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H. Funatsuki · H. Kuroda · M. Kihara · P. A. Lazzeri
E. Müller · H. Lörz · I. Kishinami

# Fertile transgenic barley generated by direct DNA transfer to protoplasts

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**Abstract** We report the generation of transgenic barley plants via PEG-mediated direct DNA uptake to protoplasts. Protoplasts isolated from embryogenic cell suspensions of barley (*Hordeum vulgare* L. cv 'Igri') were PEG-treated in a solution containing a plasmid which contained the neomycin phosphotransferase (NPT II) gene under the control of the rice actin promoter and the nos terminator. Colonies developing from the treated protoplasts were incubated in liquid medium containing the selective antibiotic G418. Surviving calli were subsequently transferred to solid media containing G418, on which embryogenic calli developed. These calli gave rise to albino and green shoots on antibiotic-free regeneration medium. NPT II ELISA revealed that approximately half of the morphogenic calli expressed the foreign gene. In total, 12 plantlets derived from NPT-positive calli survived transfer to soil. Southern hybridization analysis confirmed the stable transformation of these plants. However, the foreign gene seemed to be inactivated in plants from one transgenic line. Most of the transgenic plants set seed, and the foreign gene was transmitted and expressed in their progenies, which was ascertained by Southern hybridization and NPT II ELISA.

**Key words** Barley (*Hordeum vulgare* L.) · Protoplast · Regeneration · Transformation

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H. Funatsuki[1] (✉) · H. Kuroda · M. Kihara · I. Kishinami
Plant Bioengineering Research Laboratories,
Sapporo Breweries Ltd. 37-1, Kizaki, Nitta, Gunma, 370-03, Japan

P. A. Lazzeri
Department of Biochemistry and Physiology,
Rothamsted Experimental Station, Herpenden, Herts, AL5 2JQ, UK

E. Müller · H. Lörz
Institut für Allemeine Botanik, AMP II, University of Hamburg,
Ohnhorststraße 18, D-22609 Hamburg, Germany

*Present address:*
[1] Genetic Resource Utilization Laboratory, Hokkaido National Agricultural Experiment Station, Fukuro 2, Engaru, Hokkaido, 099-04, Japan

## Introduction

Genetic engineering of monocots is of special interest as these species include a number of agronomically important crops such as cereals (Lörz et al. 1988). As they are not readily transformed by *Agrobacterium*, various transformation methods have been proposed and attempted (Potrykus 1990). Among them, two approaches, particle bombardment and protoplast transformation, have been extensively used until now. The former method has lately yielded transgenic plants in many grass species (for a review see Vasil 1994). However, the protoplast approach has had relatively less success except in rice and a few reports in maize (Vasil 1994).

Barley is an important crop as a brewing material as well as an animal feed. Therefore great efforts have been made to produce transgenic barley by the two methods described above (Lörz and Lazzeri 1992), which has led to very recent success using the particle bombardment technique (Jähne et al. 1994; Ritala et al. 1994; Wan and Lemaux 1994; Hagio et al. 1995). However, the generation of transgenic plants using the protoplast system has not previously been reported, although fertile plants have been recovered from protoplasts (Jähne et al. 1991; Funatsuki et al. 1992; Golds et al. 1994; Kihara and Funatsuki 1994), and stably transformed cell lines have been obtained via PEG-induced direct DNA uptake into protoplasts (Lazzeri et al. 1991).

In this article, we report the production of fertile transgenic barley plants by direct DNA delivery to protoplasts.

## Materials and methods

### Protoplast transformation

Three regenerable cell suspension lines (ILR-C3, ILR-C5, ILR-C7; Funatsuki and Kihara 1994) of barley (*Hordeum vulgare* L. cv 'Igri') were used as protoplast sources. Protoplasts were isolated and washed as described previously (Funatsuki and Kihara 1994) using LKW solution [2:1 (v:v) LWS (Lazzeri et al. 1992):HBW (Funatsu-

ki et al. 1994)]. Transformation was performed basically according to Lazzeri et al. (1992). From 0.5 to 1.5 million protoplasts were suspended in 250 µl of Ca-SP solution containing 100 mM $CaCl_2$, 0.6 M sorbitol, 5 mM MES (pH 5.8) and 50 µg of plasmid DNA. Subsequently 600 µl of PEG solution [40% PEG 1500 (Wako, Osaka) dissolved in Ca-SP solution without plasmid, pH 7.0] was added. The suspension was allowed to stand for 15 min with occasional gentle shaking and was diluted with 15 ml of LKW solution. Protoplasts were collected by centrifugation (50 g, 8 min) and were then embedded in molten L1 medium (Lazzeri et al. 1991) containing 1.25% agarose before being cultured with feeder cells as described by Funatsuki et al. (1992). As a too-high plating density results in a decrease in plating efficiency and regeneration frequency (Funatsuki, unpublished data), the plating density was adjusted to $5.0\times10^5$ cells/ml.

### Plasmid construct

Plasmid pAct1Dneo (E. Müller, in preparation) was used to confer antibiotic resistance to transformed cells. The plasmid contained the NPT II structural gene and the nos terminator from pRT99 (Töpfer et al. 1988) under the control of the rice actin promoter derived from pAct1DGus (McElroy et al. 1990) (Fig. 1).

### Selection and culture of transformed cells

We employed four selection methods with three levels of selection strength, summarized in Fig. 2. After 15–18 days of culture without antibiotic, feeder cells were washed away and agarose blocks were cultured in 3 ml of L1 liquid medium containing 25 µg/ml of G418 (Geneticin, Gibco) with gentle shaking (50 rpm). After 2 weeks, colonies developing on or in agarose blocks were transferred to modified L2 solid medium (mL2, Funatsuki et al. 1992) supplemented either with 2.5 mg/l of 2,4-D (in selection method 2, 3) or with 0.5 mg/l of 2,4-D and 1.0 mg/l of BAP (in selection method 0, 1) which contained zero (in selection method 0) or 20 µg/ml of G418 (in selection methods 1, 2, and 3). In selection methods 1 and 2, calli on antibiotic-containing media were removed as soon as embryogenic structures appeared, while in selection method 3, cells were exposed to antibiotic for 1 month. Embryogenic structures were subsequently transferred to mL2 medium without G418 and were cultured until shoot regeneration was observed. Regenerated shoots were transferred to hormone-free mL2 medium for further development. When strong root systems were developed, plantlets were transplanted to soil. After 2–4 weeks of acclimatization at 18°C, plants were vernalized at 4.5°C for 1 month at least and were subsequently grown to maturity at 12°C in a growth chamber.

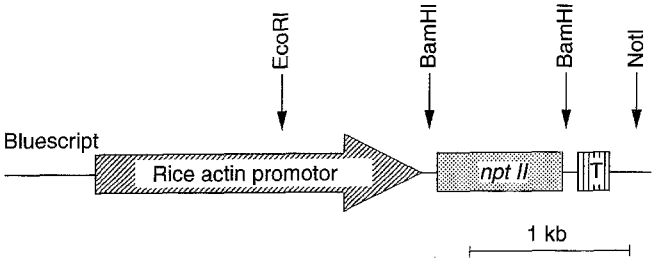


**Fig. 1** Construction of pAct1Dneo. *Arrows* show the restriction sites. The bold character '*T*' represents the terminator from the nopaline synthase gene

### NPT II ELISA

NPT II protein was detected using an ELISA kit (5 prime-3 prime, Boulder). Soluble proteins were extracted from calli or leaves in 0.1 M Tris-HCl (pH 8.0) containing 1.0 mM of PMSF with a mortar and a pestle. Protein content was determined using a protein assay kit (Bio-Rad, South Richmond), and 10–200 µg of protein was applied to each well. The content of NPT II protein was determined according to the manufacturer's instruction.

### Southern-blot hybridization

Southern-blot hybridization was performed basically following the standard protocol of Sambrook et al. (1989). Total DNA was isolated from leaf tissue of plants regenerated from $NPT^+$ calli according to Varadarajan and Prakash (1991) with the modification that ethidium bromide was added to the extraction buffer. Ten to fifteen micrograms of DNA, undigested or digested with *Eco*RI and *Nco*I endonucleases, were applied to a 1.0% agarose gel (EO10; Takara, Kyoto) and electrophorated. After transfer to nylon membrane (Hybond $N^+$, Amersham), plant DNA was hybridized with the probe prepared by the random primed labelling method using [$\alpha$-$^{32}$P] dCTP (Amersham). The probe employed was the protein-coding region of the NPT II gene (0.8-kb *Bam*HI fragment isolated from plasmid pAct1Dneo).

## Results

### Protoplast transformation and culture

Three embryogenic cell suspensions were used as protoplast sources. These lines regenerated green shoots at frequencies of over 500 (ILR-C3), 36 (ILR-C5) and 66 (ILR-C7)/$10^6$ protoplasts when cultured without transformation treatment (Funatsuki and Kihara 1994). Protoplasts isolated from these cultures were treated with PEG solution containing pAct1Dneo (Fig. 1), which carries the NPT II gene driven by the rice actin promoter (McElroy et al. 1990). PEG treatment reduced protoplast plating efficiency by 20–50% compared with control plates.

### Selection of G418-resistant calli and shoot regeneration

Agarose blocks containing 15-day- to 18-day-old protoplast-derived colonies were transferred into liquid medium supplemented with 25 µg/ml of G418.

| Selection method | 1st passage | | 2nd passage | | 3rd passage |
|---|---|---|---|---|---|
| 0 | L1 + G20 (2w) | → | mL2D0.5B1.0 | → | mL2D0.5B1.0 |
| I | L1 + G20 (2w) | → | mL2D0.5B1.0 + G25 (x) | → | mL2D0.5B1.0 |
| II | L1 + G20 (2w) | → | mL2D2.5 + G25 (x) | → | mL2D0.5B1.0 |
| III | L1 + G20 (2w) | → | mL2D2.5 + G25 (4w) | → | mL2D0.5B1.0 |

**Fig. 2** Selection and culture methods for G418-resistant calli. *L1* L1 liquid medium, *mL2* modified L2 medium, *G* G418, *D* 2,4-D, *B* BAP. The numbers following *G*, *D* and *B* show the concentrations (µg/ml). *Parentheses* represent the periods of selection passages (*w* week, *x* until embryogenic structures appeared)

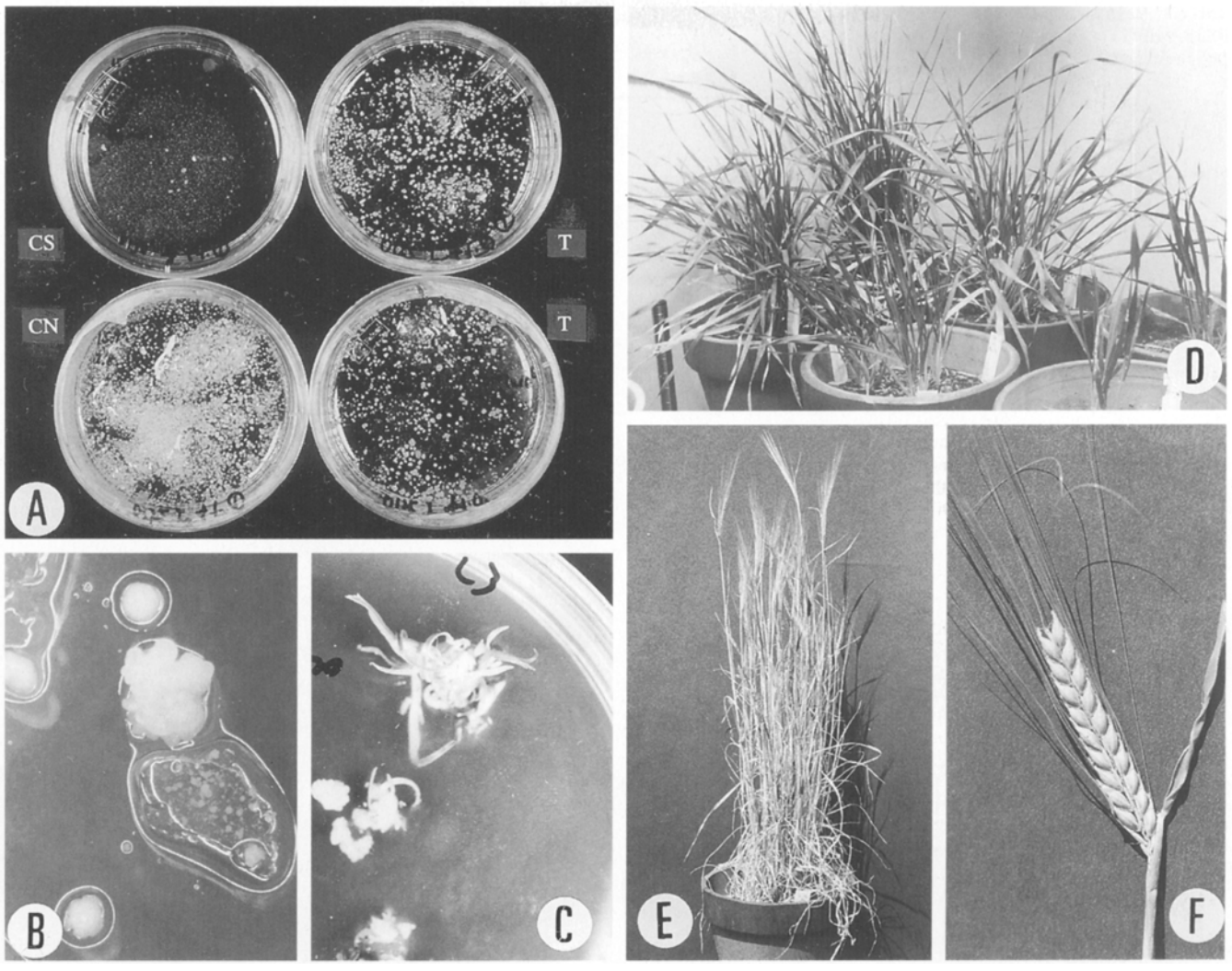


**Fig. 3A–F** Generation of transgenic barley plants. **A** Colony development after first selection passage: *CS* Control plate with G418, *CN* control plate without G418, *T* transformation-treated plate with G418. **B** Development of embryogenic structure from G418$^r$ callus. **C** Green shoot regeneration. **D** Transgenic plants grown in growth chamber. **E** Fertile transgenic plant. **F** Its spike

After 2 weeks of culture, three patterns were observed with regard to colony development; (1) no visible colony in control plates (C-plates) nor in plates containing transformation-treated protoplasts (T-plates), (2) no, or few, colonies in C-plates but many more in T-plates (Fig. 3A), or (3) numerous colonies (typically more than 50 per plate) both in C-plates and in T-plates. In cell line ILR-C5, patterns 1 and 2 were observed, while in cell lines ILR-C3 and ILR-C7, patterns 2 and 3 were often seen. In experiments showing pattern 3, further selection was not carried out as escapes were expected to account for a large proportion of the colonies. Visible colonies from T-plates and C-plates were transferred to solid media according to four different selection methods (Fig. 2). With selection method 0, which was employed only in experiments where no colony was seen in C-plates in the first selection, numerous embryogenic structures were formed on mL2 medium. With other methods, embryogenic calli developed from colonies derived from T-plates (Fig. 3B) while, with one exception, no such structure was seen in colonies from C-plates, although enlargement of bleached calli was sometimes observed. Albino or green shoots were regenerated after these embryogenic calli were transferred to antibiotic-free mL2 medium (Fig. 3C). The exceptional embryogenic callus from a C-plate formed a poor albino shoot-like structure, which, however, soon ceased growth.

## NPT II ELISA of morphogenic calli

To determine whether the G418-resistant, morphogenic calli really expressed the foreign gene, NPT II ELISA was carried out. No NPT II protein was detected in morphogenic calli obtained from selection 0 as far as we tested. As shown in Table 1, where the other selection methods were adopted, the NPT II protein was detected in 14 independent calli with green shoots and nine independent calli with albino shoots. NPT II-positive (NPT II$^+$) calli could be obtained from all cell lines used. Selection methods 1 and 2 allowed escapes

**Table 1** Results of NPT II ELISA of G418-resistant, morphogenic calli

| Exp. | Cell line | No. of protoplasts ($\times 10^6$) | Selection method[a] | No. of calli | NPT+/calli examined[b] | | Transfer to soil |
|---|---|---|---|---|---|---|---|
| | | | | | Albino | Green | |
| 1 | ILR-C7 | 0.5 | I | 4 | 1/1 | 0/3 | – |
| 2 | ILR-C3 | 1.0 | I | 9 | – | 0/2 | – |
| | | | II | 9 | 3/4 | 3/3 | 2 |
| 3 | ILR-C7 | 1.0 | I | 11 | 1/1 | 3/3 | 1 |
| | | | II | 9 | – | 0/6 | 0 |
| 4 | ILR-C5 | 2.0 | I | 1 | – | 0/1 | – |
| | | | II | 4 | | 1/4 | 1 |
| 5 | ILR-C3 | 1.0 | III | 2 | 1/1 | – | |
| 6 | ILR-C3 | 4.5 | I | 7 | 1/1 | 2/2 | 0 |
| | | | II | 9 | – | 3/3 | 3 |
| | | | III | 9 | 2/2 | 2/2 | 0 |
| Total | | | | 74 | 9/10 | 14/29 | 7 |

[a] See Fig. 2
[b] No. of NPT II-positive calli / no. of calli which gave rise to albino or green shoots

**Table 2** Summary of the analyses of transgenic lines

| Line | No. of plants | NPT II ELISA[a] | | Southern analysis | Fertility[b] |
|---|---|---|---|---|---|
| | | Callus | Leaf | | |
| T-19 | 4 | 1.0–2.0 | – | + | + |
| T-20 | 4 | 2.0< | 1.0–2.0 | + | + |
| T-21 | 1 | 1.0–2.0 | 1.0–2.0 | + | – |
| T-45 | 1 | 0.5–1.0 | 0.5–1.0 | + | + |
| T-49 | 2 | 2.0< | 2.0< | + | + |

[a] Content of NPT II protein (μg) in 1 mg total soluble protein; –, < 0.5
[b] +, fertile; –, sterile

at similar frequencies, while all morphogenic calli proved NPT II$^+$ from selection method 3. However the prolonged culture of embryogenic structures on mL2 medium with G418 yielded albino shoots at a higher frequency and resulted in the inhibition of further growth of the green shoots. Calli from selection method 2 regenerated more rooted plants than did those from selection method 1.

## Analyses of transformed plants

Of 14 plantlets derived from seven independent NPT II$^+$ lines which were transferred to soil, 12 plants from five lines survived the transfer (Table 2, Fig. 2D). Total DNA was isolated from the leaf tissues of these plants, and the presence and the integration of the foreign gene was examined by Southern hybridization (Fig. 4, Table 2). As the rice actin promoter has been proved to be functional, even when deleted to the *Eco*RI site (Fig. 1, McElroy et al. 1990), plant DNA was digested with the endonucleases *Not*I and *Eco*RI. Bands of the expected size of 1.9 kb were seen in all plants except for line T-20. Additional bands

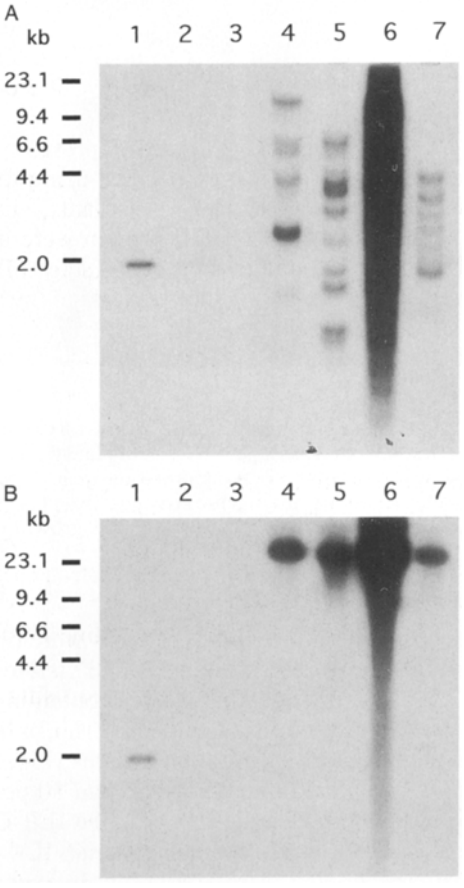


**Fig. 4A,B** Southern-blot hybridization of total DNA from transgenic plants. Total DNA was digested with *Eco*RI and *Not*I (**A**), or undigested samples were used (**B**). The *Bam*HI fragment (800 bp) of pAct1Dneo was used as a probe. *Lane 1*, pAct1Dneo (5 pg); *lane 2*, pAct1Dneo (1 pg); *lane 3*, protoplast-derived non-transformed plant; *lane 4*, plant of T-20; *lane 5*, plant of T-49; *lane 6*, plant of T-19; *lane 7*, plant of T-21

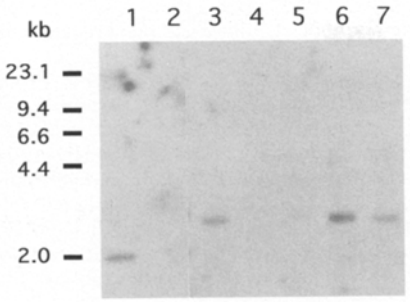


**Fig. 5** Southern-blot hybridization of total DNA from progenies of transgenic plants. Total DNA was digested with *Eco*RI and *Not*I. The *Bam*HI fragment (800 bp) of pAct1Dneo was used as a probe. *Lane 1*, pAct1Dneo (1 pg); *lane 2*, protoplast-derived non-transformed plant; *lanes 3, 6, 7*, $NPT^+$ progenies of T-20 plant; *lanes 4, 5*, $NPT^-$ progenies of T-20 plant

with larger or smaller sizes were also observed, indicating that rearrangement and/or DNA methylation of the foreign gene had occurred (Fig. 4A). When undigested samples were used, all signals were found at high molecular weight, showing the integration of the foreign gene in the barley genome (Fig. 4B). Very strong signals were detected in line T-19 in both analyses, indicating that this line carried a high copy number of the transgene.

To determine whether these plants expressed the foreign gene, NPT II ELISA was performed using their leaf tissues. In T-20, T-21, T-45 and T-49 plants, similar or slightly lower levels of the NPT II protein were detected compared with those found in their callus stage (Table 2). However, T-19 plants had undetected levels of the protein, although this line was clearly NPT II-positive at the callus level (Table 2).

### Analyses of progenies of the transgenic plants

Self-fertile plants were obtained in four transformed lines (T-19, T-20, T-45, T-49) while T-21 plants set no seed (Fig. 2E–F, Table 2). To determine whether the transgene was transmitted and expressed, NPT II ELISA was carried out using randomly chosen progenies ($T_1$ generation) of lines T-20 and T-49. Ten of fifteen progenies of line T-20 and six of twelve progenies of line T-49 proved NPT II positive. In addition, Southern analysis was performed using several T-20 progenies to confirm the presence of the transgene. As shown in Fig. 5, positive signals were detected in all $NPT\ II^+$ progenies although the band patterns differed from that of the $T_0$ plant. One $NPT\ II^-$ progeny carried no transgene while positive bands were detected in the other one.

## Discussion

We have obtained fertile, transgenic barley plants derived from transformed protoplasts. This success is mostly due to the combination of improved protocols for barley protoplast culture (Funatsuki et al. 1992; Funatsuki and Kihara 1994) and barley protoplast transformation (Lazzeri et al. 1992). The employment of relatively mild selection conditions was also of importance.

The use of highly morphogenic protoplast sources is one of the key factors for protoplast transformation systems (Potrykus 1990). The difficulty in producing cell suspensions providing highly morphogenic protoplasts is likely to prevent the generation of transgenic plants using protoplast systems in recalcitrant crops such as wheat. We have used three cell suspension lines and have obtained transgenic barley plants in all of them. Similar levels of plant regeneration from protoplasts have also been obtained in independent studies (Funatsuki et al. 1992; Kihara and Funatsuki 1994). These results suggest that the establishment of a reproducible barley transformation system using protoplasts has now become feasible, although the efficiency still needs to be improved.

To select protoplast-derived calli transformed with the NPT II gene, Lazzeri et al. (1992) gave more than two passages of selection on solid medium with 25 μg/ml and found no escape of surviving calli. Similar results have been obtained in wheat (Marsan et al. 1993). Both sets of authors have suggested that their selection conditions might be too stringent. We employed four selection methods with three levels of selection strength. Selection method 0 appeared too mild as all calli which we tested proved NPT II negative, although a screening with more samples might have detected a few positive ones. The results with selection method 3 suggested that selection on mL2 medium for 1 month was too strong. Only with selection methods 1 and 2, could transformed plants that grew to maturity be generated, indicating that this or a similar selection strength may be optimal to produce barley plants transformed with the NPT II gene. In addition, more healthy transgenic plants were recovered from selection 2 than from selection 3, although it is not certain whether the difference is significant because the comparison was based on relatively few plants. However, if this is a true effect, it would suggest that the physiological or developmental state of cells has an influence on the sensitivity of those cells to selection agents, since the two methods differed only in hormonal condition.

With regard to the generation of escapes, one unexpected phenomenon was observed; where selection method 1 or 2 was used, some untransformed cells from T-plates developed embryogenic structures on selection medium and gave rise to green shoots, which was never seen with calli from C-plates. This may be attributed to the protective effect of truly transformed cells adjacent to the escapes and suggests that a secondary screening system is needed even with a selection system which completely inhibits the growth of negative controls.

Southern analyses have clearly shown the presence and the integration of the transgene in the genome of barley plants and their progenies. However, not all transformants expressed the transgene at detectable levels in their leaf tissues even when the gene products had been detected at the callus stage. Furthermore, one line of the T-20 progenies carrying the transgene did not contain the gene prod-

uct. This inactivation of transgenes may have been the result of DNA methylation, since this process has been reported to be induced by the transgenes' own mRNA (Wasseneger et al. 1994), to increase with plant development (Borchardt et al. 1992), or to occur in subsequent generations depending on the environment (Linn et al. 1990). In addition, methylation, in combination with gene arrangement, may be partly responsible for the complex band patterns seen in Southern analyses. Further studies should examine how to control these undesirable events generated in the course of transformation procedures.

Transgenic barley plants have recently been produced by particle bombardment (Jähne et al. 1994; Ritala et al. 1994; Wan and Lemaux 1994; Hagio et al. 1995). This technique has the advantage that long-term culture is not necessary (Vasil 1994) and even intact plant tissues can be used (Christou et al. 1991; Barcelo et al. 1994; Jähne et al. 1994). However, the specialized equipment and the restriction of the use of this technique would pose problems in relation to the integration of this transformation system into practical breeding programmes. Although it is difficult to compare efficiencies between different transformation systems, based on the number of transgenic plants, the results obtained in the present study are broadly similar to those obtained in particle bombardment studies (1–32 independent regenerable transgenic lines). Therefore, it may be worthwhile to continue development of both transformation systems depending on the intended application. Furthermore, the recent success in regenerating plants from tissue-derived protoplasts of cereals (Gupta and Pattanayak 1993; Ghosh Biswas et al. 1994) should open new opportunities for the development of protoplast-mediated transformation systems.

In conclusion, we have demonstrated that the production of fertile transgenic barley is feasible by protoplast transformation.

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