

PLANT PHYSIOLOGY

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Structure of a Vicilin Protein

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On the Cover: A computer-generated schematic diagram of the structure of canavalin, the vicilin reserve protein of jack bean (*Canavalia ensiformis*), is shown against a background of canavalin crystals. The structure was determined by x-ray crystallography to a resolution of 2.5 Å and is described in the article by Ko et al. (pp. 729-744). The canavalin molecule is composed of three identical subunits, each consisting of dyad-related amino-terminal (orange) and carboxyl-terminal (purple) β barrel domains accompanied by a helix-containing extended chain (white). Canavalin displays topological similarities to another vicilin, phaseolin, and has been expressed in crystallizable form in *Escherichia coli*. Thus, it provides an ideal system for genetic engineering of vicilin proteins.

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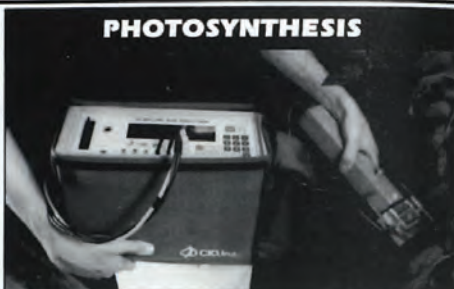
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Transformation and Regeneration of Two Cultivars of Pea (*Pisum sativum* L.)

Hartmut E. Schroeder*, Andrea H. Schotz, Terese Wardley-Richardson, Donald Spencer, and Thomas J. V. Higgins

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A reproducible transformation system was developed for pea (*Pisum sativum* L.) using as explants sections from the embryonic axis of immature seeds. A construct containing two chimeric genes, nopaline synthase-phosphinothricin acetyl transferase (*bar*) and cauliflower mosaic virus 35S-neomycin phosphotransferase (*nptII*), was introduced into two pea cultivars using *Agrobacterium tumefaciens*-mediated transformation procedures. Regeneration was via organogenesis, and transformed plants were selected on medium containing 15 mg/L of phosphinothricin. Transgenic peas were raised in the glasshouse to produce flowers and viable seeds. The *bar* and *nptII* genes were expressed in both the primary transgenic pea plants and in the next generation progeny, in which they showed a typical 3:1 Mendelian inheritance pattern. Transformation of regenerated plants was confirmed by assays for neomycin phosphotransferase and phosphinothricin acetyl transferase activity and by northern blot analyses. Transformed plants were resistant to the herbicide Basta when sprayed at rates used in field practice.

The pea (*Pisum sativum*) is an important grain legume crop plant that has gained worldwide economic importance as a source of protein for animal and human nutrition. In addition, it has well-defined genetics, and it has been commonly used as a model plant for research in plant physiology and biochemistry. The productivity and value of peas could be greatly increased by the introduction of stably inherited traits such as pest and disease resistance, herbicide resistance, and improved protein quality. These traits are not available in natural populations of near relatives of cultivated peas, but current advances in plant genetic engineering provide a potentially powerful tool for achieving these goals by another means.

The prerequisites for the transfer of foreign genes into any plant species by genetic engineering are an efficient gene delivery system, such as *Agrobacterium*-mediated DNA transfer, an effective selectable marker for transformed cells, and the ability to regenerate mature, fertile, transgenic plants from transformed tissue in culture.

Regeneration via embryogenesis or organogenesis has been described for a variety of pea explants, e.g. from immature leaflets (Mroginski and Kartha, 1981; Rubluo et al., 1984), from cotyledonary node (Jackson and Hobbs, 1990), from hypocotyls (Nielsen et al., 1991), from embryos (Kysely et

al., 1987; Natali and Cavallini, 1987; Tetu et al., 1990), from various organs of seedlings (Malmberg, 1979; Hussey and Gunn, 1984; Ezhova et al., 1985), and from protoplast cultures (Jacobsen and Kysely, 1984; Puonti-Kaerlas and Eriksson, 1988; Lehming-Mertens and Jacobsen, 1989).

Agrobacterium-mediated transformation of various pea explants has also been reported, e.g. stem explants (Lulsdorf et al., 1991), embryonic axis and epicotyl segments (Filippone and Lurquin, 1989; Puonti-Kaerlas et al., 1989), nodus explants (De Kathen and Jacobsen, 1990; Nauerby et al., 1991), and root explants and protoplasts (Schaefer and Pilet, 1991). Tumors were induced in young pea plants by wild-type *Agrobacterium* (Hobbs et al., 1990). However, no mature transgenic pea plants were regenerated from any of the above transformation systems. The only report to date of stable transformation of peas and the production of mature, flowering, transgenic pea plants is by Puonti-Kaerlas et al. (1990), who achieved regeneration by organogenesis via callus formation using a gene encoding hygromycin phosphotransferase as a selectable marker.

In this paper we report the development of a routine, reliable transformation and regeneration system for peas. The procedure has been used to introduce herbicide resistance and the expression of an antibiotic resistance gene into two cultivars of peas using an *Agrobacterium tumefaciens*-mediated delivery system. Integration of the two traits was stable, and their frequency in the first generation progeny followed the Mendelian pattern.

MATERIALS AND METHODS

Plant Material and Transformation Procedure

Pea (*Pisum sativum* L.) cv Greenfeast and cv Rondo were grown in the glasshouse, and immature pods containing seeds were harvested at 2 to 5 d beyond maximum fresh weight, at which stage the pod has begun to change from bright green to yellow and the embryonic axis is uniformly beige in color. The pods were sterilized in 70% (v/v) ethanol (1 min) followed by 1% (w/v) sodium hypochlorite (20 min) and three washes with sterile distilled water. Seeds were removed from the pods, and testas were excised. Explants for transformation were cut from the embryonic axis of these seeds. To facilitate manipulation, the embryonic axis was left tem-

Abbreviations: NPT, neomycin phosphotransferase; PAT, phosphinothricin acetyltransferase; PTT, phosphinothricin.

* Corresponding author; fax 61-6-246-5000.

porarily attached to one of the cotyledons (Fig. 1a). The root end was cut off and the remainder of the axis was sliced longitudinally into three to five segments (Fig. 1b) with a scalpel blade that was wet with a suspension of *A. tumefaciens*. Segments were then fully immersed in the bacterial suspension (10^6 cells mL^{-1}) for 30 to 40 min. Wet segments were plated on B₅ medium (Brown and Atanassov, 1985) and cultured at 26°C under fluorescent light for a 12-h photoperiod.

Plant Regeneration

After 4 d of cocultivation, explants were washed three times with sterile water containing 300 mg L^{-1} of timentin (Beecham Research Laboratories). Excess liquid was withdrawn by pipette, and explants were placed onto a callus induction medium. This medium (P1) consisted of Murashige-Skoog macro- and micronutrients (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 2 mg L^{-1} of 6-benzylaminopurine, 2 mg L^{-1} of naphthalene acetic acid, 3% (w/v) Suc supplemented with 10 mg L^{-1} of PPT (a gift from Hoechst Ltd., Melbourne, Australia), and 150 mg L^{-1} of timentin. The pH of the medium was adjusted to 5.8 before autoclaving and was solidified with 0.75% agar (Difco-Bacto).

After 15 d at 25°C under fluorescent light with a 16-h photoperiod, the explants were transferred to P2 medium, which is P1 medium with the hormone concentrations changed to 4.5 mg L^{-1} of 6-benzylaminopurine and 0.02 mg L^{-1} of naphthalene acetic acid for shoot development. Primary shoots, presumed to have arisen from preexisting meristems, were removed and discarded. The explants (Fig. 1c) were transferred to fresh P2 medium every 20 d for three or four passages, and any developing shoots (10–15 mm in length) were excised and transferred to P3 medium. P3 medium is the same as P2 medium except that the level of PPT was increased to 15 mg L^{-1} to distinguish between resistant and susceptible shoots (Fig. 1d). When the developing shoots were >20 mm in length, roots were induced on Murashige-Skoog medium containing 3% Suc and 10 mg L^{-1} of PPT following the procedure of Malmberg (1979). After a substantial root growth was established (Fig. 1e), the plantlets were transferred to culture vessels containing sand, soil, and perlite (1:1:1) and were maintained in the growth room before transfer to soil in the glasshouse, where they subsequently flowered and produced seed (Fig. 1, f and g).

Bacterial Strain and Gene Construction

The binary plasmid pSLJ1561 containing the *bar* gene encoding PAT flanked by nopaline synthase 5' and 3' sequences, together with the *nrpII* gene (encoding NPT), and flanked by cauliflower mosaic virus 35S 5' sequences and octopine synthase 3' sequences, was constructed by Gerard Bishop and kindly supplied to us by Jonathon Jones (Sainsbury Laboratory, John Innes Centre for Plant Science Research, Norwich, UK). The plasmid was mobilized from *Escherichia coli* by triparental mating to *A. tumefaciens* strain AGL1 (Lazo et al., 1991).

Enzyme Assays

To test for PAT activity in putative transformants, leaf tissue samples (30–50 mg) were homogenized in 1.5-ml microcentrifuge tubes containing extraction buffer (100 mM sodium phosphate [pH 7.0], 20 mM NaCl, 1 mM PMSF, and 1 mg mL^{-1} of BSA) in a ratio of 1:1.5 (w/v). After centrifugation for 10 min at 13,000 rpm, 16.5 μL of supernatant was added to 1 μL (0.02 μCi) of [^{14}C]acetyl-CoA (50–60 mCi/mmol; Amersham), 2.5 μL of PPT solution (0.166 M PPT in extraction buffer), and the tubes were incubated at 37°C for 30 min. Tubes were centrifuged at 13,000 rpm for 1 min, and 10- μL aliquots of the supernatant were spotted onto silica gel TLC plates. Ascending chromatography was carried out in a 3:2 (v/v) mixture of propan-1-ol and NH_4OH (25% NH_3). Plates were dipped in a solution containing 0.4% (w/v) diphenyl oxazole in 1-methylnaphthalene (α) and dried. ^{14}C -labeled compounds were detected by fluorography on x-ray film (Fuji) after an overnight exposure. NPT was detected by the dot blot method of McDonnell et al. (1987) using leaf extracts prepared as above from putative transformants.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from young leaves (Chandler et al., 1983) and prepared for blot analysis as previously described (Higgins and Spencer, 1991). A DNA fragment corresponding to the entire coding region of the NPT mRNA was ^{32}P labeled using random primers (Amersham Multiprime System).

Leaf Paint and Spray Tests with Basta

The upper surface of leaflets on 4-week-old seedlings was thoroughly wetted by painting with Basta (a commercial formulation of PPT containing 200 g L^{-1} ; Hoechst Ltd.)

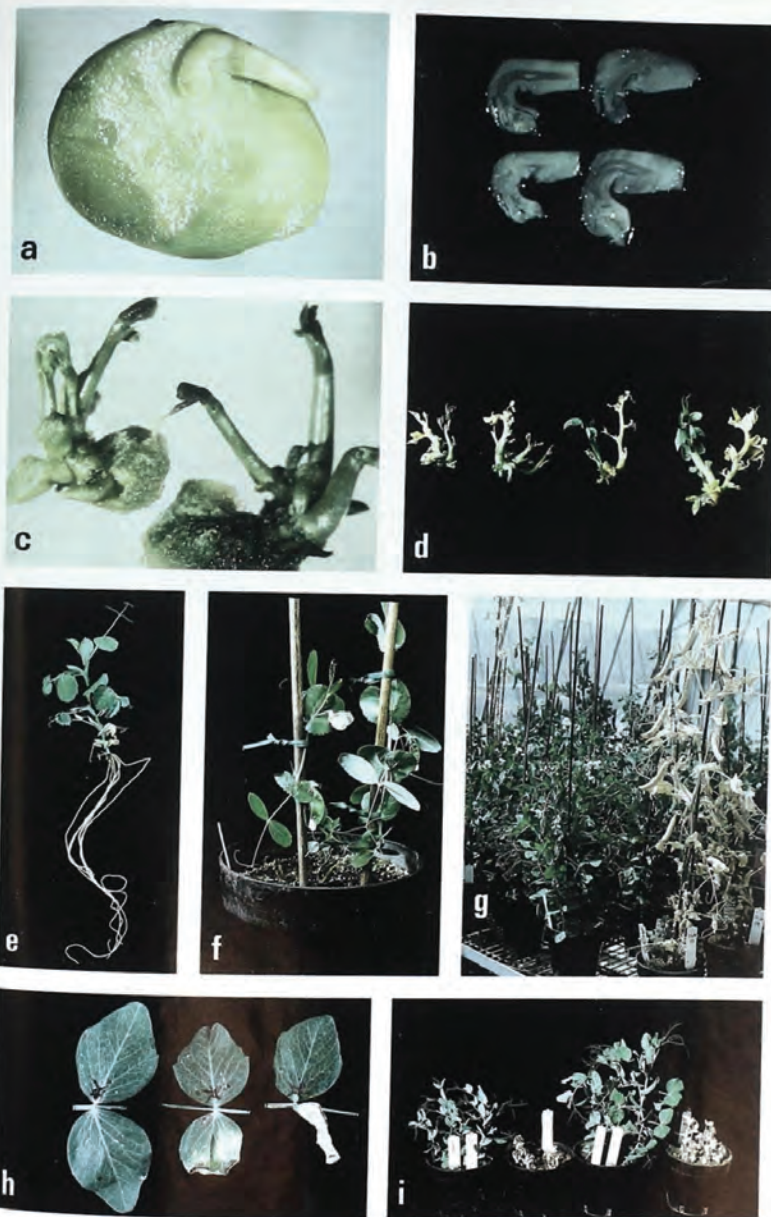


Figure 1 (facing page). Regeneration of transgenic pea plants. a, Embryonic axis attached to one cotyledon at 2 to 5 d beyond the stage of maximum fresh weight; b, explant segments derived from embryonic axis; c, multiple shoots developed on P2 medium; d, distinguishing PPT-resistant and -susceptible shoots on medium containing 15 mg L^{-1} PPT; e, rooted plantlet grown on medium containing 10 mg L^{-1} of PPT; f, transgenic plant with first flower; g, transgenic plants at various stages of development in glasshouse; h, painted leaflet test 5 d after herbicide application. The upper leaflet in each pair (marked X) was untreated. The lower leaflet of each pair was painted with Basta equivalent to 10 L ha^{-1} . The left-hand and middle leaflet pairs were from different transformed cv Greenfeast plants, and the right-hand leaflet pair was from a nontransformed cv Rondo plant; i, spray test of transgenic and untransformed plants of the two cultivars with doses equivalent to 7 $\text{L of Basta ha}^{-1}$. Photograph was taken 14 d after treatment. From left to right: transgenic cv Rondo plant; nontransformed cv Rondo plant; transgenic cv Greenfeast plant; nontransformed cv Greenfeast plant.



Figure 2. PAT activity in leaf extracts of transgenic peas, cv Greenfeast. PAT activity was assayed by TLC of the enzyme reaction products as described in "Materials and Methods." Each lane shows the reaction products obtained with 9 μ g of leaf protein. The single asterisk denotes the position of the acetyl-CoA substrate, and two asterisks denote the position of acetylated PPT product. Lane 1, Positive control, an extract from transgenic tobacco expressing the *bar* gene; lane 2, negative control, an extract from untransformed pea; lanes 3 to 12, leaf extracts from nine different transgenic peas; lane 13, the ^{14} C-labeled acetyl-CoA substrate without plant extract.

solution at a rate equivalent to 3 L of Basta ha^{-1} . The opposite leaflet of each pair was left untreated as a control (marked "X" in Fig. 1h). This treatment was repeated 3 weeks later on different leaflets of the same plants with doses equivalent to 5 and 10 L of Basta ha^{-1} . Selected Basta-resistant plants of both pea cultivars, together with control, untransformed plants of similar age, were sprayed with a dose equivalent to 7 L of Basta ha^{-1} . Plants were sprayed until there was runoff from the leaves.

RESULTS

Regeneration of Transgenic Peas

Pea explants were transformed with *A. tumefaciens* strain AGL1 harboring both *bar* and *nrpII* genes, and transgenic plants were regenerated via organogenesis as described in "Materials and Methods." After infection and cocultivation, explants developed callus and buds on P1 medium (Fig. 1c). The resultant primary shoots were discarded because we assume that they arise from preexisting shoot initials. Multiple shoots then developed during several subsequent passages on P2 medium, and these shoots were excised and transferred to P3 medium, where they could be assessed as either PPT resistant or PPT susceptible (Fig. 1d). A transgenic plant with its first flower and transgenic plants at various stages of development from flowering to seed maturity are shown in Figure 1, f and g, respectively. The time required to progress from the explant shown in Figure 1a to the mature plant in Figure 1g was approximately 9 months. Both of the cultivars that were tested, Rondo and Greenfeast, were amenable to the transformation and regeneration protocol. Between 1.5 and 2.5% of the starting explants gave rise to transformed plants.

Evidence for the expression of the *bar* gene was obtained by analysis of putative transformants for the presence of active PAT enzyme. Leaf material was assayed from nine glasshouse-grown putative transformants of cv Greenfeast, and PAT activity was detected in eight of the nine plants

(Fig. 2). The level of expression appeared to vary widely as shown by the extent to which substrate was converted to product using a constant amount of leaf protein. In one case (Fig. 2, lane 7), all of the substrate was converted to product, whereas in another (Fig. 2, lane 9), only a small proportion was converted. By assaying various dilutions of leaf extracts, we found that the level of PAT enzyme activity varied about 20-fold between the highest and lowest PAT-positive plants (data not shown).

Transgenic plants of cv Rondo were obtained with about the same frequency as cv Greenfeast. PAT activity was detected in leaves of the primary regenerants (R_0) of Rondo and in the leaves of plants from the next generation (R_1) (Fig. 3a) Northern blot analysis and NPT enzyme assays of those R_1

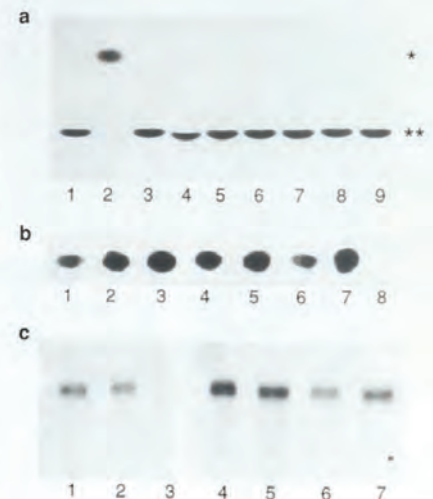


Figure 3. Expression of the *bar* and *nrpII* genes in transgenic peas. a, Expression of the *bar* gene measured as PAT activity in the leaves of three primary regenerants (R_0) of cv Rondo (lanes 3–5) and in four self-pollinated progeny (R_1) of the plant in lane 4 (lanes 6–9). Each lane shows the reaction product (marked with double asterisks) obtained with 25 μ g of leaf protein. Lane 1 contains extracts from leaves of a transgenic tobacco expressing the *bar* gene; lane 2 contains extracts from leaves of a nontransformed pea plant. b, Expression of the *nrpII* gene measured as NPT activity in extracts of leaves of three primary regenerants of cv Rondo (lanes 1–3), four R_1 progeny of the plant represented in lane 2 (lanes 4–7), and one nontransformed pea plant (lane 8). c, Expression of *nrpII* gene measured by northern blot analysis of RNA prepared from leaves of two primary regenerants of cv Greenfeast (lanes 1 and 2) and from four of the progeny (R_1) (lanes 4–7) of the Rondo plant depicted in lane 4 of Figure 3a. Lane 3 contains RNA from a nontransformed plant. The presence of NPT mRNA was detected using a ^{32} P-labeled probe specific for the NPT-coding region as described in "Materials and Methods."

Table I. Distribution of PAT and NPT activity in progeny from each of two transformed pea plants (cv Rondo)

Progeny of	PAT Activity		NPT Activity	
	Present	Absent	Present	Absent
Transformant S8 (24 progeny)	18	6	18	6
Transformant S9 (23 progeny)	17	6	17	6

and R_1 plants expressing the *bar* gene showed that the *nrpII* gene was transcribed into mRNA and translated into active NPT enzyme (Fig. 3, b and c).

Inheritance of PAT and NPT Activity

PAT and NPT activities were distributed in the next generation progeny (R_1) in a strictly Mendelian pattern with a 3:1 ratio (presence-absence), as would be expected from dominant genes at a single locus in a self-pollinating plant (Table I). In this analysis, PAT activity was detected by the painted leaf test using Basta, and NPT activity was assayed by dot blot. The results of the painted leaf test were subsequently confirmed by PAT enzyme assays.

Basta Tolerance in Transformed Peas

To evaluate whether the level of PAT activity in transformed peas was sufficient to confer resistance to the herbicide Basta, leaflets of untransformed and transgenic plants were tested for tolerance to doses equivalent to 1, 3, 7, and 10 L of Basta ha^{-1} . Preliminary tests showed that doses equivalent to 1 and 3 L of Basta ha^{-1} kill untransformed pea seedlings and mature plants, respectively. The leaflet test made use of the fact that Basta is not translocated throughout the plant and, therefore, only affects that part of the plant contacted by the spray. Figure 1h shows three pairs of leaflets in which one member of each pair was treated with a dose equivalent to 10 L of Basta ha^{-1} . The left-hand and middle leaflet pair were from different transformed plants, and the right-hand pair was from an untransformed plant. Five days after Basta application, the treated leaflet in the left-hand pair showed complete tolerance, and the middle treated leaflet showed partial tolerance. The treated leaflet from the untransformed plant was completely necrotic. When other leaflets from the middle transformed plant were retested with a dose equivalent to 5 L of Basta ha^{-1} , they showed complete tolerance to the herbicide (data not shown). Tolerance to the herbicide was also assayed on whole plants by spraying with a dose equivalent to 7 L of Basta ha^{-1} (Fig. 1i). After 14 d, transgenic plants of both cultivars showed no symptoms of herbicidal damage and grew normally to maturity, whereas the nontransgenic plants were killed.

DISCUSSION

We have developed a transformation and regeneration system that permits the introduction of foreign genes into two cultivars of peas. The system makes use of *A. tumefaciens* as the vector and the *bar* gene, which confers resistance to the herbicide PPT, as the selectable marker. Expression of

the *bar* and the accompanying *nrpII* genes was confirmed by assaying PAT and NPT enzyme activities in the putative transformants. Both genes were inherited in a 3:1 ratio in the first generation of the transformed plants, consistent with their functioning as dominant genes at a single locus in the transformed plants.

PPT is a potent inhibitor of Gln synthase in plants (Eckes et al., 1989; Krieg et al., 1990) and is available commercially as a nonselective herbicide called Basta. The *bar* gene, which confers resistance to PPT, encodes the enzyme PAT, which catalyzes the conversion of PPT to a nontoxic acetylated product (De Block et al., 1987). The *bar* gene proved to be an efficient selectable and screenable marker in pea transformation. Doses of 10 and 15 mg L^{-1} of PPT were used to select putative transgenic shoots in tissue culture, whereas mature transformed pea plants growing in the glasshouse showed resistance to spraying with Basta at levels equivalent to 5 to 10 L ha^{-1} . Either spraying of the whole plant or painting individual leaves with the herbicide gave a reliable indication of the presence and expression of the *bar* gene. Thus, in contrast to other markers, the *bar* gene offers a convenient assay for the selection of plants in soil.

A number of factors proved to be important in the consistent production of transgenic pea plants, including explant source, bacterial strain, choice of selectable marker, and the presence of hormones during cocultivation. The successful recovery of transgenic peas was highly dependent on the explant material. The optimum stage for taking embryonic axes was at 2 to 5 d (depending on the season) after the time of maximum seed fresh weight. Explants from younger or older seeds gave a much reduced yield of transformants.

In our hands, the particular strain of *A. tumefaciens* was also important. For example, strain LBA4404 was inefficient, whereas the strain AGL1 gave acceptable levels of transformation. This is in contrast to the findings of Schaefer and Pilet (1991), who found a high frequency of transformation of various pea explants regardless of the strain of *A. tumefaciens* used. AGL1 is a hypervirulent strain that facilitates DNA transfer to many dicotyledonous plants (Lazo et al., 1991). The high level of tolerance of pea tissues to kanamycin meant that this class of antibiotic, in conjunction with the *nrpII* gene, did not constitute a practical selectable marker system. An alternative system, such as that based on the *bar* gene and PPT, had to be used. This finding is in agreement with the report of Puonti-Kaerlas et al. (1990), who were able to produce transgenic peas using a gene conferring resistance to hygromycin but not with the *nrpII* gene, which confers resistance to kanamycin.

Another important factor in producing transgenic peas at a useful frequency was the presence of plant growth regulators in the cocultivation medium. The B-h medium of Brown and Atanassov (1985), which we used, contains 2,4-D and kinetin. Our observations confirm those made recently by Schaefer and Pilet (1991) that recovery of transformants is greatly reduced when these growth regulators are absent during cocultivation.

While this manuscript was in preparation, Puonti-Kaerlas et al. (1992) published a more detailed account of their procedure (Puonti-Kaerlas et al., 1990) for producing pea plants expressing the hygromycin phosphotransferase gene

in both the primary and R_1 generations. The salient differences between their transformation and regeneration procedure and the procedure reported here are the different explant materials (shoot cultures derived from the epicotyls of germinated seedlings versus embryonic axes from immature, developing seeds) and the much shorter period in tissue culture (15 months versus 9 months from explant to seed-bearing primary regenerant). The aberrations reported by Puonti-Kaerlas et al. (1992) (aborted flowers, only one or two seeds per pod, more pods per plant in the R_1 generation, nonviable seeds in some transformants) were not seen in our primary transgenic peas, possibly as a result of the much shorter period in tissue culture. We observed no flower abortions, pods contained the same number of seeds as the controls, and no nonviable seeds have been detected. Puonti-Kaerlas et al. (1992) also reported chromosome doubling in their transgenic peas and a more robust appearance (thicker stems, rounded leaves, and more lateral leaves). We have not yet made equivalent chromosome observations, but no major morphological differences have been observed between non-transformed plants and transformed plants in either the primary regenerants (R_0) or the R_1 generation.

The procedures reported here for the routine transformation and regeneration of peas, including a simple and non-destructive screening procedure, open the way for the application of genetic engineering to the improvement of this important food and feed crop. Some of the more obvious candidate genes are those conferring improved protein quality, insect pest resistance, and herbicide resistance. We have recently transferred a gene for the α -amylase inhibitor protein of *Phaseolus vulgaris* (Moreno and Chrispeels, 1989) into peas, and we are currently testing its effectiveness in conferring resistance to insect pests.

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