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Production of transgenic pea (*Pisum sativum* L.) plants by *Agrobacterium tumefaciens* – mediated gene transfer

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Summary. A transformation system that allows regeneration of transgenic pea plants from calli selected for antibiotic resistance was developed. Explants from axenic shoot cultures and seedling epicotyls were cocultivated with nononcogenic *Agrobacterium tumefaciens* strains, and transformed callus could be selected on callus-inducing media containing either 15 mg/l hygromycin or 75 mg/l kanamycin. After several passages on regeneration medium, shoot organogenesis could be reproducibly induced on hygromycin-resistant calli, but not on the calli selected for kanamycin resistance. Regenerated shoots could subsequently be rooted and transferred into the greenhouse. In addition, the effects of different callus-inducing and growth media on organogenesis were investigated. The transformation of the calli and regenerated plants was confirmed by DNA analysis.

Key words: Transformation – Pea – *Pisum sativum* – *Agrobacterium tumefaciens* – Regeneration

Introduction

Recently, the techniques for manipulating the plant genome either via direct gene transfer or by use of *Agrobacterium tumefaciens* as a vector have been extended to several agronomically important species, such as tomato, *Lycopersicum esculentum* (McCormick et al. 1986), oilseed rape, *Brassica napus* (Fry et al. 1987), rice, *Oryza sativa* (Toriyama et al. 1988; Zhang et al. 1988), potato, *Solanum tuberosum* (Shahin and Simpson 1986), rye, *Secale cereale* (De la Pena et al. 1987), and maize, *Zea mays* (Rhodes et al. 1988). The most commonly used vector for gene transfer is *Agrobacterium tumefaciens*, a soil bacterium causing the crown gall disease in higher plants. Disarmed Ti-plasmids can be used to introduce foreign genes into plant cells without interfering with the production of growth factors in the cells, and these cells may subsequently be induced to regenerate plants (for review, see Weising et al. 1988). Prerequisites for developing an efficient transformation system for any given plant species are efficient methods for transformation and selection of transformed cells, as well as a reliable in vitro culture and regeneration system. Many legume species have been shown to be susceptible to *Agrobacterium* infection but, due to problems in regeneration, few economically important grain legumes have as yet been successfully transformed using nononcogenic vectors; recovery of transgenic plants has so far been reported only in moth bean, *Vigna aconitifolia* (Köhler et al. 1987a, b) and soybean, *Glycine max* (Hinchee et al. 1988). Pea, *Pisum sativum*, has been shown to be susceptible to *Agrobacterium* infection and transformation by nononcogenic vectors (Bercetche et al. 1987; Hussey et al. 1989; Hobbs et al. 1989; Puonti-Kaerlas et al. 1989), and shoot and plant regeneration from various explants (for reviews, see Mroginski and Kartha 1984; Hammat et al. 1986), and from protoplasts (Puonti-Kaerlas and Eriksson 1988; J. Puonti-Kaerlas, unpublished data; Lehminger-Mertens and Jacobsen 1989) has been demonstrated. Thus, the basic requirements for developing a transformation system for pea already exist. Pea is an important crop plant with well-defined genetics, and many pea genes have been isolated and studied in other species that are more easily manageable in vitro. Here we report a transformation procedure for producing transgenic pea plants, which should open new possibilities both for plant breeders as a complement to traditional breeding programs and for studies on plant physiology and molecular biology using this species.

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Materials and methods

Plant material

Five cultivars of pea, cultivars Filby, Petra, Puget, Stivo, and Vreta, were used in the transformation experiments. Seeds were surface-sterilized by soaking in 20% commercial bleach (4.5% hypochlorite) for 20 min, and then rinsed several times with sterile, double-distilled water. They were germinated in darkness at 20 °C on 1.5% bacto agar plates supplemented with 0.05 *M* $CaCl_2$. After 10 days, epicotyls were excised and either used directly as explants in cocultivation experiments or maintained as shoot cultures in sterile baby food jars on MS (Murashige and Skoog 1962) medium withouth growth regulators, supplemented with 3% sucrose and solidified with 0.4% agarose. Shoot cultures and all other plant material were kept in growth chambers with 18/6 h light period (30 μE m^{-2} s^{-1}, Osram 36 W/30 warm white) and 20 °C temperature, unless otherwise stated.

Bacterial strains

The plasmid pGV1503 (Plant Genetic Systems, Ghent, Belgium), carrying the hygromycin phosphotransferase (*hpt*) gene, which confers resistance to the antibiotic hygromycin on plant cells, was transferred to *Agrobacterium tumefaciens* strain GV3101 (Holsters et al. 1980) harboring pGV2260 (Deblaere et al. 1985), by biparental mating as described by Van Haute et al. (1983). The transconjugant strain GV3101(pGV2260::pGV1503) was selected and maintained on YEB (Vervliet et al. 1975) plates with 100 mg/l rifampicin, 100 mg/l spectinomycin, and 100 mg/l carbenicillin. Similarly, plasmids pCAP212, carrying kanamycin and chloramphenicol resistance markers (Velten and Schell 1985), and pLD1, carrying kanamycin and metothrexate resistance markers (Dekeyser et al. 1989), were transferred to GV3101 harboring pGV3850 (Zambryski et al. 1983), and the transconjugant strains GV3101(pGV3850::pCAP212) and GV3101(pGV3850::pLD1) were selected and maintained on YEB plates with appropriate antibiotics. For cocultivation the bacteria were grown overnight in MinA medium (Miller 1972) on a rotary shaker (200 rpm) at 28 °C.

Transformation experiments

A transformation system based on explant cocultivation was developed for pea. Shoot cultures and epicotyls from axenically germinated seedlings were cut to pieces in 15 ml B5 medium (Gamborg et al. 1968) supplemented with 2% sucrose, 0.5 mg/l 6-benzyladenine (BA), and 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Bacteria from overnight cultures were added to a final concentration of 1×10^8 bacteria/ml and cocultivated with the plant explants for 48–72 h in the culture chamber. The explants were then washed with sterile, double-distilled water containing 500 mg/l Claforan (Hoechst), blotted dry against sterile filter paper, and transferred to callus initiation media containing 15 mg/l hygromycin B (Sigma) or 75 mg/l kanamycin sulphate (Sigma) and 500 mg/l Claforan. Claforan was omitted from the medium after 3–4 months of culture.

Callus initiation and regeneration experiments

Four different hormone combinations were tested for callus initiation efficiency. The basic medium was B5 supplemented with 3% sucrose and solidified with 0.4% agarose. The different hormone combinations tested were Ci1: 0.5 mg/l of both BA and 2,4-D; Ci2: 2 mg/l α-naphthalene acetic acid (NAA) and 1 mg/l BA; Ci3: 0.75 mg/l picloram (pic); and Ci4: 0.5 mg/l 2,4-D; (Ci = callus induction). After 1–2 months, the developing calli were excised from the explants and transferred to either callus or shoot induction media, C1: 1 mg/l BA and 0.5 mg/l NAA; C2: 0.2 mg/l indole acetic acid (IAA) and 5 mg/l BA; C3: 1 mg/l BA; C4: 0.5 mg/l pic. After 1–2 months on these media, the calli were transferred to C5: 5 mg/l of both BA and kinetin (kin), and 1 mg/l abscissic acid (ABA). The calli were subcultured every 4 weeks, and before each subculture the growth of the calli on the different media was evaluated. Shoots emerging from the calli were transferred to the shoot culture medium and maintained as shoot cultures. Roots developed spontaneously on the regenerated shoots on shoot culture medium or could be induced by transfer to root induction medium (Kublakova et al. 1988) containing half-strength MS salts, MS vitamins, 3% sucrose, and 0.18 mg/l IAA and 0.19 mg/l NAA, solidified with 0.4% agarose. The regenerated plants were first potted in jars with sterilized soil in culture chambers, after which they could be transferred to the greenhouse.

DNA analysis

Total DNA was isolated from untransformed control calli and from the calli selected for hygromycin or kanamycin resistance, basically as described by Dellaporta et al. (1983), or from leaves of the putative transgenic plants as described by Bedbrook (1981). The DNA was digested with EcoRI or HindIII, and 10-μg samples were run on 0.8% agarose gels and blotted onto supported nitrocellulose filters (Hybond C, Amersham, UK) according to the instructions of the manufacturer. The filters were probed with either the 1-kb BamHI fragment of pGV1503 containing the *hpt* gene or the 3-kb EcoRI/HindIII fragment from pLGV1103neo (Hain et al. 1985) containing the neomycin phosphotransferase II (*NPTII*) gene. The probes were labelled with ^{32}P-dCTP using a multiprime labelling kit (Amersham, UK), and hybridizations were performed according to the manufacturer's instructions. In autoradiography, Hyperfilm-MP (Amersham, UK) was used with intensifying screens at −70 °C.

Results

Callus induction and growth

The medium used for callus induction on selective plates after cocultivation with GV3101(pGV2260::pGV1503) had a distinct effect on the number of calli developing on the explants (Table 1). The greatest frequency of callus formation occurred on Ci1 (Fig. 1 a) in all three cultivars tested. On this medium as well as on Ci2, the calli produced were green and relatively fast-growing, while the few calli that grew on Ci4 were brownish and grew very slowly (Table 2). The calli on Ci3 also were brown and slow-growing. The best initial growth of calli was observed on Ci2. The best response of the cultivars tested

Table 1. The number of hygromycin-resistant calli per 200 explants produced on different callus-inducing media after cocultivation with GV3101(pGV2260::pGV1503) after 1 month. The table presents a summary of four independent experiments

Medium	Filby	Puget	Stivo
Ci 1	240	392	135
Ci 2	100	219	91
Ci 3	114	264	64
Ci 4	91	153	36

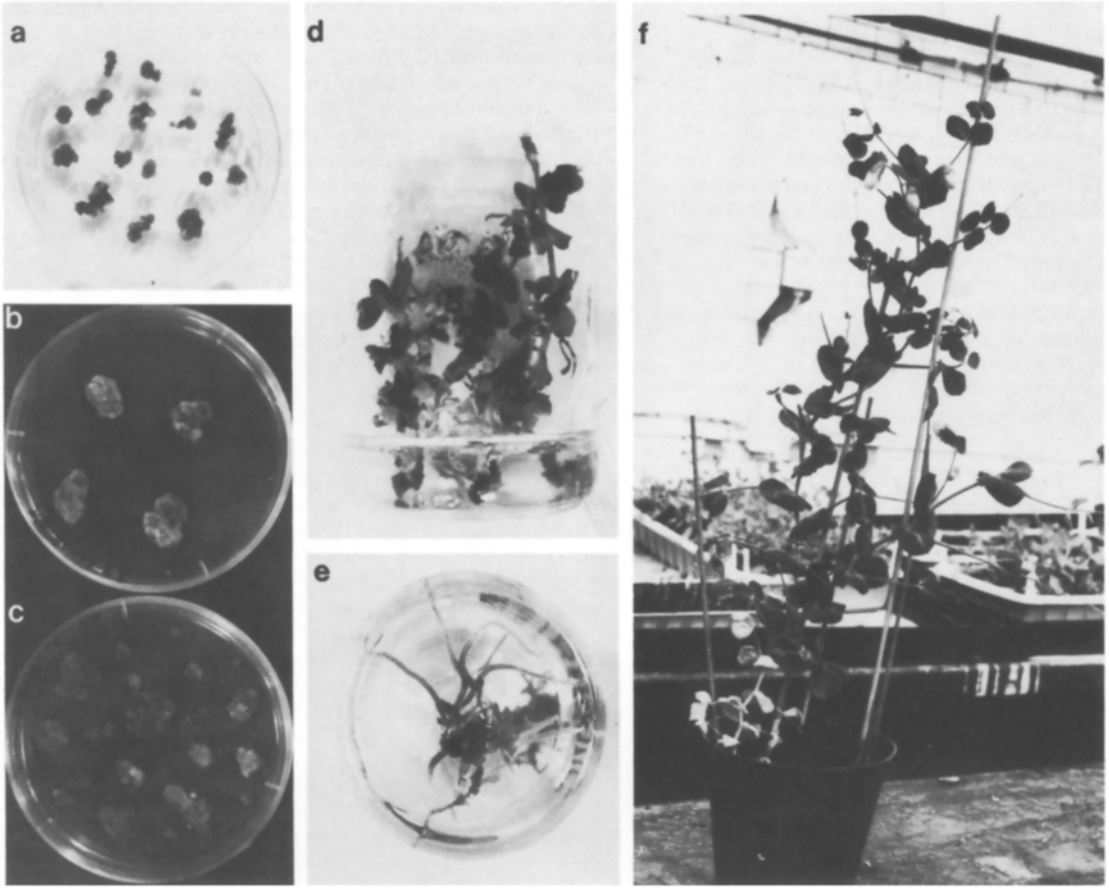

Fig. 1. **a** Stivo explants transformed with GV3101(pGV2260::pGV1503) producing callus under hygromycin selection on Ci1 medium; **b** Stivo callus transformed with GV3101(pGV3850::pCAP212) under kanamycin selection on shoot induction medium; **c** Stivo callus transformed with GV3101(pGV2260::pGV1503) under selection on shoot induction medium; **d** shoot regenerated from callus transformed with GV3101(pGV2260::pGV1503); **e** rooting of regenerated transgenic shoots; **f** transgenic Stivo plant flowering in the greenhouse

Table 2. The growth of Filby, Puget, and Stivo callus 1 month after subculturing to different callus and shoot induction media, from first to fifth subculture; a summary of seven independent experiments

Start medium Ci1	Growth on:							
1st subculture	Ci1	C1	C1	C2	C2	C3	C3	C4
Filby	+++	+++	+++	++	++	+	+	++
Puget	+++	+++	+++	++	++	+	+	+
Stivo	++	++	++	+	+	+	+	+
2nd subculture	C1	C1	C1	C2	C2	C3	C3	C5
Filby	+++	+++	+++	++	++	+	+	−
Puget	+++	+++	+++	++	++	+	+	−
Stivo	++	++	++	+	+	−	−	−
3rd subculture	C1	C4	C5	C2	C5	C3	C5	
Filby	+++	+++	++	+	±	−	−	
Puget	+++	+++	++	+	±	−	−	
Stivo	+++	+++	++	+	±	−	−	
4th subculture	C4	C5	C5	C5				
Filby	+++	++	+	±				
Puget	+++	++	+	±				
Stivo	+++	++	+	±				
5th subculture	C5	C5	C5					
Filby	++	++	+					
Puget	++	++	+					
Stivo	++	++	+					

Table 2. (Continued)

Start medium Ci2	Growth on:						
1st subculture	Ci2	C1	C2	C2	C3	C3	C4
Filby	+++	+++	++	++	+	+	+++
Puget	+++	+++	++	++	+	+	++
Stivo	+++	+++	+	+	+	+	+
2nd subculture	C1	C1	C2	C2	C3	C3	C5
Filby	+++	+++	++	++	±	+	−
Puget	+++	+++	+	+	±	+	−
Stivo	+++	+++	±	±	±	±	−
3rd subculture	C1	C4	C2	C5	C3	C5	
Filby	+++	++	++	+	−	−	
Puget	+++	++	+	+	−	−	
Stivo	+++	++	±	+	−	−	
4th subculture	C4	C5	C2	C5			
Filby	++	++	+	±			
Puget	++	++	+	±			
Stivo	++	++	±	±			
5th subculture	C5	C5					
Filby	++	++					
Puget	++	++					
Stivo	++	++					
Start medium Ci3	Growth on:						
1st subculture	Ci3	C1	C2	C2	C3	C3	
Filby	+	++	+	+	−	−	
Puget	++	++	+	+	±	±	
Stivo	+	+	±	±	−	−	
2nd subculture	C1	C1	C2	C2	C5	C5	
Filby	+	+	−	−	−	−	
Puget	+	+	−	−	−	−	
Stivo	+	+	−	−	−	−	
3rd subculture	C1	C5					
Filby	+	−					
Puget	+	−					
Stivo	+	−					
4th subculture	C5						
Filby	−						
Puget	−						
Stivo	−						
Start medium Ci4	Growth on:						
1st subculture	Ci4	C1	C2	C2	C3	C3	
Filby	+	+	+	+	±	±	
Puget	++	+	+	+	++	+	
Stivo	+	+	+	+	−	−	
2nd subculture	C1	C1	C2	C5	C3	C5	
Filby	+	+	−	−	−	−	
Puget	+	+	−	−	−	+	
Stivo	+	±	−	−	−	−	
3rd subculture	C1	C5					
Filby	+	−					
Puget	+	−					
Stivo	+	−					
4th subculture	C5						
Filby	−						
Puget	−						
Stivo	−						

+++ = green, vigorously growing callus; ++ = green, well growing callus; + = callus growing slowly; ± = hardly any growth; − = no growth, callus dying

Table 3. Production of transformed calli per 200 explants from different pea cultivars on Ci1 medium under hygromycin selection after cocultivation with GV3101(pGV2260::pGV1503) and under kanamycin selection after cocultivation with GV3101 (pGV3850::pCAP212) or GV3101(pGV3850::pLD1); a summary of four independent experiments, nt = not tested

Strain	Filby	Petra	Puget	Stivo	Vreta
GV3101 (pGV3850::pCAP212)	89	53	105	61	67
GV3101 (pGV2260::pGV1503)	240	105	392	135	155
GV3101 (pGV3850::pLD1)	158	30	NT	58	114

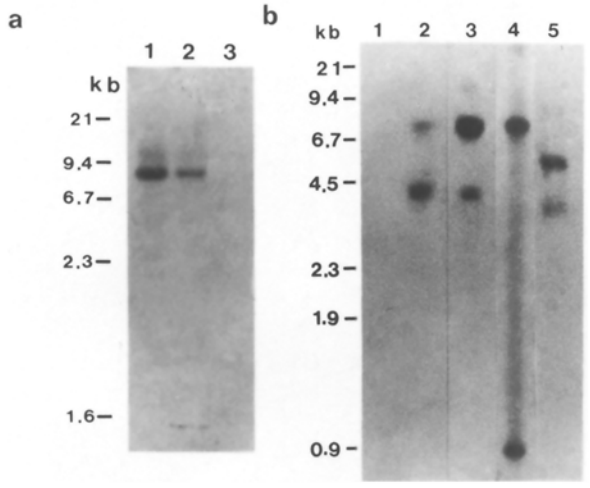

Fig. 2a and b. Southern blots of calli and plants selected for kanamycin or hygromycin resistance. **a** *lane 1* – HindIII digestion of DNA from callus transformed with GV3101 (pGV3850::pCAP212); *lane 2* – from callus transformed with GV3101 (pGV3850::pLD1); *lane 3* – from untransformed control callus. **b** *lane 1* – untransformed control; *lane 2* EcoRI digestion of DNA from a callus transformed with GV3101 (pGV2260::pGV1503); *lane 3* – EcoRI digestion of DNA from a transgenic plant regenerated from the callus in lane 2; *lane 4* – EcoRI/HindIII digestion of DNA from the same plant; *lane 5* – EcoRI digestion of DNA from a second callus transformed with GV3101 (pGV2260::pGV1503)

was obtained with Puget explants, Stivo being the least responsive.

Further growth of the calli initiated on Ci3 and Ci4 was very poor, and the survival rate upon transfer to media containing high levels of cytokinin was low (Table 2). In later stages the calli initiated on Ci1 grew better than those initiated on Ci2; in addition, the survival rate of the calli initiated on Ci1 on the high cytokinin medium was better than that of those initiated on Ci2. From these results the best culture sequence was determined to be Ci1→C1→C1→(C4)→C5.

The rate of callus production under selection was also dependent on the cultivar and the plasmid or type of selection used (Table 3). Transformation frequencies with GV3101(pGV2260::pGV1503) carrying the *hpt* gene were 1.3–3.7 times higher than with either of the strains carrying the *NPTII* gene. In all cases tested, Puget was the most responsive cultivar, while Petra was the least responsive.

Regeneration of transgenic plants

Shoot regeneration was obtained from Stivo calli (Fig. 1c and d) selected for hygromycin resistance after several passages on C5 medium. Puget calli selected for hygromycin resistance often produced roots, but the frequency of shoot formation was much lower than in Stivo calli. In the other pea cultivars tested no shoot organogenesis has been observed, nor have any shoots appeared on the kanamycin-resistant calli in Stivo or Puget, even after 20 months (Fig. 1b).

Transfer of calli to C5 medium was first possible 3–4 months after callus initiation; transfer to this medium at earlier stages resulted in the death of the calli (Table 2). One passage on C4 before transfer to C5 appeared to improve the survival and regeneration potential of Stivo callus, but was not necessary for the shoot induction. The regeneration rate of Stivo calli initiated on Ci1 was better than that of those initiated on Ci2. About 40% of the regenerated shoots rooted spontaneously when transferred to shoot culture medium; over half of the remaining 60% could be rooted by transfer to the rooting medium (Fig. 1e). Of the 28 transgenic plants thus far transferred into the greenhouse, all have developed flowers (Fig. 1f), but no seeds have been obtained to date.

DNA analysis

A Southern analysis of nuclear DNA from transformed pea material is shown in Fig. 2. Fragments hybridizing to the *NPTII* or *hpt* probes were found in all tested transgenic calli and plants, indicating stable integration of the transferred DNA into the pea genome. The 3-kb probe derived from the *NPTII* gene of pLGV1103neo hybridized to an 8.1-kb HindIII fragment from DNA of calli transformed with GV3101(pGV3850::pCAP212) as well as with GV3101(pGV3850::pLD1) (Fig. 2a). This fragment is an internal one common to both constructions.

DNA from two hygromycin-resistant calli, digested with EcoRI, gave similar patterns of hybridization to the 1-kb *hpt* gene probe, i.e., two bands in the range of 4–8 kb (Fig. 2b, lanes 2 and 5). Since the pGV1503 T-DNA contains a single EcoRI site within the *hpt* gene, this probe would be expected to hybridize to two EcoRI fragments, each extending across a T-DNA border into the plant DNA at the site of integration. The differences in sizes of the hybridizing fragments between the two transformants thus show that T-DNA integration has occurred at different locations in the genomes of the two

transformants, and that each transformant contains a single copy of T-DNA. More complex hybridization patterns observed in other transformants, using the same probe, indicate that multiple copies of the T-DNA are present in some transformants (data not shown). The presence of an internal 0.9-kb EcoRI/HindIII fragment (Fig. 2b, lane 4) confirms the structure of the T-DNA in the transgenic plants. Callus DNA and DNA from plants regenerated from the callus gave very similar hybridization patterns, indicating that the tissue culture procedure did not cause any detectable rearrangements of the T-DNA (Fig. 2b, lanes 2 and 3).

Discussion

A method for regenerating transgenic pea plants by use of cocultivation of explants from pea shoot cultures with nononcogenic *Agrobacterium tumefaciens* vectors carrying plant selectable markers was developed. Transformation was most efficient when the *hpt* gene was used as plant selectable marker. A three-step regeneration system was found to be essential for induction of shoot organogenesis in the calli selected for hygromycin resistance. The choice of selectable marker was found to be of paramount importance for the regenerative competence of the calli derived from shoot explants; hygromycin-resistant calli regenerated shoots with up to 15% efficiency, while no shoot organogenesis could be induced on medium containing kanamycin. The time required for shoot induction on the regeneration medium is as of now up to 6 months, and our attempts to reduce it have so far been unsuccessful. A similar time period was also found to be necessary for shoot regeneration from protoplast-derived callus (Puonti-Kaerlas and Eriksson 1988), although much shorter periods have been reported for regeneration from pea protoplasts via somatic embryogenesis (Lehminger-Mertens and Jacobsen 1989).

Young pea tissues, e.g., meristems or epicotyls of newly germinated seedlings, have been shown in earlier studies to be the most susceptible to infection by wild-type *Agrobacterium* strains (Hussey et al. 1989; Puonti-Kaerlas et al. 1989), but these tissues were very sensitive to the cocultivation procedure (Puonti-Kaerlas et al. 1989). Furthermore, the results obtained from inoculation studies with wild-type strains cannot be used directly to determine the best type of explant for cocultivation experiments, as the response varies between in vivo and in vitro experiments (Puonti-Kaerlas et al. 1989; Hobbs et al. 1989). In our experiments the best response was obtained by use of explants derived from axenic shoot cultures, while young epicotyls gave a significantly lower frequency of callus formation, due to poor survival of most of the explants during cocultivation (data not shown).

The response of the different pea cultivars in callus induction studies on selective medium differed between the cultivars and the types of selection applied. In all cases Puget was the most responsive cultivar, while the lowest frequency of callus formation was obtained on Petra explants. Genotype/cultivar has been shown to play a role in the susceptibility of pea to various *Agrobacterium* strains, although at present there appears to be a discrepancy between the results (Bercetche et al. 1987; Hussey et al. 1989; Puonti-Kaerlas et al. 1989; Hobbs et al. 1989), especially in the case of octopine plasmids. This may be partly explained by the use of different material in the inoculation studies. pGV2260 is a derivative of pTiB6S3, which we have earlier reported as unable to induce tumor formation in pea (Puonti-Kaerlas et al. 1989). This plasmid, however, when used as a nononcogenic cointegrate vector, gives rise to higher transformation frequencies than those obtained by the use of pGV3850, a derivative of pTiC58, which is very efficient in inducing tumors in pea. Thus, the low virulence of the B6S3 wild-type strain is probably not a result of a low efficiency in T-DNA transfer, but is more likely due to inability of the integrated T-DNA to induce tumor formation in pea.

The Southern blot experiments on transgenic calli and plants confirm the stable integration of the transferred DNA into the pea genome. The DNA patterns in the calli and the regenerated plants were similar, indicating that no rearrangements have taken place during the culture and induction of organogenesis in the calli.

In all cases tested, at least 30% more callus was obtained when *hpt* as compared to *NPTII* was used as plant selectable marker gene. At this stage we cannot differentiate between the effect of the plasmid and the effect of the selection pressure applied (kanamycin versus hygromycin) on the transformation frequencies observed when using the different vectors. In a preliminary set of experiments, however, using a vector carrying both *NPTII* and *hpt* genes as plant selectable markers, selection on hygromycin resulted in more efficient callus induction than selection on kanamycin (data not shown). One reason for this may be that the untransformed tissue is killed faster by hygromycin (about 2 weeks) than by kanamycin (about 4 weeks), and thus does not hinder the growth of transformed cells as much as when kanamycin is used as selective agent. The effect of kanamycin selection on the regenerative competence of the calli is very clear. In no case could organogenesis be induced on the calli growing on medium containing kanamycin. In control experiments, where the same calli were grown on medium without kanamycin, some organization could be observed in the calli after 3–4 months, but so far no shoots have developed from any of the calli.

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Note added in proof

In recent experiments transgenic Stivo plants derived from hygromycinresistant calli have gone through the flowering stage and set seeds at high frequency.