Methods

High rooting frequency and functional analysis of GUS and GFP expression in transgenic *Medicago truncatula* A17

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Summary

- An effective transformation method is described for *Medicago truncatula* A17, verifying its suitability as a model legume for functional genomics.
- Media and culture methods are detailed that yielded an average frequency of 35% for recovery of transgenic shoots from cotyledonary node explants and 39% for root induction and regeneration of entire plants from 419 phosphinothricin-resistant shoots.
- Fertile plants transgenic for both *35S-GFP* and *phas-GUS* were obtained in five of eight independent experiments. The presence and stable inheritance of transgenes was confirmed by GFP or GUS expression and by genomic DNA blots. GFP expression driven by the normally constitutive CaMV *35S* promoter diminished as the leaves matured. Although GUS was very strongly and uniformly expressed in seed cotyledons of most lines, one line exhibited an aberrant, patchy pattern. Additionally, weak GUS expression was evident in leaf veins from the normally stringently spatially regulated *phas* promoter.
- Stably transformed, fertile, *M. truncatula* A17 plants were generated. The unconventional expression patterns for *35S-GFP* and *phas-GUS* expression obtained in some transformants suggest the occurrence of novel epigenetic events.

Key words: *Medicago*, legume, transformation, rooting, 35S, phaseolin.

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Introduction

With over 650 genera and 18 000 species, the family Leguminoseae is the third largest family of higher plants and is second only to grasses in agricultural importance (Young *et al.*, 2003). Legumes are major sources of protein and oil for humans and livestock. Some legumes have beneficial medicinal qualities; others can be highly allergenic. While *Arabidopsis* is proving to be an excellent organism for gene discovery and functional genomics, it does not have the ability to establish symbiotic interactions with rhizobia and mycorrhizae exhibited by legumes such as *Lotus japonicus* and *Medicago truncatula.* The lack of symbiosis in *Arabidopsis* justifies the development of a legume model. In Europe, *Lotus japonicus* has been established as a valuable legume system for gene discovery. Effective systems for *Agrobacterium*-mediated transformation have been established (Handberg & Stougaard, 1992; Stiller *et al.*, 1997), enabling the use of transposon (Thykjaer *et al.*, 1995) and T-DNA (Martirani *et al.*, 1999) tagging as tools to identify novel plant genes.

M. truncatula is also emerging as a model legume system for future gene discovery. Attractive features are its small, diploid genome, self-fertility and short generation time. Rapid progress with sequencing its *c.* 500 Mbp genome and in comparative genomics is underway (Lamblin *et al.*, 2003). However, a facile system for molecular transformation is of

great importance for functional genomics. Tissue-culture-based transformation methods have been reported for four genotypes of *M. truncatula*: R108-1 (Hoffmann *et al.*, 1997; Trinh *et al.*, 1998; Scholte *et al.*, 2002), Jemalong 2HA (Thomas *et al.*, 1992; Chabaud *et al.*, 2003), Jemalong J5 (Kamaté *et al.*, 2000) and Jemalong A17 (Chabaud *et al.*, 1996; Trieu & Harrison, 1996). Among these, Jemalong A17 is of special interest because the genome of this single-seed descent line was selected for sequencing. Considerable excitement was engendered by the development of two very efficient *in planta* transformation methods, one utilizing the inflorescence and the other seedling infiltration by a medium containing *Agrobacterium* (Trieu *et al.*, 2000). These approaches were of special interest as they did not require tissue culture and could potentially be used for T-DNA insertion analysis.

Unfortunately, the *in planta* methods have not been reproduced (Somers *et al.*, 2003) and difficulty has been experienced with *M. truncatula* A17 in effectively establishing previously described tissue culture-based transformation systems (Chabaud *et al.*, 1996; Trieu & Harrison, 1996). Therefore, great need exists for a reproducible and convenient transformation method for this line. Here, we describe in detail an effective method for transformation and regeneration of *M. truncatula* A17 based on shoot organogenesis from the cotyledonary node. It has thus far been successful in producing transgenic shoots from each of eight completely independent experiments, five of which resulted in stable fertile transformants that express both *GUS* and *GFP* transgenes through the second (T_1), and in the four lines tested, the T_2 generation. Recent improvements to the regeneration protocol have yielded rooting rates approaching 50%.

Functional analysis is described for transgenic plants bearing two reporter gene constructs. In one, the *GFP* reporter is under control of the CaMV *35S* promoter, which is often regarded as constitutive (Benfey *et al.*, 1989; Battraw & Hall, 1990). In the other, the *GUS* reporter is driven by the *phas* promoter, which is under stringent spatial regulation (van der Geest *et al.*, 1995; Li *et al.*, 2001). Genomic blot analysis showed that both single copy and multiple copy plants were recovered.

Materials and Methods

Plant material

Medicago truncatula cv. Jemalong (line A17) was used for all experiments. Individual wild-type plants were grown in Redi-Earth (Scotts) in 4′ pots under glasshouse conditions of 14 h day length (23°C day, 20°C night). Plants were watered daily and once weekly with Miracle-Gro® nutrient at the concentration recommended by the manufacturer. Primary transgenic plants were grown under similar conditions in a growth chamber.

Preparation of the *Agrobacterium* suspension medium pCB302-*phas-GUS* (Fig. 1a) was electrophoretically transformed into *Agrobacterium tumefaciens* strain EHA105 or AGL1; the bacterial cells were selected on 100 mg/l kanamycin and a single resistant colony was streaked on solid YEP medium (10 g l^{-1} Bacto-peptone, 10 g l^{-1} yeast extract, 5 g l^{-1} NaCl) containing 100 mg l^{-1} kanamycin and incubated at 28°C for 2 d. After restriction digestion confirmation of the presence of the desired gene construct, liquid YEP medium (20 ml) containing 100 mg l^{-1} l kanamycin was inoculated with a single colony of *Agrobacterium* and cultured overnight at 28°C, with shaking at 250 r.p.m., to an OD600 of 1.0–2.0. The cells were then pelleted by centrifugation at 4000 × ***g*** for 10 min and resuspended to a final OD600 of 0.8 in inoculation medium, pH 5.5, consisting of SH salts and vitamins (Sigma), 20 g l^{-1} sucrose, 3 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, 100:M acetosyringone, 400 mg l^{-1} L-cysteine (Sigma), 1 mM DTT (Sigma) and 3 mM MES.

Preparation of cotyledonary explants and infection with *Agrobacterium*

Procedures for permeabilization and surface-sterilization of *M. truncatula* seeds were modified from Trieu *et al.* (2000) and the preparation of cotyledonary node explants was altered from Trieu & Harrison (1996). Mature seeds were exposed to conc. H_2SO_4 for 8 min, rinsed three times with sterile distilled water, then sterilized in 33% Chlorox® (2% NaOCl) for 10 min with gentle agitation. The seeds were thoroughly rinsed, immersed in sterile distilled water (*c.* 2 h) and arranged on 25 × 100 mm Petri dishes (15 per dish) containing a germination medium of SH salts vitamins, 20 g l^{-1} sucrose, 1 mg l^{-1} BAP in 0.8% agar (Sigma), pH 5.8. After incubation for 4 d at 25°C under fluorescent lights (16/8 h light/dark), the radicle and most of the hypocotyl were removed from the emerging seedlings by transection 1–2 mm below the cotyledonary node. The remaining tissues were bisected so that each explant had one cotyledon and half of the embryonic axis.

The explants were inoculated by immersion in the *Agrobacterium* inoculation medium described above, with gentle shaking for 30 min, then briefly blotted on sterile filter paper to remove excess *Agrobacteria*. They were then placed adaxial side face-up on cocultivation medium (inoculation medium solidified with 0.8% agar) and incubated at 25°C for 5 d with a 16/8 h (light/dark) photoperiod.

Plant regeneration and selection

Co-cultivated explants were washed twice by shaking in sterile distilled water at 100 r.p.m. (10 min each time), blotted dry on sterile filter paper and placed on 25 × 100 mm Petri dishes (*c.* 10 explants per dish) containing a regeneration medium consisting of SH salts and vitamins, 20 g l^{-1} sucrose, 3 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, 10 mg l^{-1} $AgNO_3$, 3 mM MES, 100 mg l^{-1} Claforan® (cefotaxime sodium: Hoechst-Roussel, Somerville, NJ), 500 mg l^{-1} Timentin® (ticarcillin disodium and clauvulanate potassium: GlaxoSmithKline, Research Triangle

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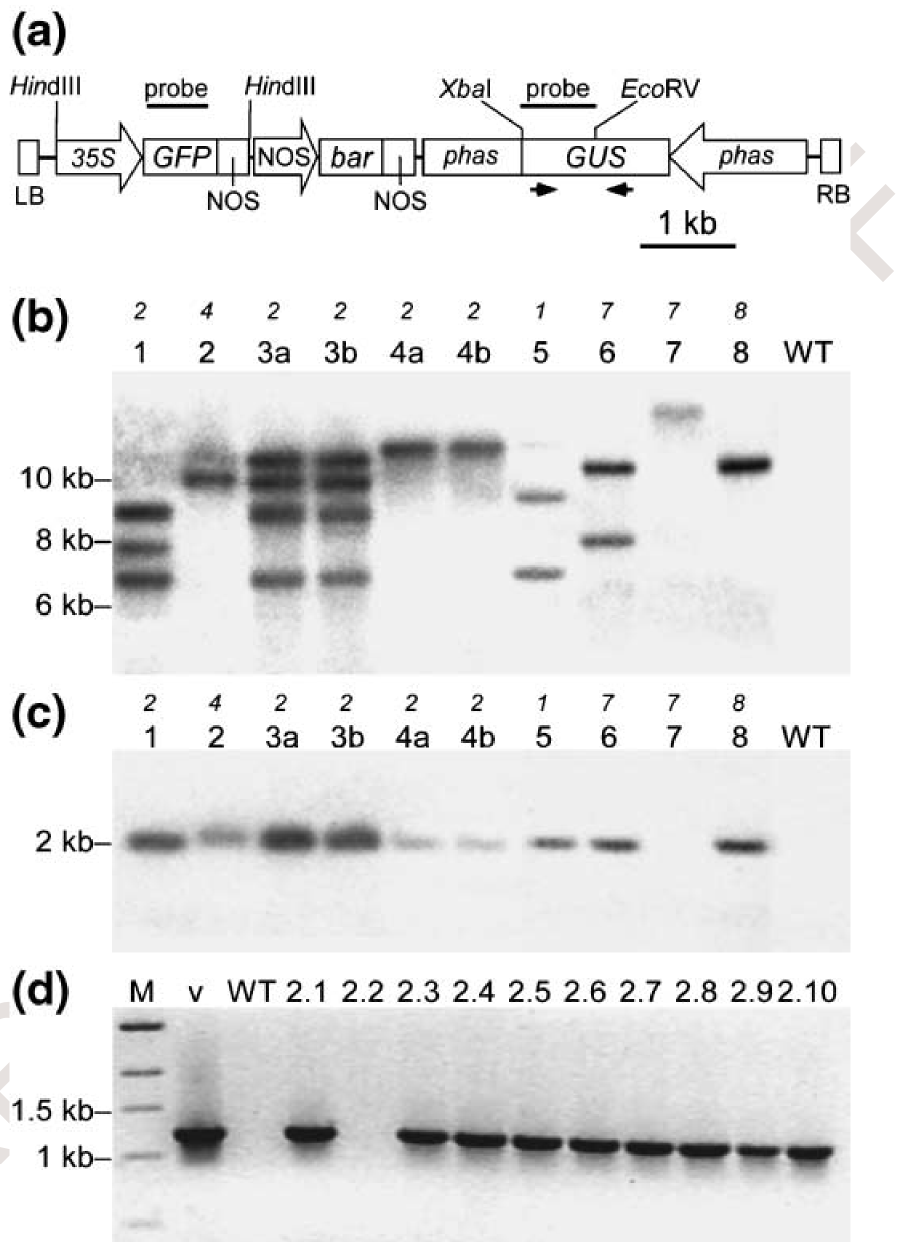

Fig. 1 Transgene construct and insertion patterns. (a) Diagram of the T-DNA region of pCB302-*phas-GUS*. The locations of the *GFP* and *GUS* probes are indicated by *lines* and primer positions by *arrows*. The *Hin*dIII sites used for the genomic blots are indicated, as are the *Xba*I and *Eco*RV sites used to obtain the *GUS* probe. (b) Genomic blot of leaf DNA from 10 T_0 plants probed for *GUS*. Plants 3a, 3b are siblings, as are 4a, 4b. WT, wild-type *Medicago truncatula* A17. The italic numerals above the lanes correspond to the experiment numbers in Table 1; the bold Roman numerals correspond to the plant line numbers. (c) The blot was stripped and re-probed for *GFP*. (d) PCR confirmation of the *GUS* insert in 10 T1 progeny of line 2 (lanes 2.1–2.10). v, binary vector pCB302-*phas-GUS*. M, 1 kb marker ladder.

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Park, NC) in 0.8% agar, pH 5.8. The explants were incubated for 15 d under the same conditions as those used for cocultivation and then transferred to selection medium (regeneration medium supplemented with 1.6 mg l^{-1} phosphinothricin (PPT) from Duchefa Biochemie, Netherlands) and transferred to fresh medium every other week. Untransformed shoots started to die after 1 wk and were removed at the time of transfer.

Induction of adventitious roots

Well developed resistant shoots (*c.* 1.5 cm) were separated from explants with a surgical blade and laid with the cut end exposed on the surface of a plant development and rooting medium consisting of SH salts and vitamins, 10 g l^{-1} sucrose, 0.5 mg l^{-1} IBA (Sigma), 100 mg l^{-1} Claforan, 300 mg l^{-1} Timentin and 0.25% Phytagel (Sigma), pH 5.8, in Phytatrays (Sigma), with 3–4 shoots per Phytatray. If no roots were induced within 1 month, the ends of the shoots were cut with a surgical blade and transferred to fresh medium. The plantlets with well-developed roots were transplanted into soil and cultured (25°C, 16/8 h photoperiod) in a growth chamber, initially with high humidity by covering with plastic bags. The plants usually started to flower after one month.

Genomic DNA blot analysis of transgenic plants

Total genomic DNA from transgenic plants was extracted using cetyltrimethylammonium bromide (CTAB) (Woodhead *et al*., 1998), digested with *Hin*dIII, loaded (10 g per lane) onto an agarose gel (1%) and, after electrophoretic separation, blotted onto Hybond-N + membrane (Amersham). The *Eco*RV-*Xba*I fragment of the T-DNA construct containing the *GUS* coding sequence was used as a *GUS* probe (Fig. 1a). A DNA fragment PCR amplified from the binary vector pCB302-*phas-GUS* with the primer pair 5′-CACTGGAGTTGT-CCCAATTCTTG-3′ and 5′-GTCTGGTAAAAGGACA-GGGC-3′ was used as a *GFP* probe (Fig. 1a). Hybridization was carried out in ULTRAhyb ultrasensitive hybridization solution (Ambion) for 14 h at 42°C. The hybridization results were recorded using a Fuji BAS 2000 phosphorimager system.

PCR analysis of *GUS* transgenic plant

Total genomic DNA was used as the template for PCR analysis with the primer pair 5′-GGTGGGAAAGCGCGTTACAAG-3′ and 5′-GTTTACGCGTTGCTTCCGCCA-3′ and 30 cycles of amplification (94°C for 1 min, 60°C for 1 min and 72°C for 2 min).

Detection of GFP expression in transgenic plants

The expression profile for intact transgenic plants was observed using a LT9700 Little Luma excitation light (Lightools, Encinitas, CA, USA) with 470 nm excitation and 500 nm emission wavelengths. Precise localization of GFP expression in transgenic plants was undertaken using a Zeiss Stemi SV11 microscope with a Zeiss AttoArc 2 light source. Excitation and emission wavelengths were 470 nm and 500 nm, respectively. Expression patterns were recorded using a Zeiss AxioCam HRc.

Detection of *GFP* transcript using RT-PCR

Total RNA was extracted from young and mature leaves of *M. truncatula* plants transgenic for *35S-GFP* using TRIzol reagent (Invitrogen) and treated with DNaseI (Invitrogen, 1 unit : 1 Φl) at 25EC for 15 min to remove DNA and then heated at 65EC for 10 min in the presence of 2.5 mM EDTA to inactivate DNase. RT-PCR reactions were performed using a OneStep-RT-PCR kit (Qiagen) according to the manufacturer's recommendations. Reverse transcription was performed at 50EC for 30 min, followed by PCR activation at 90°C for 15 min and then 25 cycles of PCR amplification (94°C for 1 min, 52°C for 1 min and 72°C for 1 min) using primer pair 5′-CACTGGAGTTGTCCCAATTCTTG-3′ and 5′-GTCTGGTAAAAGGACAGGGC-3′ for *GFP* transgene, and primer pair 5′-CTCTCCGCCACCGATTCATC-3′ and 5′-CCATCTCTCTCACTCACTGCATAC-3′ for *M. truncatula EF-I′*.

Histochemical localization of GUS activity

Embryos and leaves from transgenic plants were stained for GUS activity as described (Jefferson *et al*., 1987). Tissue samples were immersed in the GUS staining solution, vacuum infiltrated for 5 min to facilitate penetration, and then incubated at 37°C in the dark. The stained samples were then rinsed with distilled water and immersed in 95% ethanol to remove chlorophyll.

Results

Agrobacterium-mediated transformation of *M. truncatula*

Eight independent transformation experiments were performed using CaMV *35S-GFP* and *phas-GUS* constructs (Fig. 1a) inserted into binary vector pCB302 (Xiang *et al*., 1999) in *Agrobacterium* strain EHA105 (Hood *et al*., 1993) for experiments 1–3, and in strain AGL1 (Lazo *et al*., 1991) for experiments 4–8 (Table 1). In these experiments, 100–150 explants were inoculated with *Agrobacterium* suspension. Phosphinothricin (PPT)-resistant shoots were induced in 3% to 15% of the explants, with an average of 6% of the explants treated. More than one resistant shoot was often induced from a single explant; the ratios of resistant shoots to inoculated explants ranged from 3% to 174%, with an average of 35%. The clear distinction between transgenic, PPT-resistant explants with green leaves and nontransformed with browning leaves on selection medium is shown in Fig. 2(a). The difficulty in inducing roots in initial experiments using the protocol of Trieu & Harrison (1996) is evident from the data shown for experiments 3, 5 and 6 (Table 1) in which no rooted plants were recovered. However, using the improved rooting procedure (see experimental procedures for details), considerably higher proportions of the regenerating resistant shoots form roots, as seen for experiments 1 (27%) and 8 (45%). Efficient induction of roots was very dependent on both the composition of the medium and the positioning of the cut shoot, as shown in Fig. 2(b).

In total, on the basis of GFP expression, more than 30 transgenic plants have been obtained from five independent experiments. To confirm the integration of T-DNA into the genomes of these plants, genomic DNA was extracted from 10 of the T_0 plants derived from five separate experiments (experiments 1, 2, 4, 7 and 8) and subjected to hybridization analysis using the *GUS* and *GFP* probes indicated in Fig. 1(a). The results showed that, except for line 7, the T_0 plants regenerated from PPT-resistant shoots carry at least one copy of each reporter (Fig. 1b,c). Of the 10 lines tested, eight were shown to be independent. Lines 3a, 3b and 4a, 4b were siblings, reflecting the recovery of multiple PPT-resistant shoots from a single transformation event. The hybridization fragments for *GUS* are all larger than 6 kb, as expected from the

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Table 1 Efficacy of transgenic rooted shoot recovery from cotyledonary node explants of *Medicago truncatula*

	Explants			Resistant shoots			
Expt	Inoculated	Resistant[1]	Resistant (%)	Regenerated[2]	Per inoculated explant (%)	Rooted	Rooted (%)
1	140	11	8	41	29	11	27
2	150	23	15	261	174	103	39
3	150	5	3	12	8	0	0
4	150	9	6	53	35	21	40
5	150	4	3	15	10	0	0
6	100	3	3	3	3	0	0
7	140	6	4	24	17	10	42
8	100	7	7	40	7	18	45

[1]No. of inoculated explants from which shoots resistant to 1.6 mg l^{-1} phosphinothricin (PPT) were recovered. [2]No. of PPT-resistant shoots recovered from the resistant explants.

5.8 kb distance from the *Hin*dIII site between the *nos* terminator and *nos* promoter and the right T-DNA border (Fig. 1a). The 2.0 kb *GFP* hybridization fragments reflect the distance between the two *Hin*dIII sites flanking the *35S-GFP* gene. The hybridization patterns are consistent with the presence of one copy of the transgene insert in lines 4 and 8, two copies in lines 5 and 6, three copies in line 1 and four copies in line 3. Line 2 appears to have one intact copy and a second, partial copy.

The inheritance of *GUS* in the T_1 generation of line 2 was confirmed by PCR amplification. A fragment with the predicted length of 1.2 kb was amplified from nine out of 10 T_1 plants (Fig. 1d). For lines 1, 2, 3 and 4, the presence of transgenes in T_1 and T_2 progeny was confirmed by the expression of GFP and GUS.

Diminution of *35S*-driven GFP expression in mature tissues

Expression of GFP has proven a useful visual screenable marker for plant transformation (Haseloff *et al.*, 1997). To assess its value in *Agrobacterium*-mediated transformation of *M. truncatula* A17, a *35S-GFP* fusion construct (Fig. 1a) was used. Strong green fluorescence was detected at some cut surfaces of inoculated explants 2 d after cocultivation and also in newly regenerating shoots from the cotyledonary node regions of the explants (Fig. 3a,b), permitting early identification of transformed tissues. Strong expression was also seen in young (*c.* 1 d old) leaves of transgenic lines 1 (Fig. 3c) and 2; weaker expression was evident for lines 3–6 and 8. However, GFP expression diminished in all lines as the leaves aged, as seen for the 5-d-old trifoliate leaf of line 1 in Fig. 3(d) (although fluorescence at the leaf margin sometimes remained, as shown in the inset). Interestingly, as the shoots elongated, newly emerging leaves showed high GFP expression; again, this diminished as the leaves aged. The possibility that decreased expression resulted from gene silencing was evaluated by analysis of RNA transcript level using RT-PCR. In fact, similar transcript levels were detected in extracts of young and old leaves (Fig. 4), indicating that silencing was not the primary cause of decreased GFP expression.

Petals showed high levels of expression, but expression in the calyx was low (Fig. 3e). Evaluation of GFP expression in seed tissues was difficult because high levels of fluorescence were present in untransformed plants (not shown). The eight lines examined in detail were fertile although line 1 produced only a few pods. The germination rate for line 1 (*c.* 30%) was also lower than that of the other transgenic lines, which was similar to wild type.

GFP expression was evident in all tissues of the emerging T_1 progeny seedling of line 1 shown in Fig. 3(f). Seedlings of other lines had lower overall levels of expression than line 1, but, in each case, the radicle region showed higher fluorescence than the other regions. An entire 20-d-old plant of line 1 is shown in Fig. 3(g); bright green fluorescence was seen throughout the root tissues but, in the shoot, GFP expression was limited to nodes and young leaves. As shown in Fig. 3(h,i), no green fluorescence was detected for 2-d- or 10-d-old wild-type seedlings.

Diminished spatial-specificity of GUS expression driven by the *phaseolin* promoter in transgenic *M. truncatula*

Histochemical staining was performed to detect GUS expression driven by the *phas* promoter in transgenic plants. For lines 1, 2 and 4 (Fig. 1b), blue staining was evident for embryos within a few minutes of incubation, and overnight staining resulted in intense blue staining throughout the embryo, indicating a high level of GUS expression (Fig. 5a). For line 3, discrete, dark blue spots were evident throughout the embryo (Fig. 5b), suggesting the occurrence of epigenetic silencing in this multicopy line.

Although expression from the *phas* promoter is known to be strictly confined to embryogenesis and microsporogenesis

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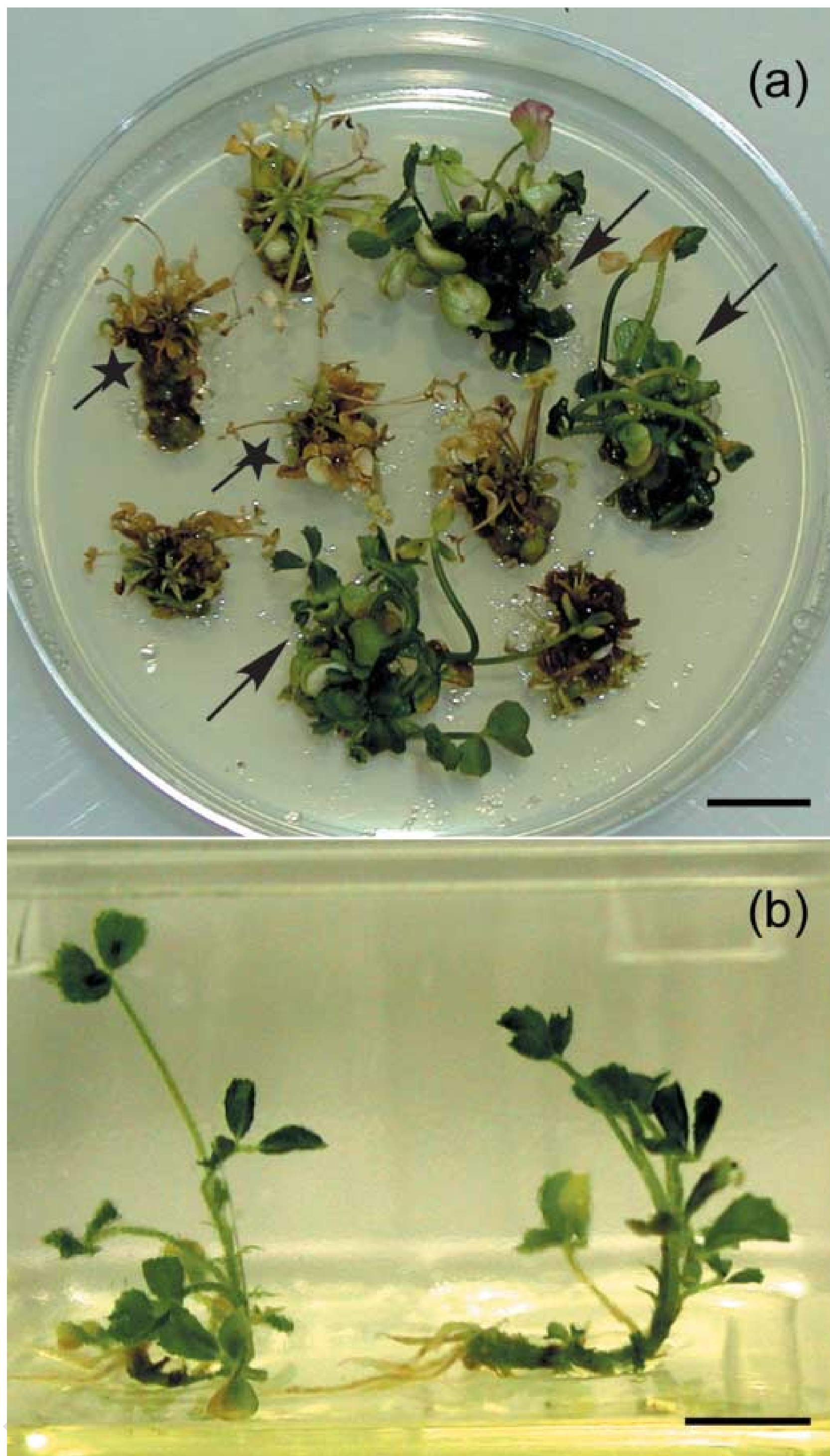

Fig. 2 Regeneration of transgenic plants. (a) Selection of regenerating *Medicago truncatula* A17 shoots on medium containing 1.6 mg l^{-1} phosphinothricin (PPT). Green, resistant shoots are indicated by arrows; brown, nontransformed shoots are indicated by starred arrows. (b) Optimal positioning of cut shoots for root induction; note that roots emerge from the upper edge of the cut surface. Both panels are for Expt 8 in Table 1. Scale bars: 1 cm in both (a) and (b).

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in beans, transgenic tobacco (Sengupta-Gopalan *et al.*, 1985; van der Geest *et al.*, 1995) and *Arabidopsis* (Chandrasekharan *et al.*, 2003), histochemical staining revealed GUS expression in the leaf veins of all the transformants (Fig. 5c); as for the embryo, line 3 exhibited spotty expression in the leaves (Fig. 5d).

GUS expression was assessed histochemically for 7-d seedlings of lines 1, 2 and 4; a representative seedling is shown in Fig. 5(e). GUS has a half-life of *c.* 50 h (Jefferson *et al.*, 1987) and residual expression was seen for tobacco seedlings 5 d after germination (Frisch *et al.*, 1995). Similarly, residual GUS expression was detected (Fig. 5e) for 7-d-old *M. truncatula*

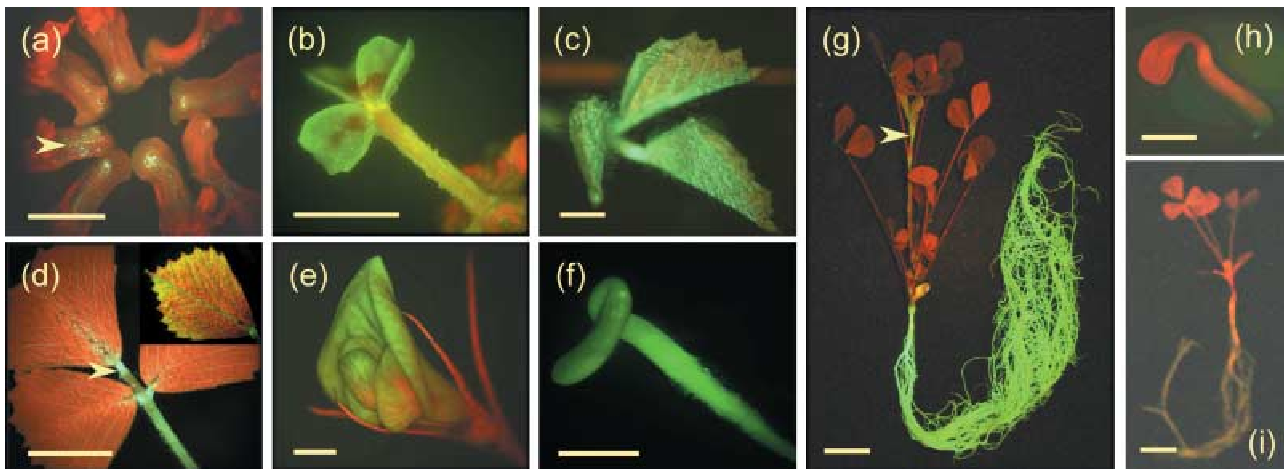

Fig. 3 GFP expression in transgenic *Medicago truncatula* A17. (a) Transient GFP expression (*arrowhead*) in explants 2 d after cocultivation. (b) Transgenic shoot 1 month after initiating regeneration. (c) Emerging trifoliate leaf after transfer of plant to soil. (d) Diminished GFP fluorescence in the mature trifoliate leaf, except at the margin of some leaves (inset); bright green fluorescence remains at the petiole nodes (arrowhead). (e) Strong fluorescence in the corolla but weak fluorescence in the calyx. (f) Ubiquitous fluorescence in germinating (2 d) T1 seedling. (g) Entire T_1 plant (*c.* 20 d) showing strong fluorescence in roots and nodal meristem (arrowhead), but no or low expression in mature leaves or stem. (h) A 2-d-old wild-type seedling. (i) A 10-d-old wild-type seedling. Scale bars: 5 mm in (a) (b) (d) (f) and (h); 1 mm in (c) and (e); 1 cm in (g) and (i).

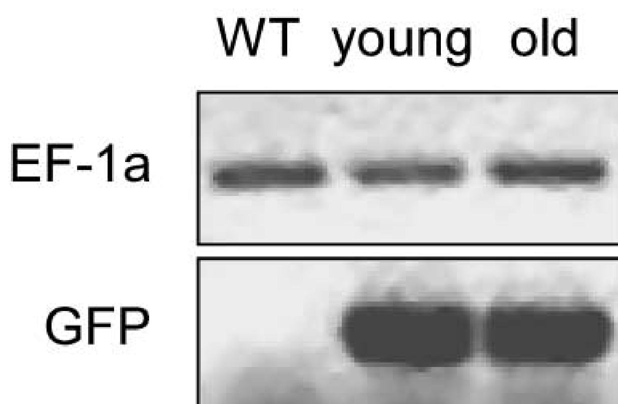

Fig. 4 RT-PCR analysis of *GFP* transcription in young and old leaves of transgenic *Medicago truncatula* A17. Bottom panel shows *GFP* transcript levels in young and old leaf tissues of transgenic plants normalized relative to that of the internal control, *EF-1′* (top panel). WT, wild-type.

seedlings of lines 1, 2 and 4. The hypocotyl and cotyledons stained strongly, moderate staining was observed for the plumular leaf and faint staining was seen in the upper parts of developing roots. No staining was observed for the cotyledonary node, the first trifoliate leaf, nor for most of the root.

Discussion

Optimization of transformation, selection and regeneration condition

The direct induction of shoots from cotyledonary nodes has been used successfully for regeneration of transgenic plants following *Agrobacterium*-mediated infection for several legume species (Somers *et al.*, 2003). Although an early report exists of *M. truncatula* A17 transformation by this approach (Trieu & Harrison, 1996), we are not aware of any subsequent articles that have utilized this system. While the work reported here is based on this earlier work, many small but important modifications were made to obtain reproducible, high frequencies for recovery of transgenic plants.

Compared with other tissue culture-based transformation methods, the cotyledonary-node approach is relatively fast and does not involve the induction of embryogenic calli and somatic embryos, which can be difficult. On the other hand, common problems with this approach are the low rates of transformation and regeneration, and recovery of transgenic plants can be less than 1% (Zhang *et al.*, 1999; Donaldson & Simmonds, 2000). In this work, up to 15% inoculated explants gave rise to PPT-resistant shoots and up to 45% PPT-resistant shoots were rooted (Table 1). Excluding experiments 3, 5 and 6, for which no rooted transgenic shoots were obtained, the average recovery of rooted shoots was 39% from a total of 419 explants. Use of the conditions detailed in the Experimental Procedures section should greatly facilitate research in gene discovery and gene function in the model plant *M. truncatula.*

In our initial attempts to establish *M. truncatula* transformation, *Agrobacterium* strain LBA4404 was used, as in the original protocol (Trieu & Harrison, 1996), but without success. *Agrobacterium* strains EHA105 (Hood *et al.*, 1993) and AGL1 (Lazo *et al.*, 1991) were then tested because of their reported virulence; both proved to be effective in transforming *M. truncatula.*

An important component of the transformation procedure is inclusion of DTT and L-cysteine in both the inoculation

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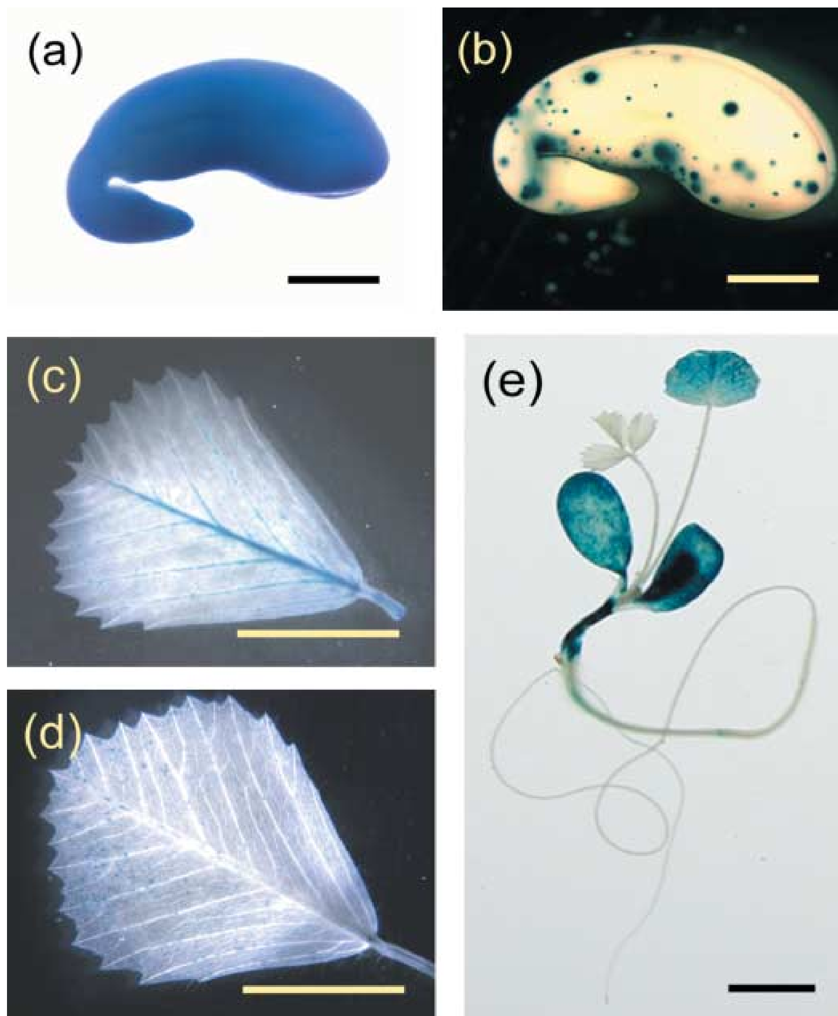

Fig. 5 GUS expression in transgenic *Medicago truncatula* A17. (a) Mature embryo exhibiting strong GUS expression in all tissues. (b) Mosaic GUS expression pattern seen in line 3. (c) GUS expression pattern typical of leaf veins in all lines except lines 3a and 3b, for which a mosaic GUS expression pattern was seen (d). (e) Entire T_1 plant of line 1 showing GUS expression in the cotyledons, the plumular leaf, and the hypocotyl and adjacent root region. Scale bars: 1 mm in (a) and (b); 5 mm in (c) (d) and (e).

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and the cocultivation media. The presence of these compounds in inoculation and cocultivation media substantially reduces tissue browning resulting from the release of phenolics (McCown *et al.*, 1968) and greatly improved transformation efficiency in soybean (Olhoft *et al.*, 2001; Olhoft *et al.*, 2003). The inclusion of these compounds improved transient GFP expression immediately after cocultivation and yielded a higher percentage of explants from which PPT-resistant shoots were regenerated.

For successful transformation of *M. truncatula*, the selection stringency must be carefully determined, and should be coupled with the strength of the promoter used to drive the resistance gene to get effective selection of transformants, that is if a weak promoter is used, a lower concentration of selection agent should be employed. The presence of 1.6 mg l^{-1} PPT in the selection medium was effective in killing untransformed shoots within 2 wk while enabling the transgenic shoots to survive and develop. To avoid untransformed shoots from escaping, selection pressure was maintained for 2 months.

In summary, the parameters we found to be of major importance in successful recovery of transgenic *M. truncatula* A17 shoots include the use of hypervirulent *Agrobacterium* strains for inoculation, the incorporation of L-cysteine and DTT in inoculation and cocultivation media to suppress tissue browning, and careful determination of antibiotic selection stringency.

Induction of adventitious roots from transgenic shoots

Rooting of the regenerating shoots has been a bottleneck for transformation of *M. truncatula* A17. In the present work, a large array of modifications were tested towards improving rooting efficiency. Changes from the conditions used by Trieu & Harrison (1996) found to be effective were: supplementation of full strength Schenk and Hildebrandt salts and vitamins (Schenk & Hildebrandt, 1972) with 0.5 mg l^{-1} indole-3-butyric acid (IBA) instead of 0.2 mg l^{-1} and reduction of the sucrose concentration from 20 g l^{-1} to 10 g l^{-1}; the use of

0.25% Phytagel as the gelling agent instead of 0.8% agar, and the exclusion of the PPT selection agent. Various combinations of IAA and NAA were tested, but they were much less effective than IBA. A procedure found to be of vital importance is that the shoots must be laid on the surface of the medium with freshly cut ends exposed in the air, as illustrated in Fig. 2(b). For shoots that did not root within 1 month, the ends were cut again and the shoot transferred to fresh medium. Recent experiments revealed that a pH of 6.5 is more effective for root induction than is pH 5.8, yielding rooting frequencies approaching 50%. Even shoots that had failed to root after a year of culture responded favourably to this procedure and the numbers of rooted shoots, and hence their percentage of resistant shoots (Table 1) is increasing.

Intriguing expression patterns of *35S-GFP* and *phas-GUS* transgenes in *M. truncatula*

The progressive loss of GFP expression in aerial parts as the plant aged was dramatic and occurred in each of the four transgenic plants studied. Consequently, transgene copy number does not appear to be a major factor. The possibility that decreased expression resulted from gene silencing was evaluated by analysis of RNA transcript level using RT-PCR, but the similar GFP transcript levels found in young (1–2 d) and old leaves (> 7 d) (Fig. 4) detracts from this notion. In detailed studies of ageing leaves of *Brassica napus*, Halfhill *et al.* (2003) found that the decrease in GFP expression was positively correlated with soluble protein content. However, this does not satisfactorily explain the rapid disappearance of GFP seen as the *M. truncatula* leaves age. Possibilities to be examined in the future include the synthesis of a quenching agent in the maturing leaves or the accumulation of a degrading agent such as a protease. The present analysis of transcript levels was not exhaustive and it also remains formally possible that gene silencing is associated with the observed loss of GFP expression.

The expression of *35S-GFP* gene fusion constructs in transgenic *M. truncatula* has previously been reported for genotypes R-108–1 and Jemalong J5 (Kamaté *et al.*, 2000) and 2HA (Chabaud *et al.*, 2003). GFP expression was evident in transgenic calli, somatic embryos, flowers, roots and germinating seedlings (Kamaté *et al.*, 2000; Chabaud *et al.*, 2003). However, no GFP expression in leaf tissue is shown in any of these reports, suggesting that, as found in our experiments, loss of expression may have occurred. Indeed, Kamaté *et al.* (2000) comment that GFP expression in embryogenic calli became weaker or ceased at embryo initiation.

From the limited number of independent transformants studied in any detail thus far, it is not possible to determine if the diminished expression observed in mature leaf tissues reflects aberrant activity of the *35S* promoter, or is a commonly occurring feature of GFP in ageing tissues that is especially apparent in *M. truncatula*. Both possibilities exist. While the *35S* promoter was originally thought to be constitutive in transgenic plants (Benfey *et al.*, 1989; Battraw & Hall, 1990), more recent studies suggest that it is subject to spatial and other fluctuations in expression (Li *et al.*, 1999) and that it may be especially vulnerable to rearrangement (Kohli *et al.*, 1999; Kumpatla & Hall, 1999). Certainly, the difference in GFP expression above and below the cotyledonary node evident in Fig. 3(g) (and seen in three additional lines) is dramatic. Further resolution of the situation will require evaluation of expression of GFP from alternative promoters and, conversely, to examine expression of GUS from the *35S* promoter.

Evidence that aberrant transgene expression in *M. truncatula* is not confined to the *35S* promoter is provided by the patchy pattern of GUS expression from the *phas* promoter in T_1 embryos of lines 3a and 3b. The nonuniform expression suggests silencing, a phenomenon not previously encountered for the *phas* promoter. This may reflect the fact that the transgene construct is present in relatively high copy number in lines 3a and 3b (Fig. 1b) since intense uniform staining, and hence GUS expression, was seen for all other lines tested (1, 2 and 4). Lack of the usual spatial stringency of expression from the *phas* promoter in transgenic *M. truncatula* was also evidenced by the weak expression of GUS in mature leaf veins (Fig. 5c,d). While this may reflect the genomic environment of the transgene, the observation of expression in several independent lines detracts from this explanation. These findings are especially interesting since it might be assumed that the *phas* promoter would behave more, rather than less, faithfully in a legume background than in evolutionarily distant species such as tobacco and *Arabidopsis*. As evidenced in Table 1, many additional transformants will be available, and their analysis will permit a more statistically sound evaluation of transgene expression patterns in *M. truncatula*.

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