Bombardment-Mediated Transformation Methods for Barley

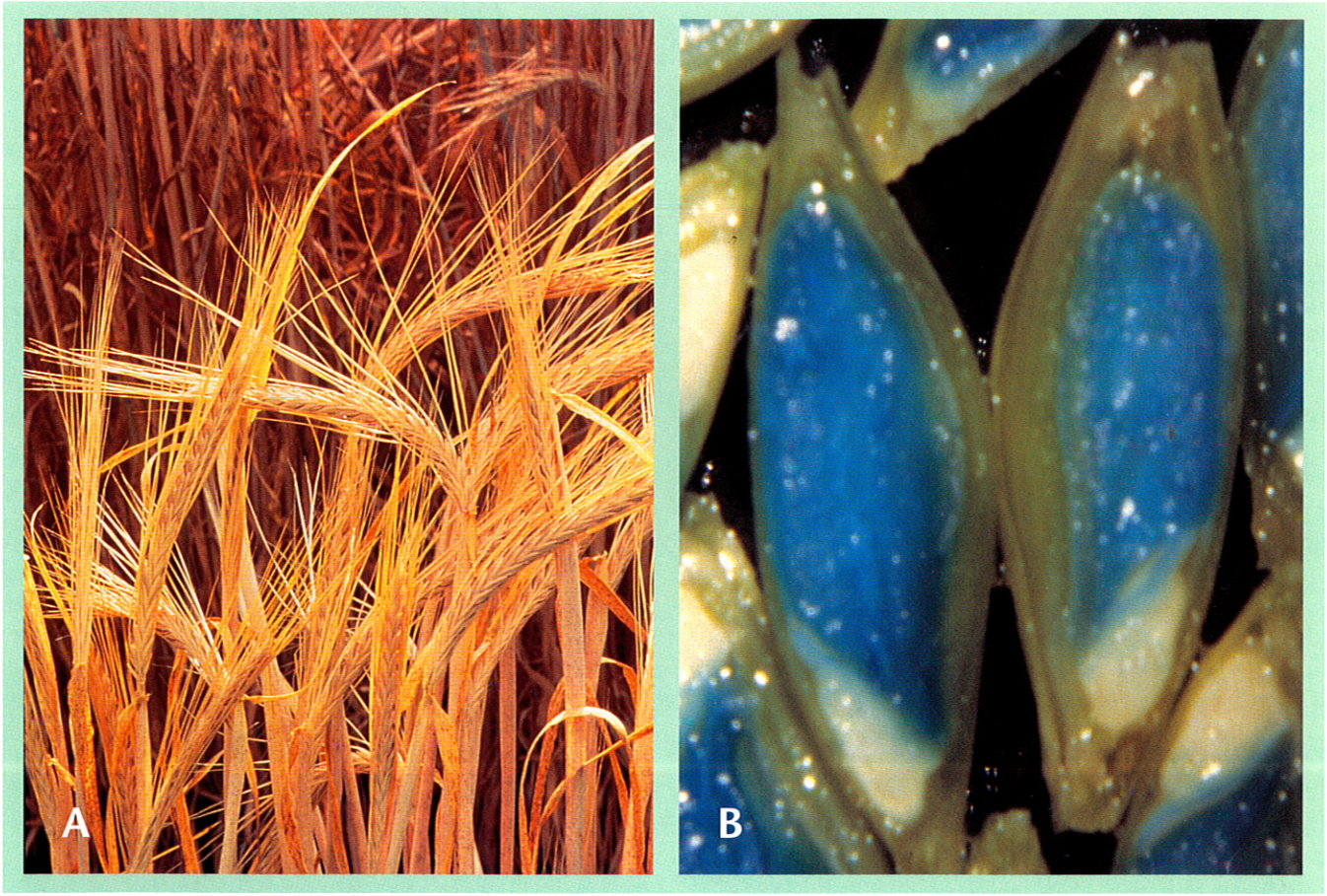

Fig. 1. A. Transgenic barley (*Hordeum vulgare* L., cv. Golden Promise). **B.** Histochemical enzyme assay for ß-glucuronidase on T_3 seeds from a stably transformed line containing *gus*A driven by a barley endosperm-specific promoter (M.-J. Cho, unpublished).

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Introduction

The analysis of plants modified by recombinant technologies has provided novel insights into questions of fundamental interest in plant biology and afforded opportunities for improvements in economically important crop species. Transformation techniques for many dicotyledonous species have become routine; these methods are most often based on the natural plant pathogen, *Agrobacterium*. Unfortunately, the transformation of cereals has been more difficult. We use the Biolistic® PDS-1000/He instrument as an efficient tool for the production of transgenic barley (*Hordeum vulgare* L.) plants (Figure 1A, B). Exogenous DNA is successfully introduced into cells from barley zygotic embryos and other explants by microprojectile bombardment (Wan and Lemaux, 1994). The bombarded immature embryos are cultured repeatedly on callus-induction medium containing a selective agent to recover stably transformed callus cultures, from which fertile transgenic plants are regenerated. On average, each 100 half-embryos yields four independent events that give rise to fertile transformed green plants.

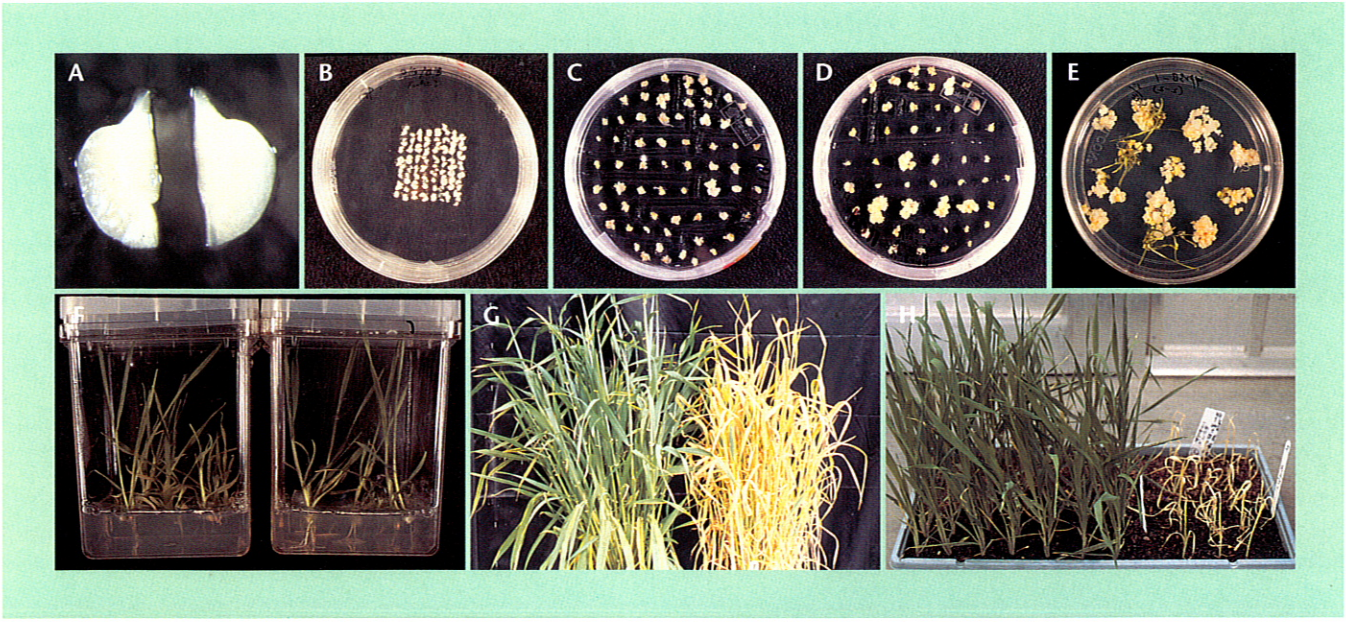

Fig. 2. **A.** Immature barley embyro after longitudinal bisection. **B.** Petri plate (10 cm diameter) showing embryos ready for bombardment. **C.** Callusing embryo explants on second round of selection. **D.** Herbicide-resistant calli on third round of selection. **E.** Barley plantlet regeneration on FHG medium with 1 mg/l 6-benzyl-aminopurine and 1 mg/l bialaphos. **F.** Green plantlets growing on rooting medium containing 1 mg/l bialaphos. **G.** Non-transformed regenerant (right) and transgenic plant (left) after two sprayings with 0.5% (v/v) Basta® herbicide. **H.** Heritability of herbicide-resistance in progeny of trangenic (left) and non-transgenic (right) plants.

Methods

Plant Materials

Plants of the barley spring cultivar Golden Promise are grown in growth chambers under a 16-h light/8-h dark period, at 12 °C and 60–80% humidity (Hunter, 1988). Light levels are set at approximately 350–400 µE at head height. Plants are fertilized with Osmocote (Sierra, 17-6-12 plus minors) at the time of planting and then weekly with 0.02% Verdi (Peter's 20-20-20 + minors).

Preparation of Immature Embryos for Microprojectile Bombardment

One day before bombardment, spikes with immature embryos about 1.5–2.5 mm in size are surface-sterilized in 20% (v/v) bleach (5.25% sodium hypochlorite) for 15 min, rinsed briefly with sterile water and washed three times for 5 minutes each. Immature embryos are bisected longitudinally through the root and shoot meristems while still inside the caryopsis, thereby preventing germination of the embryo. Both halves of the embryo are then removed and placed scutellum-side up on callus induction medium (Figure 2A). Callus induction medium is Murashige and Skoog salts (Murashige and Skoog, 1962) supplemented with 30 g/l maltose, 1.0 mg/l thiamine-HCL, 0.25 g/l *myo*-inositol, 1.0 g/l casein hydrolysate, 0.69 g/l proline, 2.5 mg/l dicamba (filter-sterilized and added after autoclaving), and solidified with 3.5 g/l Phytagel (Sigma). Each Petri dish contains as many as 100 half-embryos placed side by side in the center (Figure 2B) as close together as possible without touching so that each half-embryo maintains full contact with the media. The dishes are sealed with parafilm and placed in the dark at 24 ± 1 °C.

Plasmid Constructs

One plasmid that has been used for successful transformation is pAHC25 which contains the herbicide resistance gene, *bar*, as a selectable marker and the ß-glucuronidase reporter gene, *gusA* (Christensen and Quail, 1995). Both the selectable and screenable genes are under the control of the maize ubiquitin (*Ubi1*) promoter and first intron (Christensen *et al.*, 1992) and have a *nos* termination sequence. The selectable gene, *bar*, encodes phosphinothricin acetyltransferase (PAT), which inactivates phosphinothricin (PPT), the active component of the herbicides bialaphos (Dr. Michiaki Iwata, Meiji Seika Kaisha, Ltd., Yokohama, Japan, FAX 81-45-543-9771) and Basta (Murakami *et al.*, 1986; de Block *et al.*, 1987; Thompson *et al.*, 1987).

Bombardment Conditions

Gold particles are coated with DNA using modifications of a previously published protocol (Daines, 1990).

The protocol is

1. Prepare stock suspension of microprojectiles by mixing 60 mg of 1.0 µm gold particles (Bio-Rad) in 1,000 µl of absolute ethanol. Stock can be stored at -20 °C.
2. Vortex stock suspension for 10 sec.–1 min. Quickly remove 35 µl of the stock suspension and add to a sterile 1.5 ml microcentrifuge tube. Microcentrifuge at high speed for 30 sec. Remove ethanol with micropipette, resuspend pellet in 1 ml sterile water and microcentrifuge for 5 min. Remove water.
3. Resuspend microprojectiles in 25 µl of DNA solution (1 µg/µl). If using two plasmids, the total amount of DNA

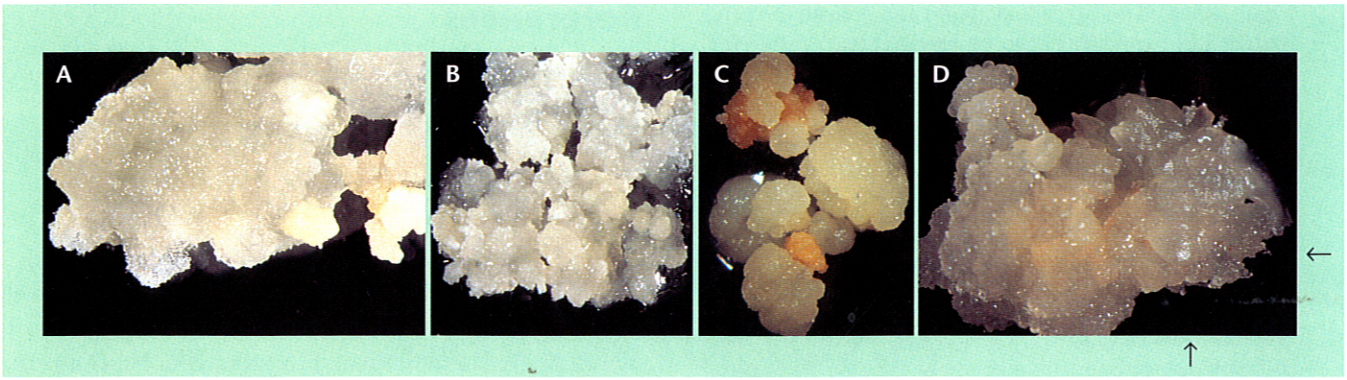

Fig. 3. A, B and C. Typical callus morphologies of non-transformed cells growing on selective medium (5 mg/1 bialaphos). None of these types have nodular structures. **D.** Transformed callus on bialaphos-containing medium. This desirable type is fast-growing, shiny, and embryogenic in appearance (for example, see intersection of two arrows).

remains 25 µg but a 1:1 molar ratio is used. Add the following, in the order specified: 220 µl of sterile water, 250 µl of 2.5 M $CaCl_2$, and 50 µl of 0.1 M spermidine (Sigma Chemical S-0266 stock solution stored at -20 °C, diluted 14 µl to 1,000 µl with water, then filter-sterilized). Mix thoroughly. Vortex on a vortex shaker (Eppendorf Model 5432) for at least 10 min at 4 °C. Microcentrifuge for 5 min and remove supernatant.

4. Resuspend DNA/microprojectile precipitate in 600 µl of absolute ethanol by pipetting up and down several times. Microcentrifuge for 1 min. Remove ethanol and resuspend pellet in 36 µl of absolute ethanol by pipetting up and down until well-dispersed.

5. Pipette 10 µl of the suspension as evenly as possible onto the center of a Mylar® macrocarrier sheet (Bio-Rad). Let ethanol evaporate. Each DNA/microprojectile suspension yields enough solution to coat three Mylar macrocarriers (0.6 mg gold and 6.9 µg DNA per bombardment).

6. Place 1,100 psi rupture disk (Bio-Rad) in unit. The distance between rupture disk and macrocarrier is 8–10 mm and between macrocarrier and stopping screen is 1 cm. Place Petri dishes containing target materials in the Biolistic PDS-1000/He device approximately 6 cm below the stopping screen (third shelf from bottom of unit). Evacuate chamber to 28–29 mm Hg and bombard the target once.

Selection of Transgenic Callus Cultures from Bombarded Immature Embryos

One day after bombardment, transfer bombarded half-embryos onto callus induction medium with 5 mg/l bialaphos (filter-sterilized and added after autoclaving). Place approximately 16 half-embryos (scutellum-side down) evenly spaced in each Petri dish. Culture half-embryos on the first selection medium for approximately 10–14 days.

At transfer to the second selection plate (5 mg/l bialaphos), all of the material from individual callusing embryos is broken into small pieces (2–4 mm) using forceps and maintained separately. Callus originating from each bombarded explant is tracked by delineating the material derived from a half-embryo with a marker pen on the Petri dish. Calli derived from different embryo halves are by definition independent events, but each embryo-half can give rise to more than one transformation event. During the subsequent two to five selection passages (each approximately 10 to 20 days, on 5 mg/l bialaphos), callus pieces showing evidence of more vigorous growth (Figures 2C/D) are transferred earlier in the subculture cycle to new selection plates. At each subsequent passage; only tissue showing vigorous growth is chosen for passage: this is done by breaking the callus into small pieces and placing on fresh selection medium. In this manner, bialaphos-resistant callus cultures are established. Non-transformed tissues (escapes) are often observed after three to five selection passages; however, these non-transgenic calli look different from true transformants in appearance. In general, non-transformed tissues appear white and watery (Figure 3A), loose and friable (Figure 3B), or round and slow growing (Figure 3C), while transformed tissues are fast-growing, shiny and nodular (embryogenic) in appearance (Figure 3D). In general, non-regenerable transformed tissues do not have nodular structures. After three or more rounds of selection, transfer resistant embryogenic callus to Petri dishes containing regeneration medium supplemented with 1 mg/l bialaphos. Tissue should be moved through selection as rapidly as possible since longer culture times result in lower regenerability and a higher incidence of albinism. Regeneration medium is FHG medium (Hunter, 1988) supplemented with 1 mg/l 6-benzyl-aminopurine solidified with 3.5 g/l Gelrite or Phytagel. Place dishes at 24 ± 1 °C under fluorescent lights (45–55 µE, 16-h light). In approximately 2–4 weeks, plantlets are observed (Figure 2E). Green plantlets, approximately 2–3 cm, are transferred into Magenta boxes containing rooting medium (callus induction medium without dicamba) with 1 mg/l bialaphos (Figure 2F). Before they grow to the top of the box, plantlets are transferred to 6-inch pots containing Supersoil (R. McLellan Co., S. San Francisco, CA) and placed in the greenhouse (16-h light period, 15–18 °C). The earlier in the selection process callus is transferred to regeneration medium, the more likely it will be that some regenerants develop from non-transformed tissue (escapes). It should be noted that green plantlets can be regenerated from non-transformed callus tissue on regeneration medium with bialaphos. These plantlets grow very slowly, however, and have thin, weak root systems or even die after two to three weeks of growth. To avoid escapes, 3 mg/L bialaphos can be used in the rooting medium.

Confirmation of Transformation

There are numerous means to confirm the transformed nature of tissues and plants.

Enzyme Assays

Determine *bar*-encoded PAT activity of callus or plant tissue using a thin-layer chromatographic assay of ^{14}C-labeled product (Spencer *et al.*, 1990). For each assay, use 12.5 µg or 25 µg of total protein extracted from callus tissue or plant leaves. This assay is valuable for screening callus lines for evidence of transformation. The presence of a signal at the position of acetylated PPT that is above background is a reliable indication of transformation. GUS activity is assayed histochemically as described (Jefferson *et al.*, 1987); presence of blue stained cells/tissues after 24-h is presumptive evidence of transformation, but is less reliable than confirmation of PAT activity.

DNA Hybridization Assay

Genomic DNA is isolated from callus or leaf tissue (Cone, 1989) and 10 µg/sample is digested with a 4-fold excess of restriction enzyme. DNA fragments are separated with agarose gel electrophoresis and transferred onto a nylon hybridization membrane (Southern, 1975). Prehybridize/hybridize according to published procedures (Wan *et al.*, 1992). With the appropriate controls, presence of a hybridizing band in a putative transformant provides the strongest evidence of transformation.

PCR Assay

Genomic DNA is isolated from callus or leaf tissue. To test for the presence of the *bar* gene in the genomic DNA, 250 to 500 ng of DNA is amplified by PCR using the primer set, BAR5F (5'-CATCGAGACAAGCACGGTCAACTTC -3') and BAR1R (5'-ATATCCGAGCGCCTCGTGCATGCG-3'), which yields a 0.34 kb *bar* fragment if template is present. Amplifications are performed in a 25 µl reaction volume with Taq DNA polymerase using a Perkin-Elmer Cetus Thermo-Cycler. In a sample PCR protocol, the regime consists of 1 min denaturation step at 95 °C, 30 sec annealing steps (5 cycles at 65 °C, 10 cycles at 60 °C, 10 cycles at 55 °C) and a 2 min extension step at 72 °C. For the final cycle, the duration of the extension step is 7 min at 72 °C. The target DNA sequence is amplified for 25 cycles and samples are then electrophoresed on a 0.8% agarose gel and examined for a 0.34 kb product. This assay provides a quick screen of tissue and is reliable provided proper controls are used. This assay does not distinguish chimeric tissue composed of a mixture of transformed and non-transformed callus tissue and does not confirm that the expression unit is intact.

Herbicide Application

Herbicide sensitivity can be checked by either spraying the entire plant with 0.5% (v/v) Basta solution plus 0.1% Tween 20 using a spray bottle on mature plants (Figures 2G) or at the 4–5 leaf stage (Figures 2H) or by painting a section of leaf blade using a cotton swab. Basta spraying of older plants may require higher levels of herbicide or multiple sprayings. Examine after one week. Plants expressing *bar* show no symptoms. Nontransformed plants or plants not expressing *bar* will show severe leaf necrosis, or die (Figures 2G/H). Herbicide sensitivity can vary depending on the age and vigor of the plant. This method provides a quick screen of plant tissue. It is reliable provided proper controls are used. The same test also can be used to challenge the progeny of transformed regenerants when examining heritability and segregation of this transgenic trait (Figure 2H).

Results

The data from several experiments performed by two different individuals are shown in Table 1. The number of independent, stable transformants varies from experiment to experiment, probably due to a variety of factors, including the condition of the donor plants and inherent variability of the bombardment process. In summary, from 952 bombarded half-embryos, 73 independent callus lines were identified as either PAT- or PCR-positive; on average, 7.7% of the bombarded half-embryos yielded independent, stably transformed callus lines.

Table 1. Production of Transgenic Plants

Bombardment	No. of Half-Embryos Bombarded	PAT$^+$ or PCR$^+$ Callus Lines	No. of Lines Yielding Plants		
			Green	Albino	None
Scientist 1					
1	75	6	5	0	1
2	55	6	4	1	1
3	24	0	0	0	0
4	35	3	3	0	0
5	124	12*	5	1	4
6	111	4	2	1	1
7	81	2	2	0	0
8	43	11	3	8	0
9	120	8	6	1	1
10	43	4	1	2	1
Subtotal	**711**	**56**	**31**	**14**	**9**
Scientist 2					
1	77	7	4	1	2
2	79	7	1	3	3
3	85	3	1	2	0
Subtotal	**241**	**17**	**6**	**6**	**5**
Total	**952**	**73**	**37**	**20**	**14**

* Two PAT$^+$ lines were contaminated before regeneration.

The results of regeneration attempts are also summarized in Table 1. Of the 73 positive callus lines, 37 lines gave rise to green plants (51%), 20 lines regenerated only albino plants (27%), and fourteen lines were nonregenerable (19%). Two PAT+ callus lines were contaminated before regeneration. More than 95% of green plantlets survived after transfer to soil in the greenhouse.

Most transgenic regenerants (T_0 plants) looked phenotypically normal and were self-fertile, although seed set varied from a few seeds per plant to nearly full seed-set. In general, callus lines with poor regenerability showed poor fertility.

Discussion

Using the protocol described here, we successfully generated fertile, transgenic barley plants by microprojectile bombardment. We observed on average a 7.7% transformation frequency, *i.e.* approximately 8 independent lines per 100 bombarded half-embryos. Of the transformed callus lines, 78% were regenerable but 20 out of the 57 regenerable lines (35%) yielded albino plants, giving an "effective" transformation frequency of approximately 4 independent lines yielding fertile green plants per 100 half-embryos bombarded. In addition, we have infrequently observed two independent events arising from a single half-embryo; the resulting callus gave rise to regenerated plants with two different Southern banding patterns.

The primary biological requirements for successful transformation are that the target cells are actively dividing, are accessible to microprojectiles, are culturable *in vitro*, and are capable of giving rise to fertile green plants. Our main efforts have been focused on the use of immature embryos. It is known from earlier studies that active cell division occurs in the epidermal and subepidermal layers of the scutellum of immature haploid barley embryos during callus induction (Kott *et al.*, 1985); however, these studies are not able to track the precise origin of regenerable callus. Therefore, we bisected immature embryos and placed the half-embryos in three orientations to introduce DNA into cells on all surfaces as well as the interior. We did not see a significant difference in the numbers of independent transformation events based on the orientation of the embryos (Wan and Lemaux, 1994). Therefore, we conclude that either all surfaces of an embryo contain division-competent cells or, even when half-embryos are placed with the embryo-axis or cut-surface facing up during bombardment, sufficient numbers of scutellar epidermal cells are exposed, transformed and give rise to callus. However, we routinely place embryos scutellum-side up for technical ease.

Although not detailed here, we have successfully transformed two other organized bombardment target tissues; young callus from immature embryos and microspore-derived embryos. From both, we obtained numerous, independently transformed, self-fertile plants (Wan and Lemaux, 1994). In the genotype of choice, Golden Promise, a large percentage of the immature embryos (90–95%) form callus on the appropriate medium. Therefore, embryos are an effective target tissue for Golden Promise. The ability to transform callus as the target tissue is important in genotypes where the callus response is low. However, albinism is a common problem in barley tissue culture (Kott and Kasha, 1984; Jähne *et al.*, 1991) and the callus approach adds time to the culturing period before bombardment. This may exacerbate the albinism problem since there is a correlation between increased albinism and longer culturing times (Kott and Kasha, 1984). Transformation of microspore-derived embryos might lead to the transformation of haploid tissues. This has the advantage of giving rise to homozygous transformed material in the first generation if the chromosomes are doubled. The disadvantage with this approach is that it is more genotype-dependent since it requires anther culture. Based on the reported frequencies, this approach appears less efficient, although no attempts were made to optimize this approach.

To achieve optimum results, we recommend the following measures:

- Using immature embryos derived from plants grown under defined growth conditions.
- Utilizing immature embryos from the cultivar Golden Promise that are 1.5–2.0 mm. This size is optimal for good callus induction and transformation in this genotype, but this may vary depending on the cultivar.
- Using bisected immature embryos. The bisection prevents germination of the embryo and results in higher quality callus.
- Optimizing the auxin source for callus induction when using other genotypes of barley. Different auxin sources such as dicamba and 2,4-D should be tested to determine the optimum for a particular genotype.
- Adjusting the bialaphos concentration may be necessary when using other genotypes of barley. For some genotypes, lower concentrations of bialaphos may be adequate for selection since higher concentrations seem to exacerbate the albinism problem.
- Checking the selection plates regularly. To speed up the selection procedure, improve regenerability and lower albinism, transfer vigorously growing callus pieces to the next selection step as soon as possible.
- Transferring the small plantlets to Magenta boxes containing sterilized peat pots with soil. This improves the vigor of the plantlets before transfer to the greenhouse. Newly transferred plantlets should be protected against high temperatures and direct sunlight.
- Using the herbicide application test to identify transformed plants that express *bar*. There is a strong correlation between the results of the PAT assay and herbicide application, *i.e.*, PAT-positive plants are resistant to Basta and PAT-negative plants are sensitive.
- Excising young embryos from T_0 (first-generation) plants before the seeds dry down and germinating them on rooting medium containing 3mg/l bialaphos. T_1 plants are available approximately 1 month earlier by culturing than if the seed is dried down (Wan and Lemaux, 1994). Embryos that germinate are actively expressing bar.

Nongerminated embryos (that might also contain the gene but are not expressing) can be rescued by transferring them to bialaphos-free medium 4 or 5 days after they are cultured on bialaphos-containing medium; some might contain nonexpressing copies of bar and others are embryos that segregate as nulls.

We have shown that the Biolistic PDS-1000/He instrument is a powerful tool for the production of fertile transgenic barley plants. We are currently using the procedure described in this bulletin to successfully generate transformed plants with a variety of introduced genes. On average, 8 out of every 100 half-embryos that we bombard give rise to a transformed callus line; 4 out of every 100 half-embryos yield fertile green plants. Additional work is in progress to improve this protocol, such as attempting to identify factors exacerbating the albinism problem. Overall, this technique is a reproducible and dependable means of producing fertile transgenic barley plants.

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Bulletin 2007 US/EG 95-0570 0396 *SIG 020996 Printed in USA*