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EVALUATION OF A COTYLEDONARY NODE REGENERATION SYSTEM FOR *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF PEA (*PISUM SATIVUM* L.)

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SUMMARY

A rapid regeneration system was used for studies of *Agrobacterium*-mediated transformation in *Pisum sativum* L. Cotyledonary node explants were inoculated with *Agrobacterium tumefaciens* strains containing binary vectors carrying genes for nopaline synthase (NOS), β-glucuronidase (GUS), and neomycin phosphotransferase (NPTII) and placed on selection medium containing either 75 or 150 mg/liter kanamycin. A GUS encoding gene (*uidA*) containing an intron was used to monitor gene expression from 6 to 21 days postinoculation. GUS activity could be observed 6 days after inoculation in the area of the explant in which regeneration occurred. Regenerating tissue containing transformed cells was observed in explants on selection medium 21 days postinoculation. Using this system, a single transgenic plant was obtained. Progeny of this plant, which contained two T-DNA inserts, demonstrated segregation for the inserts and for expression of the NOS gene in the selfed R_1 progeny. NPTII activity was observed in the R_2 generation, indicating inheritance and expression of the foreign DNA over at least two generations. Attempts to repeat this procedure were unsuccessful.

Key words: transformation; pea; *Pisum sativum*; *Agrobacterium tumefaciens*; regeneration; progeny testing.

INTRODUCTION

There has been steady progress in the transformation of crop species using *Agrobacterium tumefaciens* (*see* Gasser and Fraley, 1989 for a review). Grain legumes, however, remain recalcitrant, with definitive evidence for stable transformation provided only for soybean (Hinchee et al., 1988) and *Vigna aconitifolia* (Eapen et al., 1987). Confirmation of transformation requires physical evidence of the integrated T-DNA (Southern analysis showing junction fragments of T-DNA with plant DNA) expression of the introduced genes and stable transmission to future generations (Potrykus, 1990).

Pisum sativum L. (pea) is a grain legume widely grown for both food and feed purposes. It is of particular importance in the temperate regions because it is a high-protein crop, capable of nitrogen fixation and grows well under cool conditions. The availability of a genetic transformation system would facilitate the use of genetic engineering techniques for improving agronomic traits affecting production efficiency as well as the nutritional quality of *P. sativum*.

It has previously been shown that pea is amenable to transformation by wild type strains of *A. tumefaciens* (Hobbs et al., 1989; Puonti-Kaerlas et al., 1989), and gene expression in callus has been achieved with disarmed strains carrying selectable marker genes (Puonti-Kaerlas et al., 1989). There have been two recent reports of the production of fertile transgenic pea (*Pisum sativum* L.) plants using *Agrobacterium*-mediated transformation (Puonti-Kaerlas et al., 1990, 1992) but the method required up to a year for the production of shoots. For practical purposes, a transformation scheme utilizing a rapid regeneration system would be desirable.

Recently, a genotype independent, rapid regeneration system with little or no callus formation has been developed for pea in our laboratory (Jackson and Hobbs, 1990). It this report we describe experiments utilizing this system in an effort to rapidly produce fertile transgenic pea plants.

MATERIALS AND METHODS

Preparation of explants for inoculation. Mature seeds of *Pisum sativum* L. genotype PI244253 were surface sterilized by rinsing with 70% ethanol for 2 min followed by a 20 min soak in a 4.5% sodium hypochlorite solution (with shaking) and four rinses in sterile distilled water. The seeds were placed in sterile petri dishes containing two circular Whatman no. 1 filter papers and 10 ml of sterile distilled water. The seeds were germinated in darkness for 5 to 6 days at 25° C.

The cotyledons were removed from the seedlings and the root and main shoot were cut off as close as possible to the axillary buds in the cotyledonary nodes. The explants were prepared and inoculated by one of the following two methods:

Method 1. The axillary buds were scraped out with a scalpel and the nodes were placed on Murashige and Skoog (Murashige and Skoog, 1962) medium containing 1 mg/liter benzylaminopurine (BAP) (MS7 medium) for 1 wk, at which time any buds present were scraped off. *Agrobacterium tumefaciens* was introduced either by placing the explants in 2 ml of a 5×10^8 cells per ml *Agrobacterium* culture followed by shaking on a rotary shaker at low speed for 10 min, or by poking holes and injecting bacteria into the node region with a 1.5-ml syringe filled with the *Agrobacterium* culture and fitted with a 30-gauge needle. The explants were blotted dry between two pieces of Whatman no. 1 filter paper and placed on MS7

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medium for 3 days. The explants were then split in half (each half containing the scraped region of one axillary bud) and transferred to MS7 selection medium containing 100 mg/liter cefotaxime (Sigma, St. Louis, MO), 500 mg/liter carbenicillin (Pyopen, Ayerst), and 75 mg/liter kanamycin sulphate (Sigma).

Method 2. The explants were inoculated by stabbing and injecting bacteria into the region around the axillary buds with a 1.5-ml syringe fitted with a 30-gauge needle containing the 5×10^8 cells/ml *Agrobacterium* culture. The explants were blotted dry and placed on MS7 medium for 3 days, after which the axillary buds were scraped out and the explants were split in half and placed on MS7 selection medium containing antibiotics as per method 1, except that 150 and 250 mg/liter kanamycin as well as 15 mg/liter hygromycin were also used for selection.

Culture of explants and regeneration. Explants were kept at 25° C under a 16/8 h light/dark regimen. As regenerating shoots elongated they were removed and placed on half-strength B5 (Gamborg et al., 1968) medium for rooting. Rooted shoots were transferred to sterile vermiculite, covered to maintain high humidity, and placed in a growth chamber under a 25° C 16/8 h light/dark regimen. After 1 mo. they were uncovered and transferred to potting soil.

Agrobacterium tumefaciens. The *Agrobacterium tumefaciens* strains used were C58C1Rif^R(pGV2260) (Deblaere et al., 1985) carrying p35SGUS-INT (Vancanneyt et al., 1990) (a binary plasmid containing a gene coding for β-glucuronidase (GUS) with a plant intron inserted into it to prevent GUS expression in bacterial cells), EHA101 (Hood et al., 1986) (a disarmed derivative of A281) carrying the binary vector pUS3 (a derivative of pMON505) (Rogers et al., 1987) (Fig. 1) and EHA101 carrying the binary vector pBI1042, which carries a gene encoding hygromycin resistance (Lulsdorf et al., 1991). The three strains will be referred to as 35SGUS-INT, EHA101 (pUS3) and EHA101 (pBI1042). The bacteria were grown overnight in 10 ml of 2YT (Sambrook et al., 1989) medium plus 100 μM acetosyringone (Aldrich, Milwaukee, WI). The cultures were diluted to 5×10^8 cells/ml with 2YT and used to inoculate explants as described above.

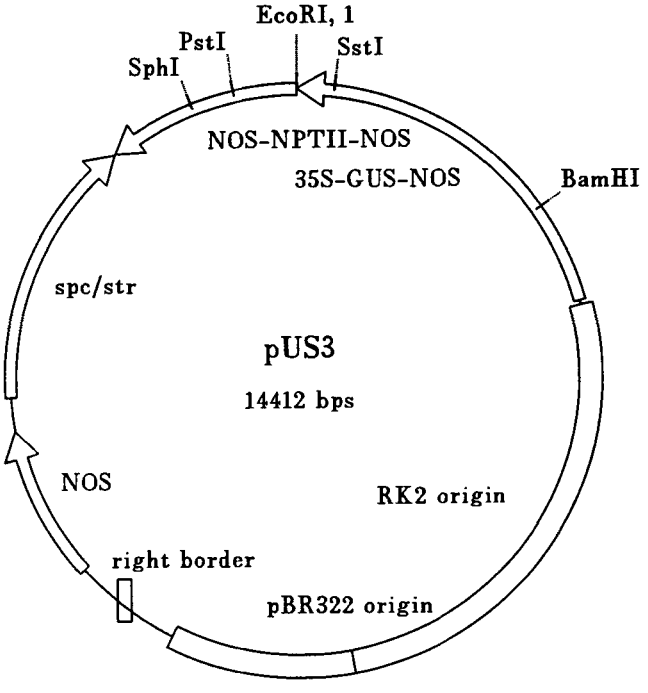

FIG. 1. Structure of pUS3. pUS3 is a binary plant transformation vector containing the *uidA* gene driven by the cauliflower mosaic virus 35S promoter and the *nptII* and *nos* genes driven by the NOS promoter of *Agrobacterium tumefaciens*. The vector also carries the nopaline T-DNA right border, a pBR322 origin of replication (ori) a wide host range origin of replication (RK2) and a bacterial selectable marker (spectinomycin resistance). pUS3 was constructed by the insertion of the HindIII-EcoRI *uidA* gene fragment of pBI221 (Clontech) into the multilinker of pMON505 (Rogers et al., 1987). The BamHI-SstI fragment of the *uidA* (1.9 kb) construct and the PstI-SphI fragment of the *nptII* (350 bp) gene were used as probes for Southern blots. The length of the 35S-GUS-NOS and NOS-NPTII-NOS genes is 3.0 and 1.4 kb, respectively. For the *nptII*, *nos*, and *uidA* genes the direction of transcription is indicated by *arrows*.

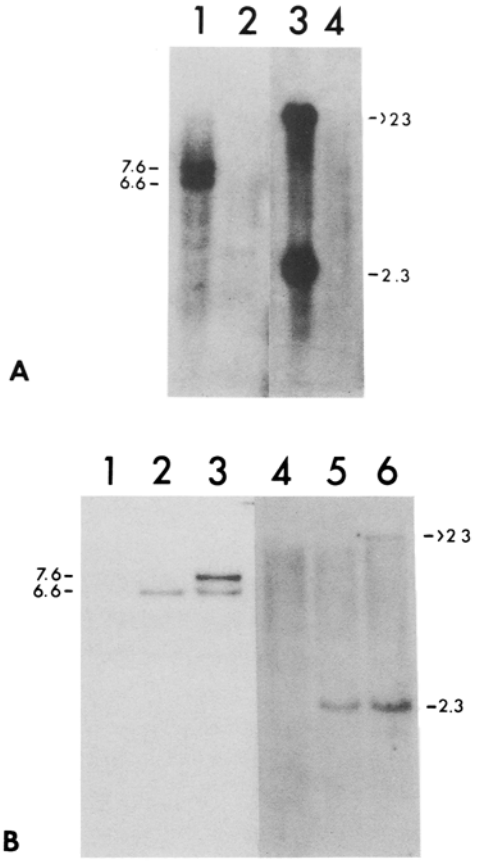

FIG. 2. Southern blots of transgenic *Pisum sativum*. All DNA was cut with EcoRI (*a*) DNA from the original regenerated (R_0) plant probed with the 350 bp *nptII* probe or the 1.9 kb *uidA* probe. *Lanes 1* and *3*, DNA from the original regenerated plant probed with *nptII* and *uidA*, respectively. *Lanes 2* and *4*, nontransformed control DNA probed with NPTII (*lane 2*) and GUS (*lane 4*). *b*, DNA from the two R_1 progeny plants showing one or two inserts when probed with *nptII* or *uidA*. *Lanes 1* and *4*, nontransformed control DNA probed with *nptII* and *uidA*, respectively. *Lanes 2, 3, 5* and *6*, DNA from the two R_1 progeny plants probed with *nptII* (*lanes 2* and *3*) and *uidA* (*lanes 5* and *6*).

NPTII assay. Assays using crude protein extracts from leaves of putative transgenic plants and controls were performed as described by Radke et al. (1988).

Nopaline assay. Leaves of transgenic plants and controls were cut up and incubated in nopaline induction medium (NIM) for 2 days. Nopaline assays were carried out as per Draper et al. (1988) except that electrophoresis was carried out for 2 h rather than 45 min.

Southern analysis. Plant DNA was extracted by the method of Dellaporta et al. (1985), digested with EcoRI, and the fragments were separated on a 1% agarose gel using TAE buffer, pH 7.5. The DNA was transferred to GeneScreen Plus (NEN DuPont) using the manufacturers instructions. Probe DNA was either the 350 bp PstI-SphI fragment from pBI121 (Clontech, Palo Alto, CA) containing a part of the coding region of the neomycin phosphotransferase (NPTII) gene or the 1.87 kb BamHI-SstI fragment from pBI121 containing the coding region of the GUS gene. Probes were labeled using a random primers labeling kit (BRL) as per the manufacturers

TABLE 1

NUMBERS OF SHOOTS PRODUCED FROM COTYLEDONARY NODE EXPLANTS OF *PISUM SATIVUM* INOCULATED WITH VARIOUS *AGROBACTERIUM TUMEFACIENS* STRAINS AND PLACED ON SELECTION MEDIUM (75 mg/liter KANAMYCIN)

Method[a]	Agrobacterium Strain[b]	No. of Explants	No. of Shoots	Average no. of Shoots per Explant	No. of Shoots Tested by Root GUS Assay	No. Positive	No. of Shoots Tested by Shoot GUS Assay	No. Pos.	No. of Transformed Shoots[c]
1	EHA101 (pUS3)	329	445	1.4	0	0	445	11	0
	35SGUS-INT	65	49	0.8	0	0	49	0	0
		394	494	1.1	0	0	494	11	0
2	EHA101 (pUS3)	50	74	1.5	0	0	74	1	1
	35SGUS-INT	50	24	0.5	0	0	24	0	0
		100	98	1.0	0	0	98	1	1

[a,b] *See* Materials and Methods; [c] As determined by Southern analysis on all shoots.

instructions. Hybridizations were carried out as described in the GeneScreen Plus instruction booklet for hybridization at 42° C.

Results

Comparison of transformation protocols. The cotyledonary node regeneration system (Jackson and Hobbs, 1990) was used throughout, and two different *Agrobacterium* inoculation protocols were followed, both of which resulted in the production of shoots on selection medium (Table 1).

Both the 35SGUS-INT and EHA101 (pUS3) strains produced shoots on selection medium (Table 1). There was no observable difference between the bathing and injection methods of inoculation for method 1 in terms of numbers of shoots produced on 75 mg/liter kanamycin and therefore the data have been combined in Table 1.

Analysis of transgenic Pisum sativum *L.* DNA was extracted from all 592 regenerated plants, digested with EcoRI, separated on an agarose gel, and examined by Southern analysis using an *nptII*

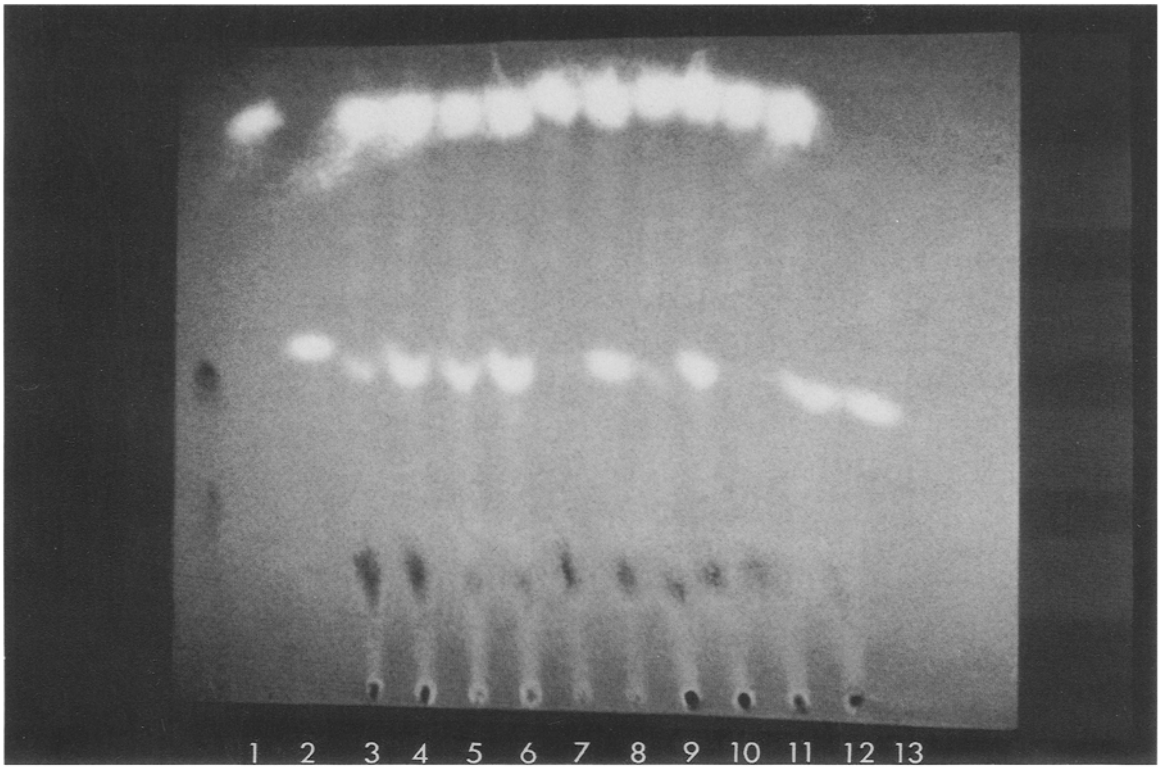

FIG. 3. Nopaline assay of R_1 progeny plants of transgenic *Pisum sativum*. *Lane 1*, arginine standard; *lanes 2* and *13*, nopaline standard; *lanes 3* and *5*, extracts from leaves of the single T-DNA insert R_1 progeny plant; *lane 3*, extract from young leaves; *lane 5*, extract from older, fully expanded leaves from the base of the plant; *lanes 4* and *6*, extracts from leaves of the single insert R_1 plant plus nopaline standard; *lane 4*, young leaves; *lane 6*, older leaves; *lanes 7* and *9*, extracts from leaves of the two T-DNA insert R_1 plant; *lane 7*, young leaves; *lane 9*, older leaves; *lanes 8* and *10*, extracts from leaves of the two insert R_1 plant plus nopaline standard; *lane 8*, young leaves; *lane 10*, older leaves; *lane 11*, leaf extract from older leaves of control plants; and *lane 12*, as *lane 11* plus nopaline standard.

probe. One plant (from an explant inoculated with EHA101 (pUS3) using method 2) gave a positive signal and contained two inserts with homology to the probe (Fig. 2). The probe was stripped and the membrane rehybridized to a labeled probe corresponding to the GUS coding region, and two bands appeared of differing sizes to those identified by the *nptII* probe. This is expected if the T-DNA is integrated into the plant DNA because the two genes are separated by an EcoRI site in this construct (Fig. 1). This plant set two seeds, which were planted, and the resulting R_1 plants were used for DNA, GUS, and nopaline analysis. One of the R_1 progeny contained one of the parental bands when its DNA was cut with EcoRI and probed with *nptII* or *uidA*, and the other contained both parental bands (Fig. 2).

For both R_1 progeny, GUS enzyme assays on leaf material showed indiscernible expression over background level (data not shown). One of the progeny (with a single insert), however, expressed nopaline in leaf tissue (Fig. 3). Nopaline expression was highest in older leaves and was only observed when the leaves were sliced and incubated in an induction medium for 2 days. This follows the established pattern for the nopaline synthase (NOS) promoter as it is more active in older leaves than in younger leaves (An et al., 1988) and is induced by wounding (An et al., 1990).

The lack of GUS activity in the single insert R_1 plant was probably due to the T-DNA being truncated in this insert. The Southern blot (Fig. 2) shows that the EcoRI fragment containing a region of homology to the *uidA* probe is only 2.4 kb in length, which corresponds to a T-DNA which is truncated in the 35S promoter of the GUS construct. The lack of nopaline activity from the R_1 plant with both inserts is similar to the observations of Hobbs et al. (1990) for tobacco, where the presence of two T-DNA inserts resulted in low expression, whereas one alone resulted in high expression. In addition, the lack of GUS activity in this plant could be due to position effects or gene rearrangements in the non-truncated insert.

R_2 progeny from the two R_1 plants were assayed for NPTII activity. Segregation for NPTII activity was observed in the progeny of the single insert plant when older leaves were used (Fig. 4) but no activity was observed in younger leaves (data not shown). The progeny were also screened by Southern analysis to confirm Mendelian segregation (Fig. 5). Out of 15 R_2 progeny of the double insert R_1 plant no clearly detectable activity of NPTII was observed.

The experiment, which resulted in the transgenic plant, was repeated with two levels of kanamycin and with hygromycin as a selectable marker. The number of shoots per explant was greatly reduced but in no case were any transgenic plants observed when all 131 plants were subjected to Southern analysis (Table 2).

Discussion

The cotyledonary node regeneration system was successfully used for the production of transgenic *Pisum sativum* L. A single transgenic plant was produced from a nodal explant isolated from a 5-day-old etiolated seedling and inoculated by injecting *Agrobacte-*

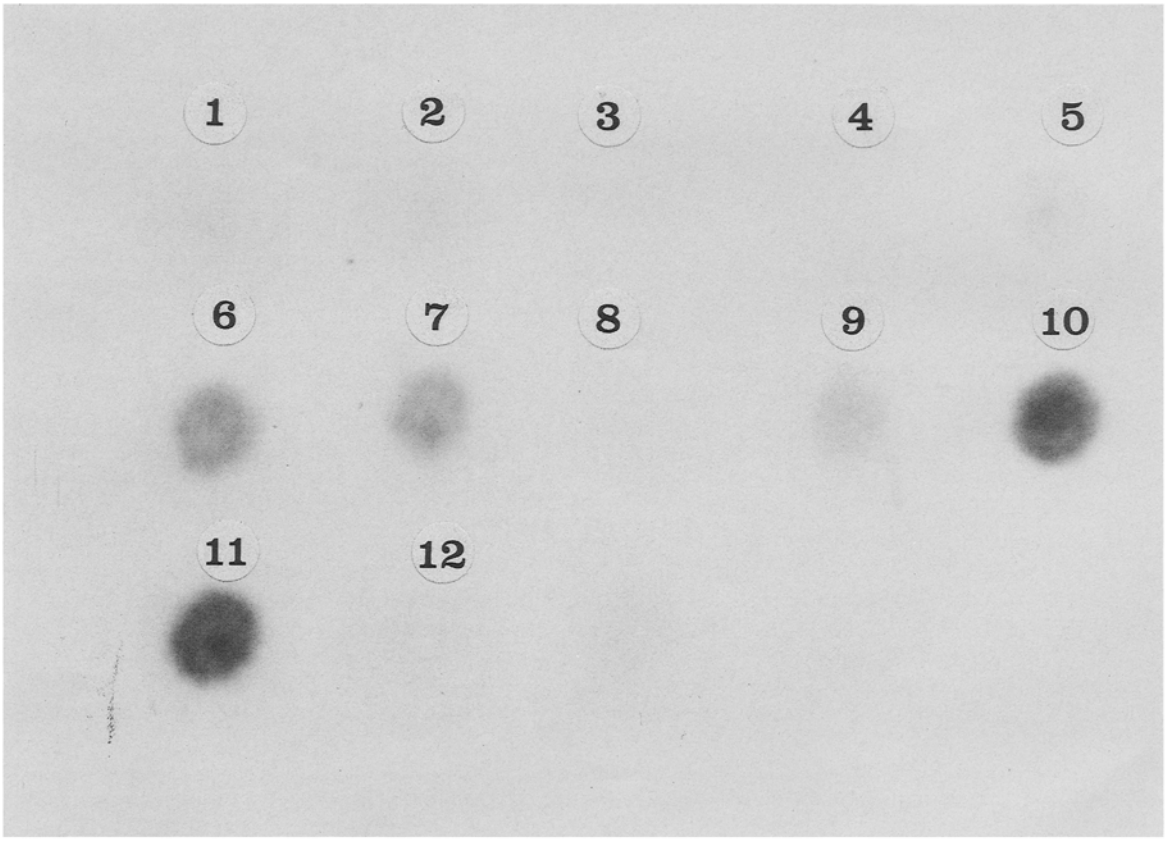

Fig. 4. NPTII assay of R_2 progeny from the single T-DNA insert R_1 plant. *Dots 1–11* are leaf extracts from older leaves whereas *dot 12* is a leaf extract from an older leaf of a nontransformed control plant.

rium into the region surrounding the axillary buds. After 3 days of co-cultivation the axillary buds were removed and the explants placed on selection medium. The transgenic plant showed integration of the T-DNA with the introduced genes being stably transmitted and expressed in the R_1 and R_2 generations. The high frequency of escape regenerants observed on selection medium was probably due to the low level of kanamycin used to allow as many transformed cells to develop into shoots as possible, regardless of level of expression of the *nptII* gene.

Hinchee et al. (1988) found that soybean cotyledonary explants were enriched for transformed cells when placed under kanamycin selection as opposed to no selection, thus affording a better opportunity of obtaining transgenic regenerants with a high expression level. We have raised the kanamycin level to 150 or 250 mg/liter or used hygromycin (15 mg/liter) in experiments using the same method as that which produced the transformed plant (method 2) in an attempt to increase the proportion of transformed cells in the explants; however, no additional transgenic plants were obtained.

The production of transgenic plants, for both research and breeding purposes, is an essential first step in the application of biotechnology to the improvement of a crop species. To achieve this, transformation systems should aim for a rapid recovery of large numbers of fertile transgenic plants, little or no callus which might induce genetic variation, and little dependence on genotype so that plant types can be chosen for their agronomic background and compatibility with *Agrobacterium* strains (Hobbs et al., 1989). The pea regeneration system used by Puonti-Kaerlas et al. (1990) for transformation studies is genotype dependent and has a long tissue culture phase (greater than 6 mo.).

Using the method reported here it was possible to produce a single transgenic pea plant whose progeny contained and expressed the foreign gene. From explantation and inoculation to rooted shoot required only 1 to 2 mo. As the regeneration system is genotype independent, the potential exists to expand transformation to other genotypes. We have been unable to repeat the production of transgenic pea plants using this system. Probably only a small number of cells in the explant are capable of both transformation and regeneration, and these cells may not be readily available to *Agrobacterium* using our system or not efficiently transformed by the strains used. Clearly, additional work on improving the efficiency of cellular transformation, coupled with the identification of the responsive tissues in the explant so that they can be effectively targeted, will be needed before a practical transformation system using cotyledonary node explants is developed. One possible technique to increase transformation frequency is to have a short callus phase. This would allow more efficient selection, as well as possibly increase the number of target cells, because more transformed cells may be capable of forming callus than are capable of direct regeneration. Experiments to develop such a system are currently underway.

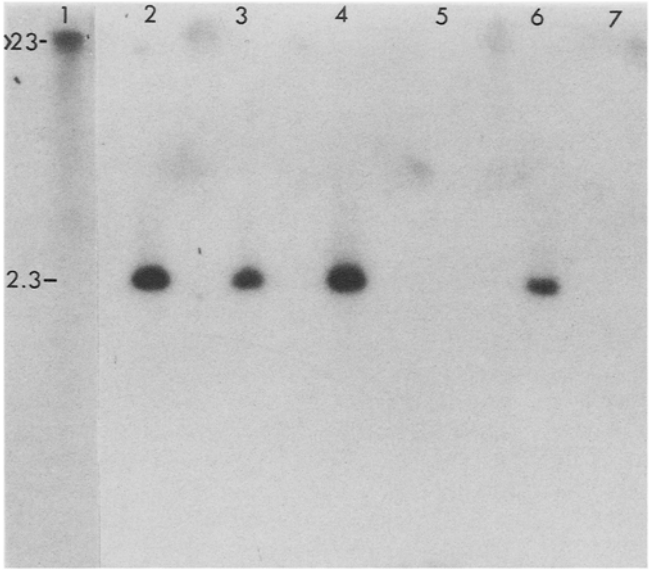

FIG. 5. Southern blot showing segregation among R_2 progeny of the single insert R_1 plant. The blot was probed with the 1.9 kb *uidA* probe. *Lane 1*, uncut DNA from an R_2 progeny plant showing integration of the foreign genes into high molecular weight DNA; *lanes 2–6*, EcoRI cut DNA from five R_2 progeny plants. Four of the plants contain the foreign gene whereas the fifth is negative, indicating segregation. *Lane 7*, EcoRI cut nontransformed control DNA.

TABLE 2

NUMBERS OF SHOOTS PRODUCED FROM COTYLEDONARY NODE EXPLANTS OF *PISUM SATIVUM* INOCULATED WITH *AGROBACTERIUM TUMEFACIENS* STRAINS AND PLACED ON SELECTION MEDIUM FOR 2 TO 4 WK[a]

Agrobacterium Strain	Selection Level[b]	No. of Explants	No. of Shoots	No. of Transformed Shoots[c]
EHA101 (pUS3)	105 kan	738	67	0
	250 kan	800	57	0
EHA101 (pBI1042)	15 hyg	200	7	0

[a] Inoculation was done using method 2; [b] mg/liter kanamycin or hygromycin; [c] determined by Southern analysis of all shoots.

Success has been achieved in the grain legume soybean using a particle gun to inject DNA-coated gold particles into meristematic tissue followed by the induction of multiple shoots (Christou et al., 1990). Although this technique is genotype independent and has proven successful in recovering transformed plants the primary regenerants are usually chimeric and the germline transformation frequency was only 0.5%. The procedure is therefore labor intensive and may not be useful for many laboratories; however, as there is currently no better system for pea transformation the use of particle gun techniques are also being investigated.

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