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RAPID COMMUNICATION

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Transgenic *Medicago truncatula* plants obtained from *Agrobacterium tumefaciens*-transformed roots and *Agrobacterium rhizogenes*-transformed hairy roots

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Abstract *Medicago truncatula*, barrel medic, is a forage crop that has been developed into a model legume. The development of new transformation methods is important for functional genomic studies in this species. Based on *Agrobacterium tumefaciens*-mediated transformation of root explants, we developed an effective system for producing *M. truncatula* (genotype R108) transgenic plants. Among the four *A. tumefaciens* strains (AGL1, C58C1, EHA105 and LBA4404) tested, EHA105 and AGL1 were most effective in regenerating transgenics. Callus induction frequency from root explants was 69.8%, and plantlet/shoot regeneration frequency was 41.3% when EHA105 was used. Transgenic nature of the regenerated plants was confirmed by PCR and Southern hybridization analyses. Progeny analysis revealed stable Mendelian meiotic transmission of transgenes. Because *M. truncatula* is particularly useful for the study of root endosymbiotic associations, we further developed a plant regeneration system from *A. rhizogenes*-transformed hairy roots of *M. truncatula*. Fertile true transgenic plants were regenerated from the hairy roots, thus allowing the assessment of gene functions at the whole plant level. Segregation analysis revealed that the hairy root genes could be segregated out in the progenies. By coupling *A. rhizogenes*-mediated hairy root transformation and the regeneration system reported here, once potential genes of interest are identified, the transformed hairy roots carrying such genes could be directly regenerated into plants for more detailed characterization of the genes.

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Keywords *Agrobacterium tumefaciens* · *Agrobacterium rhizogenes* · Genetic transformation · Hairy root · *Medicago truncatula* · Root explant

Abbreviations 2,4-D: 2-Dichlorophenoxy-acetic acid · BAP: 6-Benzylaminopurine · EST: Expressed sequence tag · GUS: β-Glucuronidase · PCR: Polymerase chain reaction · PPT: Phosphinothricin

Introduction

Legumes are widely grown for grain and forage purposes, their worldwide economic importance being second only to grasses (Graham and Vance 2003). *Medicago truncatula*, commonly known as "barrel medic" because of the shape of its seed pods, is commercially used as a forage legume in Australia. In recent years, *M. truncatula* has been recognized as an excellent model for legumes in view of its small, diploid genome, self-fertility and short life cycle (Barker et al. 1990; Bell et al. 2001). Its genome is highly conserved with alfalfa and pea and moderately conserved with soybean and other legumes (Cook 1999; Young et al. 2005). A number of research programs have been committed to *M. truncatula*, leading to a wealth of genomic resources, e.g., >226,000 expressed sequence tag (EST) sequences (http://www.tigr.org), oligo and Affymetrix microarray chips, physical and genetic maps and large scale gene-space sequencing that is scheduled for completion by the end of 2006 (Young et al. 2005). As a legume, and unlike the most studied model plant, Arabidopsis, *M. truncatula* establishes symbiotic relationships with nitrogen fixing Rhizobia. In addition, roots of *M. truncatula* are also colonized by beneficial arbuscular mycorrhizal fungi (Harrison and Dixon 1993; Bell et al. 2001). The availability of a large amount of sequence information makes the identification and isolation of genes much easier than ever before; however, functional characterization of the genes remains a challenge.

The development of efficient transformation methods is of great importance for functional genomics. In legume species, the production of transgenic plants has most commonly been based on infection by disarmed *Agrobacterium tumefaciens* (Somers et al. 2003). Transgenic *M. truncatula* plants have been obtained by *A. tumefaciens*-mediated transformation of leaf or cotyledonary explants (Trieu and Harrison 1996; Hoffmann et al. 1997; Trinh et al. 1998; Scholte et al. 2002; Chabaud et al. 2003; de Sousa Araújo et al. 2004; Zhou et al. 2004). *In planta* transformation methods were also tested in *M. truncatula*; unfortunately, these protocols have not been reproduced (Somers et al. 2003; Zhou et al. 2004).

To facilitate the study of root endosymbioses with nitrogen-fixing nodule bacteria and mycorrhizal fungi in *M. truncatula*, protocols for generating transformed hairy roots by *Agrobacterium rhizogenes*-mediated transformation were developed (Boisson-Dernier et al. 2001; Rodríguez-Llorente et al. 2003; Limpens et al. 2004). Different from *A. tumefaciens*, *A. rhizogenes* generates adventitious, genetically transformed hairy roots at the site of inoculation in many dicots. Upon expression of the *root locus* (*rol*) genes carried on the root inducing (Ri) plasmid-derived T-DNA, roots are formed of which a certain number will be co-transformed with the binary vector that contains the gene of interest (Limpens et al. 2004). *A. rhizogenes*-mediated transformation allowed the production of "composite plants", a term derived from the fact that transformed roots are induced on a non-transformed plant (Boisson-Dernier et al. 2001).

The generation of "composite plants" by *A. rhizogenes*-mediated transformation has been used as a rapid tool to test gene and promoter functions in roots (Boisson-Dernier et al. 2001; Limpens et al. 2003, 2004; Vinardell et al. 2003; Boisson-Dernier et al. 2005; Xiao et al. 2005). However, because the shoot part is not transformed, the effects of genes and promoters at the whole plant level cannot be assessed, and the transgenic trait cannot be transmitted to the progeny. In addition, not all roots are transformed; the transformed roots can also be chimeric (Limpens et al. 2004).

To date, there have been no reports on the regeneration of fertile plants from either *A. tumefaciens*-mediated root transformation or from *A. rhizogenes*-transformed hairy roots in *M. truncatula*. Here, we report (1) development of a convenient *A. tumefaciens* transformation system using root explants and (2) regeneration of true transgenic plants from *A. rhizogenes*-transformed hairy roots. Transgenes were stably integrated in the plant genome and inheritance of the transgenes was characterized in detail.

Materials and methods

Sterilization and germination of seeds

Seeds of *Medicago truncatula* genotype R108-1 (Hoffmann et al. 1997) were scarified in concentrated sulfuric acid for 5 min and rinsed three times with 4°C water. The scarified seeds were surface sterilized with 30% (v/v) commercial Clorox bleach containing 0.1% (v/v) Tween 20 for 8 min and washed three times with sterile water. Surface-sterilized seeds were plated onto sterile filter paper in a petri dish containing a small amount of sterile water to cover the seeds. The dish was first placed in the dark overnight, and then transferred a growth room at 25°C under fluorescent light (140 $\mu E\ m^{-2}\ s^{-1}$) at a photoperiod of 16 h. For hairy root transformation, 2-day-old seedlings were infected with *A. rhizogenes*. For root transformation, 3-day-old seedlings were transferred to plastic vessels containing MSO medium: MS medium (Murashige and Skoog 1962) (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) supplemented with 1% (w/v) sucrose and solidified with 0.25% (w/v) gelrite (Sigma, St. Louis, MO, USA). The seedlings were grown on MSO medium for three or more weeks until enough roots were formed and then used as donor plants for obtaining root or leaf explants.

Agrobacterium tumefaciens strains and binary vector

Four *Agrobacterium tumefaciens* strains were tested in this study: AGL1 (Lazo et al. 1991), C58C1 (Deblaere et al. 1985), EHA105 (Hood et al. 1993) and LBA4404 (Hoekema et al. 1983). The binary vector used was pCAMBIA3301, which carries a chimeric phosphinothricin acetyltransferase gene (*bar*) (de Block et al. 1987) and a β-glucuronidase gene (*gusA*) (Jefferson et al. 1987), both under the control of CaMV 35S promoter (http://www.cambia.org). The *gusA* reporter gene contains a catalase intron inside the coding sequence to ensure that expression of glucuronidase activity is derived from eukaryotic cells, not from expression by residual *A. tumefaciens* cells. The vector pCAMBIA3301 was introduced into the four *A. tumefaciens* strains by the freeze-thaw method (Chen et al. 1994).

Single colonies of *A. tumefaciens* were transferred to liquid LB medium (Qbiogene, Montreal, Canada) containing 50 $mg\ l^{-1}$ kanamycin (PhytoTechnology Laboratories). The cultures were grown at 28°C with shaking until the OD_{600} reached about 1.0. Cells were then pelleted by centrifugation at 2,400*g* for 10 min and resuspended in liquid MTR-1 medium containing MS medium (PhytoTechnology Laboratories) supplemented with 5 $mg\ l^{-1}$ 2,4-D (PhytoTechnology Laboratories), 0.5 $mg\ l^{-1}$ 6-benzylaminopurine (BAP) (PhytoTechnology Laboratories), 3% (w/v) sucrose and 19.6 $mg\ l^{-1}$ acetosyringone (ACROS Organics, Morris Plains, NJ, USA). The density (OD_{600}) of the resuspended *Agrobacterium* was adjusted to 0.4–0.5.

Infection of root explants by *A. tumefaciens*, selection and plant regeneration

Donor plants grown in the culture vessels were carefully removed from the MSO medium and excess gelrite cleaned off from the roots. The roots were placed in a

petri dish and cut into ~1 cm segments. The root segments were incubated with the four *Agrobacterium* strains for 30 min on a rotary shaker at 30 rpm. After incubation, the root segments were transferred onto MTR-1 medium solidified with 0.8% (w/v) agar (Agar–Agar, Sigma) and co-cultivated for 2 days in dark at 25°C. Control root segments were included in each experiment.

To test which part of the root was most responsive for callus induction, a root was divided into three sections S1, S2 and S3 (Fig. 1) and infected with EHA105. The S1 section was close to the root-shoot junction, S2 was the mid-root section and the S3 section included the root tip.

After co-cultivation, the root segments were transferred onto MTR2 medium: MS basal medium (Phyto-Technology Laboratories) supplemented with 5.0 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BAP, 3% (w/v) sucrose, 5.0 mg l^{-1} phosphinothricin (glufosinate ammonium, PPT. Sigma-Aldrich, Seelze, Germany), 250 mg l^{-1} cefotaxime (Agri-Bio, North Miami, FL, USA) and solidified with 0.8% (w/v) agar. The cultures were placed in the dark at 25°C. Calluses formed on MTR-2 medium were transferred onto MTR-3 medium for plantlet/shoot regeneration. The MTR-3 medium consists of MS basal medium supplemented with 2% (w/v) sucrose, 2.5 mg l^{-1} PPT, 250 mg l^{-1} cefotaxime and solidified with 0.8% (w/v) agar. Cultures on MTR-3 medium were grown at 25°C under fluorescent light (140 μE $m^{-2}s^{-1}$) at a photoperiod of 16 h and subcultured every 2–3 weeks onto fresh medium.

Plantlets/shoots formed on MTR-3 medium were transferred to plastic vessels containing MSO rooting medium. After roots were developed on MSO medium, the regenerated plants were transferred to soil and grown in growth chambers (260 μE m^{-2} s^{-1}, 16 h day/8 h night at 24/20°C) for 2 weeks. Then the established plants (T0) were transferred to the greenhouse (390 μE m^{-2} s^{-1}, 16 h day/8 h night at 24/20°C). For shoots that did not form roots on MSO, the lower part of the shoots were dipped in rooting powder (Brooker Chemical Co., Hollywood, CA, USA) and then directly transferred to soil. Seeds were harvested from the greenhouse plants.

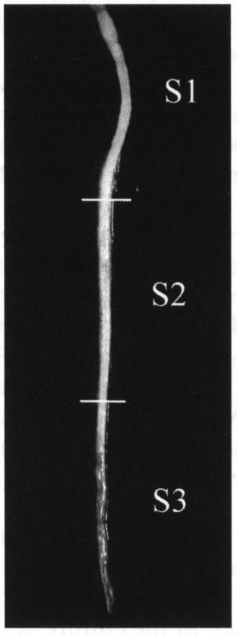

Fig. 1 Different root sections used for *Agrobacterium tumefaciens*-mediated transformation

Induction of hairy roots by *A. rhizogenes* and plant regeneration from transformed hairy roots

Agrobacterium rhizogenes strain ARqual (Quandt et al. 1993; Boisson-Dernier et al. 2001) was used for hairy root induction. Binary vector pCAMBIA3301 was introduced into the ARqual1 strain by the freezing-thaw method (Chen et al. 1994). The pCAMBIA3301 vector was the same as that used for *A. tumefaciens* transformation. Transformed *A. rhizogenes* were grown on LB-Agar medium (Qbiogene, Montreal, Canada) with kanamycin selection at 28°C for 2 days.

Radicles of 2-day-old seedlings were cut approximately 3 mm from the root tip with a sterile scalpel. The seedlings were inoculated at the radicle sections with the *A. rhizogenes* strain ARqual (Boisson-Dernier et al. 2001). The inoculated seedlings were placed on slanted agar containing a modified Fahraeus medium (1 l containing 132 mg $CaCl_2$, 123 mg $MgSO_4$, 122 mg KH_2PO_4, 286 mg Na_2HPO_4, 5 mg FeEDTA, 40 mg NH_4NO_3, supplemented with 0.1 mg of $MnSO_4$, $CuSO_4$, $ZnSO_4$, H_3BO_3 and Na_2MoO_4) in petri dishes (Boisson-Dernier et al. 2001). After 2 days, the seedlings were transferred to fresh Fahraeus medium supplemented with 5.0 mg l^{-1} PPT.

Hairy roots produced 4–6 weeks after inoculation were cut into ~1 cm segments, and the segments were placed onto MTR-2 medium for callus induction. Each hairy root was cut into a few segments, and the segments from the same root were marked under the dish. The cultures were placed in the dark at 25°C.

Calluses formed on MTR-2 medium were transferred onto MTR-3 medium for plant regeneration. Cultures on MTR-3 medium were grown under fluorescent light (140 μE m^{-2} s^{-1}) at a photoperiod of 16 h and subcultured every 2–3 weeks onto fresh medium.

Similar to the procedure used for root transformation, plantlets/shoots formed on MTR-3 medium were transferred onto MSO medium and later transferred to growth chambers and the greenhouse.

Molecular analyses of transgenic plants

Polymerase chain reaction (PCR) analysis

The MagAttract® 96 Plant kit (Qiagen, Valencia, CA, USA) was used for purification of DNA from greenhouse-grown plants. For the analysis of plants derived from

A. tumefaciens-mediated root transformation, the *bar* and *gusA* transgenes were amplified by PCR. For the analysis of plants derived from *A. rhizogenes* transformed hairy roots, the *bar* and *rolA* genes were amplified. The following sets of oligonucleotide primers were used: *bar*: 5′-CCGTACCGAGCCGCAGGAAC-3′ (forward) and 5′-CAGATCTCGGTGACGGGCAGGAC-3′ (reverse); *gusA* AACAGTTCCTGATTAACCACAA ACC-3′ (forward) and 5′-GCCAGAAGTTCTTTTTCCAGTACC-3′ (reverse); *rol*A: 5′-ACGGTGAGTGT GGTTGTAG-GTTC-3′ (forward) and 5′-CGTGCGTATTAATCC CGTAGGTTT-3′ (reverse). The expected PCR products were 444 bp for *bar*, 634 bp for *gusA* and 400 bp for *rol*A. The total volume of reaction mixtures was 50 µl, including 400 ng genomic DNA, 0.5 µl of each primer (50 µM), 1 µl of dNTP mix (10 mM each), 10 µl green GoTaq® reaction buffer (5×) and 0.25 µl GoTaq® DNA polymerase (Promega, Madison, WI, USA). For positive controls, 5 ng plasmid DNA was added to the reaction mix. Cycling parameters for *bar* and *gusA* amplification began with an initial hot start at 94°C for 2 min, then 30 cycles of denaturation (94°C; 40 s), annealing (60°C; 30 s) and extension (72°C; 60 s), followed by a final extension of 5 min at 72°C. PCR amplification products were analyzed by electrophoresis in 1% agarose/ethidium bromide gels. Cycling parameters for *rol*A amplification was similar except that the annealing temperature was 58°C. When large numbers of samples were analyzed for the progenies, PCR amplification products were loaded into 96-well E-gel (Invitrogen # G7008-02) using a multi-channel pipettor and detected after electrophoresis.

Southern hybridization analysis

Genomic DNA was isolated from freeze-dried leaf material of greenhouse-grown plants following the CTAB procedure (Lichtenstein and Draper 1985). DNA was digested with the restriction enzyme *Hin*dIII which only cleaves once in the multiple cloning site located between *bar* gene and *gusA* gene in the binary vector pCAMBIA3301. Twenty microgram DNA from each sample was digested overnight and loaded in each lane. The hybridization probe was obtained by PCR amplification of pCAMBIA3301 DNA using the *bar* primers. The 444 bp hybridization probe was [^{32}P] dCTP-labeled using the RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA, USA), and unincorporated nucleotides were removed by passing through ProbeQuant™ G-50 Micro Columns (Amersham Biosciences, Piscataway, NJ, USA). Gel electrophoresis, DNA blotting and hybridization were carried out following standard protocols (Sambrook et al. 1989).

β-Glucuronidase (GUS) staining

Histochemical assay of GUS activity was carried out on seedlings and leaves of transgenic *M. truncatula*. The materials were submerged in a substrate solution containing 100 mM sodium phosphate, pH 7.0, 10 mM Na-EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% (w/v) X-Gluc and 0.1% (v/v) Triton X-100 and incubated at 37°C overnight (Mendel et al. 1989). GUS expression was visualized after the tissues were destained by soaking and washing several times in 70% ethanol.

Results

Effects of *A. tumefaciens* strains on root transformation and plant regeneration

A large number of root explants (>500) were infected with each of the *A. tumefaciens* strains AGL1, C58C1, EHA105 and LBA4404 (Table 1). After co-cultivation with the different strains, the root segments were transferred onto selection medium containing 5 mg l^{-1} PPT (Fig. 2a). Our previous experiments have shown that 5 mg l^{-1} PPT is lethal to root explants. Expression of the *bar* transgene results in the acetylation of PPT and thus renders plant cells resistant to this herbicide. Calluses resistant to PPT were obtained in 4–5 weeks from the transformed roots (Fig. 2b). No calluses were formed from the non-transformed control root segments. The frequencies of resistant callus formation were in the range of 65.8–76.3% (Table 1). The effects of different *Agrobacterium* strains on the formation of resistant calluses were not significant (Table 1). However, when the strain LBA4404 was used for transformation, the calluses formed grew slower than those formed using the other strains.

The resistant calluses were transferred onto regeneration medium and green plantlets/shoots were produced in 5–6 weeks (Fig. 2c–f). Both somatic embryogenesis and organogenesis have been observed in the regeneration process. The effects of the different *Agrobacterium* strains were obvious at the regeneration stage. The regeneration frequencies for AGL1, C58C1 and EHA105 transformed calluses were 33.1–41.3%, which were significantly higher than the regeneration frequency (10.6%) obtained with strain LBA4404 (Table 1). The regeneration frequency of EHA105 transformed calluses was higher than that of C58C1, but not significantly different from that of AGL1. Because calluses obtained with EHA105 had the highest frequency of plantlet/shoot regeneration, EHA105 was used in later experiments involved in root transformation.

After transferring the green plantlets/shoots to rooting medium, roots were developed in about 4 weeks (Fig. 2g) and the plants were ready to be transferred to soil and grown in the growth chambers. Plants were established in soil after 2 weeks of growth in the chambers. The time from transformation to soil-established plants generally took 15–17 weeks (Fig. 2i). It should be noted that while on regeneration medium in the culture dishes, some of the plantlets had already developed good root systems (Fig. 2f); these plantlets could normally survive their direct transfer to soil and thus save 4 weeks of rooting time. It also happened that some shoots failed to

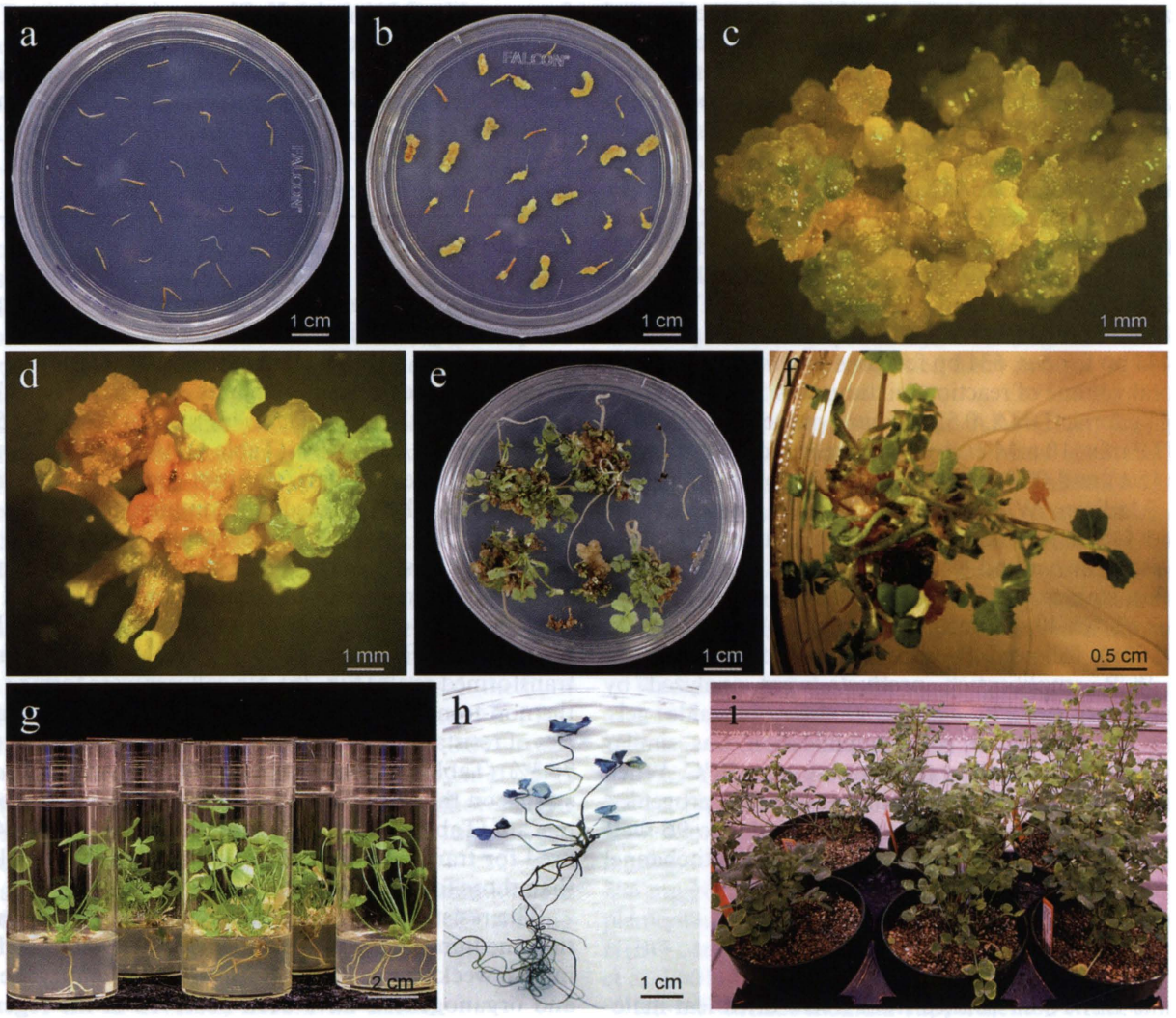

Fig. 2 Transgenic *Medicago truncatula* plants obtained after *A. tumefaciens*-mediated transformation of roots. **a** Root segments after cocultivation with *A. tumefaciens*. **b** Calluses formed from root segments 4 weeks after *A. tumefaciens*-mediated transformation and PPT selection. **c, d** Calluses started to regenerate 2 weeks after transferring them onto regeneration medium. **e, f** Plantlet/shoot regeneration 5 weeks after transferring the root-derived resistant calluses onto regeneration medium. **g** Transgenic *M. truncatula* plants with well developed roots obtained 4 weeks after transferring the plantlets/shoots to the rooting medium. **h** GUS staining of a transgenic plant. **i** Greenhouse-grown transgenic *M. truncatula* plants obtained 16 weeks after *A. tumefaciens*-mediated transformation of roots

Table 1 Effect of *Agrobacterium tumefaciens* strains on root transformation

Agrobacterium tumefaciens strain	Number of root explants plated	Frequency of resistant callus formation (%)[1]	Frequency of plantlet/shoot regeneration (%)[2]
AGL1	1,195	67.6 a	39.2 ab
C58C1	537	65.8 a	33.1 b
EHA105	1,082	69.8 a	41.3 a
LBA4404	610	76.3 a	10.6 c

Values followed by a different letter were significantly different at $P<0.05$

[1] Number of resistant calluses/number of plated explants

[2] Number of plantlets or shoots/number of resistant calluses

produce roots after several weeks on rooting medium; to solve this problem, the lower part of the shoots was dipped into rooting powder and then directly transferred to soil. About 90% of the dipped shoots produced roots and survived in soil.

Even though multiple plantlets/shoots were often produced on a single callus, to make certain that all plants were independent transformants, only one rooted plant from each callus was transferred to soil. Seeds were harvested about 2.5 months after transplanting the plants to the greenhouse.

Effects of different root sections on transformation efficiencies

To test the effects of different portions of roots on callus induction and plant regeneration, explants from the three root sections (S1, S2, S3) were transformed with *A. tumefaciens* and selected on PPT containing media. The callus induc-

tion and regeneration frequencies were similar for the three sections, no significant difference was observed (Table 2).

Molecular analysis of *A. tumefaciens* transformed plants and transgene inheritance in T1 progenies

Polymerase chain reaction screening of the regenerated greenhouse-grown plants revealed in all cases the expected *bar* and *gusA* bands (Fig. 3a), indicating that the selection was effective. Because the *bar* and *gusA* primers were designed to amplify fragments with different lengths, the transgenes (*bar* and *gusA*) were amplified simultaneously (Fig. 3a). This procedure not only saved time and chemicals, but also reduced the chance of obtaining false positives. Southern hybridization analysis was performed using DNA isolated from the T1 plants. As shown in Fig. 3b, when DNA was digested by *Hin*dIII, an enzyme that has only one cleave site in the T-DNA region, hybridization bands of different sizes were observed, demonstrating that the plants were independent transformants and that the transgene was stably integrated into the plant genome.

All the T0 transgenics produced viable seeds. T1 seedlings from six independent T0 lines (confirmed by Southern analysis) were tested for segregation of the *bar* transgene based on PCR analysis (Table 3). Chi-square analysis was performed to determine goodness-of-fit for a 3:1 ratio. The segregation ratios of all the lines had a best fit of 3:1, suggesting that the transgene was integrated as a single locus and inherited to T1 progenies following a Mendelian pattern.

Recovery of transgenic plants from *A. rhizogenes*-transformed hairy roots of *M. truncatula*

One week after inoculation with the low-virulence *A. rhizogenes* strain ARqual, hairy roots began to appear from the cut radicles and continue to grow to form "composite plants" (Fig. 4a). Hairy root segments from the "composite plants" were placed on PPT containing callus induction medium (Fig. 4b). Calluses resistant to PPT were produced from the hairy root segments in 4–5 weeks (Fig. 4c, d). Because not all the hairy roots were co-transformed with the *bar* gene, some of the roots did not form callus under continuous PPT selection (Fig. 4c). Segments from the same root were essentially the same; they either all produced calluses or formed no calluses at all (Fig. 4c). The frequency of callus formation from hairy roots was 71.6%.

Upon transfer to regeneration medium, the resistant calluses produced multiple green plantlets/shoots (Fig. 4e–g). The frequency of plantlet/shoot formation from the hairy roots was 45.1%. Because some of the roots did not form callus, the plantlet/shoot formation frequency was 62.9% if calculated based on the number

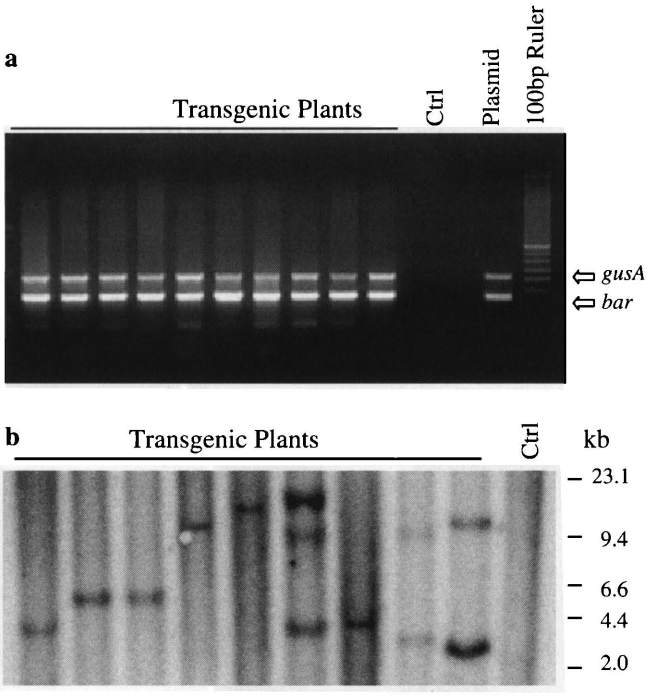

Fig. 3 Molecular analyses of T0 transgenic *M. truncatula* plants obtained from *A. tumefaciens* transformed roots. **a** Polymerase Chain Reaction (*PCR*) screening of DNA samples from greenhouse-grown plants. *Ctrl* Untransformed plants serving as control. *Arrows* indicate the expected *bar* and *gusA* bands. **b** Southern hybridization of DNA blot containing *Hin*dIII digested genomic DNA isolated from greenhouse-grown plants and hybridized with the *bar* probe. *Ctrl* Untransformed plants serving as control

Table 3 Segregation of the transgene in progenies of root-derived transgenic *M. truncatula* plants obtained by *A. tumefaciens* transformation

Transgenic line	Total number of progenies checked by PCR	Number of bar positive progenies	Number of bar negative progenies	Best fit	*P* value
T1670-6	110	84	26	3:1	0.74
T1670-7	24	18	6	3:1	1.00
T1670-9	49	37	12	3:1	0.93
T1670-12	50	41	9	3:1	0.25
T1670-25	38	29	9	3:1	0.85
T1670-27	46	36	10	3:1	0.61

P value was calculated based on chi-square test

Table 2 Effect of different root sections on transformation frequency

Root section	Number of root explants plated	Frequency of resistant callus formation (%)[1]	Frequency of plantlet/shoot regeneration (%)[2]
S1	715	74.3 a	38.0 a
S2	686	73.9 a	32.2 a
S3	642	75.1 a	39.1 a

Values followed by a different letter were significantly different at $P<0.05$

[1] Number of resistant calluses/number of plated explants

[2] Number of plantlets or shoots/number of resistant calluses

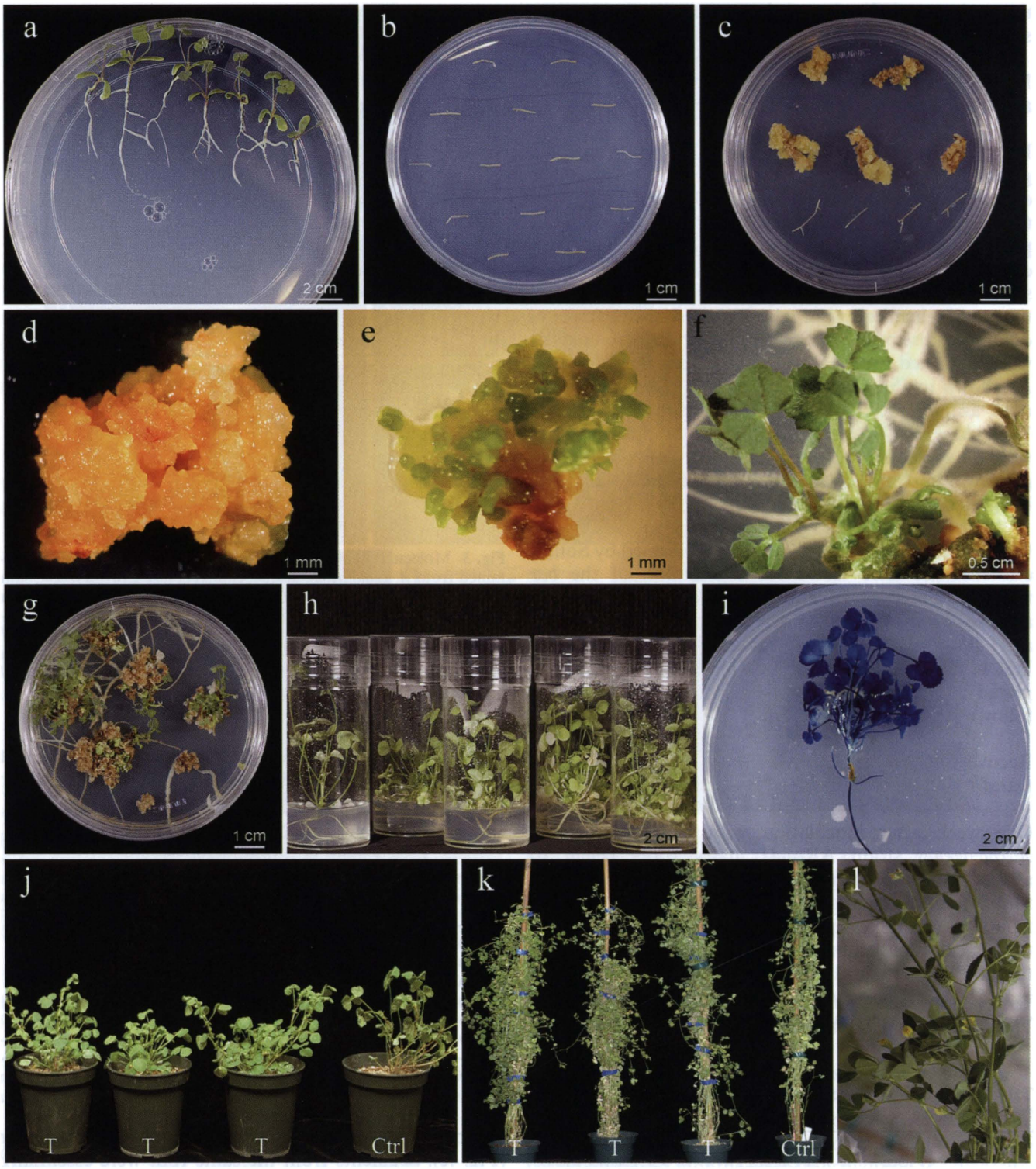

Fig. 4 Transgenic *M. truncatula* plants regenerated from *A. rhizogenes* transformed hairy roots. **a** Composite plants obtained 4 weeks after *A. rhizogenes*-mediated transformation. **b** Hairy root segments plated on callus induction medium containing PPT. **c**, **d** Resistant calluses formed from hairy root segments 5 weeks after placing them onto callus induction medium. **e** Calluses started to regenerate 2 weeks after transferring them onto regeneration mNSedium. **f**, **g** Plant/shoot regeneration 5 weeks after transferring the hairy root-derived resistant calluses onto regeneration medium. **h** Transgenic *M. truncatula* plants with well developed roots obtained 4 weeks after transferring the plantlets/shoots to the rooting medium. i GUS staining of a transgenic plant regenerated from hairy root. **j** **l** Greenhouse-grown transgenic *M. truncatula* plants obtained from *A. rhizo-genes* transformed hairy roots

of callus producing hairy roots (number of plantlet/shoot producing hairy roots divided by the number of callus producing hairy roots). Rooted in vitro plants were obtained after transferring the regenerants onto MSO medium (Fig. 4h), which survived their transfer to soil (Fig. 4j). The greenhouse-grown transgenic plants were slightly bushier than the control plants; however, the difference between transgenic and control plants was not drastic (Fig. 4j, k). No obvious difference in root phenotype was observed between hairy root-derived plants and control plants. Like the control plants, the hairy root-derived plants flowered and produced seeds (Fig. 4k, l).

Molecular analysis of plants derived from *A. rhizogenes* transformed hairy roots and segregation analysis of the *bar* and *rolA* genes in progenies

Polymerase chain reaction analysis of the regenerated plants was performed with primers designed to amplify the *rolA* gene from the Ri T-DNA and the *bar* gene from the binary vector. The PCR results showed the presence of the *bar* and *rol*A bands of the expected sizes in the corresponding transgenic samples and their absence in the negative controls, indicating that all the hairy root-derived plants contained both the *bar* and *rol*A genes (Fig. 5a, b). Southern hybridization analysis further confirmed the stable integration of the *bar* gene in the regenerated T0 plants (Fig. 5c). In contrast to "composite plants" in which only roots are transformed and GUS-stainable, the whole plantlets regenerated from the hairy root-derived calluses stained blue (Fig. 4i).

Progenies of eight hairy root-derived plants were analyzed by PCR to determine the transmission of the *bar* and *rolA* genes. Based on chi-square test for individual genes, most of the plants showed 3:1 segregation for either *bar* or *rolA* (Table 4), indicating single locus integration of the genes. A few plants had 15:1 segregation for *bar* or *rolA* (Table 4), indicating integration for each of the transgene at two locations in the plant chromosome. No segregation ratio of 9:3:3:1 was found when the two genes were analyzed together, suggesting that integration did not occur at independent locations in the chromosome and the genes were linked. Out of the eight transgenic lines tested, one line (R1672-9) showed tight linkage of the two genes, in which no segregation of *bar* or *rolA* was observed (Table 4). Segregation of *bar* and *rolA* was observed in all the other seven lines. In particular, six lines produced plants that were *bar* positive and *rolA* negative (bar+/rolA-, Table 4), demonstrating the possibility of segregating out the hairy root forming gene (*rol*) in the progenies.

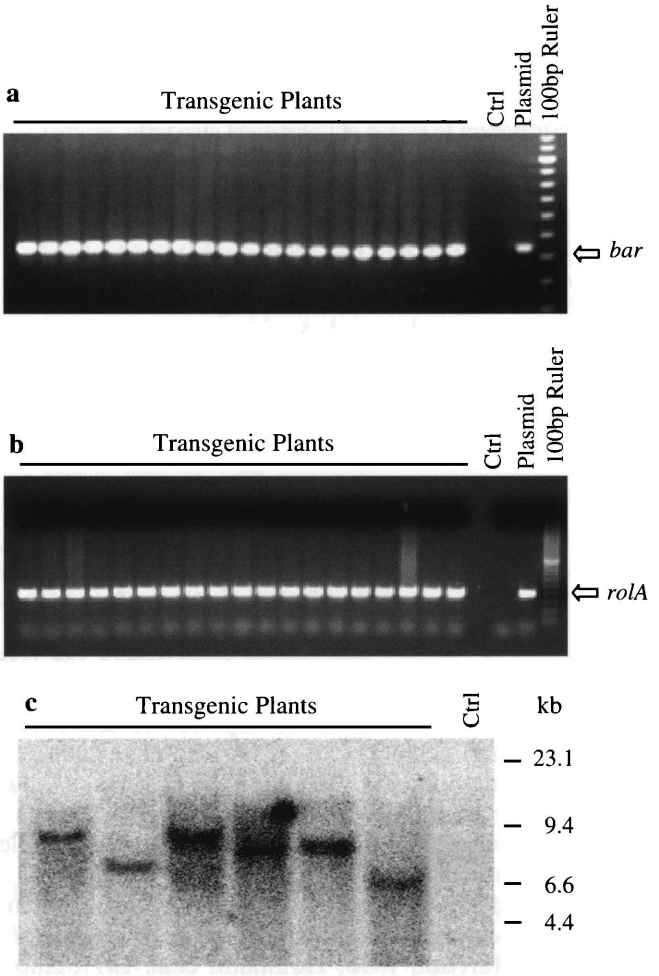

Fig. 5 Molecular analyses of T0 transgenic *M. truncatula* plants obtained from *A. rhizogenes* transformed hairy roots. **a, b** Polymerase chain reaction (*PCR*) screening of DNA samples from greenhouse-grown plants. *Ctrl* untransformed plants serving as control. *Arrows* indicate the expected *bar* and *rolA* bands. **c** Southern hybridization of DNA blot containing *Hin*dIII digested genomic DNA isolated from greenhouse-grown plants and hybridized with the *bar* probe. *Ctrl* untransformed plants serving as control

Discussion

In previous reports, leaf and cotyledon have been generally used as explants for *A. tumefaciens*-mediate transformation of *M. truncatula* (Somers et al. 2003). Here we present a new transformation system using roots as explants for *A. tumefaciens* infection. Most of the root segments could be transformed leading to the formation of resistant calluses, and 41% of the resistant calluses produced plantlets/shoots. Such transformation efficiency is sufficient for most transgene expression studies. Greenhouse-grown transgenic plants were obtained in 4 months and the transgene was inherited following a Mendelian pattern to the T1 generation. The efficiency and the time required to produce transgenics in our root transformation system are very comparable to that of leaf transformation (Hoffmann et al. 1997; Chabaud et al. 2003; de Sousa Araújo et al. 2004). Practically, the use of root as explant is more convenient than using leaf, because two to three times more explants can be obtained from the same in vitro donor plants. It is even more time saving if both root and leaf from the same donor plant are used as explants for transformation; this

Table 4 Segregation of the transgenes *bar* and *rolA* in progenies of hairy root-derived transgenic *M. truncatula* plants obtained by *A. rhizogenes* transformation

Transgenic line	Total number of progenies checked by PCR	bar +/ rolA +	bar+/ rolA −	bar −/ rolA +	bar −/ rolA −	Best fit for *bar* (*P* value)	Best fit for *rolA* (*P* value)
R1672-4	74	65	2	3	4	15:1 (0.25)	15:1 (0.51)
R1672-7	47	33	1	13	0	3:1 (0.67)	15:1 (0.24)
R1672-8	57	41	4	5	7	3:1 (0.19)	3:1 (0.32)
R1672-9	61	49	0	0	12	3:1 (0.34)	3:1 (0.34)
R1672-10	64	40	1	2	21	3:1 (0.04)	3:1 (0.08)
R1672-11	48	37	2	1	8	3:1 (0.50)	3:1 (0.50)
R1672-12	73	65	3	0	5	15:1 (0.83)	15:1 (0.10)
R1672-15	64	43	0	1	20	3:1 (0.11)	3:1(0.24)

P value was calculated based on chi-square test

has become a common practice in our laboratory. In addition, root transformation offers the opportunity to produce transgenic plants using root-specific promoters for selectable and root-related traits.

Different *A. tumefaciens* strains were tested for leaf transformation of *M. truncatula* cv. Jemalong (genotype 2HA), AGL1 was found to be superior to LBA4404, C58pMP90, and C58pGV2260 (Chabaud et al. 2003). In root transformation, EHA105 gave the best results, but the difference is not significant between EHA 105 and AGL1. Therefore, both EHA 105 and AGL1 are suitable for root transformation.

Rooting of the regenerated shoots has been a problem for leaf and cotyledon transformation in *M. truncatula* (Trieu and Harrison 1996; Hoffmann et al. 1997; Zhou et al. 2004). For shoots that do not root in a month, a common practice is to cut the end and transfer the shoots to fresh medium. However, we had previously observed that some cotyledon-derived shoots failed to root after more than 8 months with routine transfer on rooting medium. Rooting of root- or hairy root-derived transformants tends to be less of a problem than cotyledon- or leaf-derived transformants. To solve the rooting problem, we discovered that by simply dipping the non-rooting shoots into rooting powder and transferring them to soil, the shoots could produce roots in soil and grow normally thereafter. The method was even effective for rooting of some long-term cultured shoots. This is a simple yet important tip for obtaining greenhouse-grown transgenic plants.

To date, transgenic *M. truncatula* plants have only been routinely obtained through *A. tumefaciens*-mediated transformation. By inducing callus from the hairy roots and subsequent differentiation, we successfully developed a plant regeneration system from *A. rhizogenes* transformed hairy roots of *M. truncatula*. True transgenic plants could be easily established in the greenhouse, thus allowing the study of rhizosphere interactions to be carried out under soil conditions and the effects of the transgenes be evaluated at the whole plant level. Seeds harvested from the transgenic plants could be easily stored allowing for future studies to be carried out either in vitro or in soil. This is the first report on regeneration of fertile plants from *A. rhizogenes* transformed hairy roots of *M. truncatula*.

Transformed hairy roots have been widely used for the identification and characterization of genes related to nodulation (Ane et al. 2004; Bersoult et al. 2005; Frendo et al. 2005; Kaló et al. 2005; Limpens et al. 2005; Vieweg et al. 2005) and mycorrhizal symbiosis (Ane et al. 2004; Karandashov et al. 2004; Elfstrand et al. 2005; Isayenkov et al. 2005; Maldonado-Mendoza et al. 2005; Vieweg et al. 2005). The system has also been used to identify genes involved in root development (Ivashuta et al. 2005) and tannin biosynthesis (Sharma and Dixon 2005) and to characterize promoters (Rodríguez-Llorente et al. 2003; Boisson-Dernier et al. 2005; Xiao et al. 2006). If coupled with the regeneration system reported here, once potential genes of interest are identified, the transformed hairy roots carrying such genes could be directly regenerated into plants for more detailed functional analysis of genes. Furthermore, segregation analysis revealed that it is possible to segregate out the *rolA* gene in the progenies, thus allowing characterization of the genes of interest without the presence of hairy root forming genes.

In summary, we have developed a new and convenient *A. tumefaciens* transformation system for *M. truncatula* using roots as explants. Based on the root regeneration protocol, we further developed a plant regeneration system from transformed hairy roots. This technique offers the possibility to regenerate fertile transgenic plants following the screening of transgenic roots of interest obtained by rapid and easy *A. rhizogenes* transformation. These novel transformation and plant regeneration systems provide powerful tools for functional testing of genes in this important model legume species.

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References

Ane J-M, Kiss GB, Riely BK, Penmetsa RV, Oldroyd GED, Ayax C, Levy J, Debelle F, Baek J-M, Kalo P, Rosenberg C, Roe BA, Long SR, Denarie J, Cook DR (2004) *Medicago truncatula* DMI1 required for bacterial and fungal Symbioses in legumes. Science 303:1364–1367

Barker DG, Bianchi S, Blondon F, Dattée Y, Duc G, Essad S, Flament P, Gallusci P, Génier G, Guy P, Muel X, Tourneur J, Dénarié J, Huguet T (1990) *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*-legume symbiosis. Plant Mol Biol Rep 8:40–49

Bell CJ, Dixon RA, Farmer AD, Flores R, Inman J, Gonzales RA, Harrison MJ, Paiva NL, Scott AD, Weller JW, May GD (2001) The Medicago genome initiative: a model legume database. Nucleic Acids Res 29:114–117

Bersoult A, Camut S, Perhald A, Kereszt A, Kiss GB, Cullimore JV (2005) Expression of the *Medicago truncatula DMI2* gene suggests roles of the symbiotic nodulation receptor kinase in nodules and during early nodule development. Mol Plant Microbe Interact 18:869–876

de Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gosselé V, Movva NR, Thompson C, van Montagu M, Leemans J (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J 6:2513–2518

Boisson-Dernier A, Chabaud M, Garcia F, Becard G, Rosenberg C, Barker DG (2001) *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Mol Plant Microbe Interact 14:695–700

Boisson-Dernier A, Andriankaja A, Chabaud M, Niebel A, Journet EP, Barker DG, Carvalho-Niebel Fd (2005) *MtENOD11* gene activation during rhizobial infection and mycorrhizal arbuscule development requires a common AT-rich-containing regulatory sequence. Mol Plant Microbe Interact 18:1269–1276

Chabaud M, Carvalho-Niebel Fd, Barker DG (2003) Efficient transformation of *Medicago truncatula* cv. Jemalong using the hypervirulent *Agrobacterium tumefaciens* strain AGL1. Plant Cell Rep 22:46–51

Chen H, Nelson RS, Sherwood JL (1994) Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotechniques 16:664–670

Cook DR (1999) *Medicago truncatula*—a model in the making! Curr Opin Plant Biol 2:301–304

Deblaere R, Bytebier 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

Elfstrand M, Feddermann N, Ineichen K, Nagaraj VJ, Wiemken A, Boller T, Salzer P (2005) Ectopic expression of the mycorrhiza-specific chitinase gene *Mtchit 3-3* in *Medicago truncatula* root-organ cultures stimulates spore germination of glomalean fungi. New Phytol 167:557–570

Frendo P, Harrison J, Norman C, Jiménez MJH, van de Sype G, Gilabert A, Puppo A (2005) Glutathione and homoglutathione play a critical role in the nodulation process of *Medicago truncatula*. Mol Plant Microbe Interact 18:254–259

Graham PH, Vance CP (2003) Legumes: importance and constraints to greater use. Plant Physiol 131:872–877

Harrison MJ, Dixon RA (1993) Isoflavonoid accumulation and expression of defense gene transcripts during the establishment of vesicular-arbuscular mycorrhizal associations in roots of *Medicago truncatula*. Mol Plant Microbe Interact 6:643–654

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–180

Hoffmann B, Trinh TH, Leung J, Kondorosi A, Kondorosi E (1997) A new *Medicago truncatula* line with superior in vitro regeneration, transformation, and symbiotic properties isolated through cell culture selection. Mol Plant Microbe Interact 10:307–315

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208–218

Isayenkov S, Mrosk C, Stenzel I, Strack D, Hause B (2005) Suppression of allene oxide cyclase in hairy roots of *Medicago truncatula* reduces jasmonate levels and the degree of mycorrhization with *Glomus intraradices*. Plant Physiol 139:1401–1410

Ivashuta S, Liu J, Liu J, Lohar DP, Haridas S, Bucciarelli B, VandenBosch KA, Vance CP, Harrison MJ, Gantt JS (2005) RNA interference identifies a calcium-dependent protein kinase involved in *Medicago truncatula* root development. Plant Cell 17:2911–2921

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

Kaló P, Gleason C, Edwards A, Marsh J, Mitra RM, Hirsch S, Jakab J, Sims S, Long SR, Rogers J, Kiss GB, Downie JA, Oldroyd GED (2005) Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. Science 308:1786–1789

Karandashov V, Nagy R, Wegmüller S, Amrhein N, Bucher M (2004) Evolutionary conservation of a phosphate transporter in the arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci USA 101:6285–6290

Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation-competent Arabidopsis genomic library in *Agrobacterium*. Bio/technology 9:963–967

Lichtenstein C, Draper J (1985) Genetic engineering of plants. In: Glover DM (eds) DNA cloning. IRL Press, Oxford, pp 67–119

Limpens E, Franken C, Smit P, Willemse J, Bisseling T, Geurts R (2003) LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. Science 302:630–633

Limpens E, Ramos J, Franken C, Raz V, Compaan B, Franssen H, Bisseling T, Geurts R (2004) RNA interference in *Agrobacterium rhizogenes*-transformed roots of Arabidopsis and *Medicago truncatula*. J Exp Bot 55:983–992

Limpens E, Mirabella R, Fedorova E, Franken C, Franssen H, Bisseling T, Geurts R (2005) Formation of organelle-like N2-fixing symbiosomes in legume root nodules is controlled by *DMI2*. Proc Natl Acad Sci USA 102:10375–10380

Maldonado-Mendoza IE, Dewbre GR, Blaylock L, Harrison MJ (2005) Expression of a xyloglucan endotransglucosylase/hydrolase gene, *Mt-XTH1*, from *Medicago truncatula* is induced systemically in mycorrhizal roots. Gene 345:191–197

Mendel RR, Mueller B, Schulze J, Kolesnikov V, Zelenin A (1989) Delivery of foreign genes to intact barley cells by high-velocity microprojectiles. Theor Appl Genet 78:31–34

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Quandt HJ, Pühler A, Broer I (1993) Transgenic root nodules of *Vicia hirsuta*: a fast and efficient system for the study of gene expression in indeterminate-type nodules. Mol Plant Microbe Interact 6:699–706

Rodríguez-Llorente ID, Pérez-Hormaeche J, Dary M, Caviedes MA, Kondorosi A, Ratet P, Palomares AJ (2003) Expression of MsPG3-GFP fusions in *Medicago truncatula* 'hairy roots' reveals preferential tip localization of the protein in root hairs. Eur J Biochem 270:261–269

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York

Scholte M, d'Erfurth I, Rippa S, Mondy S, Cosson V, Durand P, Breda C, Trinh H, Rodriguez-Llorente I, Kondorosi E, Schultze M, Kondorosi A, Ratet P (2002) T-DNA tagging in the model legume *Medicago truncatula* allows efficient gene discovery. Mol Breed 10:203–215

Sharma SB, Dixon RA (2005) Metabolic engineering of proanthocyanidins by ectopic expression of transcription factors in Arabidopsis thaliana. Plant J 44:62–75

Somers DA, Samac DA, Olhoft PM (2003) Recent advances in legume transformation. Plant Physiol 131:892–899

de Sousa Araújo S, Duque ASRLA, dos Santos DMMF, Fevereiro MPS (2004) An efficient transformation method to regenerate

a high number of transgenic plants using a new embryogenic line of *Medicago truncatula* cv. Plant Cell Tissue Organ Cult 78:123–131

Trieu AT, Harrison MJ (1996) Rapid transformation of *Medicago truncatula*: regeneration via shoot organogenesis. Plant Cell Rep 16:6–11

Trinh TH, Ratet P, Kondorosi E, Durand P, Kamate K, Bauer P, Kondorosi A (1998) Rapid and efficient transformation of diploid *Medicago truncatula* and *Medicago sativa* ssp falcata lines improved in somatic embryogenesis. Plant Cell Rep 17:345–355

Vieweg MF, Hohnjec N, Küster H (2005) Two genes encoding different truncated hemoglobins are regulated during root nodule and arbuscular mycorrhiza symbioses of *Medicago truncatula*. Planta 220:757–766

Vinardell JM, Fedorova E, Cebolla A, Kevei Z, Horvath G, Kelemen Z, Tarayre S, Roudier F, Mergaert P, Kondorosi A, Kondorosi E (2003) Endoreduplication mediated by the anaphase-promoting complex activator CCS52A is required for symbiotic cell differentiation in *Medicago truncatula* nodules. Plant Cell 15:2093–2105

Xiao K, Liu J, Dewbre G, Harrison M, Wang Z-Y (2006) Isolation and characterization of root-specific phosphate transporter promoters from *Medicago truncatula*. Plant Biol (in press)

Xiao K, Zhang C, Harrison M, Wang Z-Y (2005) Isolation and characterization of a novel plant promoter that directs strong constitutive expression of transgenes in plants. Mol Breed 15:221–231

Young ND, Cannon SB, Sato S, Kim DJ, Cook DR, Town CD, Roe BA, Tabata S (2005) Sequencing the genespaces of *Medicago truncatula* and *Lotus japonicus*. Plant Physiol 137:1174–1181

Zhou X, Chandrasekharan MB, Hall TC (2004) High rooting fr sequency and functional analysis of GUS and GFP expression in transgenic *Medicago truncatula* A17. New Phytol 162:813–822