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TECHNICAL ADVANCE

Transformation of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*

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Summary

Two rapid and simple *in planta* transformation methods have been developed for the model legume *Medicago truncatula*. The first approach is based on a method developed for transformation of *Arabidopsis thaliana* and involves infiltration of flowering plants with a suspension of *Agrobacterium*. The second method involves infiltration of young seedlings with *Agrobacterium*. In both cases a proportion of the progeny of the infiltrated plants is transformed. The transformation frequency ranges from 4.7 to 76% for the flower infiltration method, and from 2.9 to 27.6% for the seedling infiltration method. Both procedures resulted in a mixture of independent transformants and sibling transformants. The transformants were genetically stable, and analysis of the T_2 generation indicates that the transgenes are inherited in a Mendelian fashion. These transformation systems will increase the utility of *M. truncatula* as a model system and enable large-scale insertional mutagenesis. T-DNA tagging and the many adaptations of this approach provide a wide range of opportunities for the analysis of the unique aspects of legumes.

Introduction

The past two decades have seen major advances in plant transformation, and a wide range of species can now be genetically transformed (Christou, 1995; Siemens and Schieder, 1996). These technologies have had considerable impact both on basic scientific research, where they have enabled advances in understanding plant processes, and on agricultural biotechnology, where the engineering of crops with specific traits can now be achieved.

In most cases transgenic plants are produced by methods which include the transformation of individual plant cells followed by regeneration of whole plants from those transformed cells (Christou, 1995; Fraley *et al.*, 1983; Potrykus, 1991). Although these approaches work well for some species, in others it has proven difficult to regenerate whole plants from those tissues susceptible to transformation, and in these cases transgenic plants cannot be produced. There are only a few species for which transformation systems avoiding tissue culture-based regeneration systems are available. One of these is the model plant *Arabidopsis thaliana*, for which a variety of *in planta* methods have been developed (Bechtold *et al.*, 1993; Chang *et al.*, 1994; Clough and Bent, 1998; Feldmann and Marks, 1987; Katavic *et al.*, 1994; Richardson *et al.*, 1998). The development of these simple and rapid transformation systems was a major advance for *Arabidopsis* research and has contributed greatly to the success of this model system.

Leguminous crops such as peas, soybean, bean, alfalfa, peanut and chickpea have widespread economic importance as protein sources for both animals and humans, and in association with rhizobia they are also an essential component of the global nitrogen cycle. In addition, legumes are a rich source of secondary metabolites, many of which are rare or absent in *Arabidopsis* and other genetic model plants. Molecular genetic analysis of legumes and trait improvement via biotechnological approaches are both hampered by the size and complexity of the legume genomes, and the generally intractable transformation systems (Bingham *et al.*, 1975; Cheng *et al.*, 1997; Dillen *et al.*, 1997; Hinchee *et al.*, 1988; Kim and Minamikawa, 1996; Schroeder *et al.*, 1993). To circumvent some of these obstacles, a number of researchers interested in legume biology have selected *Medicago truncatula* Gaertn. (barrel medic) as a model legume for molecular genetic analyses (Barker *et al.*, 1990; Cook *et al.*, 1995; Harrison and Dixon, 1993). This diploid, autogamous species has a relatively small genome and a rapid life cycle, and can be transformed and regenerated, although the methods are still labor intensive (Chabaud *et al.*, 1996; Hoffmann *et al.*, 1997; Thomas *et al.*, 1992; Trieu and Harrison, 1996).

The utility of *M. truncatula* as a model legume could be further improved if an easier and more efficient transformation method, similar to the *in planta* methods used for *Arabidopsis,* was available. Furthermore, development of *in planta* transformation of *M. truncatula* may facilitate the application of this technology to other agriculturally significant legume species.

Here, two *in planta* transformation methods for *M. truncatula* are described. The first method is derived from one of the *Arabidopsis* protocols and involves infiltration of flowering plants. The second involves infiltration of seedlings and permits simultaneous transformation of very large numbers of individuals.

Results

Transformation of M. truncatula *by vacuum infiltration of flowering plants*

This transformation procedure was developed from the *Arabidopsis in planta* flower transformation procedure (Bechtold *et al.*, 1993). The main difference for *M. truncatula* was the inclusion of a vernalization treatment which induces *M. truncatula* to flower early (M. Chabaud and E. Journet, personal communication). This treatment was essential, and infiltration of non-vernalized plants failed to give rise to transformants (data not shown). Briefly, *M. truncatula* seeds were imbibed and incubated at 4°C for 2 weeks, during which time they germinated. The seedlings were then planted and grown to flowering. As a result of the vernalization treatment they flower 3–4 weeks after planting, while they are still relatively small. During the initial period of flowering, when the plants had flower buds and a few open flowers, they were submerged in a suspension of *Agrobacterium* and the bacteria were vacuum-infiltrated into the plant. Following infiltration the plants were grown for a week and then infiltrated again with *Agrobacterium*. After two infiltration treatments the plants appeared unhealthy and many of the leaves died; however, they were still able to produce some seed. Transformants (T_1) were selected from the progeny of the infiltrated plants. Binary vectors carrying the *bar* gene were used for the transformation and transformants were selected by spraying the T_1 progeny with a phosphinothricin (PPT)-containing herbicide.

Following the first successful transformation experiment, TS1-3 (Table 1), in which three sets of transgenic plants were obtained, three additional experiments were undertaken. Thirty-six plants were infiltrated in each experiment and the seed from these plants was collected as a single pool. Overall, the number of seeds collected was low, as was the viability; however, in these experiments (T86-1, T86-2 and T86-4) between 13 and 76% of the T_1 seedlings were resistant to the selective agent, PPT (Table 1). Seedlings arising from a control experiment in which the plants were infiltrated with the infiltration media lacking *Agrobacterium* did not give rise to any PPT-resistant seedlings (data not shown). Transformants were not recovered from experiments in which the binary vector contained the *nptII* gene as a selectable marker (data not shown). Although kanamycin has been used successfully as a selective agent in *M. truncatula* tissue culture transformation procedures (Chabaud *et al.*, 1996), the seedlings display a high level of resistance to kanamycin and transformants could not be distinguished easily.

Southern blot analyses of transformants produced via infiltration of flowering plants

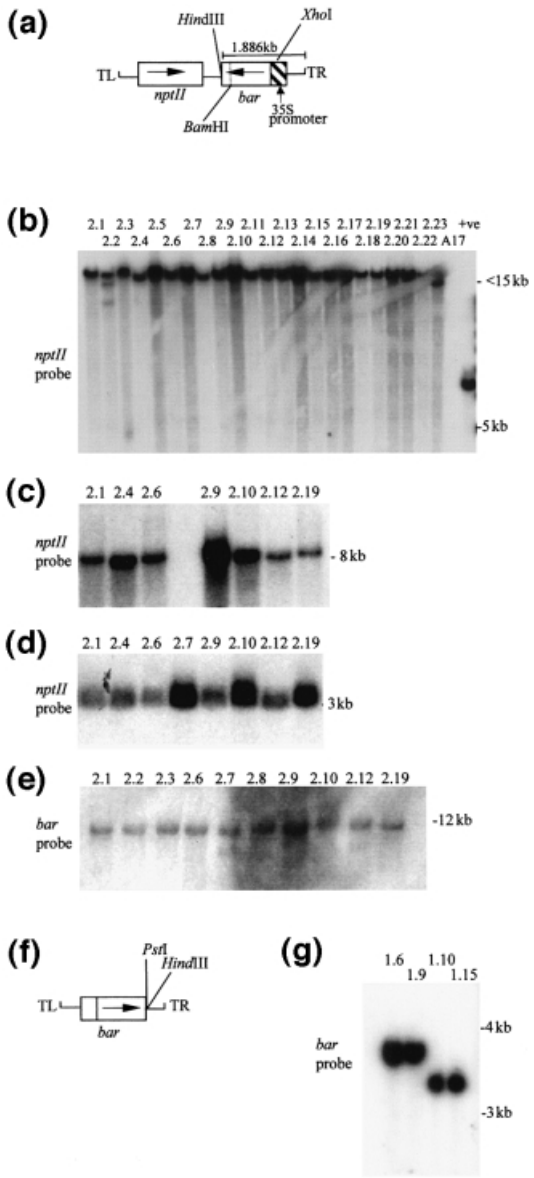
To confirm that the T-DNA was present in the genomes of the transformants, DNA was extracted from the majority of the transgenic plants from experiments T86-1, T86-2 and T86-4 and analyzed on Southern blots. All of the transformants resistant to PPT contained at least one copy of the *bar* gene integrated into the genome. In the cases where the T-DNA also contained the *nptII* gene, for example experiment T86-2 (Figure 1a), the transformants also contained at least one copy of the *nptII* gene. A selection of these Southern blots is shown in Figure 1.

DNA from 23 transformants arising from experiment T86-2 (Table 1) was digested with *Hin*dIII, blotted and hybridized with an *nptII* probe to obtain a left border analysis (Figure 1b). In each case, the probe hybridized to DNA from the transformants and not to wild-type *M.*

Table 1. Transformation experiments (infiltration of flowering plants)

Experiment	Construct and *Agrobacterium*	Selectable marker gene	No. of plants infiltrated	No. of seed collected	No. of seedlings germinated	No. of seedlings resistant to PPT	Percentage transformation[a]
TS1	pSLJ525/ASE1	*bar*	36	ND	ND	2	ND
TS2	pSKI006–Mt4/ASE1	*bar*	36	ND	ND	16	ND
TS3	pSLJ525/ASE1	*bar*	—	ND	ND	3	ND
T86-1	pSKI006/ASE1	*bar*	36	460	99	21	21.2
T86-2	pSLJ525/ASE1	*bar*	36	470	182	23	12.6
T86-4	pBIN*mgfp-ER-bar*/EHA105	*bar*	36	85	17	13	76.4

ND, not determined.
[a]The majority of the transformants were analyzed by Southern blot analysis.



truncatula (A17) DNA. Surprisingly, the majority of the transformants appeared to have identical hybridization patterns suggesting that there may be a large number of sibling transformants (Figure 1b). Additional left border analyses of a selection of these transformants (Figure 1c–e) revealed that this was the case, and only three of the 23 transformants were independent (Figure 1b). From the Southern blot analysis shown in Figure 1(b), the three independent transformants all have hybridizing fragments of around 15 kb which is somewhat unusual, since *Hin*dIII sites are expected to occur more frequently than this. Based on the corresponding ethidium bromide-stained gel, the DNA was completely digested. High molecular

Figure 1. Southern blot of transformants prepared via infiltration of flowering plants.
(a) Schematic representation of pSLJ525. TR, right border of T-DNA; TL, left border of T-DNA; *bar*, phosphinothricin acetyl transferase gene; *nptII*, neomycin phosphotransferase type II gene. Arrows indicate the direction of transcription of the genes. Restriction sites for enzymes used in the Southern blot analysis are shown.
(b) DNA from 23 transformants from experiment T86-2 (pSLJ525) (lanes 1–23), digested with *Hin*dIII and hybridized with the *nptII* gene for a left border analysis. Lanes 24 and 25 contain wild-type *M. truncatula* (A17) digested with *Hin*dIII and the SLJ525 binary vector (+ve), respectively.
(c) DNA from seven transformants from experiment T86-2 (Lanes 1–7) digested with *Hin*dIII and *Xho*I simultaneously and hybridized with a *nptII* probe for a left border analysis.
(d) DNA from eight transformants from experiment T86-2 (Lanes 1–8) digested with *Hin*dIII and *Bam*HI simultaneously and hybridized with a *nptII* probe for a left border analysis.
(e) DNA from 10 transformants from experiment T86-2 (Lanes 1–10) digested with *Xho*I and *Eco*RI simultaneously and hybridized with a *bar* probe for a left border analysis.
(f) Schematic representation of pSKI006. TR, right border of T-DNA; TL, left border of T-DNA; *bar*, phosphinothricin acetyl transferase gene. Arrows indicate the direction of transcription of the *bar* gene. Restriction sites for enzymes used in the Southern blot analysis are shown.
(g) DNA from four transformants from experiment T86-1. DNA from transformants 1.6 and 1.9 was digested with *Hin*dIII, while DNA from transformants 1.10 and 1.15 was digested with *Pst*I. The blot was hybridized with a *bar* probe for a left border analysis. These transformants are siblings.

Table 2. Segregation analysis (PPT resistance) of progeny from a selection of transformants prepared by infiltration of flowering plants

Progeny from transformants	Number of plants resistant to PPT	Number of plants sensitive to PPT	Chi-square test against ratios (*P*)		
			3R:1S	15R:1S	63R:1S
T86-1.3	83	15	*	–	–
T86-2.3	98	32	**	–	–
T86-2.5	92	2	–	**	**
T86-2.11	106	45	**	–	–

*$P \geqslant 0.01$; **$P \geqslant 0.05$; ¯$P \leqslant 0.01$.

weight *bar*- or *nptII*-hybridizing fragments were seen in DNA from some transformants, but not in others. For example, DNA from transformants from experiment T86-1 (Figure 1f) was digested with *Hin*dIII or *Pst*I and hybridized with the *bar* gene. The hybridizing fragments are 3.8 and 3.5 kb, respectively (Figure 1g). The majority of the transformants from this experiment were siblings, so only four transformants are shown.

Based these analyses it is clear that in *M. truncatula* flower infiltration transformation gives rise to independent transformants and siblings. From the analyses shown in Figure 1(c–g), three transformants out of 23 (13%) were independent. Analyses of transformants from three additional experiments further confirm these results, and the percentage of independent transformants ranged from 13 to 23% (data not shown).

Analysis of T_2 *progeny from transformants produced via the flower infiltration procedure*

The progeny from four transformants from experiments T86-1 and –2 were grown and analyzed for resistance to PPT (Table 2). In three cases the progeny segregated for resistance to PPT, indicating that the original transformants were hemizygous. However, in one case, all of the progeny were resistant, suggesting that the original transformant was homozygous. The T-DNAs appear to be inherited in Mendelian fashion, and chi-square analyses of the segregation ratios of progeny from transformants T86-1.3, T86-2.3 and T86-2.11 are consistent with the presence of a single copy of the T-DNA in each transformant. Southern blot analyses are also consistent with this result and suggest that T86-2.3 and T86-2.11 are siblings (Figure 1b and data not shown).

Transformation of M. truncatula *by vacuum infiltration of seedlings*

The seedling infiltration transformation procedure was developed from previous *M. truncatula* transformation procedures (Chabaud *et al.*, 1996; Trieu and Harrison, 1996) coupled with the *in planta* transformation approaches developed for *Arabidopsis* (Bechtold *et al.*, 1993). The initial stage of the procedure is similar to that of the flower transformation method. The seeds are imbibed and germinated at 4°C. Two weeks later the young seedlings are removed from the cold and infiltrated with *Agrobacteria* suspended in an infiltration media (Trieu and Harrison, 1996). Following infiltration, the seedlings are incubated on a co-cultivation medium (Trieu and Harrison, 1996), and finally planted and allowed to set seed. As with the previous method, transformants are selected in the following generation. In terms of performing the infiltration procedure, this approach is much easier than infiltration of flowering plants. The seedlings are small and 300–400 seedlings can be infiltrated simultaneously in a relatively small volume of *Agrobacterium* suspension.

Nine seedling infiltration transformation experiments were carried out, and transgenic plants were obtained from the eight experiments in which the binary vectors contained the *bar* gene as the selectable marker (Table 3). In the initial experiment (T84) the length of the vacuum step was varied slightly; however, this did not make a significant difference (Table 3), and in all of the subsequent experiments seedlings were subjected to two consecutive infiltrations each for 1 min. In the eight successful transformation experiments, the transformation frequency ranged from 2.9 to 27.6%, with an average of 9.4% of the progeny seedling showing resistance to PPT (Table 3). As with the flower transformation experiments, we were unable to use vectors carrying the *npt*II gene as a selectable marker.

Southern blot analyses of transformants produced via infiltration of seedlings

To confirm the presence of the T-DNA in the genomes of the transformants, Southern blot analyses were performed. All of the PPT-resistant transformants analyzed contained one or more copies of the *bar* gene integrated into the genome and, where appropriate, at least one copy of the *npt*II gene. A selection of these analyses is shown in Figure 2. Genomic DNA from transformants from experiments T84-1 and T84-2

Table 3. Transformation experiments (infiltration of seedlings)

Experiment	Construct and *Agrobacterium*	No. of plants infiltrated	No. of seed collected	No. of seedlings germinated	No. of seedlings resistant to PPT	Percentage transformation[a]
T84-1	pBI121-*bar* /EHA105	40	1326	329	22	6.7
T84-2	pBI121-*bar* /EHA105	40	1263	302	16	5.3
T84-3	pBI121-*bar* /EHA105	40	1214	173	5	2.9
T87-1	pGA482-*bar*/EHA105	40	ND	217	10	4.6
T87-2	pGA482-*bar* /EHA105	40	ND	502	89	17.7
T87-3	pKYLX71Gus/EHA105	40	ND	ND	**	ND
T87-4	pBIN*mgfp-ER-bar* / EHA105	40	ND	382	13	3.4
T87-7	pBIN*mgfp-ER-bar* and pKYLX71Gus/EHA105	40	ND	210	58	27.6
T88	pSKI015/Gv3101	70	ND	565	40	7.1

ND, not determined.
[a]The majority of the transformants were analyzed by Southern blot analysis.

was digested with *Hin*dIII, blotted and probed with an *nptII* gene probe for a right border analysis (Figure 2b). This revealed that all of the plants contained at least one copy of the *nptII* gene and five plants contained multiple copies. Five of the nine transformants from T84-1 showed unique *nptII* hybridization patterns, indicating that they are independent transformants. The remaining four transformants, 1.14, 1.16, 1.19 and 1.20, share the same hybridization pattern and are probably siblings. Transformant 2.13 also contains a hybridizing band of a similar size, but despite the apparently identical Southern blot this transformant cannot be a sibling as it arose from a separate experiment. To demonstrate that transformants arising from different experiments are different, DNA from transformants 1.2 and 1.11 arising from experiment T84-1, and transformants 3.1 and 3.5 arising from experiment T84-3, were digested with *Xho*I and *Eco*RI, blotted and hybridized with a *bar* probe for a right border analysis (Figure 2c). The hybridizing fragments from each of these transformants are clearly different, confirming that transformants arising from different infiltrated plants are independent. In this particular case, transformants arising from the same experiment (e.g. T84-3.1 and T84-3.5) are also independent. DNA from transformants from the T84 experiments was also cut and hybridized with the *uidA* gene for a left border analysis. Surprisingly, this analysis revealed that the transformants did not contain the *uidA* gene (data not shown) which should lie between the *bar* cassette and the left border of the T-DNA (Figure 2a). Rearrangement of the left section of the T-DNA, possibly due to the two adjacent, inverted 35S promoters, could have resulted in the deletion of the *uidA* gene. Although the *uidA* gene is missing, all of the transformants contained a copy of the *bar* gene (Figure 2b); however the bar cassette is slightly larger than expected, further supporting rearrangement of the left end of the T-DNA. The lack of the *uidA* gene in all of the T84 transformants could be explained by the T-DNA rearrangement occurring in the *Agrobacterium,* possibly during preparation of the culture for infiltration. The same culture of *Agrobacterium* was used for experiments T84-1, T84-2 and T84-3.

Additional analyses of transformants produced by the seedling infiltration method are shown in Figure 2(e). DNA from seven transformants from the T87-1 experiment was digested with both *Hin*dIII and *Bgl*II and hybridized with an *nptII* probe for a right border analysis. All the transformants contained DNA hybridizing to the *nptII* gene, and six showed unique hybridization patterns indicating that they are independent transformants (Figure 2e, upper panel). All of the transformants from the T87-1 experiments also contained the expected 1.6 kb *Hin*dIII/*Bgl*II fragment containing the *bar* gene (Figure 2e, lower panel).

Based on the transformants included in these Southern blots, 67% of transformants from T84-1 and 86% from T87-1 are predicted to be unique transformants (Figure 2).

In a number of transformation procedures it has been observed that co-transformation of a second T-DNA occurs with a fairly high frequency (De Neve *et al.*, 1997). To determine if co-transformation might occur in this system, a transformation experiment (T87-7) using a mixture of *Agrobacterium* strains containing the binary vectors, pBIN*mgfp-ER-bar* and pKYLX71-Gus, was carried out (Table 3). Fifty-eight transformants carrying pBIN*mgfp-ER-bar* were selected with PPT, and these were then tested for β-glucuronidase activity to determine the presence of the second T-DNA from pKYLX71-Gus (Table 3). None of the transformants showed any β-glucuronidase staining, suggesting that co-transformation of the second T-DNA did not occur. Transgenic *M. truncatula* carrying pKYLX71-Gus prepared previously by tissue culture transformation (Trieu and Harrison, 1996) was used as a positive control and showed strong β-glucuronidase staining.

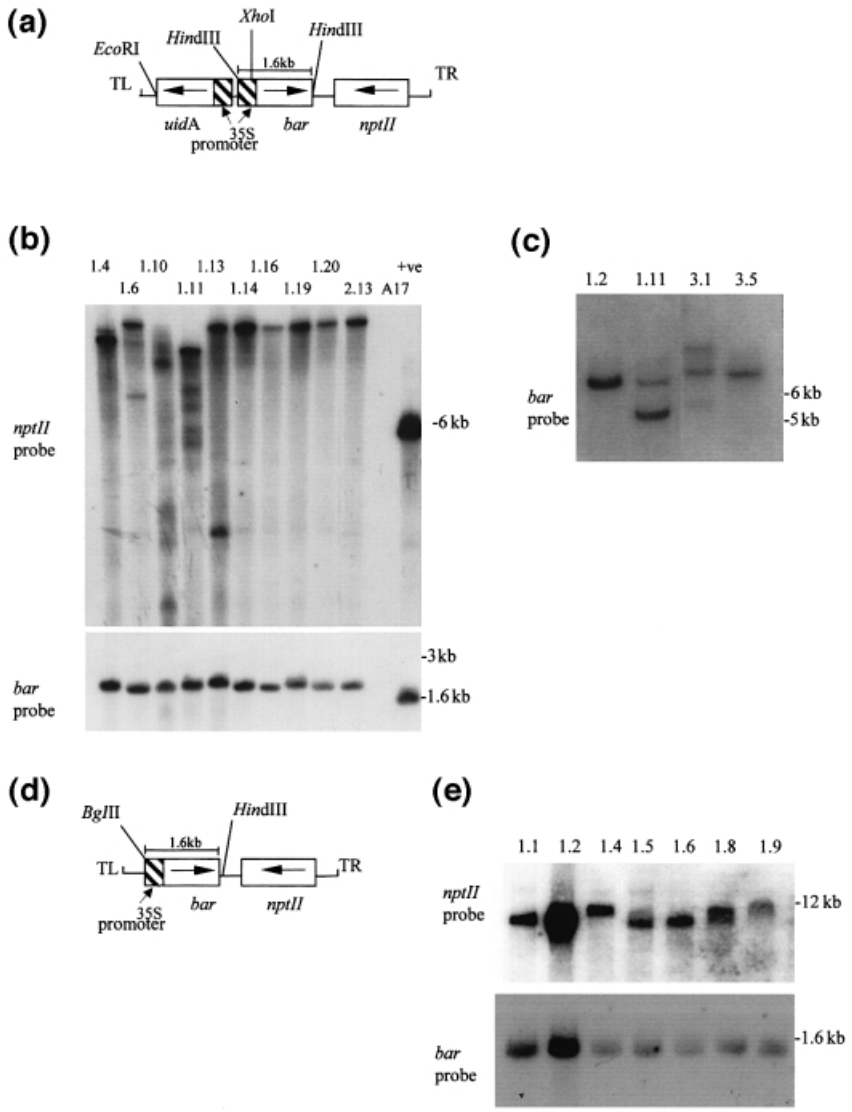


Figure 2. Southern blot of transformants prepared via infiltration of seedlings.
(a) Schematic representation of pBI121-*bar*. TR, right border of T-DNA; TL, left border of T-DNA; *bar*, phosphinothricin acetyl transferase gene; *nptII*, neomycin phosphotransferase type II gene; *uid*A, β-glucuronidase gene. Arrows indicate the direction of transcription of the genes. Restriction sites for enzymes used in the Southern blot analysis are shown.
(b) DNA from nine transformants from experiment T84-1 (pBI121-*bar*) (lanes 1–9) and one transformant from T84-2 (lanes 10), digested with *Hin*dIII. The blot was hybridized with the *nptII* gene to obtain a right border analysis (upper panel) and the *bar* gene (lower panel). Lanes 12 and 13 contain wild-type *M. truncatula* (A17) DNA digested with *Hin*dIII and the pBI121-*bar* binary vector (+ve), respectively.
(c) DNA from two transformants (1.2 and 1.11) from experiment T84-1 and two transformants (3.1 and 3.5) from experiment T84-3, digested with *Eco*RI and *Xho*I simultaneously and hybridized with the *bar* gene for a right border analysis.
(d) Schematic representation of pGA482-*bar*. TR, right border of T-DNA; TL, left border of T-DNA; *bar*, phosphinothricin acetyl transferase gene; *nptII*, neomycin phosphotransferase type II gene. Arrows indicate the direction of transcription of the genes. Restriction sites for enzymes used in the Southern blot analysis are shown.
(e) DNA from seven transformants from experiment T87-1 (pGA482-*bar*) digested with *Hin*dIII and *Bgl*II simultaneously and hybridized with the *nptII* gene for a right border analysis (upper panel) and *bar* gene (lower panel).

Analysis of T_2 *progeny from transformants produced via the seedling infiltration procedure*

The progeny of four transformants from the T84-1 and T84-2 experiments were grown and analyzed for resistance to PPT (Table 4). Three of the T_2 families segregated for resistance to PPT: therefore, as seen with the flower infiltration procedure, the original transformants were hemizygous (Table 4). Chi-square analyses of these segregation ratios suggest the presence of one copy of the transgene, an interpretation that is also supported by Southern analyses (Figure 2b). The progeny of the fourth

Table 4. Segregation analysis (PPT resistance) of progeny from a selection of transformants prepared by infiltration of seedlings

Progeny from transformants	Number of plants resistant to PPT (R)	Number of plants sensitive to PPT (S)	Chi-square test against ratios (*P*)		
			3R:1S	15R:1S	63R:1S
T84-1.14	83	0	–	*	**
T84-1.19	44	20	**	–	–
T84-1.20	38	14	**	–	–
T84-2.6	44	21	**	–	–

*$P \geqslant 0.01$; **$P \geqslant 0.05$; ¯$P \leqslant 0.01$.

transformant (T84-1.14) were all resistant to PPT, suggesting that the transformant is homozygous.

Discussion

A variety of *in planta* transformation procedures have been developed for *Arabidopsis*, including seed transformation, transformation by application of *Agrobacterium* to cut shoots, and transformation by infiltration or application of *Agrobacterium* to flowers (Bechtold *et al.*, 1993; Chang *et al.*, 1994; Clough and Bent, 1998; Feldmann and Marks, 1987; Katavic *et al.*, 1994). In contrast, few *in planta* approaches have been developed for other plant species (Chen *et al.*, 1998; Chowrira *et al.*, 1995), and the application of the *Arabidopsis* flower infiltration method to two other species, *Brassica napus* and *Beta vulgaris,* was unsuccessful (Siemens and Schieder, 1996). The two *in planta*, infiltration-based transformation methods reported here have been used successfully to transform *M. truncatula* and may also be suitable for other species. Compared with earlier tissue culture-based transformation methods for *M. truncatula* (Chabaud *et al.*, 1996; Hoffmann *et al.*, 1997; Thomas *et al.*, 1992; Trieu *et al.*, 1997), both of these *in planta* transformation procedures are easier technically, do not require tissue culture phases, and are generally faster. The flower transformation method is similar to the procedure described for the transformation of *Arabidopsis*, while the seedling transformation method is novel and has not been described previously. The efficiency of transformation for both methods was high. An average of 36% (three experiments) of the progeny from the plants subjected to flower transformation were transformants, while 9.4% (eight experiments) of the progeny of the infiltrated seedlings were transformed. These frequencies are high in comparison with *Arabidopsis*, where the transformation frequency ranges from 0.1 to 3% (Bechtold *et al.*, 1993; Clough and Bent, 1998). However, in contrast to *Arabidopsis* transformants, *M. truncatula* transformants appear to be a mixture of independent and sibling transformants. In particular, the flower transformation method gives rise to a large number of siblings. Based on those transformants tested by Southern blot analysis, the percentage of independent transformants was between 13 and 23% for transformants arising from the flower infiltration procedure, and between 67 and 86% for the transformants arising from the seedling transformation procedure. Southern analyses also revealed that some transformants had lost the sequences lying between the selectable marker and the left border. Loss of the left borders during transformation has been reported previously (Porsch *et al.*, 1998; van der Graaff *et al.*, 1996) and it appears to be important to place the gene of interest between the right border and the selectable marker.

Both of the *in planta* transformation procedures described here have worked successfully with a range of binary vectors, providing that the T-DNA contains the *bar* gene as a selectable marker. A variety of *A. tumefaciens* strains have been used, including EHA105, ASE1 and GV3101, and the only strain that was unsuccessful was LBA4404 (data not shown), a strain widely used in the *M. truncatula* tissue culture transformation methods (Chabaud *et al.*, 1996; Trieu and Harrison, 1996).

In the flower infiltration and seedling infiltration procedures, the amount of seed collected from the infiltrated plants was low (Tables 1 and 3). Despite this, it was still possible to obtain transformants. Consequently, it may be possible to adapt these approaches to other legumes, or non-legume species that do not have prolific seed production. In particular, the seedling transformation method may be useful for transformation of large species which otherwise would be difficult to submerge in *Agrobacterium*.

The mechanism underlying transformation in these two approaches is unknown. In *Arabidopsis*, the *in planta* shoot-transformation procedure gives rise to independent and sibling transformants, whereas both the flower infiltration and seed imbibition methods give rise to independent, hemizygous transformants (Bechtold *et al.*, 1993; Feldmann and Marks, 1987; Katavic *et al.*, 1994). Two possible mechanisms have been proposed. The first involves transformation of cells in the L1 layer and then

the replacement of cells from the L2 layer with L1 layer cells. The L2 layer then gives rise to sporogenous tissues. The second hypothesis is that the bacteria remain alive within the intercellular spaces of the plant tissue, and later gametophyte progenitors, gametophytes or fertilized embryos are transformed (Bechtold *et al.*, 1993; Feldmann and Marks, 1987). Recent analyses support the latter hypothesis (Bechtold and Pelletier, 1998). Transformants from the *M. truncatula* procedures appear to be a mixture of independent and sibling transformants and, surprisingly, the flower transformation method gives rise to a much larger number of sibling transformants than the seedling infiltration method. In the seedling infiltration procedure, the agrobacteria are in contact with the plant from the very early stages of development, so it seems more likely that transformation of meristem cells could occur, giving rise to multiple sibling transformants. The different level of independent versus sibling transformants obtained in the two methods suggests that the mechanisms of transformation may differ. The high proportion of siblings arising from *M. truncatula* flower transformation also suggests that the mechanism of transformation in *M. truncatula* flower infiltration is different to that occurring in *Arabidopsis* flower infiltration. The underlying mechanisms in *M. truncatula* are not known, but we speculate that in the flower infiltration procedure the targets of transformation are the meristem cells of the axillary buds that later develop into peduncles and inflorescences. Transformation results in chimeric plants with transformed sectors, and transformants arising from seeds produced in these sectors would be siblings. The L1/L2 layer cell replacement mechanism outlined by Feldman and Marks (1987) could give rise to chimeric sectors, and is also predicted to result in mostly hemizygous sibling transformants, as observed here. Transformation of the germ-line cells might also occur but at a much lower frequency. In the seedling infiltration procedure, we suggest that transformation of germ-line cells (Bechtold *et al.*, 1993) occurs more frequently, resulting in a greater proportion of independent transformants. Again, at this stage we can only speculate on reasons for a possible difference in mechanism in the two methods. Maybe the developmental age and health of the plants, or the titre of bacteria within the plant, influence the mechanism of transformation. The plants infiltrated at the seedling stage certainly appear healthier and produce more seed than those infiltrated at the flowering plant stage (Tables 1 and 3). Further analyses, including the use of *Agrobacterium* strains carrying reporter constructs to allow localization of the bacteria and identification of the initial transformed cells, will be needed to unravel the underlying mechanisms.

Medicago truncatula is an annual medic that is grown as pasture legume in a number of regions including Mediterranean areas, South Africa and Australia (Crawford *et al.*, 1989). This species was originally proposed as a model legume in 1990. Since then, the infrastructure and resources for this system have expanded and a number of research advances, including insight into two plant-microbe symbioses, have been made (Harrison and Dixon, 1994; Penmetsa and Cook, 1997; Pichon *et al.*, 1992). Although the current transformation approaches (Chabaud *et al.*, 1996; Hoffmann *et al.*, 1997; Trieu and Harrison, 1996) are suitable for the generation of small numbers of transgenic plants, they cannot be used to generate the large numbers of lines required for gene-tagging approaches. Insertional mutagenesis or gene tagging has been one of the most valuable research tools in a range of systems, including *Arabidopsis* (Feldmann, 1991), where it has facilitated many of the advances in our understanding of plant development. The *in planta* transformation systems described here, in particular the seedling infiltration method, now make large-scale insertional mutagenesis feasible in *M. truncatula*. T-DNA tagging and adaptations such as activation tagging (Walden *et al.*, 1994) will provide a powerful complement to the current approaches for analysis of the unique biochemical and physiological features of legumes.

Experimental procedures

Plant growth conditions

Medicago truncatula Gaertn 'Jemalong' (line A17) was used for all the experiments. Plants were grown in 11 cm diameter pots, in Metro-mix 250 or 350 (Scott, Marysville, OH, USA), nine plants per pot. The light level was 150 μmol m^{-2} s^{-1} with 18 h light/25°C and 6 h dark/22°C. The plants were fertilized with Miracle-Gro when necessary.

Agrobacterium *strains and binary vectors*

The following *Agrobacterium tumefaciens* strains and binary vectors were used in these experiments: *A. tumefaciens* strain ASE1 carrying the binary vectors pSLJ525 (Jones *et al.*, 1992) or pSKI006 (Kardailsky *et al.*, 2000) (www.salk.edu/LABS/pbio-w/); *A. tumefaciens* strain EHA105, carrying pBI121-*bar* or PKYLX7-Gus (Franklin *et al.*, 1993), or pBIN*mgfp-ER-bar*, or pGA482-*bar*; *A. tumefaciens* strain Gv3101 carrying pSKI015 (Kardailsky *et al.*, 2000). The addition of the *bar* gene to a number of vectors was achieved as follows. A *Hin*dIII/*Hpa*I fragment containing the 35S-*bar*-OCS 3′ sequences cassette was excised from pSLJ525 and inserted between the *Hin*dIII and *Hpa*I sites of pGA482 to create pGA482-*bar*. The same *Hin*dIII/*Hpa*I fragment was used to produce pBI121-*bar* and pBIN*mgfp-ER-bar*. For each of these vectors, the *Hpa*I site was converted to a *Hin*dIII site by the addition of an *Hpa*I-*Hin*dIII linker and then the *Hin*dIII fragment was then inserted into the *Hin*dIII site of pBI121 or pBIN*mgfp-ER* (Haseloff *et al.*, 1997) to create pBI121-*bar* and pBIN*mgfp-ER-bar*, respectively.

Sterilization and vernalization of M. truncatula *seeds*

Medicago truncatula seeds were soaked in concentrated sulphuric acid for approximately 10 min, rinsed three times with sterile distilled water and then sterilized in 30% chlorox, 0.1% Tween 20 for 5 min with gentle agitation. The seeds were then washed three times in sterile distilled water and spread on 0.8% water agar (Sigma agar-agar) in petri plates (10 × 100 mm), approximately 25 seeds per plate. The plates were wrapped with parafilm and aluminum foil. The seeds were then vernalized by incubating at 4°C for 2 weeks (M. Chabaud and E. Journet, personal communication).

Preparation of Agrobacterium *for flower infiltration*

A liquid culture of *Agrobacterium* was grown at 28°C in YEP medium (Bacto-peptone 10 g, yeast extract 10 g, NaCl 5 g) containing the appropriate antibiotics for the strain, to an OD_{600} of 1.6-1.8. The cells were pelleted by centrifugation, 5 min, 8000 r.p.m. (Sorval SA600 rotor) at room temperature, and resuspended in flower infiltration media (0.5× MS salts, 1× Gamborg's vitamins, 0.04 μM BAP, 0.02% Silwet77 (OSI specialities, Inc., Danbury, CT, USA), pH 5.7.

Preparation of Agrobacterium *for seedling infiltration*

15 ml TY (tryptone 5 g, yeast extract 3 g, $CaCl_2.2H_2O$ 0.88 g, pH 7.) containing the appropriate antibiotics were inoculated with *Agrobacterium* and grown overnight at 28°C with shaking at 250 r.p.m., to an OD_{600} of 1.6. The cells were then pelleted by centrifugation for 5 min, 8000 r.p.m. (Sorval SA600 rotor) at room temperature. The cells were resuspended in 30 ml (2× volume of culture) of seedling vacuum-infiltration medium and used immediately. Seedling vacuum infiltration medium is based on the M2A media (Trieu and Harrison, 1996) and contains 1× PDM (Plant Development Medium) salts and vitamins (Chabaud *et al.*, 1996), sucrose 10 g l^{-1}, BAP 15 μM, NAA 0.5 μM, acetosyringone 100 μM, adjusted to pH 5.8.

Transformation of M. truncatula *by vacuum infiltration of flowering plants*

Vernalized seedlings were grown until the plants had small flower buds and a few opened flowers. This occurs approximately 4 weeks after planting. The plants were watered heavily on the day before infiltration took place. To infiltrate the plants, the pots were inverted and the above-ground portion of the plant submerged in a container filled with a suspension of *Agrobacterium* in flower infiltration medium. Usually the soil was held in the pot by the roots, but if the soil appeared loose the pot was packed with cotton wool to ensure that the soil did not fall out. The pot and tray were placed in a vacuum chamber and a vacuum drawn to 25 inches Hg and held for 3 min. The vacuum was released very rapidly and the procedure repeated once. Following two exposures to vacuum, some of the plant leaves became dark and water-soaked. The pots were removed from the *Agrobacterium* and placed on their sides in a tray to prevent *Agrobacterium* from entering the soil. The tray and pots were transferred to a growth chamber set at 18°C, 95% humidity, 16 h days with four cool, white lights (Sylvania, F48T12, 115 W). The plants were incubated in the chamber for a week. After 2-3 days the pots were placed in an upright position again. The pots were not watered during this time. After a week, the infiltration process was repeated and the plants returned to the growth chamber for a week. In some cases the plants required water during the second week and this was applied carefully to the bottom of the pots. After the second week the plants were returned to normal growing conditions and allowed to set seed. Transformants were selected in the subsequent generation.

Transformation of M. truncatula *by vacuum infiltration of seedlings*

Vernalized seedlings were removed from the cold room and transferred to petri plates (10 × 100 mm) containing approximately 10 ml *Agrobacterium* per seedling vacuum infiltration medium suspension. The medium coats, but does not completely submerge, the seedlings. The petri plates were placed in a vacuum chamber and a vacuum drawn to 25 inches Hg. This was held for 1 min and then released rapidly. The vacuum step was repeated once more and then the seedlings blotted on sterile filter paper (3MM, Whatman International Ltd, Maidstone, UK) and spread onto seedling co-cultivation medium, approximately 20 seedlings per petri plate (10 × 100 mm). Seedling co-cultivation medium is based on M2C medium (Trieu and Harrison, 1996) and contains 1× PDM salts and vitamins (Chabaud *et al.*, 1996), sucrose 10 g l^{-1}, acetosyringone 100 μM, agar-agar 0.75% (Sigma), pH 5.8. The plates were incubated in a growth chamber at 20°C, 16 h days, 90% humidity with two fluorescent lights (Sylvania 115 W, F48T12/CW/VHO) for 2 days. Following incubation the seedlings were rinsed briefly in distilled water and planted into pots containing Metro-Mix 350 with 10 seedlings per pot. The pots were covered with a plastic dome and placed in the growth conditions. The dome was removed after 1 week. The plants began to flower about 24 days later and seed was collected.

Selecting M. truncatula *transformants with PPT*

Seeds collected from the infiltrated plants were treated with acid as described, but were not surface-sterilized. They were placed on damp filter paper in petri plates and incubated at 4°C for 3–4 days to help germination. They were then planted in Metro-Mix 350 in large trays, grown to the first trifoliate stage and sprayed thoroughly with Ignite (AgrEvo, Wilmington, Delaware, USA) diluted to contain PPT at 80 mg l^{-1}. The seedlings were initially covered with a plastic dome that was removed after 3–4 days. The non-transformed seedlings began to die in approximately 5 days. Two weeks later the seedlings were sprayed with 560 mg l^{-1} PPT. Transformants are completely resistant to this level of PPT. Transformants were labeled numerically and the number of the transformation experiment included to distinguish between transformation experiments.

Southern blotting

DNA was extracted from the transgenic plants by standard approaches (Dellaporta *et al.*, 1983). Southern blotting and hybridization were carried out as described previously (Church and Gilbert, 1984). The *bar* probe consisted of a 424 bp fragment, amplified from the *bar* gene using primers 5′-GAGCCCAGAACGACGCCCG and 3′-TATCCGAGCGCCTCGTGC. The *nptII* probe consisted of a 766 bp fragment amplified from the *nptII* gene using primers 5′-GAACAAGATGGATTGCACGC-3′ and 5′-GAAGAACTCGTCAAGAAGGC-3′. The probes were labeled as described previously (Trieu and Harrison, 1996).

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