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Stable transformation of barley via PEG-induced direct DNA uptake into protoplasts

P. A. Lazzeri, R. Brettschneider, R. Lührs and H. Lörz

Institute for General Botany (AMP II), University of Hamburg, Ohnhorststrasse 18, W-2000 Hamburg 52, FRG

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Summary. Protoplasts isolated from a barley cell suspension (cv Dissa) were transformed with plasmid DNA containing the neomycinphosphotransferase II (*NPT*) and β-glucuronidase (*GUS*) genes, using polyethyleneglycol (PEG) to induce DNA uptake. Transformed microcalli were selected in media containing G418 sulphate. *NPT* activity was detected in all antibiotic-resistant cell lines, but not all *NPT*-positive cell lines had *GUS* activity. Southern analysis confirmed the presence of sequences homologous to the *NPT* and *GUS* genes in DNA of G418-resistant callus.

Key words: *Hordeum vulgare* – Protoplasts – DNA uptake – Stable transformation – Cereals

Introduction

Barley is one of the world's major cereals and, consequently, there is interest in the use of genetic engineering techniques for its improvement. However, a trait it has in common with other cereals is that it is not readily amenable to *Agrobacterium*-mediated transformation, so that other transformation methods must be considered (Lazzeri and Lörz 1988; Potrykus 1990). Of the various methods proposed for cereal transformation, to date two, namely, direct DNA uptake by protoplasts (Paszkowski et al. 1984) and particle bombardment (Klein et al. 1987), have unequivocally yielded stably-transformed plants whose progeny inherit the transferred gene(s) (Shimamoto et al. 1989; Gordon-Kamm et al. 1990).

The major constraint on the use of direct DNA uptake into protoplasts for cereal transformation has been the relative difficulty of grass protoplast culture and regeneration (Lörz et al. 1988; Vasil 1988). While this remains a problem, the last years have seen considerable progress, in that plants have been regenerated from protoplasts of most of the major cereals (rice: Fujimura et al. 1985; maize: Rhodes et al. 1988a; wheat: Vasil et al. 1990; barley: A. Jähne, P.A. Lazzeri, H. Lörz, submitted; Yan et al. 1990), and these successes have been quickly followed by the first reports of transgenic cereal plants generated via protoplast transformation (Rhodes et al. 1988b; Toriyama et al. 1988). As a transformation method, particle bombardment is theoretically tissue and genotype independent. In practice, however, the majority of experiments in which stable transformation of cereal cells has been achieved have used suspension cells as target tissue, the same source material as is used for protoplast isolation, so at present both methods are largely dependent on the efficiency of producing embryogenic suspensions from the species to be transformed.

In barley, a number of laboratories have now reported transient gene expression using several different techniques. Protoplasts from cell suspensions, mesophyll, or aleurone tissue have been transformed by either PEG- or electroporation-mediated DNA uptake (Junker et al. 1987; Teeri et al. 1989; Lee et al. 1989, respectively), and expression of the *NPT* or *CAT* genes has been monitored over periods of 1–5 days. Particle bombardment has been used to transform suspension cells and immature zygotic embryos (Mendel et al. 1989; Kartha et al. 1989). In the first study, cotransformation of two plasmids containing the *NPT* and *GUS* genes was shown, while in the second, *CAT* was used as the reporter gene. Creissen et al. (1990) also used particle bombardment to deliver plasmid constructs containing tandem dimers of wheat dwarf virus (WDV) into microspore-derived embryos of barley, and subsequently detected virus replication in bombarded cultures. The same authors also made cocul-

tivation experiments using *Agrobacterium* strains with WDV-containing plasmids inserted into their T-DNA, again using microspore-derived embryos as the target tissue. Viral infection was detected in cocultivated tissues, but at lower frequencies than in bombardment experiments. Two other transformation techniques that have been used in barley are DNA uptake by imbibing embryos (Töpfer et al. 1989) and the electrophoresis of DNA into germinating seeds (Ahokas 1989). Both techniques allowed reporter gene detection for periods of hours (Ahokas 1989) or days (Töpfer et al. 1989) after treatment. Although these various experiments show the feasibility of transferring and expressing foreign DNA in barley cells, none presents evidence for stable transformation, which is clearly essential for genetic manipulation to be considered.

As a step towards the development of a transformation system for barley, we report here the transformation of suspension cell protoplasts and the selection of stably transformed cell lines.

Materials and methods

Cell suspensions

Protoplasts were isolated from suspension cells of the *Hordeum vulgare* L. cv Dissa (cell line D2; Lührs and Lörz 1988). Suspensions were cultured in L1 medium with 2 mg/l 2.4-D (Table 1), in 100-ml Erlenmeyer flasks that were shaken at 100 rpm, in dim light, at 25 °C. Suspensions were subcultured three times per week; at each subculture, excess cells were removed to leave approximately 2.5 g fresh weight per flask and the old medium was replaced with 10 ml of fresh medium. Under the culture conditions used, the cell suspensions consisted of small aggregates (0.1–1 mm in diameter) of isodiametric, cytoplasm-rich cells and contained few elongated or dead cells. Initially, the suspension medium contained 60 g/l glucose as carbon source, but it was subsequently found that maltose was preferable, as it gave more consistent cell growth and less compact cell aggregates.

Protoplast isolation

Protoplasts were isolated 2 or 3 days after subculture. Two or 2.5 g fresh weight of cells were incubated with 20 ml filter-sterilized enzyme solution and shaken at 40 rpm at 25 °C. The enzyme solution comprised 1% cellulase Onozuka RS (Yakult, Tokyo), 0.5% Macerozyme R10 (Serva), and 0.05% Pectolyase Y23 (Seishin, Tokyo) dissolved in washing (LW) solution at a pH of 5.7 and an osmolarity of 725–750 mOsm. [LW solution contains the macro- and microsalts and amino acids of L1 medium (Table 1) and 0.6 *M* Mannitol]. Protoplasts were isolated after 3 h cell digestion in enzyme solution. They were washed through 100-, 50-, and 25-µm diameter sieves with LW solution (pH 5.7, 725 mOsm) before being collected by centrifugation at $50 \times g$ for 4 min. Protoplasts were washed once in LW solution, pelleted, and then resuspended in the same solution, where they were stored at 7 °C until use (1–5 h).

Protoplast isolations made on the 2nd or 3rd day after suspension subculture were generally most successful. When protoplasts were isolated on the 1st day after subculture, yields were low and the protoplasts were fragile, while on the 5th and later days after subculture, cell walls were more resistant to digestion and protoplasts isolated were full of starch and had poor plating efficiencies.

Routinely, protoplast yields of $1-5 \times 10^6$/g fwt cells were obtained. The protoplasts were relatively homogeneous and had diameters between 15 and 30 µm. As with suspensions, the sugar used in protoplast medium was changed from glucose (90 g/l) to maltose (180 g/l) during the experiments, as the latter had been found to give higher plating efficiencies.

Protoplast transformation procedures

Six different PEG brands/molecular weights – Merck 1,500, Serva 6,000, Serva 6,000 pure, Merck 6,000 molecular biology grade, Sigma 8,000, Aldrich 8,000 – were tested (at 20% final concentration) for their effect on protoplast plating efficiency, using the Negrutiu et al. transformation protocol (1987).

Three different PEG-mediated DNA uptake procedures were used, adapted from those of Krens et al. (1982), Negrutiu et al. (1987), and the fusion protocol of Hein et al. (1983). In each case, 1×10^6 protoplasts were suspended in 0.5 ml of the appropriate salt/osmoticum solution, and 50 µg of supercoiled plasmid DNA (at a concentration of 1 µg/µl in 10:1 TRIS:EDTA buffer) was added, followed by 1 ml of 40% Merck 1500 PEG solution, added dropwise, with gentle shaking (thus, final PEG concentration ~25%). The mixture was shaken at intervals over 15 min, followed by stepwise dilution with 2-ml aliquots of salt/osmoticum solution, to a final volume of 10 ml. Protoplasts were then pelleted by centrifugation ($50 \times g$, 5–7 min).

For the Krens transformation method, F medium (Krens et al. 1982), pH 5.6, was used to suspend the protoplasts before transformation and later to dilute the transformation mixture, while the PEG was dissolved in F medium at pH 7.0. For the Negrutiu method, protoplasts were initially suspended in MaMg solution (50 m*M* $MgCl_2$, pH 5.6; Negrutiu et al. 1987); PEG was dissolved in 0.4 *M* mannitol, 0.1 *M* $Ca(NO_3)_2 \cdot 4\,H_2O$, pH 8.0; and the transformation mixture was diluted with LW solution.

For the Hein method, protoplasts were suspended in LW solution, the PEG solution was as for the Negrutiu method, and the dilution solution was 0.275 *M* $Ca(NO_3)$, pH 6.0. All transformation solutions were adjusted to 700 mOsm with mannitol and sterilized by ultrafiltration. The DNA used for all transformations was the plasmid pRT99GUS (Töpfer et al. 1988), which contains the *NPT* and *GUS* gene coding regions, each under the control of separate 35S promoters and termination sequences from the cauliflower mosaic virus. The size of the plasmid was 6.7 kb.

Protoplast culture and selection

After transformation, protoplast pellets were suspended in 2.5-ml aliquots of L1 medium containing 2 mg/l 2.4-D, either 90 g/l glucose or 180 g/l maltose, and 10 g/l Sea Plaque agarose (medium osmolarity adjusted to 700 mOsm with mannitol). Suspended protoplasts were pipetted into 60-mm diameter plastic petri dishes (Greiner, tissue culture quality, protoplast density therefore 4×10^5/ml). Cultures were incubated at 25 °C under continuous low light for 11–15 days, before the application of selection pressure. To determine protoplast plating efficiencies, microcalli were counted either after 15 days of culture or at the time of transfer to selection, when this was earlier than 15 days.

For selection, agarose segments cut from 60-mm dishes were transferred to 90-mm petri dishes, and 7.5 ml of L1 suspension medium (~270 mOsm) containing 33 g/l G418 sulphate (Gibco) was added (thus, final antibiotic concentration 25 mg/l). Cultures were incubated on a rotary shaker at 40 rpm under dim

Table 1. Compositions of L1 and L2 media[a]

	L1	L2
Macro salts (mg/l):		
NH_4NO_3	750	1,500
KNO_3	1,750	1,750
KH_2PO_4	200	200
$MgSO_4 \cdot 7H_2O$	350	350
$CaCl_2 \cdot 2H_2O$	450	450
Micro salts (mg/l):		
$MnSO_4 \cdot H_2O$	15	
H_3BO_3	5	
$ZnSO_4$	7.5	
KI	0.75	
$Na_2MoO_4 \cdot H_2O$	0.25	
$CuSO_4 \cdot 5H_2O$	0.025	
$CoCl_2 \cdot 6H_2O$	0.025	
FeNaEDTA:	as MS medium[b]	
Vitamins (mg/l):	vitamins, minor sugars, sugar alcohols, coconut water, as Kao medium[c]	inositol 200; thiamine HCl 10; pyridoxine HCl 1; nicotinic acid 1; Ca pantothenate 1; ascorbic acid 1
Amino acids (mg/l):	Gln 750, Pro 150, Asn 100	
Sugars (g/l):	glucose 60 or maltose 60[d]	maltose 30
2,4-D (mg/l):	2.0	2.5
Osmolarity (mOsm):	~500 or ~270[e]	~190
pH:	5.6	

[a] Media were sterilized by ultrafiltration. For solid cultures, double-concentrated media were mixed with molten, double-concentrated agarose solution
[b] Murashige and Skoog (1962)
[c] Kao (1977)
[d] Maltose substituted for glucose during the course of the experiments
[e] For media with glucose or maltose, respectively

light at 25 °C for 20 days, after which the selection medium was replenished and the dishes were returned to the shaker. After 40 days selection in liquid medium, microcalli ≥1 mm in diameter were transferred individually to the surface of L2 medium (Table 1) containing 2.5 mg/l 2.4-D and 25 mg/l G418, and solidified with 4 g/l S1A agarose (Sigma). Calli were maintained on this medium by monthly subculture.

NPT and GUS assays, Southern analysis

Calli showing sustained growth after at least two passages (60 days) on solid selection medium were sampled at random to test for *NPT* and *GUS* activity. *NPT* assays were performed by the dot blot method of McDonnell et al. (1987), with the exception that an extraction buffer containing 200 m*M* TRIS-HCl, 0.5% SDS, and 20 mg/l BSA, pH 7.1, was used instead of the published solution. *GUS* assays, both histochemical and fluorimetric, were performed according to the protocol of Jefferson (1987). For histochemical assays, 100–500 mg fwt of callus pieces was tested per experiment. DNA was isolated from callus tissue by the method of Mettler (1987). Restriction digests were performed according to enzyme manufacturers' instructions. Subsequent restriction fragment separation and hybridizations were performed according to Maniatis et al. (1982). Washed filters were exposed to Kodak XAR-5 X-ray film at −70 °C.

A 0.8-kb HindIII-BamHI fragment from the plasmid pHP23 (J. Pazskowski, ETH Zürich, Switzerland) was used as the probe for the *NPT* gene, while a 2.0-kb HindIII-BamHI fragment from the plasmid pRT99GUS was used as the probe for the *GUS* gene.

Results

Protoplast culture, transformation

In control protoplast cultures, the first divisions could be observed after 3 days, with most cells dividing on the 4th or 5th day. Plating efficiencies for control cultures varied between 1 and 6%, with the average around 3%.

When different PEG brands or molecular weights were tested for their effect on protoplast division using the Negrutiu protocol, all reduced plating efficiency, giving values between 61 and 83% of the control. The two high-purity grades of PEG tested gave the better protoplast plating efficiencies, while the 8,000 molecular weight PEGs caused more extreme protoplast aggregation and gave the lowest plating efficiencies. High-molecular-weight PEG solutions were difficult to handle, however, because of their viscosity; therefore, Merck 1500 PEG, which gave an intermediate plating efficiency (69% of control), was used in subsequent experiments.

Protoplasts reacted differently to the three different transformation protocols used and their reaction also varied between isolations. Larger protoplasts, with less dense cytoplasm, were prone to burst during PEG treatment, while smaller, cytoplasm-rich protoplasts tended to aggregate.

In general, protoplast aggregation was more extreme with the Krens protocol and occurred least with the Negrutiu protocol. Aggregated protoplasts could usually be dispersed by gentle agitation during the posttransformation dilution, but in isolations that previously showed a degree of spontaneous fusion, PEG treatments produced very stable aggregates. This effect was also seen when protoplasts were isolated at higher enzyme incubation temperatures (28–33 °C). While protoplasts in small aggregates (~5–15 protoplasts) were still able to divide when embedded in agarose, those in larger aggregates (25+ protoplasts) frequently degenerated. If aggregates were not efficiently dispersed by the dilution procedure, they were suspended in LW solution for some hours at 7 °C to allow separation and then later suspended in agarose. After transformation treatments, protoplast plating efficiencies varied between ~40 and 80% of the control values (Table 2). Of the three transformation protocols used, the Krens method gave the highest plating efficiencies, but there was clearly variation between isolations in the ability of protoplasts to withstand the transformation procedures.

Table 2. Effect of different transformation protocols on protoplast plating efficiency

Transformation protocol	Control plating efficiency	Treated plating efficiency	Treated/ control
Krens[a]	2.3%	1.7%	0.74
Krens	1.1%	0.9%	0.82
Krens	2.3%	1.8%	0.78
Negrutiu[b]	3.2%	1.5%	0.47
Negrutiu	5.4%	3.4%	0.63
Negrutiu	3.0%	1.3%	0.43
Hein[c]	3.6%	1.9%	0.53
Hein	2.7%	1.4%	0.52

[a] Krens et al. (1982)
[b] Negrutiu et al. (1987)
[c] Hein et al. (1983)

Although transformation treatments reduced overall plating efficiency, the time of onset of protoplast division was similar in both control and transformed protoplasts.

Selection

G418 was used as selection agent after several compounds (G418, Kanamycin, Paramomycin, Hygromycin, and Methotrexate) had been tested for their inhibition of barley microcallus growth. G418 (at 25 mg/l) and Hygromycin (at 50 mg/l) were most efficient (data not shown); G418 was chosen for further use because of the ease of assaying (*NPT*) enzyme activity in transformed tissue. The time of application of selection pressure was critical for efficient selection. Microcalli larger than 8–16 cells in diameter became highly antibiotic resistant, while premature transfer of cultures to selection conditions (microcalli four to eight cells) resulted in no calli surviving in selected dishes and in drastically reduced plating efficiencies (5–10% of usual) in control dishes. Slowly shaking (40 rpm) selection cultures markedly improved the growth rate of control and transformed microcalli, without reducing selection efficiency.

Selection medium was replaced after 20 days. If this was not done, nontransformed microcalli were able to grow and escape selection, particularly in cultures with good plating efficiencies and therefore high microcallus densities.

After 40 days, calli surviving in liquid selection were transferred to solid medium, again containing 25 mg/l G418. At this stage, two classes of calli were distinguished – compact, yellow colonies and friable, bleached colonies. The latter were judged to be "escapes," but for consistency all calli ≥ 1 mm in diameter were subcultured.

Selected calli (cell lines) showed variable responses on solid medium. Some lines grew at a rate comparable to control callus, some grew only slowly, and others initially grew well but later ceased growth. Those lines which after three passages showed sustained growth similar to that of controls were maintained subsequently. The efficiency of the initial liquid selection varied considerably between experiments, with between 3 and 100% of selected calli continuing growth on solid medium (Table 3).

Table 3. Selection efficiency in four experiments

Protocol	No. of calli transferred from liquid selection[a]	No. of lines growing on solid selection[b]	No. of lines NPT tested/ no. positive
Krens	74	31 (41%)	11/11
Negrutiu	14	3 (21%)	2/2
Negrutiu	8	8 (100%)	3/3
Hein	32	1 (3%)	1/1

[a] Calli with diameter ≥ 1 mm after 40 days in culture
[b] After three 4-week passages

Table 4. *GUS* activity in transformed (*NPT*-positive) cell lines

Line[a]	Histochemical test		Enzyme activity[b]	Southern analysis[c]
	No. positive	/ No. tests		
2a/6	1	3	0.20	positive
2a/53	1	2	0.27	positive
3b/6	0	6	0.12	positive
6a/40	0	3	0.11	positive
6a/42	2	2	0.48	positive
6a/45	0	2	0.14	positive
5b/2	5	5	1.44	positive
6/2	3	3	1.40	positive
6/6	3	3	7.32	positive
6/8	2	2	3.27	positive
Controls	0	6	0.12 ± 0.03	

[a] Cell lines from three experiments
[b] nmol MU hr^{-1} mg $protein^{-1}$
[c] For presence of *NPT* gene

NPT and GUS assays, transformation efficiency

Randomly chosen antibiotic-resistant lines were tested for *NPT* enzyme activity by the dot blot method (Fig. 1c). All lines tested (~ 25) gave positive results. There appeared to be good correlation between levels of *NPT* activity and callus growth rate under selection, with vigorous lines giving the most intense signals. Twenty-one *NPT*-positive cell lines were tested for *GUS* expression by the histochemical assay. Blue cells, indicating *GUS* activity, were seen in ten lines. Among the positive lines different forms of expression were seen. In lines with the most blue cells, approximately 5–15% of the callus

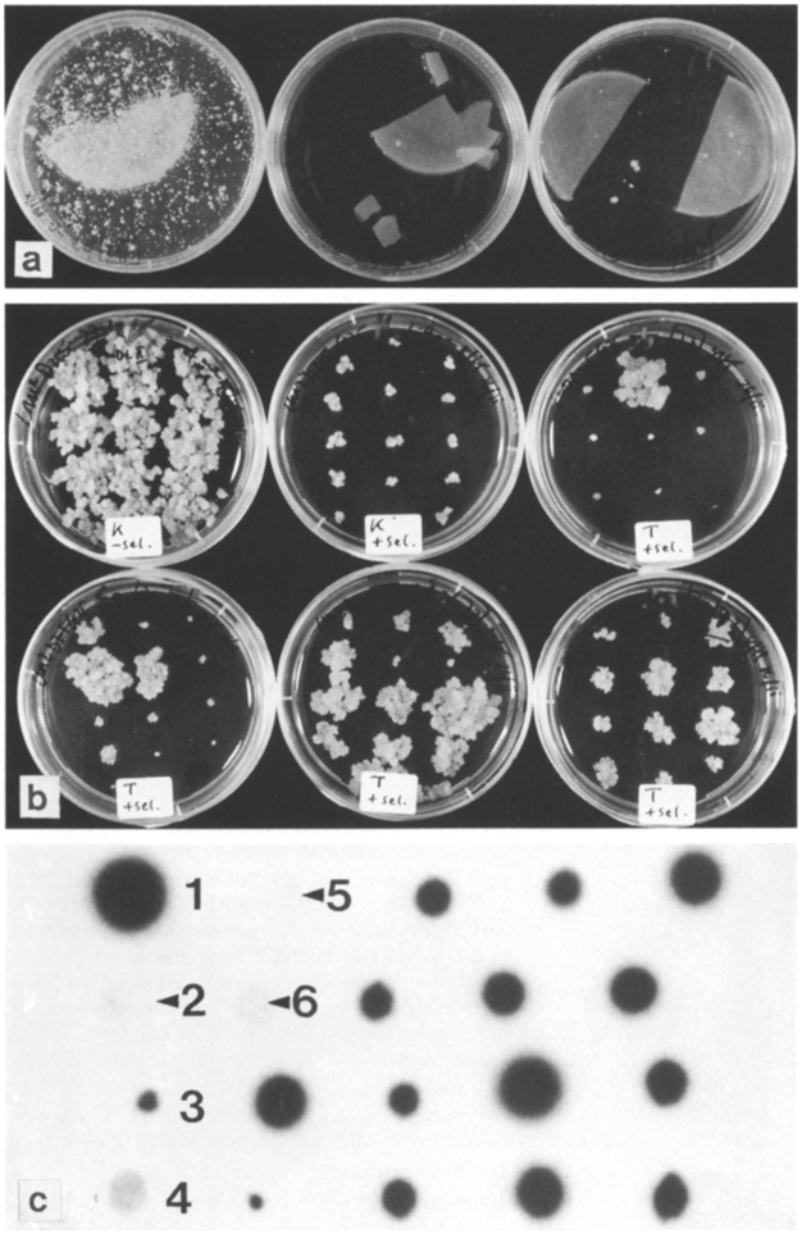

Fig. 1 a–c. Antibiotic selection for transformed cells and assay for *NPT* activity in resistant cell lines. **a** Selection of agarose-embedded microcalli in liquid medium. *Left* to *right:* nontransformed microcalli without selection; non-transformed microcalli with 25 mg/l G418; transformed microcalli with 25 mg/l G418. **b** Culture of callus on solid medium. From *top left:* control (non-transformed) callus without selection; control callus with selection (25 mg/l G418); remaining dishes, putatively transformed calli picked from liquid selection, on medium containing G418. **c** *NPT* dot blot test of resistant cell lines. *1 Vigna* callus transformed with the *NPT* gene. *2* Control *Vigna* callus. *3* Tobacco callus transformed with the *NPT* gene. *4* Control tobacco callus. *5* and *6* Control barley callus. All other spots are independent G418-resistant barley cell lines

showed coloration, and *GUS* expression was consistent in repetitive tests. In other lines, however, isolated groups of blue cells as few as 5–50 in number were seen, and in some tests no blue cells were observed among many callus pieces.

Levels of *GUS* activity (measured by fluorimetric assay) varied considerably among the cell lines judged *GUS*-positive from the histochemical test (maximum 7.3, minimum 0.2 nmol MU h^{-1} mg $protein^{-1}$) (Table 4); however, these data correlated with the frequency and consistency of blue color production in transformed callus. Lines in which blue cells had never been observed (e.g., 3b/6, 6a/40, 6a/45) had enzyme activity levels similar to those of control tissues.

Of the three transformation methods used, the Krens method gave the highest frequency of transformants (1.3 in 10^4) (Table 5), but as the different protocols were compared in four independent experiments, a direct comparison of efficiency is not possible, due to the known variation in protoplast "quality" between isolations.

Southern analysis

DNA isolated from selected callus lines was restricted with the enzyme PstI, which excises a 0.8-kb *NPT* gene

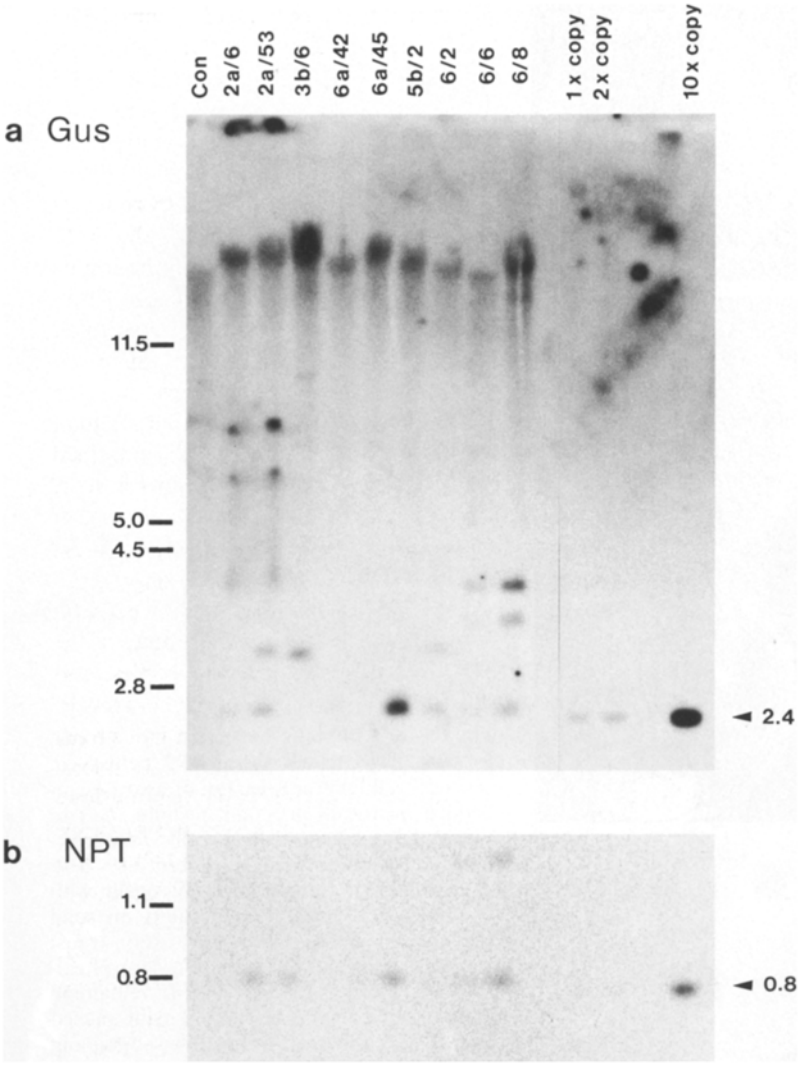

Fig. 2a and b. Southern blot analysis of DNA from G418-resistant cell lines. Genomic DNA (10 μg lane), isolated according to Mettler (1987) was digested with PstI. The DNA gel blot was first hybridized to a ^{32}P-labelled HindIII-BamHI *NPT* fragment of pHP23 (**b**) and then, after probe removal, hybridized to a HindIII-BamHI *GUS* fragment of pRT99GUS (**a**). For copy number reconstruction, amounts of pRT99GUS (1.7 pg, 3.4 pg, 17 pg) digested with PstI were loaded, to give equivalents of one, two, and ten copies per haploid barley genome, respectively

Table 5. Transformation frequency in four experiments

Protocol	No. of protoplasts treated	Control plating efficiency	No. of resistant clones[a]	Freq. of resistant clones[b]
Krens	10×10^6	2.3%	31	13 in 10^5
Negrutiu	8×10^6	3.0%	3	1.3 in 10^5
Negrutiu	10×10^6	3.2%	8	2.5 in 10^5
Hein	4.5×10^6	1.4%	1	1.6 in 10^5

[a] After minimum of three passages on solid medium with 25 mg/l G418
[b] As a fraction of the total dividing protoplasts

fragment and a 2.4-kb. *GUS* gene fragment from the plasmid pRT99GUS.

When hybridized with the HindIII-BamHI *NPT* probe, PstI-restricted DNA of all lines tested was seen to contain the expected 0.8-kb fragment (Fig. 2b). From copy number reconstruction, the lines tested appeared to contain between one and ten copies of the 0.8-kb fragment per haploid genome.

Hybridization of DNA from selected lines with the HindIII-BamHI *GUS* gene fragment showed that, with one exception (6a/45), all the lines tested contained sequences homologous to the probe (Fig. 2a). Line 3b/6 showed only a fragment larger than the expected (2.4-kb) band, while the other lines all appeared to contain the 2.4-kb fragment. Lines 2a/53, 6/2, 6/6, and 6/6 contained also larger fragments in addition to the 2.4-kb fragment.

When unrestricted DNA from selected cell lines was probed with the *NPT* gene fragment, hybridization was only seen in high-molecular-weight regions, indicating integration of the plasmid into genomic DNA.

Discussion

The experiments reported here demonstrate the transformation of barley protoplasts by direct DNA uptake and

add barley to the list of major crops for which integration and expression of foreign genes has been reported.

The two most common methods for inducing DNA uptake by protoplasts are electroporation and PEG treatment. As toxicity of commercial PEGs may be problematic for their use with some protoplasts (Kao and Saleem 1986), we examined the influence of various PEGs on barley protoplasts. The results suggest that purified grades of PEG indeed have lower toxicity, but the clearer finding was that higher molecular weight PEGs caused more protoplast damage. Different PEG molecular weights were not directly compared for their activity in promoting DNA uptake, but stably transformed lines have been obtained with PEGs of molecular weights of 1,500, 4,000, and 6,000 (unpublished data).

In our experiments, transformed cell lines were recovered with all three transformation protocols used. The major difference between the three protocols is in the solution used to suspend the protoplasts prior to the addition of DNA and PEG, with the Krens protocol using a Ca^{++} solution, the Negrutiu protocol a Mg^{++} solution, and the Hein protocol the more complex LW washing solution. Although the Krens protocol appeared to be harsher on the protoplasts, causing more aggregation, this was generally a reversible process, and the Krens method gave the highest protoplast plating efficiencies and the best transformation frequency. Several studies have compared the effectiveness of Ca^{++} and Mg^{++} ions in PEG-mediated transformation (e.g., Negrutiu et al. 1987; Maas and Werr 1989) and found Mg^{++} to be superior, but it is likely that the optimal cation/PEG combination will vary with plant species.

The selection system used in our experiments, i.e., the *NPT* gene driven by the CaMV 53S promotor, conferring resistance to G418, has previously been used to select transformants in *Lolium* (Potrykus et al. 1985) and rice (Toriyama et al. 1988), and is now seen to function adequately in barley. The effectiveness of the initial liquid selection was nevertheless clearly dependent on microcallus size and density (protoplast plating efficiency). Consequently, primary selection efficiency varied considerably between experiments. The secondary (solid) selection was more efficient; all antibiotic-resistant lines tested for *NPT* activity were clearly positive. The fact that no escapes were detected may suggest that the selection pressure was too stringent; it is possible that slow-growing lines that were discarded were transformants with lower levels of *NPT* activity, especially as control callus showed negligible growth under the selection conditions.

GUS activity among transformed cell lines was inconsistent; by the histochemical assay, *GUS* activity was detected in ca. half of the lines tested, while with the more sensitive fluorescent assay, six of ten lines tested had activity levels more than double that of the control tissue. The absolute levels of *GUS* activity among lines that produced blue cells varied between 0.2 and 7.3 nmol MU h^{-1} mg $protein^{-1}$ (average 2.05 ± 0.97). These values compare with averages of 93 ± 13 for rice and 8.2 ± 3.5 for maize callus transformed with the *GUS* gene controlled by the CaMV 35S promoter (Peng et al. 1990; Lyznik et al. 1989).

From Southern analysis, the DNA of G418-resistant calli contained sequences that hybridized to the *NPT* probe, giving bands of 0.8 kb. These data and the results from *NPT* activity assays indicated the presence of full-size functional copies of the *NPT* gene in selected lines. From analysis with the *GUS* probe, however, line 6a/45 appeared not to contain *GUS* gene sequences, and line 3b/6 showed only integration of a fragment larger than the intact gene. The other lines examined all contained fragments of 2.4 kb, corresponding to the intact *GUS* gene. These data correlate with *GUS* activity data in that lines 6a/45 and 3b/6 had no detectable activity, while the other lines had shown *GUS* expression. *GUS* copy number did not appear to correlate with levels of activity, however, as most lines contained one or two copies of the intact gene; only 5b/2 clearly had multiple copies, and this line did not have one of the highest expression levels.

The transformation efficiencies obtained in our experiments, between 1 and 10×10^{-5} of microcalli selected, or $0.4-3 \times 10^{-6}$ of total protoplasts treated, are lower than those reported for PEG transformation of maize (5×10^{-5} of total protoplasts; Lyznik et al. 1989) or rice (9×10^{-5} of total protoplasts; Yang et al. 1988). However, our results were obtained with protoplasts having relatively lower plating efficiencies and without optimization of the transformation protocols. By modification of protoplast culture procedures, plating efficiencies up to 20% can be achieved (data in preparation); this, in combination with an optimized transformation protocol, should allow higher transformation efficiencies.

The cell line transformed in the present experiments (D2, Lührs and Lörz 1988) is now over 3 years old and is no longer morphogenic. However, there has recently been success in regenerating plants from barley suspension protoplasts, both in our laboratory and elsewhere (A. Jähne, P. A. Lazzeri, H. Lörz, submitted; Yan et al. 1990), so the procedures described here may now be applied to morphogenic protoplasts with the aim of producing transgenic barley plants.

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