Plant Cell Reports (1999) 19:13–19

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Comparison of different methods for plant regeneration and transformation of the legume *Galega orientalis* Lam. (goat's rue)

Received: 11August 1997 / Revision received: 6 April 1998 / Accepted: 1 March 1999

Abstract For the first time, regeneration and transformation have been achieved from the legume *Galega orientalis* Lam. (goat's rue). Two different regeneration protocols are described, one based on direct shoot induction from meristems and the other involving callus induction and shoot induction from callus with the plant growth regulator thidiazuron (TDZ). Different media and explants were evaluated. Three different transformation methods were compared: cocultivation with four different *Agrobacterium tumefaciens* strains, electroporation of embryos and apical meristems and particle bombardment of embryos. TDZ-promoted shoot induction on calli from immature embryos gave the best results. Transformation using this regeneration protocol was most successful with particle bombardment. Stable transformation has yet to be proven.

Key words *Agrobacterium* · Particle bombardment · Whole-plant electroporation

Abbreviations *BAP* 6-Benzylaminopurine · *CaMV* Cauliflower Mosaic Virus · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *D-Glc* D-glucose · *GUS* β-Glucuronidase · *IAA* Indoleacetic acid · *MS* Murashige & Skoog · *NAA* Naphthaleneacetic acid · *NPTII* Neomycin phosphotransferase II · *PPT* Phosphinothricin acetyl transferase · *TDZ* Thidiazuron

Communicated by K. Glimelius

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Introduction

Galega orientalis (goat's rue) is a promising perennial nitrogen-fixing forage legume, especially suitable for temperate regions. *G. orientalis,* and the related species *G. officinalis,* form indeterminate root nodules in interaction with their microsymbiont *Rhizobium galegae* (Lipsanen and Lindström 1988). This symbiosis displays extreme host specificity. *Rhizobium* from other species do not infect *Galega*, and *R. galegae* is found to infect only *Galega* (Lindström 1989). The factors imposing this specificity are not yet known, but since *G. orientalis* in general is resistant to microorganisms, and hence plant diseases, disease tolerance and the host specificity might be correlated to a specific plant defence mechanism. This makes *G. orientalis* particularly suitable for ecological farming.

An efficient transformation and regeneration system will enable molecular and biochemical analysis of this plant. This would also open the way for application of genetic engineering to improve this future pasture legume. Stable transformation has been reported for a number of legumes using the common transformation methods such as *Agrobacterium tumefaciens* and *A. rhizogenes* transformation, particle bombardment and electroporation of protoplasts (see reviews by Christou 1994; Atkins and Smith 1997). Some promising alternative transformation methods have also been carried out on legumes using whole plants, such as *Agrobacterium* transformation of seedling meristems (e.g. in *Glycine max*: Chee and Slightom 1995; in *Lotus japonicus*: Oger et al. 1996; in *Lupinus angustifolius*: Atkins and Smith 1997) and electroporation of apical meristems (e.g. in *G. max*, *Pisum sativum*, *Vigna unguiculata* and *Lens culinaris*: Chowrira et al. 1995, 1996). With these new methods, the tissue culture step is almost completely avoided, the process is faster and somaclonal variation is minimized. Yet despite those recent successful results, legumes are still considered to be a recalcitrant group for tissue culture and transformation.

Here, we report for the first time two different regeneration protocols for *G. orientalis*. In the first, direct shoot induction was obtained from nodal tissue, while callus was induced from different explants prior to shoot induction in the otherin the other. Three different transformation methods were also evaluated: cocultivation using four different *A. tumefaciens* strains, electroporation and particle bombardment. The efficiency of the methods was evaluated by comparing transient expression by histochemical staining of β-glucuronidase (*GUS*).

Materials and methods

Bacterial strains and vector construct

Four different *A. tumefaciens* strains were used. The disarmed octopine strain, LBA 4404 containing the disarmed *Ti* plasmid pAL 4404 and the binary vector pBI 121 (Clontech, USA) containing the *GUS* gene without an intron and the neomycin phosphotransferase II (*ntpII*) gene, conferring resistance to kanamycin. The three other *Agrobacterium* strains were the "supervirulent" disarmed l,l-succinamopine strain AGL1 (Lazo et al. 1991) containing the disarmed Ti plasmid pTiBo542ΔT, the disarmed l,l-succinamopine strain EHA 105 (Hood et al. 1993) containing the disarmed Ti plasmid pEHA 105, and the disarmed nopaline helper strain MOG 301 (Hood et al. 1993) containing the disarmed Ti vector pMOG 301. In these three strains, the binary vector pMOG 410 (Hood et al. 1993) carrying the *GUS* gene containing an intron (not splicable by prokaryotes) driven by the CaMV 35S promoter and with CaMV 35S terminator was introduced.

Plant material

Seeds from *G. orientalis* were obtained from Prof. K. Lindström, Helsinki University, Finland. These seeds represented a large variety of genotypes since no specific cultivars have yet been established. To obtain immature embryos as well as nodal tissues, flower buds and pods, seeds were sown in soil and put in the greenhouse in a 22/18 °C day/night temperature regime. Normal daylight was supplemented with helium lamps. No fertilizer was used to advance flowering. Crossing was made randomly. After three weeks the green pods were collected and sterilized in 40% commercial bleach (5% active ingredient) for 40 min. The immature embryos were dissected out and used for regeneration. Nodes and flower buds were sterilized in 10% commercial bleach with a few drops of Tween 20 for 10 min and washed three times with sterile distilled water.

For in vitro material, mature seeds were sterilized in 30% commercial bleach with a few drops of Tween 20, for 30 min, followed by 1 min in 70% ethanol and washed three times with sterile distilled water. Seeds were germinated on 0.7% agar plates and grown for 1 week in the dark at 24 °C. After 1 week, seedlings were transferred to B5 medium (Gamborg et al. 1968) in Magenta boxes (Sigma, USA) and were cultured at 24 °C under a 16/8 h day/night photoperiod, with an average light intensity of 40 μmol s^{-1} m^{-2} (Grolux lamps, Sylvania, Germany).

Direct shoot induction

For each type of explant, medium or hormone combination, at least 30 but usually 50 explants were used. All experiments were repeated twice.

Explants

The following explants were used as plant material for growth rate experiments and direct shoot induction: 3-mm sections of cotyledons, hypocotyls, petioles, nodes, roots, stems and leaves obtained from in-vitro-grown seedlings, 1 week as well as 1 month old. From greenhouse-grown plants (3 months old), 3-mm sections of leaves, stems, nodes, petioles and flower buds at different developmental stages were used. One-week-old in-vitro-grown seedlings with the cotyledons removed were also tested for shoot induction.

Media

First, the general growth response was tested in different media. Of 18 different media tested, 5 were selected in terms of growth response as judged visually: B5 medium, B5-2 × N, that contained twice the amount of nitrogen than ordinary B5, B5-casein (Nolan et al. 1989) that contained 250 mg l^{-1} casein and one-third of the amount of FeEDTA compared with ordinary B5, PGO (De Greef and Jacobs 1979) and PC-L2 (Phillips and Collins 1979).

Subsequently, shoot induction was compared using six different hormone combinations in the five media:; no hormones; 9.1 μm zeatin and 5.7 μm indoleacetic acid (IAA); 9.1 μm zeatin and 1.0 μm IAA; 8.9 μm 6-benzylaminopurine (BAP) and 1.0 μm zeatin; 4.5 μm BAP; and 8.9 μm BAP.

A pulse treatment was also tested. Twenty nodes from 3-month-old greenhouse-grown plants were incubated in very high concentrations of kinetin or BAP (900 μm) in MS medium (Murashige and Skoog 1962) supplemented with sucrose, for 2 or 5 h. After the hormone pulse, the nodes were placed on B5 medium supplemented with casein, sucrose, 9.1 μm zeatin and 1.0 μm IAA. The number of shoots was counted after 4 weeks. This experiment was repeated once.

General growth conditions

Cultures were grown in vitro at 24 °C under long-daylight conditions with a 16/8 h day/night photoperiod, and an average light intensity of 40 μmol s^{-1} m^{-2}. All material was transferred to new media every month. When the induced shoots were about 10 mm, they were transferred to root induction media, B5 medium with 0, 2.7 μm or 5.4 μm naphthalene acetic acid (NAA). Regenerated plants were first sown in vermiculite and put in the greenhouse at 16 °C and normal daylight supplemented with helium lamps, and after 3 weeks transferred to a mixture of soil and vermiculite. Plants were inoculated with 2 ml of *R. galegae* (Hambi 1174) suspension, approximately 1×10^8 cells ml^{-1}, to obtain nodules.

Shoot induction via a callus phase

For each hormone combination, at least 40 explants were used for each tissue type, while for embryos at least 10 were used, in this set of experiments. Experiments were repeated twice.

Explants

The following explants were compared: immature and mature embryos, 3-mm sections of 1-week- and 1-month-old in-vitro-grown plants. The immature and mature embryos were divided into three pieces: root, apical meristemmeristem and cotyledon.

Media

The tissue pieces were placed on callus medium consisting of B5 medium supplemented with 30 g l^{-1} sucrose and as hormones, either 9.0 μm 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.4 μm

BAP, 18 μm 2,4-D and 4.4 μm BAP, or 9.0 μm 2,4-D and 8.8 μm BAP were tested. Cultures were kept in the dark at 24 °C. Subculturing was every 4th week. No shoots were observed in the media used above. Thus, after 3 months, all induced calli were transferred to B5 medium containing 30 g l^{-1} sucrose, 5.0 μm thidiazuron (TDZ), 0.5 μm IAA and 10% coconut water (Sigma, USA) to induce shoots. Cultures were henceforth grown under light (16/8 h day/night photoperiod, with an average light intensity of 40 μmol s^{-1} m^{-2}).

General growth conditions

Shoot induction from callus was made under the same growth conditions described previously for shoot induction from explants. Subsequent shoot elongation was obtained with 1.4 μm gibberellic acid. Culture conditions, root induction and transfer to the greenhouse were done as above.

Particle bombardment

A particle bombardment device built according to Finer et al. (1992) was used for the biolistic delivery. With this device, the following parameters were used: helium flow = 5 l min^{-1}, vacuum = 0.8 atm, release time = 60 ms, and distance between DNA-tungsten holder and target = 170 mm. Tungsten particles were coated with DNA from the plasmid pMOG410, according to Vain et al. (1993). Ten mature embryos precultured for 1 day in the dark at 24 °C before bombardment were placed in the centre of a Petri dish with MS medium for each bombardment. After bombardment, embryos were cultured in the dark at 24 °C. In total, 100 embryos were bombarded and the embryos were bombarded once.

Agrobacterium cocultivation

The different *A. tumefaciens* cultures were grown in LB medium with antibiotics, according to recommendations, and with NaCl reduced to 0.1%. The bacteria were harvested at the exponential growth phase, and resuspended in MS medium to 1×10^9 cells ml^{-1}. The four different *A. tumefaciens* strains were used to infect either nodes of 1-month-old in-vitro-grown plants (LBA 4404) or 1-week-old in-vitro-grown seedlings, where only the cotyledons were cut off. Nodes or meristems were damaged by piercing or particle bombardment prior to cocultivation with the *A. tumefaciens* suspension. Three hundred nodes were used in each of five experiments and about 30 seedlings were used for each *A. tumefaciens* strain. These experiments were repeated four times. A cocultivation period of 4 days was seen to be optimal in terms of GUS staining and was routinely used.

Plant electroporation

The electroporation system from Equibio equipped with a cuvette with a 10-mm electrode gap was used to electroporate different types of plant tissue. The parameters which gave the highest survival rate of 1-week-old in-vitro-grown seedlings were one exponential decay pulse at 300 V, capacity 900 μF, electrode distance 10 mm, and shunt resistor at 201 ohm. This was used in all further experiments. Different kinds of plant material were used: mature embryos, 1-week-old in-vitro-grown seedlings, and 8-week-old greenhouse-grown plants. At least 40 explants were used for each tissue type. The protocol used for electroporation was from Chowrira et al. (1995, 1996). Embryos and seedlings were checked for transient expression after 4 days. The greenhouse-grown plants were screened for survival during the following weeks. The number of plants where new shoots started to develop was counted. Those new shoots were checked for *GUS* expression 35 days after electroporation.

Evaluation of transformation methods

GUS expression was assayed histochemically according to Jefferson et al. (1987). After 3–5 days, transformed tissue was incubated for 24 h at 37 °C. After cocultivation with *A. tumefaciens,* shoots were selected on B5 medium supplemented with 100 mg l^{-1} kanamycin (as determined by a concentration range) and 250 mg l^{-1} cefotaxime. Shoots were checked for *GUS* expression after 4 months on selection medium. No bacterial growth was observed at that time.

Results and discussion

Direct shoot induction

Explants

The influence of starting material on the capability for shoot production can be seen in Table 1. The best results were obtained with young seedlings where the cotyledons had been removed, with a 92 ± 4% shoot induction and with an average of 1.5 ± 0.3 shoots per explant. The first shoots appeared after 5 days. Nodal tissue also gave fairly good results. From the nodes from 1-week-old plants, shoots were induced from 79 ± 3% of the explants. The frequency of explants from which shoots could be induced decreased and the induction also took longer with increasing age of the starting material. But even nodes from mature greenhouse-grown plants could be used to induce shoots (Fig. 1a). Generally two, sometimes up to four shoots were induced on each explant. After primary shoots were excised, another set of shoots was induced. A few shoots were induced from hypocotyls and cotyledons of 1-week-old in-vitro-grown seedlings and from stems, hypocotyls and cotyledons from 1-month-old in-vitro-grown plants.

Table 1 Frequency (% ± SD) of different explants with shoot induction. All explants were induced on B5 medium supplemented with 9.1 μm zeatin and 1.0 μm IAA, except for the seedlings whose cotyledons were removed, where no hormones were added

Explant type	One-week-old in-vitro-grown seedlings	One-month-old in-vitro-grown seedlings	Greenhouse-grown plants	One-week-old in-vitro-grown seedlings with cotyledon cut off
Node	79 ± 3	64 ± 6	51 ± 6	92 ± 4
Hypocotyl	14 ± 1	10 ± 3	–	–
Cotyledon	6 ± 3	5 ± 1	–	–
Stem	–	2 ± 1	3 ± 2	–
Petiole	0	0	0	–
Leaf	–	0	0	–
Flower bud	–	–	0	–
Root	0	0	0	–

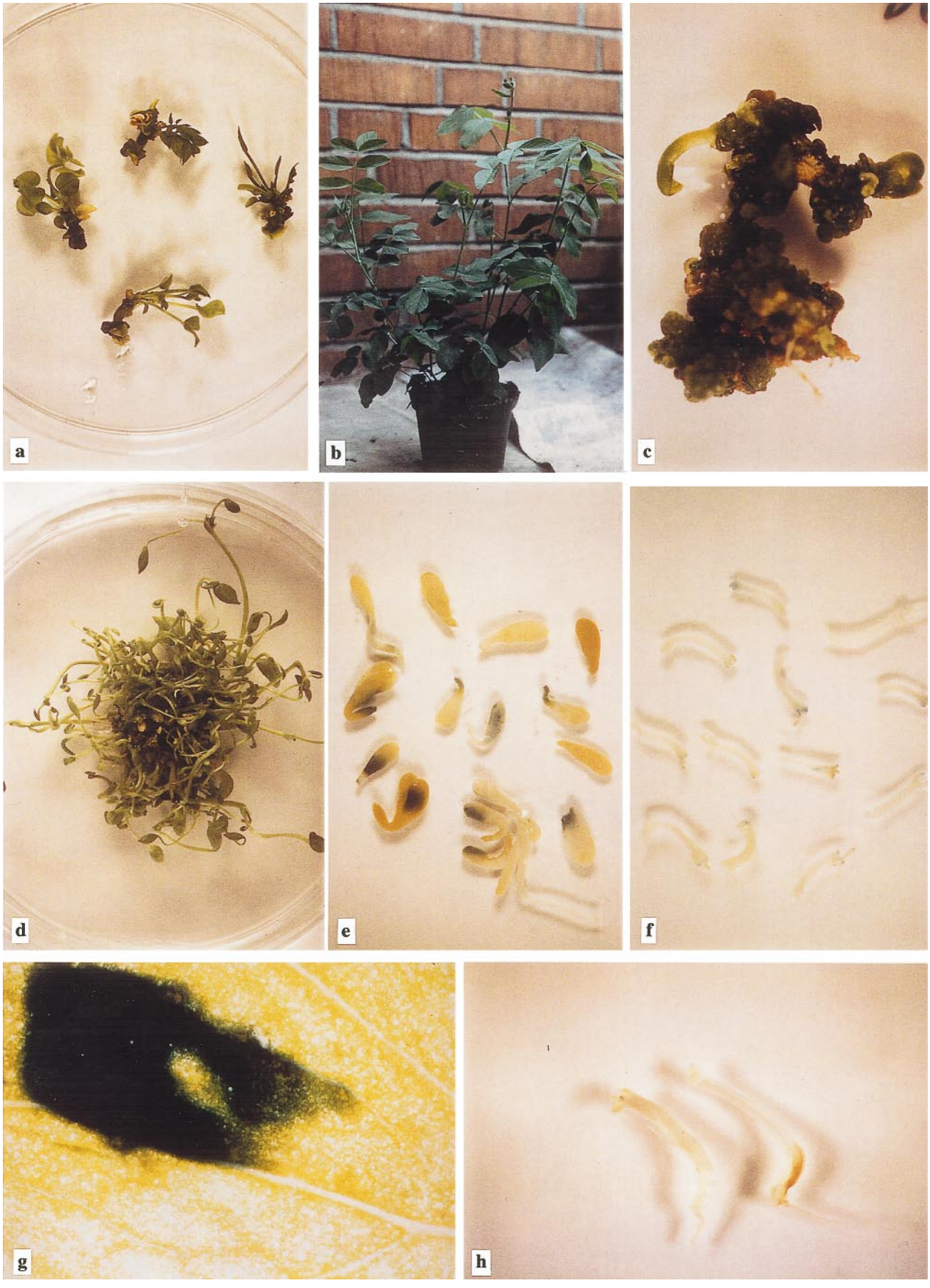
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Fig. 1 **a** Direct shoot induction from nodes from greenhouse-grown plants on B5 medium without hormones. **b** Regenerated *Galega* plant. **c** Shoot induction from callus on shoot development medium with 5.0 μm TDZ, 0.5 μm IAA and 10% coconut water. Green nodular callus with multiple shoot formation from yellowish callus induced from an immature embryo. **d** Shoot elongation obtained with 1.4 μm gibberellic acid. **e** Transient *GUS* expression from mature embryos after particle bombardment. **f** Transient *GUS* expression from seedlings after transformation with *Agrobacterium tumefaciens* strain AGL1. **g** Chimeric *GUS* expression in leaf of a shoot initiated after electroporation. **h** Transient *GUS* expression from an electroporated seedling (*left*) and control (*right*)

Media

All tissue types showed the same general response to the different media. Ordinary B5 medium gave the best growth and shoot induction, followed by B5 medium supplemented with casein. The lowest growth rate was obtained with PC-L2 medium. No increase in either the frequency of shoot-inducing calli (79%) or the number of induced shoots (2.5) was obtained by adding any combination of hormones to the media (Table 2). The pulse treatment with high cytokinin concentrations gave a negative response, with only one induced shoot per incubation (4/40). In conclusion, young seedlings with cotyledons removed cultured on B5 media without any supplements gave the best results in terms of direct shoot induction.

General growth conditions

Using B5 media supplemented with 5.4 μm NAA resulted in rooting of 70 ± 6% of the shoots. Rooting on B5 medium supplemented with 2.7 μm NAA or without any NAA could be obtained, but at a lower rate. All of the in-vitro-regenerated plants transferred to the greenhouse survived. Regenerated plants were phenotypically normal and no difference in nodulation or fertility was observed (Fig. 1b). The whole procedure took 2–3 months from start until a regenerated plant could be transferred to the greenhouse.

Callus induction

Explants

All tested explants showed swelling as well as early signs of callus formation at the cut regions within the first weeks of culture. The damaged tissue gave a faster growth response, as reported in clover (Beattie and Garrett 1995). Shoot induction was mainly obtained from callus initiated either from meristems or from mature or immature embryos. Among these, immature embryos gave the highest frequency of shoots, with the apical meristem showing the best results. Calli induced from cotyledons or roots from the embryo were always inferior in terms of shoot induction. Table 3 shows the results obtained with calli induced from embryonic tissue.

Table 2 Comparison of different hormone concentrations and combinations in B5 media. Nodes from 1-month-old in-vitro-grown plants were used

Hormones	Shoot-inducing calli (% ± SD)	Number of shoots per callus ± SD
9.1 μm zeatin and 1.0 μm IAA	64 ± 6	2.0 ± 0.1
8.9 μm BAP	30 ± 8	1.6 ± 0.1
8.9 μm BAP and 1.0 μm IAA	50 ± 2	2.1 ± 0.4
No hormones	80 ± 4	2.5 ± 0.1

Table 3 Shoot induction from apical meristems from embryos on B5 medium supplemented with 30 g l^{-1} sucrose, 5.0 μm TDZ, 0.5 μm IAA and 10% coconut water

Hormones during callus phase	Immature embryos		Mature embryos	
	Shoot-inducing calli (% ± SD)	Number of shoots per callus ± SD	Shoot-inducing calli (% ± SD)	Number of shoots per callus ± SD
9.0 μm 2,4-D 4.4 μm BAP	60 ± 14	28 ± 10	40 ± 12	14 ± 8
18.0 μm 2,4-D 4.4 μm BAP	28 ± 10	8 ± 3	–	–
9.0 μm 2,4-D 8.8 μm BAP	37 ± 8	11 ± 4	–	–

Media

During callus induction, no significant differences were observed among the different hormone concentrations. At the end of the first subculture, 77 ± 9% of all the explants had produced yellowish calli regardless of hormone concentration, although the morphology differed between the concentrations. No shoots were induced by any of the tried hormone combinations. However, after transfer to the media containing TDZ and to light, differences in the frequency of shoot induction could be observed among the cultures, depending on the medium on which the calli had been induced (Table 3). Calli induced with 9.0 μm 2,4-D and 4.4 μm BAP became dark green and showed multiple shoot bud induction, starting after the first week (Fig. 1c). Calli induced on this medium gave a shoot induction of 60% ± 14. On average, 28 ± 16 shoots, but sometimes up to 60 shoots, could be induced from the same embryo. Shoots were also induced on calli from other media when transferred to the TDZ-containing medium, although after a longer time and at lower frequency. During the whole process, frequent transfers were important to prevent blackening of the tissue.

TDZ is reported to be a new strong shoot inducer for different legumes, including pea, chickpea, lentil (Malik and Saxena 1992), white clover (Beattie and Garrett 1995) and tepary bean (Dillen et al. 1996) and has now also been shown to give high shoot induction in *Galega*.

General growth conditions

The majority of the developed shoots were phenotypically normal and could be easily elongate with 1.4 μM gibberillic acid (Fig. 1d). No albino shoots were observed. However, a few shoots appeared abnormal and had a lower rooting frequency. With the normal-looking shoots also, rooting took longer and occurred at a lower frequency compared to shoots induced directly. However, in this case, rooting could be improved using Gelrite as a solidifying agent instead of plant agar. Using this protocol, it took 6 months to transfer a regenerated plant to the greenhouse.

Particle bombardment

Ten percent (11/100) of the mature embryos bombarded with tungsten particles coated with the plasmid pMOG 410 were scored as GUS positive (Fig. 1e).

Agrobacterium transformation

Comparing the four different *A. tumefaciens* strains, the hypervirulent AGL1 strain gave the best results, with a third of the explants showing transient expression (Fig. 1f). The results were reproducible. After 4 months on selection media, 3 shoots out of 13 (infected with LBA 4404) still showed chimeric *GUS* expression.

Electroporation

The best results were obtained by electroporating apical meristems from 8-week-old greenhouse-grown plants. All shoots that developed after the treatment were assayed 35 days after electroporation. A third (13/40) of the obtained shoots showed a chimeric positive GUS staining (Fig. 1g). The survival rate of the meristems was 85%, similar to previous results with pea (Chowrira et al. 1996). Using 1-week-old in-vitro-grown plants, cut as in *Agrobacterium* transformation experiments, 7 out of 50 showed at least one site of *GUS* expression (Fig. 1h), while only 1 out of 40 mature embryos showed strong transient expression.

Comparison of transformation methods

For a transformation system that can be used for practical applications, several questions need to be addressed. How will the in vitro culture and transformation methods affect the original plant material, e.g. in terms of induced somaclonal variation? Equally important is probably the subsequent behaviour of the introduced trait: how will it be transmitted through the generations, where in the plant genome has it been introduced, as this will influence the risk of gene silencing, how many copies of the transferred gene are likely to have been introduced? Here we have described different methods for plant regeneration and transformation. Although all three tested transformation methods were capable of introducing new genes into the cell nucleus of the plant material, there are several aspects that need to be taken into consideration in choosing a combination of plant regeneration system and transformation method.

Particle bombardment was shown to be applicable for transformation of *G. orientalis*. Particle bombardment in combination with the highly efficient shoot induction from callus with TDZ might be a promising approach for *Galega* transformation. The drawback of this approach is the need for a tissue culture stage. Furthermore, it is possible that with particle bombardment, more unstable insertions may occur, as insertions mainly take place by random cross-over (Christou 1997).

The most recent transformation methods developed for legumes rely on transformation being carried out in planta so that tissue culture is minimized or avoided (Atkins and Smith 1997). Here we have described two different methods using the intrinsic regeneration capability of specific plant organs: nodal tissue which could be transformed by *Agrobacterium* cocultivation, and apical meristems into which new genetic material could be introduced by electroporation.

Three chimeric shoots were obtained from nodal tissue cocultivated with *A. tumefaciens* strain LB 4404. In this first experiment, the transformation efficiency was, however, quite low as judged by transient expression. Later experiments showed another strain, AGL1, to give a much better result, with a third of the explants showing transient expression. The possibility of using *A. tumefaciens* cocultivation combined with direct shoot induction from nodal tissue without any tissue culture step is very interesting. The more directed and stable method for introduction of a genetic trait is probably transformation using the Ti-plasmid of *A. tumefaciens*. The Ti-plasmid is known to have a preferential mechanism of insertion, which will probably also affect in which region of the genome insertion will occur (e.g. Risseuw et al. 1997; Zupan and Zambryski 1997).

Transformed chimeric shoots were also obtained by electroporation of meristems on whole plants. Here too, a tissue culture stage is avoided. The transforma-

tion rate corresponds to previously published electroporation experiments with different legumes (Chowrira et al. 1995, 1996). Various legumes have been transformed with electroporation of intact tissue (Dillen et al. 1995; Chowrira et al. 1995, 1996). The disadvantages with electroporation of whole plants are that it requires high concentrations of DNA (600 μg pMOG410 were used for 40 plants) and that chimeric transformants are obtained in the first generation. To obtain complete transformants, the seeds from the chimeric plants have to be selected on selection media. Electroporation of whole plant tissue is a very new method, and might result in unstable patterns of inheritance of the new trait, given that electroporation of protoplasts is known to result in a high frequency of multicopy insertions (e.g. Jarl and Rietveld 1996).

Acknowledgements This research was supported by The Nordic Joint Committee for Agricultural Research. We thank R.A. Ludwig, USA, for the strain AGL1. We also want to thank MOGEN, Holland for the strain MOG 301, EHA 105 and plasmid pMOG 410. I. de Jong is gratefully thanked for providing excellent technical assistance.

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