (Samac et al. 2004), and *M. truncatula* (Auriac and Timmers 2007). In *Arachis* nodules, GFP fluorescence was significantly low, detectable only by powerful laser excitation. This appears to be due to fluorescence quenching rather than 35S promoter inactivity because GUS expression driven by the same promoter in the same tissue was normal. Such quenching of GFP fluorescence in invaded cells has been reported earlier (Auriac and Timmers 2007). We have successfully used other fluorophores, such as SYTO9 (Auriac and Timmers 2007; Haynes et al. 2004) for staining the invaded zone or DAPI and PI (Limpens et al. 2005) for investigating the symbiosome distribution in the infected cells of the transgenic *Arachis* nodule (Fig. 4M and N).

The *Arachis* nodules are of determinate aeschynomenoid type (Boogerd and Rossum 1997). For more than 97% of the transgenic nodules, there were no differences in morphology and development when compared with wild-type nodules. However, consistently, 2 to 3% of the transgenic nodules were found to bear nodular roots which was never observed in wild-type plants (Fig. 4G). Even in the natural soil where the wild type and the composite plant was invaded and colonized by a similar contingent of symbiont (Fig. 5), the transformed plant developed 2 to 3% of nodules with apical root growth. Because nodular roots are never seen in wild-type nodules of *Arachis* spp., it is the transforming principles in the *rol* genes in *Arachis* transgenic ("hairy") roots that appear to have influenced the hormonal landscape inside the nodules to cause such a developmental response. Similar observations were reported by Akasaka and associates (1998), too, where transformation was carried out by *Agrobacterium rhizogens* MAFF-02-10266 (mikimopine type) containing the plasmid pRi1724 compared with *A. rhizogens* R1000 (agropine type) containing plasmid

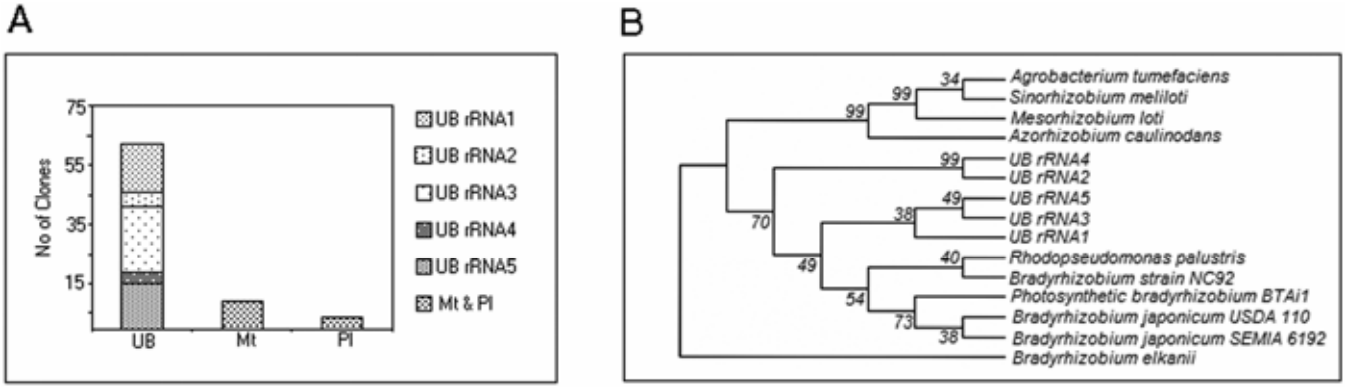

Fig. 5. 16S rDNA profile for the infected zone metagenome of nodules developed in composite plants grown in natural soil. **A,** No of clones representing the 16s rDNA from mitochondria, plastid, and uncultured *Bradyrhizobium* sp. (*Arachis*) (1 to 5). **B,** Phylogenetic tree of the members of the *Rhizobiaceae.* Tree was generated based on a CLUSTALW alignment and then using Maximum parsimony method (MEGA). Numbers above the branches represent the percentages of 1,000 bootstrap replications.

pRiA4b, used by us. This indicates that, regardless of the *Agrobacterium* strain that is used for transformation, transformed nodules develop this ability to generate apical roots. It is interesting to note that nodules formed by actinomycetes normally feature nodular roots (Gherbi et al. 2008). Thus, the unusual structures in the transgenic *Arachis* nodules may not be aberrant structures but may, in fact, represent a part of a spectrum of form in symbiotic nodules formed by the nodulating eurosids (Dudley et al. 1987). Similar morphological alterations of transformed root nodules in soybean have been previously reported by Bond and Gresshoff (1993), where multiple active meristems caused the soybean nodules to become elongated and branched, resembling indeterminate nodules of *Pisum sativum* and *M. truncatula*.

In summary, a reproducible and efficient transformation procedure for *Arachis* spp. has been developed. Despite the large genome and recalcitrance to transformation, investigation of the molecular mechanism of the nonclassical nodulation features of *Arachis* spp. by knocking down or overexpressing genes of interest can now be undertaken, which would contribute to our understanding of the molecular mechanism of rhizobial invasion and nodule formation in legumes of the aeschynomeneae tribe.

MATERIALS AND METHODS

Plants.

Seed of *Arachis hypogea* JL-24 (a popular cultivar in India), *A. hypogea* Spanish bunch (ICG 3700), *A. hypogea* valencia (ICG 2716), *A. hypogea* Virginia bunch (ICG 6284), *A. hypogea* Virginia runner (ICG 5262), *A. fastigiata* (ICG 332), *A. vulgaris* (ICG 4729), and *A. peruviana* (ICG 10036) were obtained from the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT; Andhra Pradesh, India).

Strains and binary vectors.

Agrobacterium rhizogenes R1000 was provided by C. Somerville of Standford School of Medicine, Department of Plant Biology. Binary vectors pK7WGF2/pK7GWIWG2D(II) and pCAMBIA2301 were purchased from the Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology, Ghent University, Belgium and CAMBIA, Canberra, Australia, respectively. Binary vectors were introduced into *A. rhizogenes* R1000 by the freeze-thaw method, as described by Höfgen and Willmitzer (1988). Culture of *A. rhizogenes* R1000 was grown in *Agrobacterium*-specific yeast extract broth medium at 28°C. Medium was purchased from Highmedia. *Bradyrhizobium* sp. (*Arachis*) NC92 (IC 7001) strain was obtained from ICRISAT.

Transformation with *A. rhizogenes*.

The embryonic axes of *Arachis hypogea* JL-24 dry seed were surface sterilized for 15 min with sodium hypochlorite solution (3% active chlorine) containing 0.01% Tween 20 and rinsed five times with sterile deionized water. *Agrobacterium rhizogenes* R1000 culture was prepared in liquid YEP media, resuspended in liquid MS basal medium, and incubated in the presence of acetosyringone at 10 mg/liter for 45 min. The embryonic axes were then co-cultivated with *Agrobacterium rhizogenes* and plated in half-strength MS medium (Highmedia) with kanamycin (USB, Cleveland) at 25 mg/liter and acetosyringone (Sigma, Mumbai, India) at 10 mg/ml at 25°C in darkness.

Hairy root emergence and plant cultivation.

At 10 to 12 days after inoculation, the plantlets were subcultured twice at 7-day intervals in solid half-strength MS medium containing carbenicillin at 100 mg/liter and kanamycin at 25 mg/liter and kept in darkness at 25°C for growth of hairy roots. Plantlets were then subjected to a normal photoperiod of 16 h of light and 8 h of darkness for 2 days and then the composite plants were potted in fresh sterile vermiculite for nodulation assays.

Nodulation of transgenic hairy roots and acetylene reduction assay.

Composite plants were inoculated with *Bradyrhizobium* sp. (*Arachis*) NC92 culture grown in yeast extract mannitol medium. Plants were watered every 3 days with Hogland nitrogen-free medium (Uheda et al. 2001). Nodulation was quantified three weeks onward after inoculation. Nitrogen fixation of standard and transgenic hairy roots was assayed using nitrogenase acetylene reduction (Vessey 1994).

Cryosectioning of nodules for confocal microscopy.

Sample preparation was done according to Haynes and associates (2004). Sections were prepared using a cryocutter (Leica, Ryswyk, The Netherlands) after fixing the sample in plastic resin (Leica). Sections of 5 to 20 μm were prepared and taken in polylysine (Sigma) coated slides, followed by rehydration and staining (when indicated) with DAPI (USB) or a Live/Dead bacteria staining kit (Invitrogen) for PI and SYTO9. Sections were mounted with Antifade Gold (Invitrogen).

GUS and GFP expression and visualization.

Histological assay for checking GUS gene expression was performed according to Jefferson and associates (1987). Briefly, hairy roots were vacuum infiltrated with 0.1 M sodium phosphate buffer containing 0.25% sodium dodecyl sulfate (SDS) and 1 mM X-Gluc and then incubated for 12 h at 37°C. Expression of GUS and GFP in whole tissues or half-sectioned nodules were analyzed by light and fluorescence stereomicroscopy, respectively. Cryosections of transgenic nodules were analyzed using a confocal microscope (ZEISS AXIOVERT equipped with a LSM510) Argon LASER with 488-nm laserline. GFP and SYTO9 fluorescence was imaged using the following settings: 488-nm LASER, HFT405/488, NFT490, BP505-550). Settings for PI were Argon 543-nm LASER, HFT488/543, NFT545, BP560-615 and, for DAPI, were Argon 405-nm LASER, HFT405/488, NFT490, BP420-480. Images were processed using Zeiss image browser version 4.2 and Adobe Photoshop CS2.

Low-vacuum scanning electron microscopy sample preparation.

Nodules were washed and fixed by a mixture of formalin, alcohol, and acetic acid and were then dehydrated by a series of alcohol-gradient steps. They were then transferred to 100% chloroform through a gradient of alcohol/chloroform (3:1, 1:1, and 1:3) mixtures at room temperature. Paraplast (Sigma) was dissolved in this chloroform at room temperature for embedding the tissue, and then incubated at 37 and 60°C for 24 h each. The sample was properly oriented before taking out the block to room temperature. Then, 20-μm sections were prepared using microtome (Reichert, Germany) and placed on suitably cut polylysine-coated slides. Paraplast was removed with four changes of Xylene (1 h each) and rehydrated. The slides were analyzed using a FEI Quanta-200 MK2 low-vacuum scanning electron microscope (Bal 1990).

RNA isolation and RT-PCR.

RNA isolation, RT-PCR, and cloning were done as described by Raichaudhuri and associates (2006) and Nag and associates (2005). The following sets of forward:backward (fwd:bkwd) primers were used for the indicated genes: i) fwd, AhActin

5′GAAATGTGCCCCTTATCAGATATAC3’ and bkwd, AhActin 5′GTTTGAGAGCATAGCGTGCTGTGCTG3′; ii) fwd, AhSymRK 5′ATACCTGTTGTACGACCACT3′ and bkwd, AhSymRK 5′ATGGAGAAGATCTGGCATCA 3′; iii) fwd, RolB 5′GCTCTTGCAGTGCTAGATTT 3′ and bkwd, RolB 5′GAAGGTGCAAGCTACCTCTC3′; iv) fwd, GFP 5′CCGGGGTGGTGCCCATCCTGGTC3′ and bkwd, GFP 5′ CCTTCAGCTCGATGCGGTTCACC 3′; and v) fwd, AhCCaMK 5′GCCAAAGATGTTCAAATGGACCCT 3′ and bkwd, AhCCaMK 5′ CCTGAGACTTTCAATTTCCTCTTC 3′ Clones were sequenced using BD-mixture (Applied Biosystems, India).

Real-time PCR.

Approximately 200 ng of total RNA was reverse transcribed by using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) and oligo(dT)17. Quantitative PCR was performed by using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.). The primer sets for AhCCaMK, AhSymRK, and AhActin are indicated above. The PCR program consisted of 1 cycle at 95°C for 5 min, 40 cycles at 95°C for 10 s and 52°C for 30 s, ending with 1 cycle at 95°C for 1 min. The results were normalized with AhActin expression levels.

Preparation of DNA from infected zone.

Nodules were surface sterilized, treated with 0.25% SDS, and repeatedly washed with sterile distilled water. They were half sectioned and the infected zones were collected with a sterile needle, DNA was prepared according to standard protocol described by Sambrook and associates (1989). 16S rDNA was amplified by 5′-TAACACATGCAAGTCGAACG-3′ and 5′-ACGGGCGGTGTGTAC-3′ as forward and backward primer, respectively, cloned, and sequenced following standard protocol.

Construction of phylogenetic tree.

For the phylogenetic and molecular evolutionary analyses, the alignment was performed by using the CLUSTALW and Maximum Parsimony algorithms of *MEGA* version 4 (Tamura et al. 2007). The following sequences (with GenBank accession numbers in parentheses) were analyzed: uncultured *Bradyrhizobia* sp. *Arachis* 1 to 5 (EU982409, EU982410, EU982411, EU982412, and EU982413); *Mesorhizobium loti* (BA000012); *Bradyrhizobium* sp. (*Arachis*) strain NC92 (U12900); *Sinorhizobium meliloti* (AL591688); *Bradyrhizobium japonicum* SEMIA 6192(AY904772); *A. tumefaciens* (AB102735); *Azorhizobium caulinodans* (AP009384); *B. japonicum* USDA 110 (BA000040); *Bradyrhizobium* BTAi1 (CP000494); *Rhodopseudomonas palustris* (CP001096); and *B. elkanii* (AB272335).

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