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Inheritance of a bacterial hygromycin phosphotransferase gene in the progeny of primary transgenic pea plants

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Summary. An analysis of the progeny of primary transgenic pea plants in terms of transmission of the transferred DNA, fertility and morphology is presented. A transformation system developed for pea that allows the regeneration of fertile transgenic pea plants from calli selected for antibiotic resistance was used. Explants from axenic shoot cultures were co-cultivated with a non-oncogenic *Agrobacterium tumefaciens* strain carrying a gene encoding hygromycin phosphotransferase as selectable marker, and transformed callus could be selected on callus-inducing media containing 15 mg/l hygromycin. After several passages on regeneration medium, shoot organogenesis could be reproducibly induced on the hygromycin resistant calli, and the regenerated shoots could subsequently be rooted and transferred to the greenhouse, where they proceeded to flower and set seed. The transmission of the introduced gene into the progeny of the regenerated transgenic plants was studied over two generations, and stable transmission was shown to take place. The transgenic nature of the calli and regenerated plants and their progeny was confirmed by DNA and RNA analysis. The DNA and ploidy levels of the progeny plants and primary regenerants were studied by chromosome analysis, and the offspring of the primary transformants were evaluated morphologically.

Key words: Transformation – *Pisum sativum* – *Agrobacterium tumefaciens* – Regeneration – Transgenic plants – Progeny

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; BA, 6-benzyladenine, *hpt*, hygromycin phosphotransferase gene; IAA, indole acetic acid, kin, kinetin; NAA, α-naphtalene acetic acid; picloram, 4-amino-3,5,6-trichloropicolinic acid

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Introduction

Agrobacterium tumefaciens is a soil bacterium causing crown gall disease in higher plants. The transfer of a segment (T-DNA) from the Ti-plasmid of virulent bacterial strains into the plant nuclear genome and the expression of the genes located in the T-DNA cause a neoplastic growth of the transformed cells resulting in tumour formation (for reviews see Gheysen et al. 1985; Zambryski 1988). Disarmed Ti-plasmids can be used to introduce foreign genes into plant cells without interfering with the production of growth factors in the transformed cells, and these cells may subsequently be induced to regenerate plants (for a review see Weising et al. 1988). Recently, the techniques for manipulating the plant genome 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), oil seed rape, *Brassica napus* (Fry et al. 1987) and potato, *Solanum tuberosum* (Shahin and Simpson 1986). Prerequisites for developing an efficient transformation system for any given plant species are efficient methods for transformation and selection of transformed cells compatible with 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 commercially important grain legumes have as yet been successfully transformed using non-oncogenic vectors. Recovery of transgenic plants and stable transmission of the introduced genes into the progeny of the transgenic plants have so far been reported only in soybean, *Glycine max* (Hinchee et al. 1988). Pea, *Pisum sativum*, is an important crop plant in the temperate regions and especially interesting as a potential alternative crop for the cereals due to its symbiotic nitrogen-fixing capacity and its high protein content. Pea

has been shown to be susceptible to *Agrobacterium* transformation by non-oncogenic vectors (Puonti-Kaerlas et al. 1989; De Kathen and Jacobsen 1990; Lulsdorf et al. 1991), and fertile transgenic plants have been regenerated from callus selected for hygromycin resistance (Puonti-Kaerlas et al. 1990). Here we report a method for plant regeneration from transgenic callus and the analysis of the R_1 and R_2 progeny of the original R_0 regenerant transgenic plants. A stable inheritance of the transferred DNA in the progeny is demonstrated.

Materials and methods

Plant material

Pea cvs 'Puget' and 'Stivo' 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 propagated as shoot cultures in sterile baby-food jars on MS (Murashige and Skoog 1962) medium without growth regulators, supplemented with 3% sucrose and solidified with 0.4% agarose or 0.3% Gelrite gellan gum (Chemical Dynamics Corp). Shoot cultures and all other plant material were kept in growth chambers with 18/6 h light/dark period (50 $\mu E\ m^{-2}\ s^{-1}$, Osram 36 W/30 warm white) and 20 °C temperature unless otherwise stated.

Bacterial strains

The plasmid pGV1503 (Plant Genetic Systems, Gent, Belgium) carrying the hygromycin phosphotransferase gene (*hpt*), which confers hygromycin resistance to plant cells (Van den Elzen et al. 1985), was transferred to *Agrobacterium tumefaciens* strain GV3101 (Holsters et al. 1980) harbouring 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 appropriate antibiotics. For co-cultivation the bacteria were grown overnight in MinA medium (Miller 1972) on a rotary shaker (200 rpm) at 28 °C.

Transformation of pea explants

A transformation system based on explant co-cultivation developed for pea (Puonti-Kaerlas et al. 1990) was used. Shoot cultures derived from axenically germinated seedlings were cut into approximately 3-mm pieces in 5 ml B5 medium (Gamborg et al. 1968) supplemented with 2% sucrose, 0.5 mg/l BA and 0.5 mg/l 2,4-D. Bacteria from overnight cultures were added to a final concentration of 1×10^8 bacteria/ml in a final volume of 15 ml and co-cultivated 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 medium containing 15 mg/l hygromycin B (Sigma) and 500 mg/l Claforan.

Callus initiation and regeneration of transgenic plants

Callus was induced on the explants after co-cultivation under selection on B5 medium supplemented with 3% sucrose and solidified with 0.4% agarose or 0.3% Gelrite, and containing 0.5 mg/l of both BA and 2,4-D as well as 15 mg/l hygromycin B and 500 mg/l Claforan. After 1–2 months the developing calli were excised from the explants and transferred to callus medium (B5 with 1 mg/l BA, 0.5 mg/l NAA and 15 mg/l hygromycin B and 500 mg/l Claforan). At the subsequent subcultures the calli were transferred first to B5 medium with 0.5 mg/l picloram and 15 mg/l hygromycin B and 500 mg/l Claforan, and then to shoot induction medium (B5 with 5 mg/l of both BA and kin, 1 mg/l ABA and 15 mg/l hygromycin B and 500 mg/l Claforan). The calli were subcultured every 4 weeks, and Claforan was omitted from the medium after 3–4 months of culture.

When the shoots emerging from the calli were 1–2 mm long, they were transferred to the shoot culture medium to elongate and normalize. Roots either 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, 0.18 mg/l IAA and 0.19 mg/l NAA, solidified with 0.4% agarose or 0.3% Gelrite. When the roots were 2–5 cm long, the plantlets were transferred directly into pots in the greenhouse and covered for the first week with a mesh cloth. After 10–15 weeks in the greenhouse the plants proceeded to flower and subsequently set seed.

Resistance tests of the offspring and inheritance of hpt

Mature seeds were collected from the R_0 primary transgenic plants and grown in the greenhouse for analysis. Before sowing, the seeds were rubbed gently with sandpaper to facilitate water uptake during imbibition and germinated on moist filter paper for 2–4 days. When the radicle emerged the seeds were planted in the greenhouse. The third uppermost leaf from the R_1 and control plants with 8–11 leaf pairs was used in screening for hygromycin resistance by spotting a drop of 1% hygromycin B solution in 0.1% SDS onto the leaf by means of a cotton wool pad wrapped around a toothpick. The appearance of local lesions on the leaves at the point of application indicating sensitivity to hygromycin was monitored after 1–2 weeks. The spot test was confirmed by testing the capacity of the leaf explants to produce callus on selective medium. Leaves were surface sterilized by immersion in 15% commercial bleach with a few drops of Tween 20 for 15 min and subsequently rinsed several times with sterile, double-distilled water. They were cut to pieces and placed on callus-inducing medium containing 15 mg/l Hygromycin B. Explants from untransformed control plants were also included. The R_1 generation was selfed to study the transmission and segregation of the transferred gene in the R_2 progeny.

DNA analysis

Total DNA was isolated as described by Bedbrook (1981) from untransformed control plants and from the transgenic calli and plants as well as progeny plants, both from those selected for hygromycin resistance and from those sensitive to hygromycin. The DNA was digested with *Hin*dIII, and 10 µg samples of both digested and undigested DNA were run on 0.8% agarose gels and blotted onto supported nitrocellulose filters (Hybond C, Amersham) according to the instructions of the manufacturer. The filters were probed with the 1-kb *Bam*HI fragment of pGV1503 containing *hpt*. The probes were labelled with ^{32}P-dCTP by random priming (Boehringer), and hybridizations were performed as described (Amersham). In autoradiography, the filters were exposed to X-ray film between intensifying screens at −70 °C.

RNA analysis

Total RNA was extracted from the R_1 and R_2 seedlings and non-transformed controls as follows. Leaves were homogenized

in liquid nitrogen, transferred to centrifuge tubes containing equal volumes of extraction buffer (100 m*M* TRIS-HCl pH 8.5, 100 m*M* NaCl, 20 m*M* EDTA, 1% w/v SDS, 2% v/v β-mercaptoethanol) and 1:1 phenol : chloroform. After vortexing, the homogenate was centrifuged for 5 min at 9,500 *g*, and the aqueous phase was re-extracted with phenol : chloroform. After precipitation by isopropanol, the nucleic acids were resuspended in water, from which the RNA was precipitated with LiCl. After resuspension in water, the RNA was reprecipitated with Na-acetate and ethanol and finally dissolved in water. Samples of RNA (15 µg per lane) were run on 1% agarose-formaldehyde gels as described (Maniatis et al. 1982) and blotted onto supported nitrocellulose filters (Hybond C, Amersham) according to the instructions of the manufacturer. The hybridization and autoradiography were as described for the DNA filters.

Chromosome analysis

Root tips from 6- to 10-day-old seedlings were collected and treated for 4 h in saturated α-bromonaphthalene solution and fixed for 24 h in 1 : 3 95% ethanol : glacial acetic acid (Pijnacker et al. 1986). They were then used for chromosome analysis by Feulgen staining (Gould 1984).

Morphological evaluation of the offspring

The morphology of the R_1 and R_2 plants was compared to that of the parental controls by counting the number of nodes and measuring the height of the plants at the onset of flowering. The length of the first and second leaflets from the leaves of the first flowering node and from the node immediately beneath it were measured, and their length/width ratio was determined. The width of the stem of the internode below the first flowering node was measured as well. The number of pods and seeds per plant was determined to evaluate the fertility of the transgenic clones. In the results only a summary of the statistical analysis of the data is presented. The data is available on request.

Results

Regeneration of transgenic plants

Shoot regeneration was obtained from 'Stivo' and 'Puget' calli selected for hygromycin resistance after several passages on shoot induction medium (Fig. 1 a). 'Puget' calli produced roots frequently, but the frequency of shoot formation was much lower in 'Puget' (0.2%) than in 'Stivo' (1–15%) calli. The time required for shoot induction on the regeneration medium was 4–9 months. About 40% of the regenerated shoots obtained after 6 months rooted spontaneously when transferred to shoot culture medium; over half of the remaining 60% could be rooted by transfer to the rooting medium (Fig. 1 b). The shoots arising from calli older than 8 months were progressively more difficult to root.

Fertility of the R_0 regenerants

Plants from six independent 'Stivo' lines regenerated from transgenic calli were transferred to the greenhouse to set seed. All lines produced flowers in amounts comparable to those produced by the control plants (data not shown), but three of the lines (S1, S4 and S6) did not produce any seeds. Two of the latter lines (S1 and S6) aborted their flowers, and one (S4) produced pods with no viable seeds. One of the flower-aborting lines (S6) also differed from the control plants morphologically by longer internodes, leaf form (narrower, more oblong) and flower form (longer corolla and stamens and pistils, Fig. 2 a). Three of the six lines (S2, S3 and S5) produced viable seeds with good germination capacity. The number of pods per plant did not differ from the controls, but the number of seeds obtained was less, as usually only one seed per pod developed fully into maturity. Of the

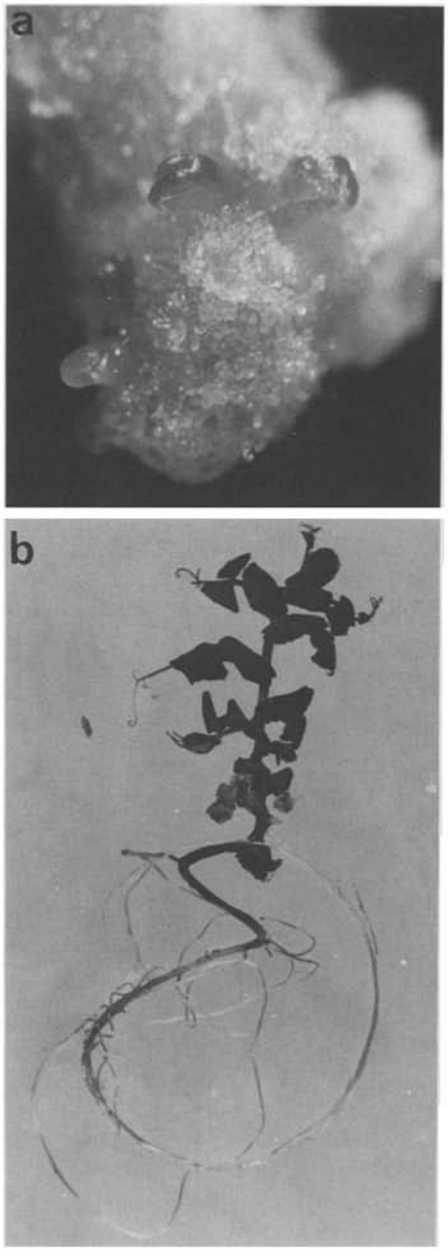

Fig. 1. **a** 'Stivo' transgenic callus regenerating shoots on selective medium; **b** rooted transgenic shoot

Fig. 2. **a** 'Stivo' R_0 regenerants, from the *left* to the *right*, clones S2, S3 and S6; **b** transgenic R_1 (*left*) and R_2 (*right*) plants of 'Stivo' clone S2

collected fully developed seeds 96% could be germinated and transferred to the greenhouse. In clone S2, 30 R_1 plants could be obtained after selfing 6 R_0 plants.

Resistance tests

The leaf spot test was designed as a rapid screening method for hygromycin-resistant plants in the greenhouse, and its accuracy was confirmed by testing the ability of leaf explants to produce callus on selective medium. Leaves from sensitive progeny plants, like the control leaves, developed local lesions 2–4 mm in diameter 1–2 weeks after the application of hygromycin onto the leaves. No lesions were observed in the leaves of primary regenerants even when a 2% hygromycin solution was used. The lowest concentration to induce lesions in the control leaves was 0.2%. In total, 34 plants of clones S2 and S3 were examined both by the spot test and by the callus induction assay. When cultured, leaf explants from all plants showing resistance in the spot test were also able to produce callus on the selective medium, whereas explants from controls and from plants judged to be sensitive were not.

Table 1. Segregation of hygromycin resistance in the R_2 progeny of the hygromycin-resistant R_1 plants in clone S2

Plant number	Resistant seedlings	Sensitive seedlings
S2 : 2	1	1
S2 : 7	9	0
S2 : 8	5	1
S2 : 9	8	4
S2 : 12	1	2
S2 : 14	11	0
S2 : 15	1	2
S2 : 16	8	0
S2 : 19	4	1

Inheritance of hygromycin resistance

R_1 plants from 'Stivo' clones S2 and S3 were tested for hygromycin resistance. In the spot and callus induction test 23 of the S2 progeny plants were resistant to hygromycin and 7 were sensitive. The R_1 progeny of clone S3 contained 1 sensitive and 3 resistant plants. The segregation pattern of hygromycin resistance was studied in the R_2 offspring of resistant R_1 plants in clone S2 (Table 1). No resistant seedlings were detected in the progeny of the sensitive plants. Thus, hygromycin resistance was stably transmitted into the offspring of the primary transformants as a single dominant Mendelian trait, even in the absence of selection pressure.

DNA and RNA analyses

DNA from hygromycin-resistant callus, R_0 regenerants and plants in the R_1 and R_2 generations was analysed to examine the copy number and integration pattern of the introduced *hpt*. Fragments hybridizing to the *hpt* probe were found both in the undigested high-molecular-weight DNA (data not shown) and in the digested samples in all of the tested transgenic calli and plants, indicating stable integration of the transferred DNA into the pea genome, with stable transmission into the offspring of the primary regenerants (Fig. 4a). In the progeny plants analysed, all the plants resistant to hygromycin also contained the transferred DNA integrated into the genome, and the pattern of integration coincided with that of their parent

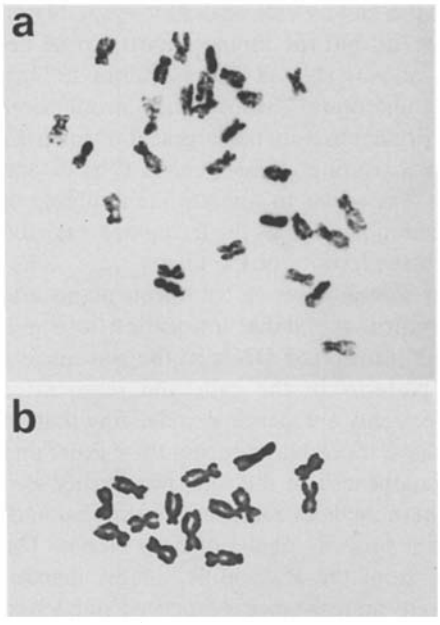

Fig. 3. **a** Root tip squash of a tetraploid transgenic plant; **b** root tip squash of a diploid parent plant

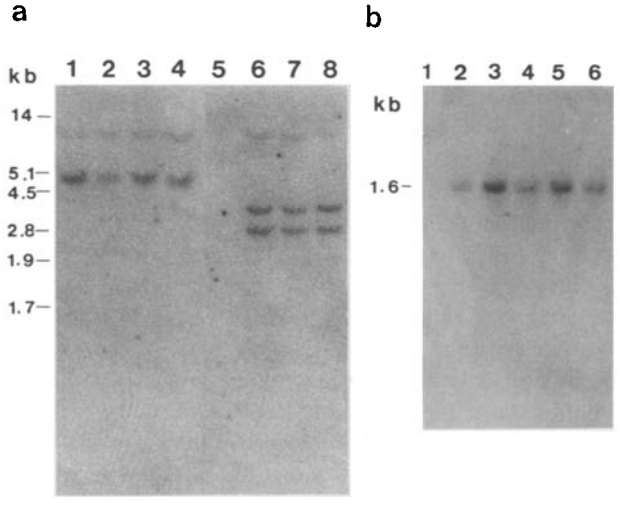

Fig. 4. **a** Southern analysis of *Hin*dIII-digested DNA samples from transgenic and control peas. *Lanes 1–4* transgenic callus, a R_0, R_1 and R_2 plant of 'Stivo' clone S2, respectively; *lane 5* untransformed control; *lanes 6–8* transgenic callus, a R_0 and R_1 plant of 'Stivo' clone S3, respectively. **b** Northern analysis of RNA from transgenic pea plants. *Lane 1* Untransformed control, *lanes 2–4* R_2 plants of clone S2, *lanes 5 and 6* R_1 plants of clone S3

plants as well as with their callus of origin. None of the hygromycin-sensitive offspring plants tested contained *hpt*. In no case could a phenotypic loss of the marker gene activity be detected.

In the DNA digested with *Hin*dIII, one band of about 4.9 kb in clone S2 and two bands of about 3.6 and 2.8 kb in clone S3 hybridized to the 1-kb *hpt* probe. Since pGV1503 T-DNA contains a single *Hin*dIII site outside *hpt*, this probe would be expected to hybridize to one *Hin*dIII fragment that extends across a T-DNA border into the plant DNA at the site of integration. The difference in size and number of the hybridizing fragments between the two clones thus shows that T-DNA integration has occurred at different locations in the genomes of the transformants and that clone S2 contains a single copy and S3 two copies of the T-DNA.

To confirm that the resistance to hygromycin was contingent on the expression of *hpt* in the transformed plants, RNA analysis was performed. In the Northern blots (Fig. 4b) an RNA transcript of 1.6 kb was recognized by the *hpt* probe in all of the transgenic offspring tested, while no signal was detected in the sensitive progeny plants or in the control plants.

Chromosome analysis

'Stivo' clones S2 and S3 were analysed by chromosome counting of root tip squashes, and both of them were shown to be tetraploid (Fig. 3a). In total 4 R_1 and 12 R_2 plants were analysed. To determine when the tetraploidization event had taken place, the parental material used for the shoot cultures was analysed as well. The results (Fig. 3b) show that the parental material was diploid, thus indicating that the polyploidization must have occurred during tissue culture.

Morphological evaluation of the offspring

The flowering time in the R_1 and R_2 offspring was 11–12 weeks from sowing, when the plants had 16.8 (±4.0)–20.0 (±2.6) nodes and the controls had 19.0 (±2.6) nodes (Fig. 2b). At this time the height of the controls was 29.0 (±3.0) cm and that of the transgenic offspring plants was between 29.2 (±7.0) and 35.0 (±5.6) cm. The regenerated material did not deviate significantly from the parental control material for these characteristics; nor did the leaf length of the leaflets in the flowering node or in the second leaflet of the transformants differ greatly from the controls. For other characteristics, however, the transgenic plants differed significantly from the controls. The leaf length of the first leaflet in the flowering node of the transformants and controls was 2.6 (±0.2) cm–3.5 (±0.7) cm and 3.2 (±0.2) cm, respectively, and that of the second leaflet, 2.3 (±0.5) cm–2.9 (±0.6) cm and 2.4 (±0.3) cm, respectively. The length of the first and second leaflet in the node below the flowering node was 3.8 (±0.6) cm and 2.5 (±0.1) cm, respectively, in the parental controls and 3.1 (±0.5) cm–3.9 (±0.6) cm and 2.2 (±0.3) cm–2.7 (±0.5) cm, respectively, in the transgenic offspring. The leaf shape, as indicated by the length/width ratio [1.7 (±0.2) and 1.5 (±0.6) in the first

and second leaflet, respectively, of the flowering node of the controls and 1.4 (±0.1) and 1.3 (±0.1)–1.4 (±0.2), respectively, in the transformants], was more rounded in the transgenic plants and their offspring. There was also variation in these characteristics between the offspring clones derived from the same callus, and plants not differing significantly from the controls in any of the parameters measured were found as well. The offspring of the primary transformants were, however, more robust than the controls. This was apparent, e.g. in the width of the stem, which ranged between 4.0 (±0.7) cm and 6.1 (±0.7) cm in the transformants and was 3.4 (±0.5) cm in the controls. The transformants also produced significantly more lateral shoots. The number of pods per plant was 50 (±17)–88 (±16) and 16 (±5) in the transgenic and control plants, respectively, but as the number of seeds per pod was considerably less in the transgenic plants, the total number of seeds per plant, 63 (±23)–83 (±33) in the transformants, did not differ significantly from the controls.

Discussion

A method for regenerating fertile transgenic pea plants by use of co-cultivation of explants from pea shoot cultures with non-oncogenic *Agrobacterium tumefaciens* vectors carrying a plant selectable marker gene was developed (Puonti-Kaerlas et al. 1989, 1990). The introduced gene, after selfing the primary transformants, was shown to be stably transmitted into the progeny over the two generations tested.

The plants obtained were fertile, but in chromosome analysis they were shown to be tetraploid. The reasons for doubling of the chromosome number may be the long culture period when high amounts of growth regulators are present in the culture medium. Polyploidization of pea cells in culture depends on the presence of cytokinins and auxins in the medium (Matthysse and Torrey 1967), and changes in ploidy levels as a result of tissue culture are also common in other plant species (D'Amato 1977) including, e.g. potato (Karp 1986; Sree Ramulu 1986) and tomato (Evans 1986). Tetraploid and aneuploid (Ahmed et al. 1987; Natali and Cavallini 1987a, b) pea plants have been regenerated from callus along with diploid plants, both via shoot organogenesis and somatic embryogenesis (Kysely et al. 1987), so evidently the tendency for endopolyploidization is high in pea. Other authors have reported the regeneration of only diploid plants, even when both polyploid and aneuploid cells were present in the cultures (Rubluo et al. 1984; De and Roy 1985). The first cell divisions in culture are usually diploid, and the doubling of C values takes place during later divisions (De and Roy 1985; Natali and Cavallini 1987a). The number of cells with 4C and higher C values increases during the culture (De and Roy 1985; Natali and Cavallini 1987a), but the timing appears to be dependent on the cultivar (Natali and Cavallini 1987a). Although highly aberrant C values and chromosome numbers may be present in calli, the largest abnormalities are selected against during organogenesis (Natali and Cavallini 1987a). Pea seems to tolerate the doubling of the chromosome number well, as the tetraploid stage did not interfere with the fertility of the plants.

The Southern blot analyses on transgenic plants and their progeny confirm the stable integration into and inheritance of the transferred DNA in the pea nuclear genome. DNA patterns in the calli, the regenerated plants and their progeny are identical, indicating that no rearrangements have taken place during the culture and induction of organogenesis in the calli, nor during seed formation. Northern analysis confirms the expression of *hpt* in the resistant progeny plants of both clones. The segregation data from the R_1 and R_2 plants demonstrates that hygromycin resistance is inherited stably as a single dominant Mendelian trait and suggests that chromosome doubling has taken place before the integration of *hpt* into the pea nuclear DNA. No loss of the transferred gene or gene activity was observed even in the absence of selection over the two offspring generations tested.

Morphologically, the tetraploid R_1 and R_2 plants did not deviate much from the controls, except for being more robust, having more rounded leaves and producing more pods and lateral shoots. The variation between the two examined clones and the controls was slight, thus showing that little somaclonal variation could be detected in the plants on the phenotypic level. More aberrant plants were observed in the R_0 population, but part of the morphological changes in this generation may have been of epigenetic origin. As many of the variant plants did not produce any seeds, it was not possible to differentiate between epigenetic and somaclonal changes. In soybean, transgenic plants obtained by use of particle bombardment also have been shown to be morphologically stable (Christou et al. 1989), whereas in townsville stylo morphologically aberrant plants were detected in the R_2 generation (Manners 1988). In pea, no such phenomena were observed, but as the number of seeds obtained was not very large, it may be that such changes have been selected against during seed development.

In some plants non-transformed escapes have been regenerated together with transgenic plants (Hinchee et al. 1988; Manners 1988; Pickardt et al. 1991), but in this study no false positives were found. The long culture period with continuous selection up to the time of shoot elongation of the regenerated shoots may have minimized the risk of untransformed cells surviving within the calli. The risk of chimaera production could not be bypassed, as complex multicellular structures were used as

explants, but in the two clones analysed no indications of chimaeras were detected.

The time required for shoot induction on the regeneration medium is as yet up to 9 months, which makes the method time-consuming and increases the risk for genomic changes in culture. A more rapid regeneration system has been described for pea (De Kathen and Jacobsen 1990), but the transgenic plants obtained by that method were chimaeric and sterile. So far we have been able to obtain fertile transgenic plants from two pea cultivars. The bottle-neck in pea transformation is the regeneration step, and more work is in progress to improve the regeneration system of pea.

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