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Agrobacterium-Mediated Transformation of Subterranean Clover (*Trifolium subterraneum* L.)[1]

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We have developed a rapid and reproducible transformation system for subterranean clover (*Trifolium subterraneum* L.) using *Agrobacterium tumefaciens*-mediated gene delivery. Hypocotyl segments from seeds that had been allowed to imbibe were used as explants, and regeneration was achieved via organogenesis. Glucose and acetosyringone were required in the co-cultivation medium for efficient gene transfer. DNA constructs containing four genes encoding the enzymes phosphinothricin acetyl transferase, β-glucuronidase (GUS), neomycin phosphotransferase, and an α-amylase inhibitor were used to transform subterranean clover. Transgenic shoots were selected on a medium containing 50 mg/L of phosphinothricin. Four commercial cultivars of subterranean clover (representing all three subspecies) have been successfully transformed. Southern analysis revealed the integration of T-DNA into the subterranean clover genome. The expression of the introduced genes has been confirmed by enzyme assays and northern blot analyses. Transformed plants grown in the glasshouse showed resistance to the herbicide Basta at applications equal to or higher than rates recommended for killing subterranean clover in field conditions. In plants grown from the selfed seeds of the primary transformants, the newly acquired gene encoding GUS segregated as a dominant Mendelian trait.

Subterranean clover (*Trifolium subterraneum* L., subclover) is native to the Mediterranean regions and is grown as a pasture legume mainly in Australia and the countries around the Mediterranean. To a lesser extent, it is also grown in the Americas, Africa, New Zealand, and Japan (Johnstone and McLean, 1987). In Australia, subterranean clover is the major pasture legume and is grown on more than 16 million ha of mainly acidic and infertile lands. As a pasture crop and because of its ability to fix nitrogen, subterranean clover is a major contributor to the productivity of the meat, wool, dairy, and wheat industries in Australia (Johnstone and McLean, 1987). It also has the potential to be grown in other temperate regions of the world.

A number of grain and pasture legumes are now amenable to gene transfer by genetic engineering using the *Agrobacterium*-mediated gene delivery system. These include soybean (Hinchee et al., 1988), pea (Puonti-Kaerlas et al., 1990; Schroeder et al., 1993), *Stylosanthes* (Manners, 1988), *Medicago sativa* (Deak et al., 1986; Schroeder et al., 1991), *Lotus corniculatus* (Webb et al., 1990), and white clover (White and Greenwood, 1987). To date, subterranean clover has not been reported as being amenable to infection by *Agrobacterium* spp., and no gene transfer experiments on this species have been reported. Because of the importance of subterranean clover in Australian agriculture, we set out to develop a gene transfer system for this clover so that useful genes can be introduced by genetic engineering techniques. A regeneration system for subterranean clover has recently been developed (Heath et al., 1993) using hypocotyl tissues from germinating seeds. We have adapted this procedure and used it in conjunction with a disarmed strain of *Agrobacterium tumefaciens* to deliver four genes to subterranean clover. This is the first report of an efficient transformation and regeneration system for subterranean clover. It produces transgenic plants, ready for transfer to the glasshouse within 16 weeks.

MATERIALS AND METHODS

Agrobacterium Strain and Vector Plasmids

Agrobacterium tumefaciens strain AGL1 (Lazo et al., 1991) containing a disarmed Ti plasmid was transformed with the binary plasmid pMCP3, which contained four chimeric genes within the T-DNA (Fig. 1). The *uidA* gene (Jefferson et al., 1986) encoding GUS was reconstructed to contain a 5′ upstream region (nucleotides 6909–7437) from the CaMV gene encoding the 35S RNA (Pietrzak et al., 1986). The 3′ flanking DNA sequence was also derived from CaMV (nucleotides 7439–7632; Pietrzak et al., 1986). The *bar* gene (De Block et al., 1987) encoding PAT was as described by Jones et al. (1992) with a 5′ upstream region derived from the 35S RNA gene of CaMV and a 3′ region from the octopine synthase gene. The *nptII* gene encoding NPTII contained 5′ and 3′ regions derived from the nopaline synthase gene of *A. tumefaciens* and its construction has been described by An et al. (1985). The gene encoding α-AI (Moreno and Chrispeels, 1989) from the seeds of *Phaseolus vulgaris* was modified to contain a 5′ upstream and a 3′ region derived from the phytohemagglutinin gene (Altabella and Chrispeels, 1990). Initial experiments involved the use of the binary plasmid pKIWI 105, which contained an *nptII* gene driven by the

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Abbreviations: α-AI, α-amylase inhibitor; CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; IBA, indolebutyric acid; NAA, naphthalene acetic acid; NPTII, neomycin phosphotransferase II; PAT, phosphinothricin acetyl transferase; PPT, phosphinothricin; TDZ, thidiazuron.

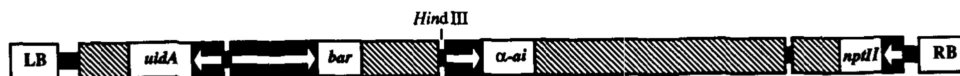

Figure 1. Schematic diagram of the four genes inserted between the left and right borders of the plant transformation vector, pMCP3. The *uidA* gene was flanked by a 5′ and 3′ region of CaMV 35S RNA gene (total length, 2.5 kb), the *bar* gene was controlled by the CaMV 35S promoter and flanked by an octopine synthase 3′ end (total length, 2.7 kb), the *α-ai* gene was flanked by 5′ and 3′ ends of the phytohemagglutinin gene (total length, 4.7 kb), and the *nptII* gene was flanked by 5′ and 3′ ends of the nopaline synthase gene (total length, 2.2 kb). The components of this four-gene construct are drawn to scale. The single *Hin*dIII restriction enzyme site, located between the left (LB) and right (RB) borders, is shown.

nopaline synthase promoter and the *uidA* gene driven by a CaMV 35S promoter (Janssen and Gardner, 1989).

Source and Preparation of Explants

Seeds of various cultivars of subterranean clover were obtained from the Western Australian Department of Agriculture and from Cleanseeds Pty Ltd. (Bungendore, New South Wales, Australia). Seeds were surface sterilized by immersing in water for 2 min, 70% ethanol for 5 min, and 5% sodium hypochlorite for 20 min, followed by washing with sterile water six to seven times. The seeds were then allowed to imbibe overnight in water at 16°C in the dark. The lower hypocotyl together with the radicle (length approximately 2–3 mm, hereafter called the primary explant) from seeds that had been allowed to imbibe (Fig. 2A) was excised with the aid of a dissecting microscope.

Infection of Explants with *Agrobacterium* and Regeneration of Plantlets

Stocks of *A. tumefaciens* (AGL1) harboring a binary plasmid were grown in Luria broth (1% Bacto tryptone, 1% NaCl, 0.5% Bacto yeast extract [pH 7.5]) with 20 μg/mL rifampicin to maximum cell density and diluted with an equal volume of 30% glycerol before freezing at −80°C. Working cultures were made by growing the *Agrobacterium* in modified Luria broth medium without antibiotics and in which the NaCl was reduced to 0.1%, and 10 mM D-Glc and 20 μM acetosyringone were added. The bacteria were grown for 24 h at 28°C, with shaking, to a concentration of 2×10^9 to 5×10^9 cells/mL and used for inoculation of the primary explants. After an inoculation period of 30 to 40 min, the primary explants were transferred to the co-cultivation medium consisting of L2 minerals, organic A (Phillips and Collins, 1984), iron (Murashige and Skoog, 1962), and 3% Suc. pH was adjusted to 5.8 before agar was added (0.8%, BBL obtained from Becton Dickinson, Cockeysville, MD) and prior to autoclaving. The medium was supplemented with 2.5 μM TDZ (Schering, Berlin, Germany), 1.6 μM NAA, 10 mM Glc, and 20 μM acetosyringone, all from filter-sterilized stocks. The primary explants were co-cultivated with *Agrobacterium* by inserting the root pole of the explants in an upright position in the agar. A co-cultivation period of 7 d was used routinely. All subsequent tissue culture steps were at 16 h/8 h light/dark cycles with a photon fluence rate of 100 to 200 μE m^{-2} s^{-1} from Phillips cool-white fluorescent light. The day and night temperatures were 25 and 20°C, respectively. After co-cultivation for 7 d, primary explants, now approximately 12 mm in length, were washed with cefotaxime (500 mg/L) and cut 1 to 2 mm below the first cut, and the root segment was discarded. These secondary explants, called hypocotyl segments, were transferred with the newly cut surface in contact with the regeneration medium consisting of L2 minerals and organic A (Phillips and Collins, 1984), iron (Murashige and Skoog, 1962), 3% Suc, 0.8% agar (pH 5.8), 2.5 μM TDZ, 1.6 μM NAA supplemented with 50 mg/L of PPT from a technical formulation of glufosinate ammonium (Hoechst, Melbourne, Australia), and 200 mg/L cefotaxime. Surviving explants were transferred to fresh medium every 3 weeks.

After 60 to 70 d, vigorous shoots derived from the hypocotyl segments grown with selection on regeneration medium were excised and dipped for 60 s in a solution of IBA (1 mg/mL). The shoots were transferred to L2 basal medium (Phillips and Collins, 1984) without selection but supplemented with 3 mg/L IBA. Roots developed in 2 to 3 weeks and were allowed to grow for a further 1 to 2 weeks.

Transfer to the Glasshouse and Selection of Plants for Analysis

Rooted plantlets were transplanted into potting mix in the glasshouse. The mix consisted of recycled soil:leaf mulch:perlite:vermiculite in a ratio of 6:1:1:1 (base mix). To each 4200 L of base mix was added 6 kg of Aboska (N:P:K = 15.7:8:6.2), 2 kg of dolomite, 2 kg of calcium carbonate, and 20 L of blood and bone, and the pH was adjusted to 6.5 with more calcium carbonate if necessary. The plants were kept covered with plastic jars for 5 to 7 d under shade cloth and slowly acclimatized to the glasshouse environment over 2 weeks. The plantlets were maintained at 27 and 20°C during the day and night, respectively. One untransformed plant was used as a negative control, and 10 of the putative transformants were randomly selected for analysis. They belonged to four different cultivars representing three different subspecies of subterranean clover, namely cv Larisa, ssp. *yanninicum* (regenerants 3, 5, 8, 9, and 10); cv Rosedale, ssp. *brachycalycinum* (regenerants 1, 4, and 6); cv Junee, ssp. *subterraneum* (regenerant 2); and cv Trikkala, ssp. *yanninicum* (regenerant 7).

Enzyme Assays

PAT activity was assayed using the procedure of Spencer et al. (1990) with slight modification. Leaf tissue (100 mg from glasshouse-grown plants) was extracted with 800 μL of

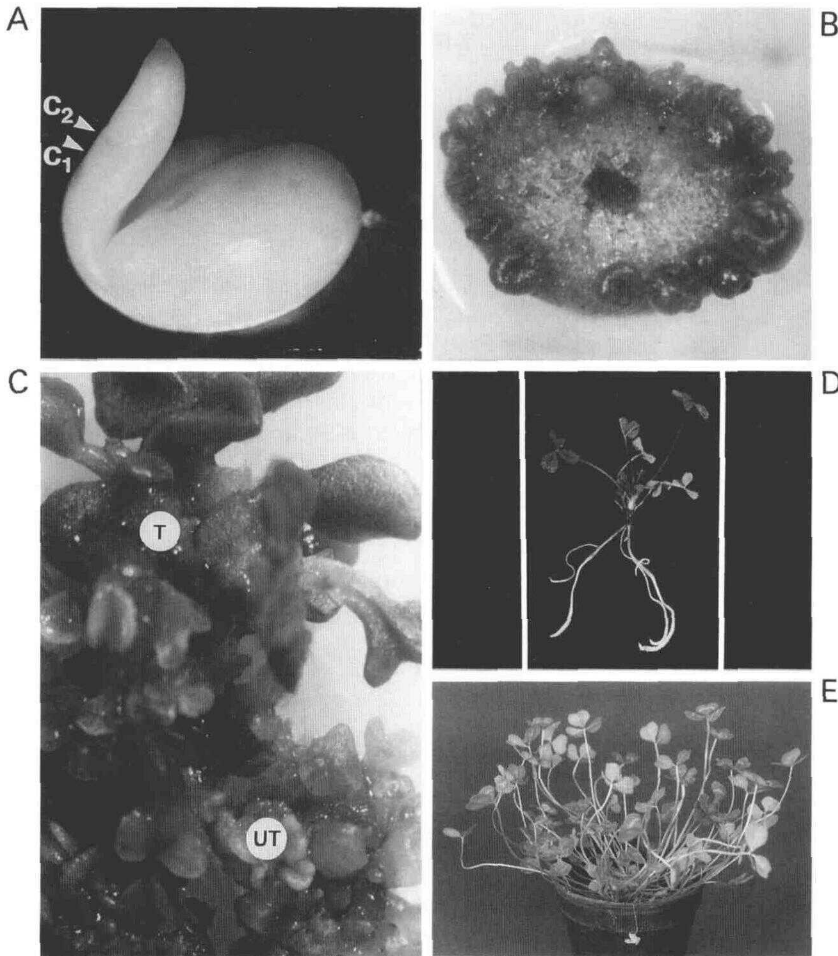

Figure 2. Regeneration of transgenic subterranean clover plants. A, Seeds were allowed to imbibe overnight in water, and the seed coats were removed. The hypocotyls were cut at position C_1 just before inoculation with *A. tumefaciens*. The lower hypocotyl plus radicle (approximately 2–3 mm long) was co-cultivated with the bacteria for 7 d. A second cut was made at position C_2, and the segment between C_1 and C_2 (approximately 2 mm long) was transferred to the regeneration medium containing PPT as described in "Materials and Methods." B, Shoot primordia emerged from the peripheral cells of hypocotyl segments within 14 d. C, Transformed (T) shoots, resistant to PPT in the selection medium, grew vigorously, whereas the untransformed (UT) shoots remained weak and died within 60 d. D, A transformed plantlet ready for transfer to the glasshouse. E, A transgenic subterranean clover plant 6 weeks after transfer to the glasshouse.

extraction buffer (100 mM Na-phosphate [pH 7.0], 20 mM NaCl, and 1.0 mM PMSF). The protein concentration in the extract was determined by the Bradford (1976) assay. The PAT enzyme reaction mixture (18 μL final volume) contained 30 μg of leaf protein, 3 μL of PPT stock (prepared by diluting 5 μL of technical grade glufosinate ammonium [2.4 M PPT] in 1 mL of extraction buffer), and 4 μL (0.02 μCi/μL) of [^{14}C]acetyl CoA (50–60 mCi/mmol; Amersham) and was incubated at 37°C for 30 min. The reaction mixture was centrifuged at 10,000*g* for 30 s, and 15 μL of the supernatant was spotted onto a thin-layer silica gel 60 plate (0.2 mm; E. Merck) and chromatographed in a 3:2 (v/v) mixture of 1-propanol and NH_4OH (28% NH_3). Plates were dipped in enhancer (0.4% 2,5-diphenyloxazole in 1-methyl-naphthalene) solution and air dried, and [^{14}C]acetylated PPT was detected by fluorography. GUS activity was determined by the procedure of Jefferson (1987), except for the addition of 20% methanol to minimize any endogenous blue coloration (Kosugi et al., 1990) in subterranean clover extracts. The NPTII enzyme assay was carried out following the procedure of McDonnell et al. (1987).

RNA Isolation and Northern Blot Analysis

Total RNA was isolated according to the method of Chandler et al.(1983), and northern blot analyses were as described previously (Higgins and Spencer, 1991). In brief, 200 mg of young leaf tissue were ground to powder with liquid nitrogen and extracted with 600 μL of extraction buffer and 800 μL of phenol:chloroform:isoamyl alcohol (25:24:1). RNA was precipitated with 2 M LiCl and reprecipitated with 2 volumes of ethanol. The *nptII* probe (approximately 1.9 kb of *Bam*HI-*Hin*dIII fragment) was obtained from pGA492 (An, 1986). The *uidA* probe (534 bp of *Hin*cII fragment) was isolated from pRAJ275 (Clontech Laboratories, Palo Alto, CA). The *bar* fragment used as a probe was made by using the PCR to amplify the *bar*-coding sequence between +223 and +533 (De Block et al., 1987) using the oligonucleotide primers: 5′-TGC ACC ATC GTC AAC CAC TA-3′ and 5′-ACA GCG ACC ACG CTC TTG AA-3′.

The *bar, uidA,* and *nptII* probes were labeled with [^{32}P]-dCTP using the Megaprime DNA-labeling system (Amersham) following the supplier's instructions.

Genomic DNA Isolation and Southern Blotting

DNA was isolated from young leaf tissue taken from glasshouse-grown plants (Sutton, 1974). The DNA was treated with RNase followed by proteinase K. The purified DNA was digested with the restriction enzyme *Hin*dIII. The *bar* probe (described in "RNA Isolation and Northern Blot Analysis") was also used for the Southern hybridization.

Hybridization was done as described by Southern (1975), using the hybridization buffer described by Khandjian (1987).

α-AI Immunoblot Analysis

Protein (70 μg) from the seeds of putative transformants was fractionated by SDS-PAGE and transferred to nitrocellulose membranes (Higgins and Spencer, 1991). The α-AI was detected with a rabbit antibody to the protein (kindly supplied by Dr. M.J. Chrispeels). The second antibody was goat anti-rabbit IgG coupled to alkaline phosphatase (Promega).

Herbicide Tolerance in Transgenic Plants

The upper surfaces of five fully expanded leaflets of plants grown in the glasshouse were thoroughly wetted by painting with Basta (200 g/L of glufosinate ammonium; Hoechst) solution containing 2, 20, 100, 200, 600, 1200, or 2000 mg/L of PPT. Tolerance to PPT was scored 7 d after treatment of leaflets.

RESULTS

Development of Transformation and Regeneration Procedures

The regeneration system for subterranean clover reported earlier (Heath et al., 1993) has been modified by replacing the phytohormone benzylaminopurine with TDZ because the latter was found consistently to induce a higher number of adventitious shoots from hypocotyl explants, thus enabling the production of more shoots in selection medium. Adventitious shoots were formed on the periphery of hypocotyl segments grown on the regeneration medium (Fig. 2B).

Initially, pKIWI 105, a binary plasmid containing 35S *uidA* (Janssen and Gardner, 1989), was used to optimize the conditions of gene delivery by monitoring the transient expression of the *uidA* gene. When the explant tissue was totally immersed in the bacterial suspension during co-cultivation, there was no growth of primary explants, and no transient expression of GUS could be detected. When the explant tissue was co-cultivated on agar medium with the root poles embedded in the medium without any hormone (as described in "Materials and Methods"), there was active growth of the explant but very little evidence of expression of the *uidA* gene. However, when this was done in the presence of TDZ and NAA, there were many GUS-positive regions, mainly in the peripheral area of the cut surfaces of the hypocotyls. Co-cultivation was carried out with root poles embedded in agar for 1, 3, 5, and 7 d, after which the explants were washed with cefotaxime and the upper 2 mm were transferred to regeneration medium containing cefotaxime. GUS assays were carried out after a further 4 to 5 d of incubation. GUS expression increased progressively with increasing time of co-cultivation (data not shown), and a 7-d co-cultivation period was therefore used routinely for transformation.

The *nptII* gene in pKIWI 105 did not confer sufficient resistance to kanamycin to allow this antibiotic to be used as a selectable marker (data not shown). However, when explants were infected with *Agrobacterium* containing pMCP3 and transferred to regeneration and selection media containing the herbicide PPT, shoot primordia formed within 1 week from the peripheral regions of the infected hypocotyl explants (Fig. 2B). PPT-resistant shoots remained dark green with thick leaves and were healthy in appearance (Fig. 2C), whereas the untransformed shoots either died or remained pale green and grew slowly under selection. The optimal level of PPT was 50 mg/L for selection of transformed shoots. Explants on 100 mg/L died without any shoot production, whereas on 10 and 30 mg/L PPT many untransformed shoots were able to grow. Between 0.5 and 1% of the hypocotyl explants gave rise to transformed shoots without Glc or acetosyringone in the co-cultivation medium. The transformation frequency was increased to 4% when both Glc (10 mM) and acetosyringone (20 μM) were included in the co-cultivation medium.

Rooting of Transformed Shoots

Shoots formed in the presence of PPT (50 mg/L) were excised, and the cut surfaces were dipped briefly in concentrated IBA (1 mg/mL). Almost all shoots treated in this way formed roots within 2 to 3 weeks. Without this pulse of high auxin treatment, root formation required 6 to 9 weeks. Plantlets were transferred to the glasshouse 1 to 2 weeks after the initiation of roots.

Expression of the *bar* Gene

The presence of the *bar* transgene was demonstrated by Southern hybridization analysis. Genomic DNA was isolated from 9 of 10 regenerated plants and digested with the restriction enzyme *Hin*dIII. Because the *bar* gene lies between the *Hin*dIII site in the T-DNA and the right border (Fig. 1), any fragment detected by the *bar* probe should result from cutting at both the internal *Hin*dIII site and another *Hin*dIII site in the adjacent genomic DNA. In 8 of the 9 regenerated plants, fragments greater than 6.5 kb in length were detected by the *bar* probe, demonstrating not only the presence of the *bar* gene but also its integration into the genomic DNA of subterranean clover (Fig. 3). Plant 1 was negative for the foreign gene and presumably represents an escape from the selection screen. One transgenic plant (plant 5) appeared to contain three copies of the transgene, and the remainder had only one copy (Fig. 3).

To confirm the expression of the *bar* gene in transformed plants, leaves of 10 glasshouse-grown plants were tested for the presence of PAT mRNA and enzyme activity. Six of the 10 plants were positive for PAT mRNA (Fig. 4A). The message level appeared to vary widely, and all transformants that produced detectable mRNA also produced PAT enzyme (Fig. 4B). The level of PAT activity correlated in general with the levels of its mRNA. However, three transformants (plants 5, 8, and 9) showed low PAT activity, although the mRNA was below the level of detection. Plant 4, which was not analyzed by Southern blotting because of poor DNA recovery, was positive for PAT mRNA and PAT enzyme. Therefore, by including plant 4 with the 8 positive plants analyzed by Southern blotting, 9 confirmed transgenic plants were recovered from 10 selected plants.

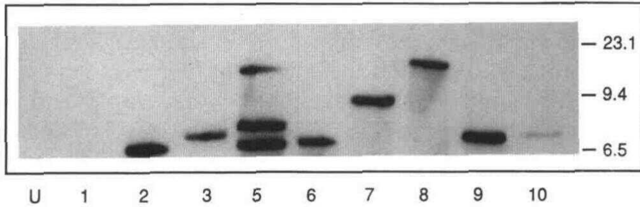

Figure 3. Southern blot analysis of DNA from 9 subterranean clover plants regenerated on medium containing PPT. Genomic DNA was digested with *Hin*dIII and probed with a fragment of the *bar* gene. DNA fragments extending from a *Hin*dIII site in the T-DNA of pMCP3 to an adjacent site in plant DNA were detected in 8 of the 9 regenerants. Because of the position of the single *Hin*dIII site in pMCP3, *Hin*dIII digestion of the DNA yielded one positive fragment from each copy of the *bar* gene inserted into the genomic DNA. The numbers on the *y* axis refer to DNA size markers (in kb) derived from *Hin*dIII digestion of λ DNA. Each lane contained 10 μg of DNA, except lane 10, which contained 2 μg. Lanes 1 to 10 contained DNA from regenerated plants 1 to 10, whereas lane U contained DNA from an untransformed plant.

The Tolerance of Transformed Plants to Basta in the Glasshouse

To determine whether the level of PAT activity in transformed subterranean clover was sufficient to confer resistance to the herbicide Basta, leaflets of untransformed and transformed plants were tested for tolerance to doses up to 2000 mg/L PPT. The leaves of untransformed plants were killed by PPT at 2 mg/L, whereas the leaves of various transformed plants survived PPT levels between 200 and 2000 mg/L (Fig. 4C). After 7 d, the treated leaflets from the untransformed plants were necrotic and dry, whereas those from transformed plants were either unaffected or showed only localized dead cell areas near the periphery of treated leaves. Treatment with 360 to 600 mg/L PPT corresponds approximately to the rates of application of Basta recommended by the manufacturer for killing subterranean clover in the field. There was a close correlation between the level of tolerance to Basta and the levels of PAT enzyme activity (Fig. 4).

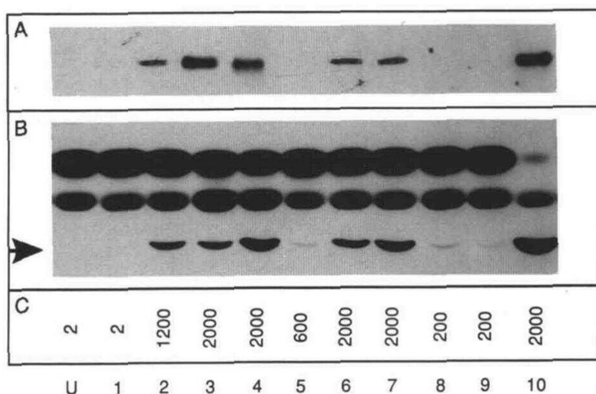

Figure 4. Expression of *bar* gene in putative transformants. A, Northern blot showing presence of PAT mRNA in young leaves of regenerated plants. B, An autoradiograph showing PAT activity in young leaves. The position of the acetylated product of the PAT reaction is indicated by the arrow. C, The herbicide tolerance level of the regenerated plants was tested by painting leaves with Basta. The figures represent the lowest level of the herbicide (PPT in μg/mL) that caused necrosis of the leaves; a visual assessment of damage was used. See the legend to Figure 3 for an explanation of lane designations.

Expression of the *uidA* and *nptII* Genes

Leaves from the 10 regenerated plants were also analyzed for the presence of GUS mRNA by northern blotting. Six of the 10 plants were positive for GUS mRNA, with a wide range of variation in levels (Fig. 5A). The GUS histochemical assay showed that those plants that were positive for mRNA were also positive for GUS enzyme. The intensity of blue coloration in the six plants varied in the different organs and with age (data not shown). The young leaves were deep blue throughout, whereas the mature leaves were blue predominantly near the vascular bundles. Roots, petiole, stem, and floral parts (calyx, corolla, androecium, and gynoecium) were also blue, with intense color near the vascular bundles. The cells of mature organs showed weak GUS activity. The *nptII* mRNA and protein were below the level of detection in all but one (plant 4) of the MCP3 transformants (data not shown).

Expression of a Gene Encoding the Bean α-AI

Protein extracted from seeds of the 10 primary transgenic plants was assayed by western blotting for the presence of α-AI. Seeds from all of the transformants (except plant 1) contained α-AI, and the expected low mol wt polypeptides were detected (Fig. 6). Therefore, unlike the *uidA*, *bar*, or *nptII* genes, the *α-ai* gene was expressed in all 9 of the confirmed transgenic plants.

Segregation of Transgenes in T_1 Progeny

Primary transgenic plants grown in the glasshouse were allowed to form seeds after self-pollination. Seeds from 4 of

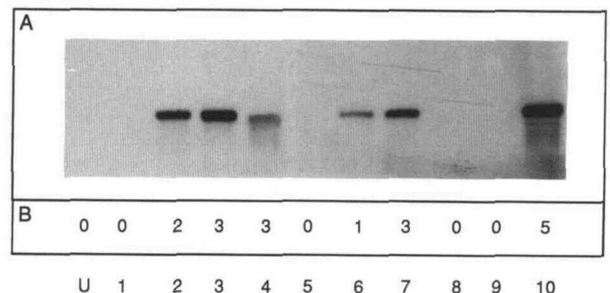

Figure 5. Expression of the *uidA* gene in young leaves of regenerated plants. A, Northern blot showing GUS mRNA in the transgenic plants. Each lane was loaded with 5 μg of total RNA. B, The intensity of GUS color was assayed following histochemical staining using a visual score. A scale of 0 to 5 was used: 0, no color; 5, most intense blue color. See the legend to Figure 3 for an explanation of lane designations.

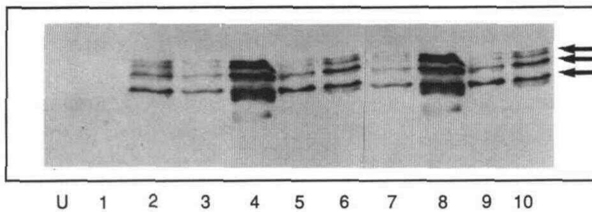

Figure 6. Expression of the gene encoding α-AI in the seeds derived by selfing 10 primary regenerants. Seeds were collected from glasshouse-grown plants and α-AI was detected in seed extracts by western blot analysis. Arrows indicate the positions of processed α-AI from bean. They range in size from 15 to 18 kD (Altabella and Chrispeels, 1990). See the legend to Figure 3 for an explanation of lane designations.

the 10 primary regenerants were germinated, and histochemical GUS assays were carried out on the seedlings. The observed ratios of GUS-positive to GUS-negative seedlings fitted a 3:1 segregation ratio (Table I), indicating that the GUS gene was transmitted to the offspring as a dominant Mendelian trait at a single locus.

DISCUSSION

We have developed a rapid and reproducible transformation system for subterranean clover that permits the introduction of foreign genes into a wide range of cultivars. In this system we made use of *A. tumefaciens* for gene delivery, and the *bar* gene, which confers resistance to the herbicide Basta (PPT), as the selectable marker. PPT is an inhibitor of Gln synthase in plants (Eckes et al., 1989; Krieg et al., 1990) and is the active ingredient in the nonselective herbicide Basta. The *bar* gene encodes the enzyme PAT, which catalyzes the conversion of PPT into a nontoxic acetylated product (De Block et al., 1987). In vitro selection for resistance to Basta has proved to be an efficient method for the transformation of subterranean clover, whereas selection on kanamycin was unsuccessful (results not shown). PPT has also been proved to be suitable for selection of transformed wheat (Vasil et al., 1992), *Atropa belladonna* (Saito et al., 1992), rice (Rathore et al., 1993), maize (Fromm et al., 1990), alfalfa (D'Halluin et al., 1990), tobacco (Dröge et al., 1992), and pea (Schroeder et al., 1993).

The conditions that were important in the production of transformed subterranean clover plants were the explant source, the use of acetosyringone, TDZ, and NAA in the co-cultivation medium, and the amount of PPT used for selection. It was important to take the hypocotyl explants from seeds that had imbibed for 16 to 20 h only. Explants taken from seeds that had imbibed for 2 d or more showed a much reduced capacity for regeneration.

The presence of growth regulators in the co-cultivation medium enhanced the recovery of transformed shoots. Peripheral cells at the cut surface of subterranean clover hypocotyls are capable of proliferation when grown in the regeneration medium containing plant hormones and may be more susceptible to infection by *Agrobacterium* as suggested by the data of Braun (1975) and Sangwan et al. (1992).

Glc and acetosyringone are known to act synergistically as virulence-inducing agents during infection by *Agrobacterium* (Cangelosi et al., 1990; Shimoda et al., 1990). The presence of these agents in the co-cultivation medium had a marked positive effect on the transient expression of the *uidA* gene in subterranean clover tissue.

The progeny from primary transgenics showed segregation of the *uidA* gene in a 3:1 Mendelian ratio, clearly indicating integration of T-DNA into the subterranean clover genome. In some segregating progeny (T_1) of transgenic plants, the cotyledon and embryonic axis tissues were negative for GUS expression, whereas the immature seed coat was positive. This is consistent with the fact that the seed coat is formed from the mother tissue (Foster and Gifford, 1959), but cotyledons and the embryonic axis are formed after fertilization.

Southern hybridization data showed that 8 of 9 regenerants analyzed contained the *bar* gene (Fig. 3) and the same 8 plants also contained the *uidA* gene (data not shown). One regenerant (plant 4) not analyzed by Southern blotting was positive for the expression of transgenes *bar*, *uidA*, and *α-ai*, as revealed by northern and western blots. This confirms that there were 9 transgenics in this set of 10 plants. Only 6 of these 9 confirmed transgenic plants were clearly positive for mRNAs and enzymes of both *uidA* and *bar* transgenes. Both of these genes were expressed either weakly or not at all in plants 5, 8, and 9. Lu et al. (1991) have observed a co-expression of 58% in transgenic carnation containing *nptII* and *uidA* genes. In contrast, the *α-AI* gene was expressed in all 9 transgenic plants. The α-AI protein in beans is synthesized as a proprotein that undergoes proteolytic cleavage that is required for its activation as an inhibitor of α-amylase (Pueyo et al., 1993). The presence of multiple immunoreactive polypeptides indicates that the α-AI proprotein also undergoes cleavage processing in transgenic subterranean clover seeds.

This system for transformation of subterranean clover is applicable to a wide range of cultivars belonging to all three subspecies of subterranean clover and yields transformed plants ready for transfer to the glasshouse within 16 weeks. This opens the way for the application of genetic engineering to the improvement of the productivity and quality of this important pasture legume.

Table I. *Segregation analysis of the uidA gene in seedlings from selfed seeds of the primary regenerants (based on histochemical GUS assays)*

Primary Regenerant No.	No. of Seedlings		χ^{2a} (3:1)	P
	GUS (+)	GUS (−)		
2	35	13	0.11	0.72
3	53	20	0.30	0.55
4	44	16	0.09	0.75
6	66	18	0.57	0.45

[a] χ^2 goodness of fit values and their probabilities were calculated according to the method of Srb et al. (1965).

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