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Generation of Large Numbers of Independently Transformed Fertile Barley Plants[1]

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A rapid, efficient, and reproducible system to generate large numbers of independently transformed, self-fertile, transgenic barley (*Hordeum vulgare* L.) plants is described. Immature zygotic embryos, young callus, and microspore-derived embryos were bombarded with a plasmid containing *bar* and *uid*A either alone or in combination with another plasmid containing a barley yellow dwarf virus coat protein (BYDVcp) gene. A total of 91 independent bialaphos-resistant callus lines expressed functional phosphinothricin acetyltransferase, the product of *bar*. Integration of *bar* was confirmed by DNA hybridization in the 67 lines analyzed. Co-transformation frequencies of 84 and 85% were determined for the two linked genes (*bar* and *uid*A) and for two unlinked genes (*bar* and the BYDVcp gene), respectively. More than 500 green, fertile, transgenic plants were regenerated from 36 transformed callus lines on bialaphos-containing medium; albino plants only were regenerated from 41 lines. T_0 plants in 25 lines (three plants per line) were analyzed by DNA hybridization, and all contained *bar*. Most contained the same integration patterns for the introduced genes (*bar*, *uid*A, and the BYDVcp gene) as their parental callus lines. Transmission of the genes to T_1 progeny was confirmed in the five families analyzed by DNA hybridization. A germination test of immature T_1 embryos on bialaphos-containing medium was useful for selecting individuals that were actively expressing *bar*, although this was not a good indicator of the presence or absence of *bar*. Expression of *bar* in some progeny plants was indicated by resistance to the herbicide Basta. The T_1 plants were in soil approximately 7 months after bombardment of the immature embryo.

The ability to transform plants through recombinant technologies has been of great value in exploring fundamental questions and enabling crop improvement. Recently, due to prolonged effort and substantial resources, the generation of stable transformants and fertile transgenic plants has been achieved in the cereals rice (Toriyama et al., 1988; Zhang et al., 1988; Zhang and Wu, 1988; Shimamoto et al., 1989; Datta et al., 1990; Christou et al., 1991; Peng et al., 1991, 1992; Cao et al., 1992; Li et al., 1993; Rathore et al., 1993), maize (Fromm et al., 1990; Gordon-Kamm et al., 1990; D'Halluin et al., 1992; Walters et al., 1992; Koziel et al., 1993), wheat (Vasil et al., 1992; Weeks et al., 1993), and oat (Somers et al., 1992). In recent years, a number of laboratories utilized a variety of approaches to introduce foreign genes into barley (*Hordeum vulgare* L.). Protoplasts, derived from mesophyll, aleurone, endosperm tissue, or cell suspensions, were used to demonstrate transient uptake and expression of exogenous DNA via either PEG- or electroporation-mediated DNA uptake (Junker et al., 1987; Lee et al., 1989, 1991; Teeri et al., 1989; Lazzeri et al., 1991). Particle bombardment was used to introduce and express foreign DNA in different barley tissues, such as suspension cells (Mendel et al., 1989), IEs (Kartha et al., 1989; Lee et al., 1991), callus tissue (Kartha et al., 1989), and endosperm (Knudsen and Müller, 1991). To date, there is only one report of stable transformation of barley, in which DNA was introduced into protoplasts via PEG-induced DNA uptake (Lazzeri et al., 1991), but no plants were recovered.

We report here the generation of self-fertile, transgenic barley plants. Exogenous DNA is introduced by microprojectile bombardment into IEs, IE-derived young callus, and MDEs. From these tissues, we generate numerous independently transformed callus lines. DNA hybridization analysis confirms the presence of two linked and two unlinked genes in most lines. Functional expression of *bar* and *uid*A is confirmed. Self-fertile transgenic barley plants are regenerated from 36 callus lines, and stable transmission of the introduced genes is demonstrated by DNA hybridization analysis. Functional expression of *bar* in T_0 and T_1 plants is also shown.

MATERIALS AND METHODS

Plasmids

The plasmid pAHC25 (constructed by A. Christensen, George Mason University, Fairfax, VA, and provided by P.H. Quail, Plant Gene Expression Center, Albany, CA) consists of the *uid*A reporter gene (Jefferson et al., 1987) and a selectable gene, *bar* (Thompson et al., 1987). Each is under the control of the maize ubiquitin (Ubi-1) promoter and first intron (Christensen et al., 1992) followed by the 3′ untranslated region and polyadenylation signal of the nopaline synthase gene (*nos*) from *Agrobacterium tumefaciens* (Bevan et al., 1983). The plasmid pBARGUS (Fromm et al., 1990) contains *uid*A driven by the maize *Adh1* promoter and intron 1 and *bar* driven by the CaMV 35S promoter and *Adh1* intron

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Abbreviations: BYDVcp, barley yellow dwarf virus coat protein; CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; IE, immature zygotic embryo; MDE, microspore-derived embryo; PAT, phosphinothricin acetyltransferase; PPT, phosphinothricin.

1. Both genes are terminated by the *nos* 3′ end from *A. tumefaciens*. The transformations also included one of two plasmids containing BYDVcp genes. One plasmid, pRsa1BYDVcp, contained the structural gene for the coat protein of a PAV serotype of BYDV from the Pacific Northwest (GenBank accession number L19504) under the control of the CaMV 35S promoter and terminated by *nos* (kindly provided by S. Wyatt, Washington State University, Pullman, WA). A second BYDVcp construct was pPAV110, which contained a cDNA encoding the coat protein from a p-PAV serotype of BYDV (Vincent et al., 1990) driven by the CaMV 35S promoter and terminated by *nos* (kindly provided by R. Lister, Purdue University, West Lafayette, IN).

Plant Materials

Plants of the barley (*Hordeum vulgare* L.) spring cultivar Golden Promise were grown in growth chambers under a 16-h light/8-h dark period at 12°C and 60 to 80% humidity (Hunter, 1988). Light levels at head height were approximately 350 to 400 μE. Seeds of a winter cultivar, Igri, were germinated in soil in the growth chamber under the same conditions. When about 10 cm in height, the seedlings were vernalized for 8 weeks under a 10-h light (10–15 μE)/14-h dark period at 4°C (Hunter, 1988). After vernalization, they were grown under the same regime as Golden Promise plants. All plants were fertilized with Osmocote (Sierra, 17–6–12 plus minors) at the time of planting and then biweekly with 0.02% Verdi (Peter's, 20–20–20).

Immature Embryos and Callus Derived from Immature Embryos

Spikes of cv Golden Promise with immature embryos about 1.5 to 2.5 mm in size were surface sterilized in 20% (v/v) bleach (5.25% sodium hypochlorite) for 5 min, rinsed briefly three times, and washed for 5 min with sterile water. Immature embryos were dissected from young caryopses and left intact or were bisected longitudinally. For induction of callus for bombardment, embryos (intact or bisected) were placed scutellum-side down on callus induction medium, which was Murashige and Skoog medium (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 Pro, and 2.5 mg/L dicamba, solidified by 3.5 g/L Gelrite (Scott, Carson, CA) or Phytagel (Sigma). Embryos were incubated at 25°C in the dark, and embryogenic callus was selected for bombardment after 2 weeks.

Anther Culture and Microspore-Derived Embryos

Spikes, wrapped by the flag leaves, were harvested from cv Igri plants when the microspores were at mid-uninucleate to early binucleate stages and surface sterilized briefly with 70% ethanol. Anthers were dissected from spikelets and 60 anthers were placed in each Petri dish (35 × 10 mm) with 3 mL of 0.3 M mannitol. The Petri dishes were sealed with Parafilm and incubated at 25°C in the dark for 3 or 4 d. Anthers were subsequently transferred into Petri dishes with 3 mL of Hunter's liquid FHG medium (a modified Murashige and Skoog medium with lower NH_4NO_3 and high Gln; Kasha et al., 1990) without Ficoll-400 and supplemented with 1 mg/L IAA and 0.2 mg/L kinetin (designated as FHG^+) and incubated as described. MDEs were visible after approximately 2 or 3 weeks and were used for bombardment after approximately 4 weeks.

Preparation of Bombardment Plates and Microprojectile Bombardment

One day before bombardment, IEs (1.5–2.5 mm) from young caryopses of cv Golden Promise were cut in half longitudinally and placed in three different orientations (scutellum-side up, scutellum-side down, or cut-surface up) on callus induction medium in the center of Petri dishes (100 × 15 mm) as shown in Figure 1A. For bombardment of callus, approximately 0.5 g of embryogenic callus (Fig. 1B) from cultured IEs was cut into small pieces (about 2 mm) and placed in the center of a Petri dish (100 × 15 mm) containing callus induction medium. MDEs were harvested from anther culture plates using a Pasteur pipet and distributed evenly in Petri dishes (100 × 15 mm) on a piece of 5-cm Whatman No. 3 filter paper supported by two 7-cm filters. Before bombardment, excess medium was removed from the filters.

Plasmid DNA was adsorbed to gold particles (1.0 μm, DuPont, Wilmington, DE) as described previously (Daines, 1990). When two plasmids were used (pBARGUS or pAHC25 and pRsa1BYDVcp or pPAV110), equal amounts (μg) of DNA from the two plasmids were mixed; this resulted in an approximately 1:2 molar ratio. All target materials were bombarded once, using a DuPont PDS 1000 He Biolistic Delivery System. The target materials were positioned approximately 13 cm below the microprojectile stopping plate; 1100-p.s.i. rupture discs were used.

Selection of Transformants

IEs and Callus

One day after bombardment, half-embryos and callus pieces were transferred individually to callus induction me-

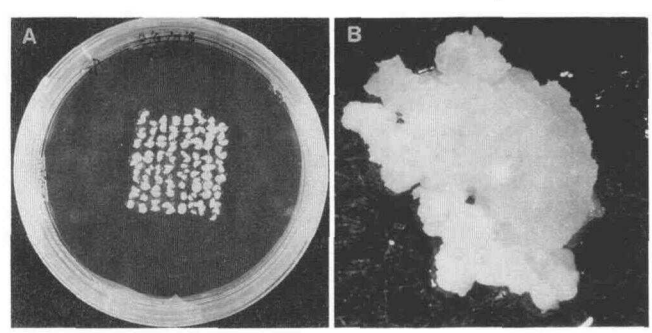

Figure 1. Immature embryos and callus tissue for bombardment. A, IEs (1.5–2.5 mm) were cut in half longitudinally from young caryopses of cv Golden Promise and placed on solid callus induction medium in three different orientations. The orientation scutellum-side up is shown here. The photograph was taken before bombardment, 1 d after dissection. B, An example of young callus developing from a dissected immature embryo of cv Golden Promise approximately 10 d after excision. For bombardment, embryogenic callus was cut into small pieces and placed as in A.

dium with 5 mg/L bialaphos; the half-embryos were cultured scutellum-side down, irrespective of their orientation during bombardment. Tissue remained on the first selection plate for approximately 10 to 14 d. At transfer to the second selection plate (5 mg/L bialaphos), individual callusing embryos or callus pieces were broken using forceps into several small pieces and maintained separately. During the subsequent two to three selection passages (each approximately 10 to 20 d, at 5 mg/L bialaphos), callus pieces showing evidence of more vigorous growth were transferred earlier to new selection plates and tissue was handled in an identical manner. All callus tissue developed originally from each piece of embryo or callus was defined as a line. Bialaphos-resistant callus lines were maintained by monthly subculture on callus induction medium with 5 mg/L bialaphos.

MDEs

Following bombardment, several drops of FHG^+ medium were added to the MDEs. After 2 or 3 d, embryos of >1.5 mm were transferred individually onto callus induction medium with 3 or 5 mg/L bialaphos. Smaller embryos remained on the filters and were transferred to selection medium when they were approximately 1.5 mm. Filter papers were washed every 2 or 3 d by repeated addition and removal of liquid FHG^+ medium. MDEs remained on the first selection medium for 10 to 20 d. MDEs showing evidence of callus formation were transferred to fresh selection medium with 5 mg/L bialaphos. During transfer, each callusing MDE was broken into a few small pieces. Further selection was as described in the previous section.

Enzyme Assays

Activity of PAT was determined using a thin-layer chromatographic assay (Spencer et al., 1990). Twenty-five or 12.5 μg of total protein extracted from callus tissue or plant leaves was used per assay. GUS activity was assayed histochemically as described (Jefferson et al., 1987); the presence of any blue cells in a callus line resulted in that line being scored as GUS-positive.

Plant Regeneration and Herbicide Application

Plants were regenerated from PAT-positive callus lines by transferring embryogenic callus to FHG medium with 1 mg/L bialaphos at 23° or 25°C under fluorescent lights (45–55 μE, 16 h/d). In approximately 2 weeks, plantlets were observed. Green plantlets, approximately 2 cm, were transferred into Magenta boxes containing plantlet growth medium (hormone-free callus induction medium) with 1 mg/L bialaphos. Before they grew to the top of the box, plantlets were transferred to 6-inch pots containing Supersoil and placed in the greenhouse (16-h light period, 15°-18°C). Regenerants grew to maturity and self-pollinated. Some of the plants were tested for their response to Basta (200 g/L PPT, Hoechst AG, Frankfurt, Germany) by spraying with a 0.5% (v/v) solution plus 0.1% Tween 20. Plants were also regenerated from wild-type callus on the media without bialaphos.

Southern Hybridization

Genomic DNA was isolated from callus or leaf tissue (Cone, 1989) and 10 μg/sample was digested with a 4-fold excess of restriction enzyme. DNA was electrophoresed in 0.8% agarose gels in Tris-borate-EDTA buffer, transferred (Southern, 1975) onto membrane (Hybond-N, Amersham, Arlington Heights, IL), and prehybridized/hybridized according to published procedures (Wan et al., 1992). For detecting sequences containing *bar*, *uid*A, and the BYDVcp gene, a 0.5-kb *Bam*HI-*Sph*I fragment from the *bar* coding region, a 1.8-kb *Bam*HI-*Sac*I fragment from the *uid*A coding region of pAHC25, and a 0.48-kb *Eco*RI fragment from the BYDVcp gene in pRsa1BYDVcp were ^{32}P-labeled using the Prime-a-Gene labeling system (Promega, Madison, WI) according to the manufacturer's instruction. Following hybridization, filters were washed as described (Wan et al., 1992) and exposed to x-ray film at −80°C. Probes were stripped (Wan et al., 1992) before the filters were rehybridized.

Gemination Test of Immature Embryos of T_1 Generation

Immature embryos (approximately 1.5–2.5 mm) were dissected from young caryopses of T_0 and nontransformed R_0 plants and plants grown from wild-type seeds, and cultured scutellum-side down on FHG medium with 3 mg/L bialaphos under the same conditions for plant regeneration. The number of germinated embryos was determined in 4 or 5 d. The seedlings from germinated embryos were transferred onto the plantlet growth medium in Magenta boxes and defined as T_1(R) plants, i.e. T_1 plants from bialaphos-resistant embryos. Embryos that did not germinate were transferred onto FHG without bialaphos; some survived and germinated into seedlings, which were defined as T_1(S) plants, i.e. T_1 plants from bialaphos-sensitive embryos. Some plants [T_1(R) and T_1(S)] from each T_1 family were grown to maturity in the greenhouse in 6-inch pots. Other seedlings were planted in soil in trays (52 × 26 × 6 cm), spaced approximately 4 cm apart. At the third- or fourth-leaf stage, they were sprayed with 0.5% Basta and resistant and susceptible plants were scored after 1 week.

RESULTS

IEs, IE-derived callus, and MDEs were bombarded with DNA from a single plasmid carrying both a screenable marker *uid*A and a selectable marker *bar*, either alone or in combination with a second plasmid carrying a BYDVcp gene. The gene *uidA* encodes GUS; *bar*, isolated from *Streptomyces hygroscopicus* (de Block et al., 1987), encodes PAT, an enzyme that acetylates and inactivates PPT, the active component of the herbicides Basta (PPT) and Herbiace (bialaphos) (Murakami et al., 1986; Thompson et al., 1987).

Bombardment and Selection of Transformants

Bombarded IEs

Ten bombardments were performed using IEs from cv Golden Promise as targets (Table I, section A). For bombardment, bisected IEs were placed on callus induction medium

Table I. *Summary of bombardment and selection experiments with IEs, callus tissue, and MDEs*

Target and Orientation	Bombardment	Construct[a]	No. of Half-IEs or MDEs	No. of Independent PAT+ Callus Lines (%)[b]	No. of Lines Yielding Plants		
					Green	Albino	None
A. IEs							
Cut-surface up	1	A + B	75	6 (8.0)	5	0	1
	2	A + B	55	6 (10.9)	4	1	1
	3	A + B	24	0 (0)	0	0	0
	4	A + B	35	3 (8.6)	3	0	0
	5[c]	A + B	124	12 (9.7)	5	1	4
	6	A + B	111	4 (3.6)	2	1	1
	7	A + B	81	2 (2.5)	2	0	0
Scutellum up	8	A + B	43	11 (25.6)	3	8	0
	9	A + C	120	8 (6.7)	6	1	1
Scutellum down	10	A + B	43	4 (9.3)	1	2	1
	Subtotal		711	56 (7.9)	31	14	9
B. Callus	1	A + B		21	0	18	3
	2	A + C		6	3	3	0
	Subtotal			27	3	21	3
C. MDEs	1	A + B	524	2 (0.4)	1	1	0
	2	A + B	645	2 (0.3)	0	2	0
	3	D	280	2 (0.7)	0	2	0
	4	D	580	2 (0.3)	1	1	0
	5	D	380	0 (0)	0	0	0
	Subtotal		2409	8 (0.3)	2	6	0
	Total			91	36	41	12

[a] Constructs A, B, C, and D represent pAHC25, pRsa1BYDVcp, pPAV110, and pBARGUS, respectively. [b] The number in parentheses is the percentage of half-IEs or MDEs yielding PAT-positive (PAT+) callus lines. [c] From this bombardment plate, two PAT+ callus lines were contaminated before regeneration was performed.

(Fig. 1A) in three orientations: cut-surface up (seven bombardments), exposing inner cell layers; scutellum-side up (two bombardments), with epidermal cells of the scutellum exposed; and scutellum-side down (one bombardment), placing the embryo axis upward. Several half-embryos were randomly chosen from each bombardment plate for GUS assay the day after bombardment. Most half-embryos had dozens to hundreds of GUS-positive foci (Fig. 2A). During the first round of selection most embryos grew or expanded; a few had visible callus development (Fig. 3A). Germination of half-embryos was much less extensive compared with that of intact embryos on bialaphos-free medium. Every half-embryo was cut into several small pieces and transferred to fresh selection medium for a second round of selection. During the second round of selection, some pieces of callus grew vigorously; the growth of others was inhibited, and in some cases pieces turned brown (Fig. 3B). To obtain callus lines devoid of nontransformed tissue, growing callus tissue was transferred onto fresh selection medium (Fig. 3C) in the same manner one or more additional times until uniform growth was obtained (Fig. 3D).

From 9 of 10 bombardment experiments, 56 independent callus lines were identified as PAT positive (Table I, section A; Fig. 4). No PAT-positive lines were obtained from bombardment experiment No. 3 (Table I, section A); however, only 24 half-embryos were subjected to selection. Most of the half-embryos bombarded in this experiment and in bombardment experiment No. 4 were used in another study. The percentage of bombarded half-embryos that yielded independent PAT-positive callus lines was 7.9 on average, with a range of 2.5 to 25.6 for the nine successful experiments. There were no dramatic differences in frequencies depending on the orientation of the embryos during bombardment.

Bombarded Callus Tissue

IE-derived embryogenic callus tissue was selected for bombardment 2 weeks after initiation (Fig. 1B). As with IE bombardments, the pieces of callus tissue assayed for GUS activity had dozens to hundreds of blue foci. In the first experiment, 21 resistant callus lines, each from a different piece of callus, were PAT positive (Table I, section B). Six callus lines from bombardment No. 2 were PAT positive.

Bombarded MDEs

The results from five bombardments with MDEs are summarized in Table I, section C. As with IEs and callus, a small number of MDEs were assayed for GUS activity after bombardment. Some MDEs had GUS-positive foci; however, no careful measurements of their frequencies were made. A total

Figure 2. Histochemical analyses of bombarded immature embryos and transgenic callus. A, One day after bombardment, several embryo pieces from each bombardment plate were assayed for histochemical GUS activity. B, PAT$^+$ callus lines were assayed for GUS activity by histochemical staining; each well contained callus from one line. C, Histochemical assay of callus from one transformed line.

of 2409 MDEs from five bombardment plates were transferred individually onto the first selection plates. After culturing for 10 to 20 d on the first selection plate, the growth of most MDEs was inhibited and callus formation was not observed. Some MDEs showed callus formation, but the growth of callus was much slower than that from nonbombarded MDEs grown without selection. During the second round of selection, some callus grew more vigorously and was subjected to further selection. After three or more rounds of selection, bialaphos-resistant callus lines were established. From four of the five bombardment plates, eight resistant callus lines that appeared embryogenic (two lines per bombardment plate) had PAT activity (Table I, section C).

In total, we obtained 91 independent PAT-positive callus lines from 15 of 17 bombardment experiments (plates) using IEs, callus tissue, or MDEs as target materials (Table I). To speed up the selection process, any callus pieces showing vigorous growth were subjected to the next round of selection immediately. We found that PAT-positive callus tissue was distinguishable from PAT-negative tissue capable of growing in the presence of bialaphos by the fact that it had embryogenic structures. Escaped tissue had a loose structure and appeared nonmorphogenic. PAT-positive callus lines were not recovered from nonbombarded tissues or tissues bombarded with gold microprojectiles only.

DNA Hybridization Analysis of PAT-Positive Callus Lines and Co-Transformation/Co-Expression Frequencies

Analysis for the Presence of bar

Sixty-seven of the 91 PAT-positive callus lines were analyzed by DNA hybridization for the presence of introduced genes; the other 24 callus lines, including 21 lines from one experiment that gave rise to only albino plants (Table I, section B, bombardment No. 1), were not subjected to further analysis. Undigested genomic DNA from the callus lines was hybridized with the *bar* probe and hybridization was seen only in the region of high mol wt DNA, providing evidence that *bar* integrated into genomic DNA, as exemplified in Figure 5A. Genomic DNA from callus bombarded with plasmid pAHC25 alone or in combination with pRsa1BYDVcp or pPAV110 was digested with *Bgl*II, which releases from pAHC25 a 1.7-kb fragment that contains *bar* and the first intron of ubiquitin. Genomic DNA of four callus lines from MDEs bombarded with pBARGUS and pRsa1BYDVcp was digested with *Hin*dIII, which releases a 2.1-kb fragment from pBARGUS that contains 440 bp of the 35S promoter, the *Adh1* intron, *bar*, and the *nos* 3′ end. Genomic DNA from all 67 PAT-positive callus lines was shown to contain *bar*-hybridizing sequences. The variation in banding pattern and copy numbers is exemplified in Figure 5B. Hybridization patterns varied from line to line. Sixty-three lines contained an expected 1.7- or 2.1-kb fragment, although most also contained extra hybridization fragments of varying sizes that could represent rearranged copies of the *bar* expression unit. Estimated copy numbers for the intact *bar* fragment ranged from 1 to >20. Four lines did not contain the expected 1.7- or 2.1-kb fragment but had one or a few predominant fragments of different sizes. Except for the callus lines that had only the 1.7- or 2.1-kb fragment, the hybridization patterns of all other lines were unique, consistent with the expectation that these lines were from independent transformation events. The hybridization patterns were not useful in deter-

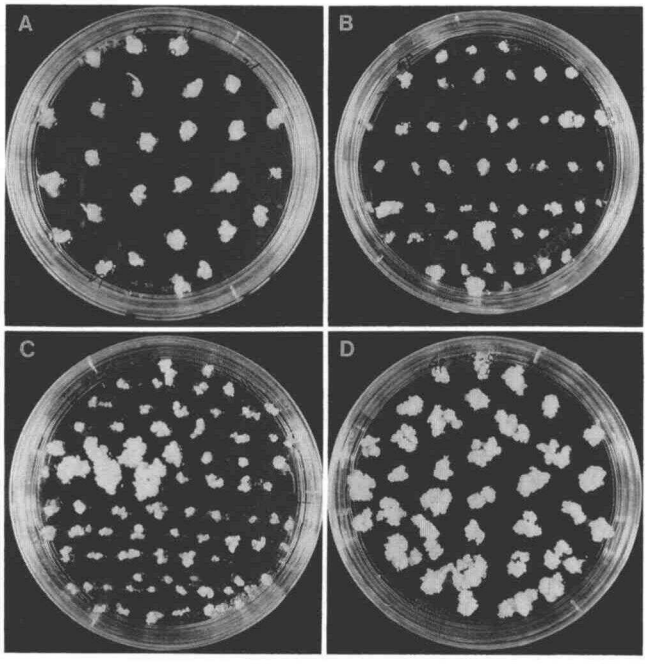

Figure 3. Selection of transformants from bombarded immature embryos. A, Immature embryo halves incubated on the first selection plate (11 d after bombardment). B, Callus from immature embryos on the second round of selection (11 d after transfer, 23 d after bombardment). Black lines demarcating the callus tissue from a single embryo can be seen on the plate. C, Callus tissue in the third round of selection (12 d after transfer, approximately 40 d after bombardment). A few pieces of callus in one line grew vigorously and appeared embryogenic. D, A bialaphos-resistant callus line on 5 mg/L bialaphos-containing medium (17 d after transfer, 2½ months after bombardment). Some callus tissue of this line was transferred to regeneration medium 2 months after bombardment.

mining whether a callus line arose from more than one independently transformed cell.

Co-Transformation and Co-Expression of a Linked, Nonselected Gene

Analysis of co-transformation of *uid*A was performed on 67 *bar*-transformed callus lines by reprobing the blots used for *bar* analysis with ^{32}P-labeled *uid*A-coding sequence. *Bgl*II excises a 4.1-kb *uid*A-containing fragment from pAHC25; *Hin*dIII releases a 7.1-kb *uid*A-containing fragment from pBARGUS. Of the 67 *bar*-hybridizing callus lines, 56 contained sequences hybridizing to the *uid*A probe as exemplified in Figure 5C, and this represents an 84% co-transformation frequency. Fifty PAT-positive callus lines were analyzed histochemically for expression of *uid*A, and 24 (48%) were GUS positive (Fig. 2, B and C). All callus lines that expressed GUS

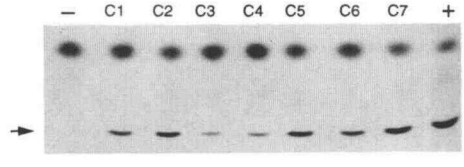

Figure 4. PAT activity in some bialaphos-resistant callus lines (C1–C7). The band corresponding to acetylated PPT is marked by an arrow; that band is absent in nonbombarded callus (–). A transgenic maize callus line (R. Williams and P.G. Lemaux, unpublished data) was included as a positive control (+).

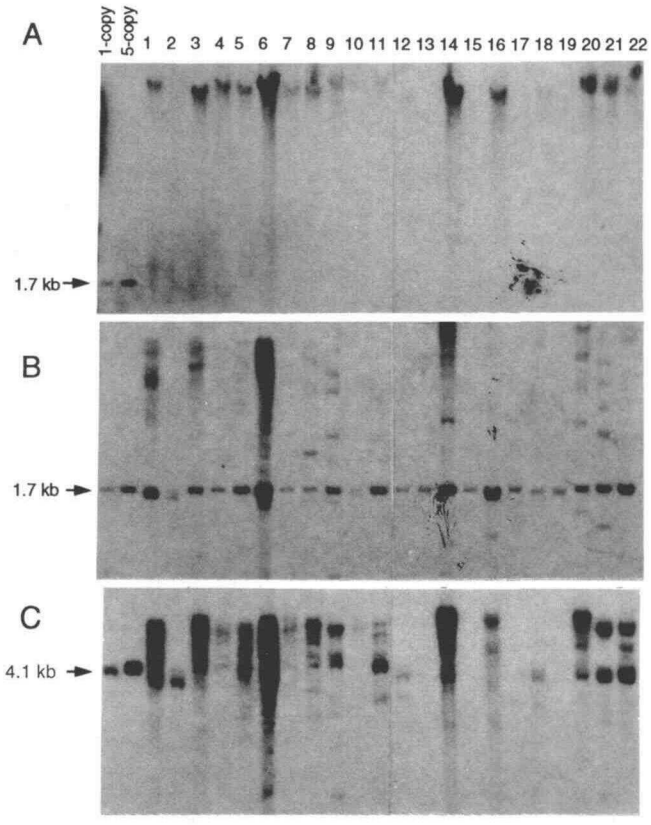

Figure 5. DNA hybridization analysis of independent PAT-positive callus lines. A, DNA gel blot of undigested genomic DNA (10 μg/lane) from 22 callus lines (lanes 1–22, all were transformed with pAHC25 and pRsa1BYDVcp) hybridized to ^{32}P-labeled *bar* probe. Plasmid DNA representing one and five copies of the introduced gene was mixed with undigested genomic DNA from nontransformed callus, digested with *Bgl*II, and included as copy number standards. The 1.7-kb band represents the intact *bar*-containing fragment and is marked by an arrow. Due to the difficulty of transferring undigested genomic DNA onto membranes, it is difficult to see the hybridization bands in some lanes. B, Genomic DNA (10 μg/lane) from the same 22 callus lines was digested with *Bgl*II and hybridized to ^{32}P-labeled *bar* probe. *Bgl*II-digested genomic DNA from nontransformed callus was included in each of the copy number standard lanes. C, Blot in B was stripped of the *bar* probe and hybridized with ^{32}P-labeled *uid*A probe. The intact unit is 4.1 kb and is marked by an arrow. Names of the callus lines in lanes 1 to 22 are as follows: 1, GP717B-33; 2, GP717B-37; 3, GP717B-59; 4, GP717B-118; 5, GP717B-121; 6, GP717B-150; 7 GP717B-158; 8, GP717B-189; 9,GP717B-197; 10, GP724B-3; 11, GP724B-4; 12, GP1016B-1; 13, GP1016B-2; 14, GP1016B-3; 15, GP1016B-4; 16, GP1016B-5; 17, GP1016B-6; 18, GP1016B-8; 19, GP1016B-12; 20, GP1016B-C1; 21, GP1016B-C2; 22, GP1016B-C4.

contained the intact 4.1-kb *Bgl*II or 7.1-kb *Hin*dIII fragment. Of the 17 lines that did not express GUS but contained hybridizing fragments, 8 had intact fragments and 9 had fragments of other sizes.

Co-Transformation of a Nonlinked Gene

Seventy-three PAT-positive callus lines were derived from tissues bombarded with two plasmids, pRsa1BYDVcp and either pAHC25 or pBARGUS (Table I). Digestion with *Bgl*II releases a 1.2-kb fragment from pRsa1BYDVcp that contains 250 bp of the *Adh1* intron 1, the BYDVcp gene, and the *nos* 3′ end. *Hin*dIII digestion releases a 1.9-kb fragment containing the entire BYDVcp gene expression unit. Of the 48 *bar*-transformed callus lines that were analyzed, 41 contained BYDVcp gene-hybridizing fragments (data not shown), an 85% co-transformation frequency. Analysis of the 14 callus lines bombarded with pAHC25 and pPAV110 (Table I, sections A and B) is still in progress. Expression of the BYDVcp gene in callus was not examined. Seed from T_0 and T_1 plants was sent to our collaborators (S. Wyatt and R. Lister) for examination of expression of the BYDVcp gene and the response of transgenic plants to BYDV infection.

Regeneration and Analysis of T_0 Plants

The results of regeneration attempts are summarized in Table I. Of the 91 PAT-positive callus lines, 36 lines gave rise to multiple green plants, as exemplified by Figure 6, A and B, and some of them also gave rise to albino plants. From 41 lines, including 18 lines from one callus bombardment plate (Table I, section B, bombardment No. 1), only albino plants were regenerated. No plants were obtained from 12 lines; 2 lines became contaminated before regeneration commenced. A range of 3 to 80 plants was obtained from each of these 36 lines. More than 90% of the plantlets survived transfer to soil in the greenhouse and more than 500 plants reached maturity (Fig. 6C). Most T_0 plants had normal morphology and were self-fertile (Fig. 6D), although seed set varied from a few seeds per plant to nearly full seed-set. The variation in seed-set appeared greater among plants from different lines than among plants from the same line. Plants from one of the two lines developed from MDEs of cv Igri set seed well and were likely diploid; plants from the second line were sterile and possibly haploid. A few plants from different lines were morphologically abnormal and unable to set seed, although they had normal siblings. Twenty-one T_0 plants from six different transgenic callus lines were sprayed with 0.5% Basta and none showed signs of necrosis (data not

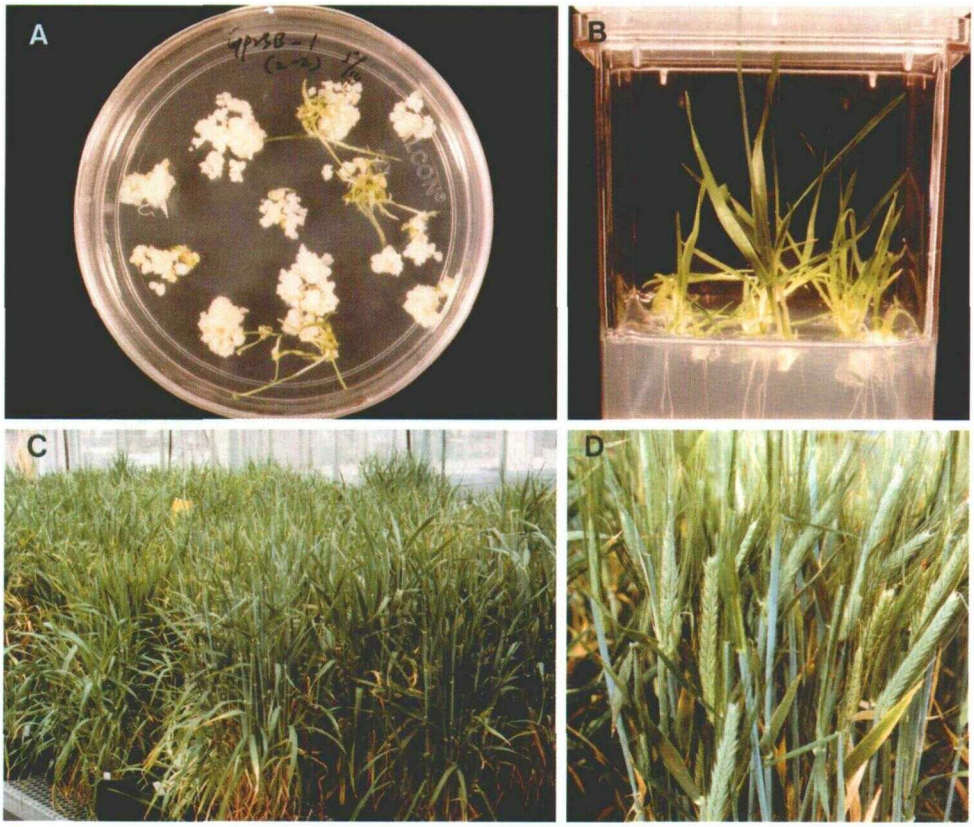

Figure 6. Regeneration and growth of T_0 plants. A, A regeneration plate with green plantlets. Green plants were regenerated from 36 PAT-positive callus lines in the presence of 1 mg/L bialaphos. B, Plantlets from A were transferred to boxes for further growth before being transferred to soil. More than 500 T_0 plants were grown to maturity in the greenhouse (C) and most had normal morphology and were self-fertile (D).

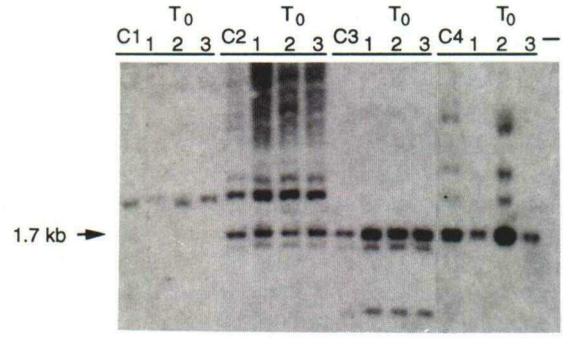

Figure 7. DNA hybridization analysis comparing callus and T_0 plants. The independently transformed callus lines (transformed with pAHC25 and pRsa1BYDVcp) are designated C1 to C4, representing callus lines GP724B-48 (C1), GP717B-189 (C2), GP717B-192 (C3), and GP724B-4 (C4). The three T_0 plants from each of the callus lines, indicated by an underline, are indicated by the numbers 1 through 3. Genomic DNA was digested with *Bgl*II and hybridized to a ^{32}P-labeled *bar* probe. The 1.7-kb intact *bar*-containing fragment is marked by an arrow. DNA from a nontransformed plant was included as a negative control (–).

shown). In contrast, plants regenerated from nontransformed callus were sensitive to concentrations of Basta as low as 0.1%.

Three T_0 plants from each of 25 transgenic callus lines were chosen randomly for DNA hybridization analysis, as exemplified in Figure 7. All T_0 plants analyzed contained *bar*-hybridizing sequences. The DNA hybridization patterns from nearly all of the T_0 plants were identical to that of the corresponding callus. The patterns of *bar*-hybridizing sequences of four T_0 plants in 3 lines, however, were different from the patterns of their parental callus lines (data not shown). The *bar* hybridization patterns of T_0 plants in 2 lines suggested that the corresponding callus lines developed from two independent transformation events that occurred in the same half-embryo or piece of callus (for example, callus line No. 4, Fig. 7). Almost all T_0 plants from callus lines that contained *uid*A and/or BYDVcp gene sequences also contained those sequences (data not shown); however, six T_0 plants in 4 lines had hybridization patterns for *uid*A different from their parental callus lines, and four plants in 2 lines had differences for the BYDVcp gene. For example, T_0 plants from one line did not have BYDVcp sequences, but the parental callus line did.

Germination Tests of T_1 IEs

Various numbers of T_1 IEs were isolated from 35 self-pollinated T_0 plants representing 18 transgenic lines and incubated on medium with bialaphos. On d 4, germinated seedlings [T_1(R)] were approximately 2 to 3 cm (Fig. 8) and were transferred to growth medium. Some embryos showed no signs of germination and were transferred to bialaphos-free medium, where some of them germinated into seedlings [T_1(S)].

The data on the germination tests are summarized in Table II. None of the 68 embryos isolated from plants grown from wild-type seeds or the 117 embryos from plants regenerated from nontransformed callus germinated in the presence of bialaphos. Embryos from 21 of 35 T_0 plants representing 10 transgenic callus lines segregated germinating and nongerminating embryos. The segregation ratios of the embryos from 9 plants representing 8 lines (Table II) were not significantly different from 3:1, a ratio indicating that there is only one expressed copy of *bar* or that all expressed copies of *bar* are at a single locus. Embryos from 14 T_0 plants representing 9 transgenic callus lines either did not germinate in the presence of bialaphos or only 1 of them germinated (GP717B-11–2 and GP717B-197–7). From 1 line (GP717B-14), embryos from 1 plant segregated germinating and nongerminating embryos, whereas none of the embryos from the other plant germinated.

Analysis of T_1 Plants

Four or more T_1(R) plants from every family (i.e. T_1 plants from one T_0 plant) analyzed in the germination test and a small number of T_1(S) plants from some families were grown to maturity in the greenhouse. Of the T_1 plants that reached maturity, whether they came from a T_0 plant with high or low frequency seed-set, all had nearly full seed-set and normal morphology. T_1(R) plants from 5 families (GP717B-4–13, GP717B-9–18, GP717B-33–1, GP717B-59–11, and GP724B-4–2; 5 or 6 plants per family) were analyzed by DNA hybridization and all had the intact 1.7-kb *bar*-containing fragment, as exemplified in Figure 9A. DNA hybridization with *uid*A and BYDVcp gene probes confirmed that these genes were also co-transmitted to the T_1 generation (data not shown). This confirmed the transmission of introduced genes to the T_1 generation. Some T_1(S) plants in 2 families were also examined along with their T_1(R) siblings by DNA hybridization. Two of the 5 T_1(S) plants in 1 family (GP717B-33–1, Fig. 9B) and all 4 T_1(S) plants in another family

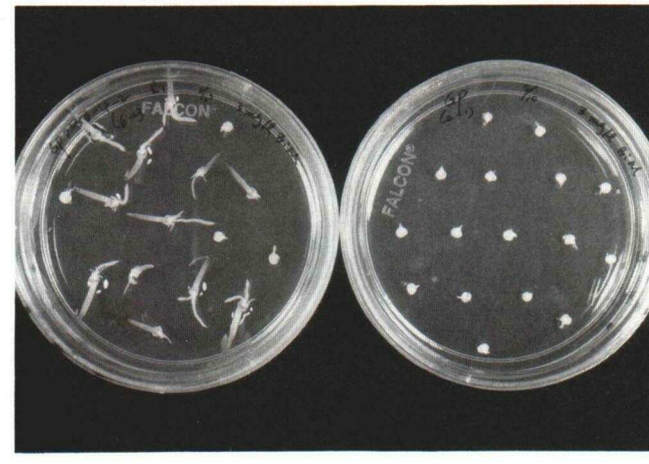

Figure 8. A germination test of T_1 immature embryos on FHG medium containing 3 mg/L bialaphos. The plate on the left contains T_1 embryos from a T_0 plant; the plate on the right contains embryos from a nontransformed R_0 plant.

Table II. *Summary of germination tests of T_1 immature embryos on FHG + 3 mg/L bialaphos*

T_0 Plant[a]	No. of T_1 Embryos		
	Total	Germinated	Nongerminated
CK1[b]	68	0	68
CK2[c]	117	0	117
GP717B-2-5	42	0	42
GP717B-4-9	47	29	18
GP717B-4-13[d]	24	16	8
GP717B-9-1	23	12	11
GP717B-9-18[d]	80	58	22
GP717B-11-1	13	0	13
GP717B-11-2	114	1	113
GP717B-14-5[d]	12	10	2
GP717B-14-8	28	0	28
GP717B-31-3	55	0	55
GP717B-32-16	37	0	37
GP717B-32-21	17	0	17
GP717B-33-1	83	51	32
GP717B-33-21[d]	25	16	9
GP717B-59-11[d]	49	36	13
GP717B-189-4	81	0	81
GP717B-192-1	31	0	31
GP717B-197-5	18	0	18
GP717B-197-6	11	0	11
GP717B-197-7	32	1	31
GP724B-4-1[d]	56	40	16
GP724B-4-2[d]	89	69	20
GP724B-47-1A	61	0	61
GP724B-47-2	42	0	42
GP724B-95-5	44	24	20
GP724B-96-5	32	10	22
GP1016B-C2-1	34	14	20
GP1016B-C2-2	54	29	25
GP1016B-C2-6[d]	26	16	10
GP1016B-C2-7	28	11	17
GP1016B-C2-8	52	28	24
GP1016B-C2-10	35	19	16
I529B-413-1	35	20	15
I529B-413-2	47	23	24
I529B-413-3[d]	27	17	10

[a] For example, in designating T_0 plants, such as GP717B-4-9 and GP717B-4-13, GP717B-4 refers to one of the transformed callus lines and 9 and 13 refer to T_0 plant numbers. [b] Plants grown from wild-type seeds. [c] Plants regenerated from nontransformed callus. [d] Analysis using the χ^2 test indicated that segregation ratios of T_1 IEs from these plants were not significantly different from 3:1 (at $a = 0.05$).

(GP717B-59–11) had *bar*-hybridization patterns identical to their T_1(R) siblings, even though they were sensitive in the germination test to bialaphos. Some T_1(R) and T_1(S) plants were grown in trays for Basta spraying. All of the T_1(R) plants were resistant to 0.5% Basta. None of the T_1(S) plants from 17 families tested was resistant (data not shown). Therefore, the inability of some IEs to germinate in the presence of bialaphos appeared to be predictive of the *bar* expression state in plants. There were three exceptions. All the T_1(S) plants in 2 families (I529B-413–2 and GP724B-95–5) and 7 of 10 T_1(S) plants in another family (GP717B-4–9) were as resistant as their T_1(R) siblings.

DISCUSSION

Although progress on barley transformation has been slower than in other cereal crops, we report here a means to generate large numbers of independently transformed barley callus lines and transgenic plants. Most of the plants were self-fertile and yielded seed. In the lines analyzed, DNA hybridization data proved that the introduced genes were stably transmitted to progeny. The *bar* gene was functional in T_0 and some T_1 plants, resulting in plants that were resistant to Basta. A gene that may contribute to resistance to BYDV was also successfully introduced into most transgenic lines and was passed to T_1 progeny.

Stable Transformation Using Various Tissue Explants

Selection of the target tissue for transformation of cereals is an important factor in achieving success. The primary

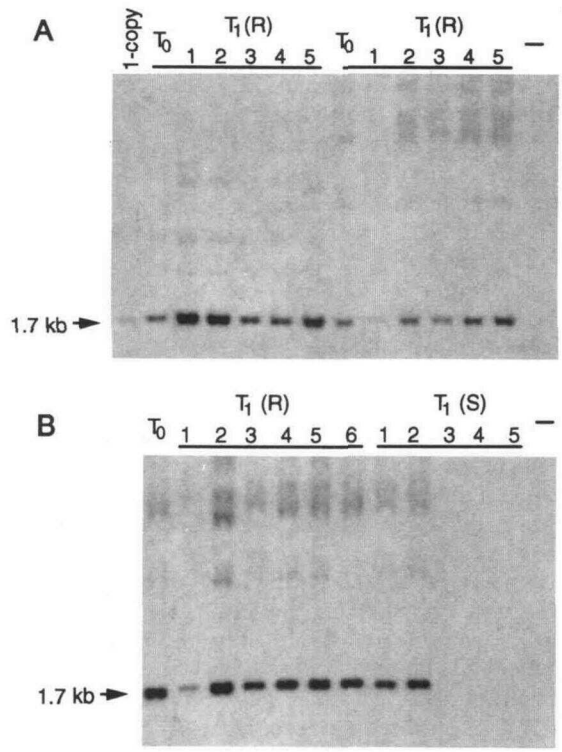

Figure 9. DNA hybridization analysis comparing T_0 and T_1 plants. A, Genomic DNA from T_0 and T_1(R) plants (transformed with pAHC25 and pRsa1BYDYcp) was digested with *Bgl*II and hybridized to a ^{32}P-labeled *bar* probe. Plasmid DNA representing one copy of the introduced gene was mixed with genomic DNA from a nontransformed plant, digested, and included as a copy number standard. DNA from a nontransformed plant was included as a negative control (–). Each set of one T_0 plant and its five T_1(R) plants is indicated by an underline. The T_0 plant in the left set is GP-724B-4–2; in the right set it is GP717B-33–1. The 1.7-kb intact *bar*-containing fragment is marked by an arrow. B, One T_0 plant (GP717B-33–1) and its six T_1(R) and five T_1(S) plants were analyzed by DNA hybridization with a *bar* probe.

requirement for an optimal target is that the tissues or cells receiving exogenous DNA are culturable in vitro, actively dividing, and capable of giving rise to fertile plants. In our study, we used successfully three different organized target tissues, IEs, young IE-derived callus, and MDEs, and obtained numerous, independently transformed, self-fertile plants from each tissue.

The main effort was focused on use of IEs. It was 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 were not able to track the precise origin of regenerable callus. Therefore, we bisected IEs and placed the half-embryos in three orientations to introduce DNA into cells on all surfaces as well as the interior. For some orientations the numbers of bombardment experiments is low; however, we did not see a dramatic difference based on the orientation of the embryo (Table I, section A). Based on this, either all surfaces of an embryo contain division-competent cells or, even when half-embryos were placed embryo-axis or cut-surface up during bombardment, sufficient numbers of scutellar epidermal cells were exposed and transformed, and gave rise to callus. No careful studies were done to determine the precise origin of the transformed cells. In later studies, we placed the embryos scutellum-side up for bombardment because it was technically easier.

The transformation frequency reported here, 7.9 independent lines per 100 IE halves, is high relative to those reported for cereals except rice (Li et al., 1993). It is likely that a combination of factors contributed to this success. One possible factor is the use of bisected embryos, which appear to form higher-quality callus than intact embryos (our unpublished observation). This improvement in callus response might be due to the partial inhibition of embryo germination, or possibly to the fact that wounding increases the ability of some cells in the IE to divide. A number of other factors also might have influenced the efficiency, including the use of bialaphos as the selective agent and the subdivision of growing callus into small pieces during selection, which results in the separation of putatively transformed from nontransformed tissue. It is unlikely that the use of the ubiquitin promoter to control *bar* had a significant impact, although this promoter has been shown to support transient expression levels significantly higher than the 35S promoter (Taylor et al., 1993). Published results in rice comparing 35S-driven *bar* with another strong promoter, rice *Act1*, indicated that both promoters produced sufficient levels of PAT to confer resistance on the transformants (Cao et al., 1992). Although limited in numbers, our transformation data with MDEs also failed to show a difference in numbers of transformants between experiments using the 35S and ubiquitin promoters to drive *bar*.

Young embryogenic callus tissue was also a good target material. We obtained the highest number of independently transformed callus lines from a single bombardment with callus (Table I, section B, bombardment No. 1), although all the plants regenerated in this experiment were albino (for discussion, see "Albinism" below). In a second, similar experiment we obtained six independent lines, three of which yielded green plants. Although we used only IE-derived callus in this study, callus tissue developed from other explants might also be useful, provided it is embryogenic and capable of regenerating green plants. Use of young callus as a target tissue opens up the possibility of successfully transforming barley cultivars in which the number of IEs yielding embryogenic callus is low.

IEs are a common explant for tissue culture in barley. Callus response and the production of regenerable tissue from this explant is observed with many common cultivars (Goldstein and Kronstad, 1986; Lührs and Lörz, 1987; Bregitzer, 1991). Almost every IE of cv Golden Promise used in this study formed embryogenic callus. We have also tested one winter type (cv Igri) and four other spring varieties (cvs Klages, Moravian III, Morex, and Sabarlis) and found that all form callus at high frequencies from immature embryos and that the callus could regenerate to yield green plants (data not shown). Given this, we believe that it is likely that the procedures described in this paper, using IEs and young callus from IEs, form the basis for genotype-independent transformation methodologies for barley.

The use of MDEs or microspores as target tissue has the advantage of potentially being able to generate homozygous transgenic plants within one generation. In this study the use of MDEs as target tissue, although not as efficient in generating transformed callus lines as IEs and callus, is to our knowledge the first report of success using this explant as a transformation target in cereals. We achieved an average success rate of 3.0 independently transformed lines per 1000 MDEs (Table I, section C). This frequency is lower than that seen with IEs, 7.9 independent lines per 100 IEs (Table I, section A), and this may be due in part to the diversity of the MDEs used for bombardment. If MDEs were selected for a certain stage or size, a higher transformation frequency might be achieved. We also carried out preliminary transformation studies with microspores of cv Igri and observed dividing GUS-positive microspores a few days after bombardment (our unpublished data). Although some bialaphos-resistant MDEs were obtained and developed into callus, they were PAT negative. Because other approaches were successful, no further studies were performed using microspores; however, we believe that, using the procedures outlined in this paper, transformation using microspores should be possible.

Co-Transformation/Co-Expression

In most of our experiments, target materials were bombarded with two different plasmids, allowing us to assess co-transformation of both linked and unlinked genes. The 84% co-transformation frequency with linked genes obtained in this study is lower than the 100% frequency reported in previous studies (Klein et al., 1989; Vasil et al., 1992). Our co-transformation frequency for unlinked genes (85%) is higher than those reported for stable nonembryogenic Black Mexican Sweet maize transformants (63%, Spencer et al., 1990) and for stable embryogenic maize lines (77%, Gordon-Kamm et al., 1990). If significant, the higher co-transformation frequency in our study might have resulted from the use of a 1:2 molar ratio of selected:nonselected genes. The high frequency of co-transformation of unlinked genes forms the

basis for an efficient means to introduce unlinked agronomically important genes into barley.

The 46% co-expression frequency of the linked genes (*bar* and *uid*A) in callus is lower than the 84% co-transformation frequency, suggesting that *uid*A in some *bar*-transformed callus lines was not expressed or was expressed at a level too low to be detected histochemically. The co-expression frequency is also lower than the 75% frequency previously reported in transformed oat calli (Somers et al., 1992).

Albinism

A total of 91 transgenic callus lines were generated in this study; however, 41 of them yielded only albino plants (Table I). Albinism is a common problem in barley tissue culture (Kott and Kasha, 1984; Kasha et al., 1990; Jähne et al., 1991). It is known that genetic factors (Foroughi-Wehr et al., 1982) and culture conditions (Ziauddin and Kasha, 1990; Kao et al., 1991) influence albinism. Therefore, it is possible that selection conditions, such as the addition of bialaphos in the medium and the ammonia released by nontransformed cells during selection (Tachibana et al., 1986), exacerbated the problem of albinism. Albinism was particularly acute and unexpected in one experiment (Table I, section B, bombardment No. 1), where all regenerable lines yielded albino plants. Repeating this experiment, we observed that half of the lines yielded green plants. This indicates that the generation or use of callus itself or the slightly older age of the selected callus lines was not the cause of albinism. The state of the donor plant or some unknown factors during that experiment might have been responsible.

Our results show that more PAT-positive callus lines from IEs (55%) were able to regenerate green plants than lines from the MDEs (25%). Unlike the IEs, MDEs were cultured in vitro prior to transformation. The longer exposure of MDEs to a variety of culturing conditions before bombardment, including submersion in liquid medium, might have affected their ability to regenerate green plants. The IEs and MDEs were derived from different cultivars, however, so the influence of genotype on albinism must also be considered.

Germination Test

The germination test, designed to speed up analysis of progeny and determine the segregation ratios of *bar*, yielded unexpected results. Although embryos from 21 of the 35 T_0 plants segregated as germinating and nongerminating, in only 9 plants were the segregation ratios close to 3:1 (Table II). In many cases no embryos germinated in the presence of the selective agent, despite the fact that their parental T_0 plant was regenerated in the presence of bialaphos and contained *bar*-hybridizing sequences. Occasionally, individual T_0 plants from the same line yielded progeny that reacted differently (e.g. most embryos from GP717B-14-5 germinated, whereas none of the embryos from a sibling plant, GP717B-14-8, did).

We analyzed some T_1(S) plants to determine whether the inability to germinate on bialaphos was indicative of the absence of *bar*. In some plants, *bar* was present (Fig. 9B), implying that, even though *bar* was expressed in callus and T_0 plants, in some cases the gene was not expressed sufficiently in the immature embryos to permit germination on bialaphos. In most cases the inability of *bar*-containing T_1 IEs to germinate was reflective of the *bar* expression state in plants; however, in three families *bar* expression was activated after embryo development in some or all of the T_1(S) plants, since the plants were not affected by Basta spraying.

Taken together, the data indicate that *bar* expression in transgenic progeny may vary from plant to plant. Expression also might depend on developmental state, since some *bar*-containing embryos were not able to germinate in the presence of bialaphos, yet they gave rise to plants resistant to Basta. These differences in expression might relate directly to the ubiquitin promoter and/or they might be influenced by positional effects. Based on DNA hybridization analysis of T_1(R) and T_1(S) plants, it seems that the germination test is useful in selecting individuals that express *bar* actively; however, it is not a good indicator of the presence or absence of *bar*. It is of interest to determine whether the expression of the selective gene is an indicator of the expression of other linked genes, thereby making the germination test useful in identifying transformants likely to reproducibly express linked genes of interest.

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