Theor Appl Genet (1994) 89: 525–533

A. Jähne · D. Becker · R. Brettschneider · H. Lörz

Regeneration of transgenic, microspore-derived, fertile barley

Received: 27 December 1993 / Accepted: 17 February 1994

Abstract We have developed a system for the biolistic transformation of barley using freshly-isolated microspores as the target tissue. Independent transformation events led, on average, to the recovery of one plant per 1×10^7 bombarded microspores. Putative transformants have been regenerated using phosphinothricin as a selective agent. R_0 plants have been transferred to soil approximately 2 months after bombardment. Integration of the marker genes *bar* and *uid*A has been confirmed by Southern analysis. The marker genes are inherited in all progeny plants confirming the expected homozygous nature of the R_0 plants.

Key words Barley microspore culture
Particle bombardment · Fertile transgenic barley plants

Introduction

Cereals have proven to be recalcitrant to genetic engineering since they do not belong to the natural host range of *Agrobacterium*. Several transformation techniques have been developed but to-date only three methods have been found suitable for obtaining transgenic cereals: transformation of totipotent protoplasts (for review see Vasil and Vasil 1992), particle bombardment of regenerable tissue cultures (for review see Sanford 1990) and, more recently, tissue electroporation (D'Halluin et al. 1993). These techniques depend on the availability of efficient and competent tissue-culture systems.

In barley considerable progress has been made in the improvement of tissue-culture systems. It is now reproducibly possible to establish embryogenic suspensions from which fertile plants can be regenerated (Jähne et al. 1991 a; Mordhorst and Lörz 1992; Huang et al. 1993). The regeneration of protoplast-derived plants has also been reported (Jähne et al. 1991 b; Funatsuki et al. 1992; Golds et al. 1994). Although the stable transformation of barley protoplasts is a successful and routinely-used method (Lazzeri et al. 1991) it has not been possible until now to combine both techniques in order to obtain stably-transformed fertile barley plants.

The application of biolistic methods appears to be more promising as they have proven suitable for the stable transformation and regeneration of several cereals such as maize (Gordon-Kamm et al. 1990), rice (Cao et al. 1992), wheat (Vasil et al. 1992), oat (Somers et al. 1992), sugarcane (Bower and Birch 1992) and barley (Wan and Lemaux 1994). For these experiments, regenerable suspension or callus cultures have been used as target tissues. The suitability of these tissues is limited because the regeneration capacity of cells gradually declines during maintenance, and the risk of somaclonal variation increases. Albinism, phenotypic abnormalities, or reduced fertility have been reported for transgenic maize (Gordon-Kamm et al. 1990), wheat (Vasil et al. 1992), oat (Somers et al. 1992) and barley (Wan and Lemaux 1994).

Therefore, it is desirable to deliver the DNA directly into primary explants having a high regeneration capacity. In cereals, scutellar tissues of rice (Christou et al. 1991), maize (Koziel et al. 1993) and wheat (Weeks et al. 1993; Becker et al. 1994) have been shown to be suitable for this purpose, but immature inflorescences of tritordeum (Barcelo et al. 1994) have also been used successfully to obtain stably-transformed plants.

In barley the most reliable and efficient regeneration system is based on the culture of microspores. For plant-breeding purposes the cultivation of whole anthers is well established for several genotypes and high numbers of regenerants can be obtained (Kuhlmann and Foroughi-Wehr 1989). However, the cultivation of isolated microspores in liquid medium has been found to be superior to anther culture since the regeneration frequency could be significantly

Communicated by G.Wenzel

A. Jähne[1] (✉) · D. Becker · R. Brettschneider · H. Lörz
Universität Hamburg, Institut für Allgemeine Botanik,
AMP II, Ohnhorststrasse 18, D-22609 Hamburg, Germany

[1] Resistenzlabor der Saaten-Union, Hovedisser Strasse 92, 33818 Leopoldshöhe, Germany

increased (Olsen 1991; Ziauddin et al. 1992; Hoekstra et al. 1993; Mordhorst and Lörz 1993). Isolated microspores are very attractive for the application of transformation techniques because they are unicellular and can easily be regenerated to homozygous, dihaploid plants, which is significant for breeding purposes. Therefore several attempts have been made to transfer DNA directly to barley microspores.

Although microspores are surrounded by the intine and exine cell walls they have been used as target cells for direct DNA uptake mediated either by PEG or by electric pulses. In this way, it has been possible to demonstrate transient expression of a reporter gene in barley (Kuhlmann et al. 1991) and maize (Fennell and Hauptmann 1992) but the regeneration of transformed plants could not be achieved.

Barley microspores have also been tested for their suitability for transformation by microinjection but this method does not seem to be very promising (Bolik and Koop 1991, Olsen 1991).

In the present study we report the reproducible transformation of barley by particle bombardment using microspores as target cells.

Materials and methods

Plant material

Experiments were carried out using the winter-type barley cv Igri. Seeds were germinated in a peat/soil mix in a growth chamber (14/12 °C day/night, 16 h photperiod, 10000–16000 lx). After 2 weeks, seedlings were vernalized for a period of 8 weeks (2 °C, 9 h light, 4000 lx). Fertilizer (0.7‰ Wuxal, N: P: K approximately 12:4:6) was applied 8 weeks after vernalization and the treatment was carried out with each watering. One to two weeks before the first spikes were harvested the plants were transferred to a controlled greenhouse (18/14 °C day/night, 16 h light, at least 18000–20000 lx) and fertilizing was increased to 1‰ Wuxal.

Culture media

The medium for isolation and culture of microspores (MS) was a modified MS medium which had a total nitrogen content of 20 mM, as described by Mordhorst and Lörz (1993). For selection of microspore cultures, the glutamine was omitted from the medium while the other components remained unchanged. Basta was used as a selective agent in concentrations of 3–5 mg/l of phosphinothricin (PPT). The regeneration medium was similar to the selection medium but the amount of maltose was reduced to 30 g/l and the 6-Benzylaminopurine (6-BAP) was omitted while the concentration of Basta ranged between 3 and 10 mg/l of PPT.

All media were adjusted to pH 5.6 with KOH and filter sterilized. For solid media, the solutions were made double-concentrated, and mixed with an equal volume of a double-concentrated, autoclaved gelling-agent solution. The following gelling agents were tested in different concentrations:
Type I-A-Agarose (Sigma): 1.0%, 0.8%, 0.6%, 0.4%.
SeaPlaque Agarose (FMC): 2.0%, 1.5%, 1.0%.
GelRite (Roth): 0.2%, 0.4%.

Isolation of microspores

The isolation of microspores was performed according to the protocol of Mordhorst and Lörz (1993). The viability of microspores was determined by staining with fluorescein diacetate (Widholm 1972).

Culture of microspores

Several culture systems were tested in order to find the appropriate plating method suitable for particle bombardment. The freshly-isolated microspores were either plated on filters or on solid medium. When the first method was used, about 5×10^5 viable microspores were plated on a filter (Schleicher and Schüll, 0.2 µm pore size) and after 1 h 1 ml of liquid medium was added. For culture on solid medium, the microspores were plated either in droplets or spread on the surface of the induction medium. Cultures were left for about 1 h on the clean bench prior to bombardment. For further culture, microspores were incubated in the dark at 26 °C.

Selection of microspore cultures

Two to three weeks after bombardment, cultures which contained visible microcalli (50–80 µm diameter) were transferred to selection medium. The microcalli of each plate were divided and transferred to media containing 3, 4 and 5 mg/l of PPT. Some microcalli were transferred to a non-selective medium without glutamine in order to determine the regeneration frequency of the microspores. Cultures were incubated for a further week in the dark at 26 °C before they were transferred to a room with 16 h light at 24 °C. After about 1–2 weeks in the light, regenerating plantlets were selected and cultured on regeneration media containing 3–10 mg/l of Basta. Rooted plantlets were transferred to soil, vernalized (for conditions see above), and grown to maturity in the greenhouse.

Plasmid construct

For particle bombardment, the plasmid pDB1, which has been described previously (Becker et al. 1994), was used. This plasmid contains the *uid*A gene under the control of the actin-1 promoter, and the *bar* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1).

DNA precipitation and microprojectile bombardment

Plasmid DNA was adsorbed to gold particles of an average size between 0.4 to 1.2 µm (Heraeus, Karlsruhe, Germany) as described previously (Becker et al. 1994). The particles were resuspended in either 60, 120 or 240 µl of ethanol. For each bombardment, 3.5 µl of the suspended DNA-coated particles were spread onto the surface of the macrocarrier. The particle gun employed in these experiments was a PDS 1000/He gun (BioRad, München, Germany).

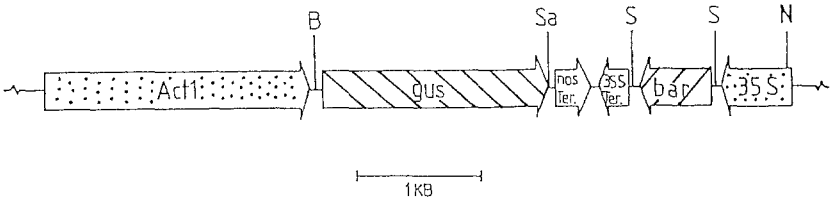

Fig. 1 Schematic representation of plasmid pDB1 used in the transformation experiments

Histochemical and fluorometrical GUS assays

GUS activity was determined either histochemically, as described by McCabe et al. (1988), or fluorometrically, according to the protocol of Jefferson (1987). Transient GUS activity in bombarded microspores was analysed histochemically by incubating microspores for 24 h at 37 °C in staining buffer 2 days after bombardment. The GUS activity of leaves was determined histochemically and fluorometrically. Chlorophyll of histochemically-stained leaves was extracted by incubation in absolute ethanol for 2 h at 65°C.

Herbicide application

All selected regenerants, as well as all R_1 plants, were sprayed with an aqueous solution of BASTA containing 150 mg/l of PPT and 0.1% Tween 20.

DNA isolation and Southern-blot analysis (Southern 1975)

As described by Dellaporta et al. (1983), total genomic DNA was isolated from leaf tissue of selected regenerants and their progeny.

Twenty micrograms of genomic DNA, uncut, digested with *Sal*I or with *Bam*HI/*Sac*I (to cut out the *bar* and *uid*A gene) and with *Nco*I (to determine the number of integration sites per genome), was separated by electrophoresis in 0.8% agarose (25 V overnight). DNA was blotted to Hybond N membranes according to a protocol described by Amersham, England. The detection of introduced DNA was performed using a modified protocol of the non-radioactive digoxigenin chemiluminescent method of Neuhaus-Url and Neuhaus (1993). The modifications are described by Becker et al. (1994). Hybridization signals were visualized on X-Omat AR film (Kodak, Rochester, USA).

PCR-labelling of probe DNA

Probe DNA was labelled as described previously (Becker et al. 1994).

Results

Culture of microspores and regeneration

For using the particle gun it is necessary to plate the microspores on a dry surface. Therefore, different plating methods have been tested. One possibility is to plate the microspores on filters. In experiments where liquid induction medium has been added after 1 h, to reach a final density of 5×10^5 microspores/ml, no further development of the microspores was observed. A more convenient method is culture on solid medium. On agarose-solidified media, the frequency of initial divisions was very low and the development of aggregates occured only rarely, whereas microspores from the same preparation cultured in liquid medium regenerated to plants with high frequencies. Therefore, different agents and varying concentrations for the solidification of media have been tested but only on induction media containing 0.2% GelRite did the microspores divide reproducibly and develop to embryos (Fig. 2 A). The microspores have been plated in droplets because the plating density had to be very high to trigger microspore development (Fig. 2 B). Plating efficiencies of up to 3% could be achieved when 2×10^6 microspores were spread on induction medium (3-cm diameter) whereas no divisions occurred when less than 5×10^5 microspores have been cultured. However, for optimization of particle-bombardment conditions it is useful that as many dishes as possible are available. Therefore, the droplet culture was used although it was not possible to determine plating efficiencies. Densities of $2–5\times10^5$ microspores/droplet led to optimal results. The regeneration frequency displayed a very high variability, strongly depending on the growth conditions of donor plants. In eight independent experiments regeneration frequencies between 18 and 153 plants per 1×10^5 viable microspores were obtained (Table 1). On average 82 green plants per 1×10^5 microspores were regenerated.

Table 1 Regeneration frequency of microspores (MS) cultured on solid induction medium (eight independent experiments)

Exp. no.	Total no. of cultured MS	Green plants/ 1×10^5 MS	Albino plants/ 1×10^5 MS
1	3.3×10^5	121	4.2
2	2.1×10^5	153	2.3
3	3.5×10^5	18	0.8
4	3.8×10^5	56	0.8
5	3.8×10^5	49	0.8
6	3.3×10^5	114	5.1
7	2.8×10^5	103	0
8	2.2×10^5	87	0.5

Table 2 Successfully-used parameters for particle bombardment of barley microspores

Parameter	Optimum
Distance between	
A: rupture disk and macrocarrier	2.5 cm
B: macrocarrier and stopping screen	0.8 cm
C: stopping screen and sample	5.5 cm
Gas pressure	1 100–1 800 PSI
Partial vacuum	27 inch Hg
Particles	Gold (size 0.4–1.2 µm)
Particle amount	58 µg

Parameters for particle bombardment

Optimization of bombardment conditions for barley microspores has been based on the analysis of transient expression of the *uid*A gene. Microspores have been bombarded with the plasmid pDB1 (Fig. 1) carrying both the *uid*A and the *bar* marker genes. The highest numbers of *uid*A-expressing microspores was achieved when the parameters shown in Table 2 were used for particle bombardment. An average of 1% of the bombarded microspores expressed the *uid*A gene in the histochemical assay (Fig. 3 A). The number of blue microspores was not significantly influenced by the helium gas pressure. Within a range of 1 100–1 800 PSI high expression levels have been found. However, lower (450–900 PSI) or higher (2 000–2 200 PSI) pressures resulted in a reduced number of transient GUS

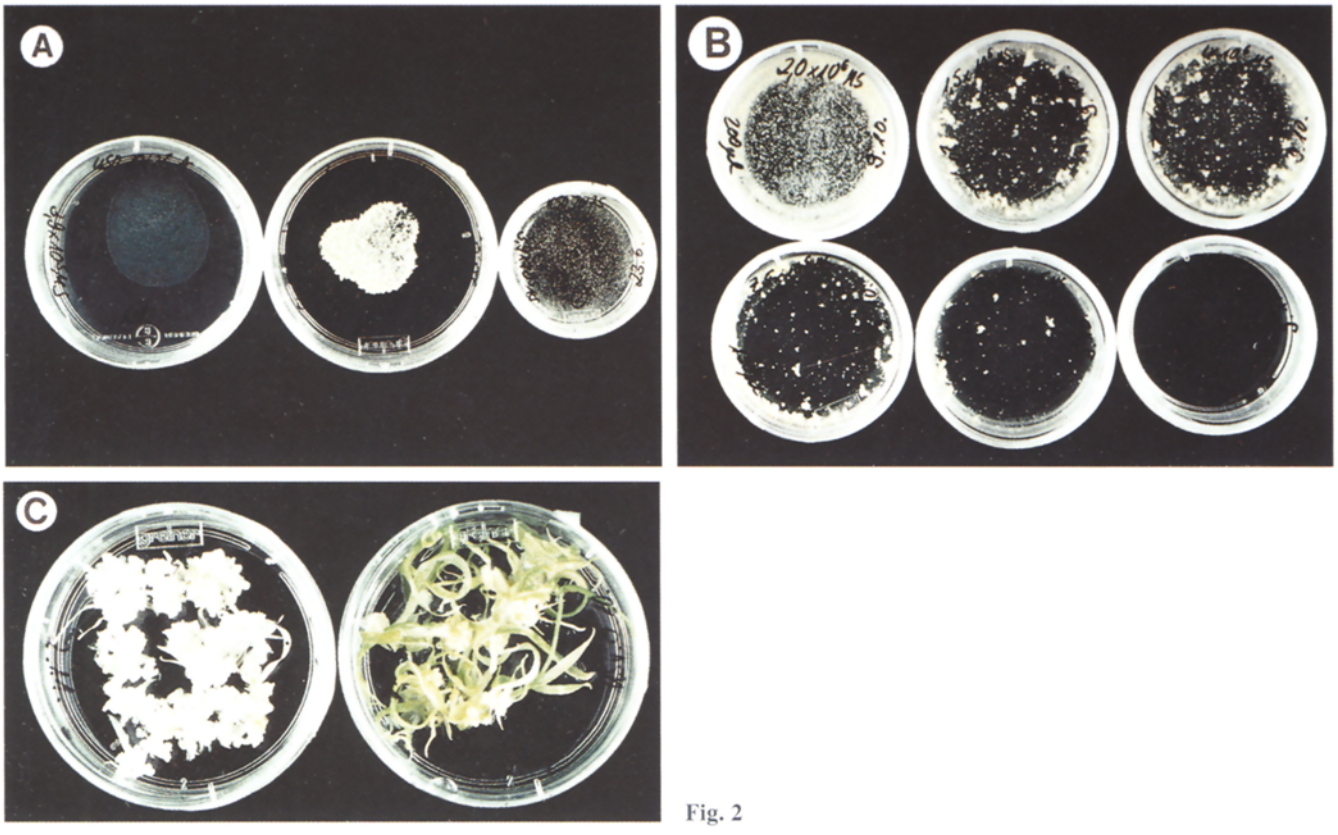

Fig. 2

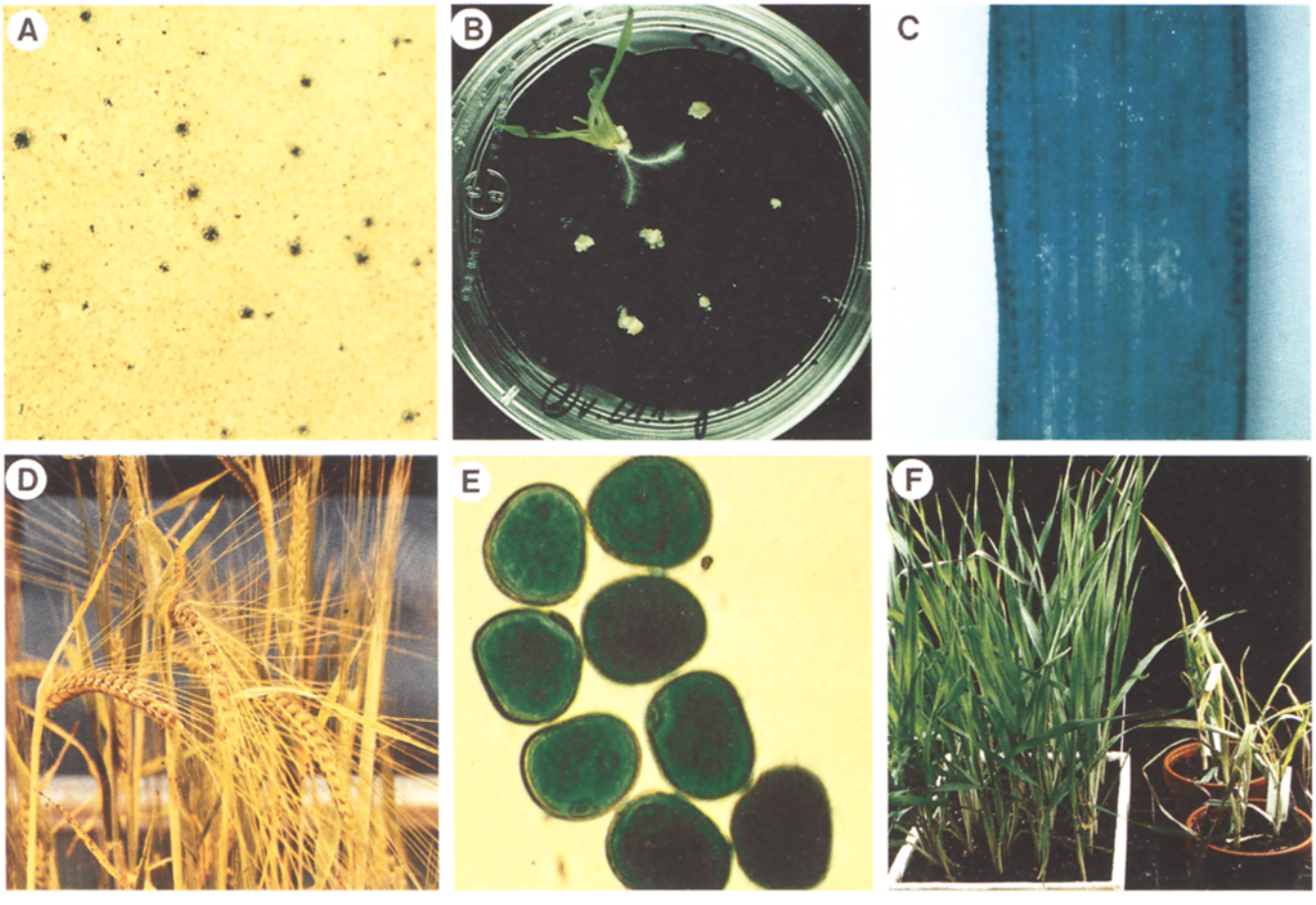

Fig. 3

signals. In the case of higher gas pressures the viability of microspores, which was determined by staining with FDA, decreased. Gas pressures lower than 2000 PSI had no detectable influence on microspore viability. Three different amounts of particles were tested (116, 58 and 29 µg gold per bombardment). With increasing amounts of particles a higher number of transient GUS signals was achieved. No influence on microspore viability was observed using 58 and 29 µg of gold per bombardment. An amount of 116 µg of gold led to a slight decrease of viability. For stable transformation experiments 58 µg gold per bombardment was chosen.

Table 3 Summary of nine transformation experiments

Exp. no.	Total no. of bombarded microspores	Gas pressure	Selection pressure	Total no. of selected plants	Activity	
					GUS	PAT
1	2.2×10^6	1550 PSI	5 mg/l PPT	1	+	+
2	4.2×10^6	1550 PSI	3 mg/l PPT	2	–	+
3	5.1×10^6	1800 PSI	4 mg/l PPT	1	–	+
4	5.5×10^6	1300 PSI	4 mg/l PPT	1	+	+
5	4.6×10^6	1800 PSI	4 mg/l PPT	1	+	+
6	1.5×10^6	1550 PSI	4 mg/l PPT	1	–	+
7	4.1×10^6	1550 PSI	4 mg/l PPT	3	–	+
8	3.8×10^6	1300 PSI	5 mg/l PPT	1	+	+
9	2.6×10^6	1100 PSI	3 mg/l PPT	1	–	+

Selection of transformants

Barley microspores plated on solid medium containing 420 mg/l of glutamine have been bombarded with gold particles coated with the plasmid pDB1 1–2 h after isolation. Bombarded microspores have been cultured for 2 weeks in the dark at 26°C. During this time proembryogenic structures developed which were then transferred for 2 additional weeks to a glutamine-free induction medium containing 3–5 mg/l of PPT. A concentration of 3 mg/l of PPT was sufficient to completely inhibit the development of non-bombarded control calli.

In order to evaluate the effect of glutamine-free induction medium, calli have also been transferred to non-selective media where the glutamine was added or omitted, respectively. Surprisingly, the addition of glutamine to the induction medium resulted in a negative effect on the regeneration capacity of the microspore cultures. In ten independent experiments the plant regeneration capacity was significantly higher on glutamine-free induction medium (Fig. 2 C).

After 1 week of culture on selection medium in the dark, the plates were transferred to light. Regenerating plantlets were then transferred to hormone-free regeneration medium (Fig. 3 B). Plantlets showing GUS activity in their leaves (Fig. 3 C) have been cultured on regeneration medium containing the same concentration of PPT as during callus induction. For plantlets which were negative in the histochemical GUS assay the concentration was increased to 8–10 mg/l of PPT. Seven to eight weeks after isolation and bombardment of the microspores putative transformants were transferred to soil. In nine independent experiments 12 plants were selected. However, from 19 bombardments it was not possible to select any plant. From each experiment, bombarded microspores have been plated on non-selective, glutamine-free induction medium in order to test the regeneration frequency. The selection of putative transformants has always been correlated with high regeneration frequencies of the microspores. In all experiments resulting in no plant regeneration at all the regeneration frequency of the cultures was extremely low. Table 3 summarizes the results obtained in nine successful transformation experiments. In total, these experiments and the non-successful ones produced, on average, one transformed plant per 1×10^7 microspores. However, when only the successful experiments are taken into account an average of one transgenic plant per 2.8×10^6 microspores was regenerated.

Analysis of R_0-plants

All selected regenerants have been tested for GUS activity by staining leaf pieces histochemically. Only four of the regenerants have proven to be GUS-positive. In GUS negative plants no activity was detectable using the fluorometrical assay, either.

About 1 month after vernalization regenerants have been screened for phosphinothricinacetyltransferase (PAT) activity by spraying whole plants with an aqueous solution of the herbicide BASTA. One week later plants were scored and none of the 12 regenerants showed any necrosis, indicating the functional activity of PAT.

Five transformed R_0 plants have been analysed by Southern-blot hybridisation for the presence of the introduced genes. No hybridisation signals could be detected in non-transformed control plants to the *uid*A or *bar* coding regions. Genomic DNA was digested either with *Sal*I, which excised the 550-bp *bar* fragment, or with

Fig. 2A–C Barley microspore culture. **A** Influence of gelling agent on culture response of microspores from the same isolation. Left: induction medium solidified with 0.8% Type I-A Agarose (Sigma); middle: induction medium solidified with 0.2% GelRite; right: liquid induction medium. **B** Influence of plating density on culture response of microspores from the same isolation. Decreasing densities from 2×10^6 microspores (upper row, left) to 3×10^5 (lower row, right) per dish (3 cm diameter). **C** Influence of glutamine on regeneration frequency of microspore-derived aggregates. Left: medium with 420 mg/l of glutamine; right: medium without glutamine

Fig. 3A–F Stages in the production of transgenic barley plants. **A** Transient GUS activity in microspores 48 h after bombardment. **B** Regeneration of a barley plant under selection conditions using 5 mg/l of PPT. **C** GUS activity in leaf tissue of transgenic barley. **D** Fertile transgenic barley plant. **E** GUS activity in pollen grains of a homozygous R_0 plant. **F** BASTA-resistant progeny of transgenic barley plants. Left: resistant transgenic progeny; right: sensitive control plants 1 week after herbicide application

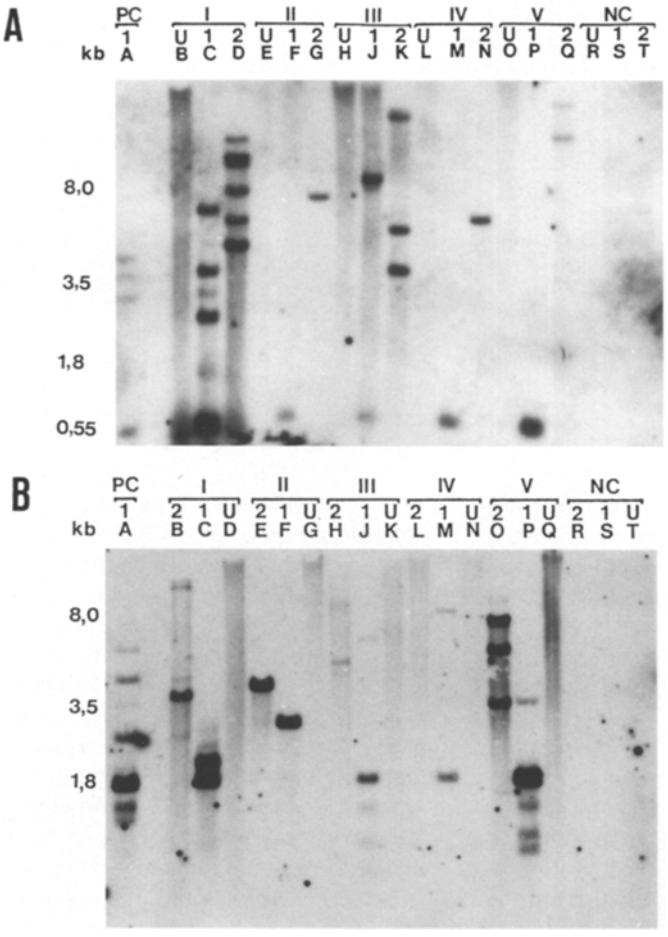

Fig. 4A, B Integration of the *bar* (Panel **A**) and *uid*A (Panel **B**) genes in the genome of five R_0 plants analysed by Southern blots. All plants are PAT positive. Plants I and V are GUS positive, plants II, III and IV show no detectable GUS activity. *Lanes* labelled "*U*" show undigested genomic DNA (20 µg/lane); *lanes 1* show DNA digested either with *Sal*I (Panel **A**) or with *Bam*HI/*Sac*I (Panel **B**) to cut out the introduced gene; *lanes 2* show DNA digested with *Nco*I to determine the number of integration sites per genome. *NC*, negative barley control; *PC*, positive wheat control. Hybridization was performed using a DIG-11-dUTP-labelled *bar* (Panel **A**) or *uid*A (Panel **B**) probe

*Bam*HI/*Sac*I, to release the 1.8-kb *uid*A fragment. Genomic DNA digested with *Sal*I hybridized to DIG-labelled *bar* fragments appears to contain intact copies of the *bar* gene (Fig. 4, Panel A, lanes C, F, J, M, P). The number of integration sites or copies per genome, as determined by *Nco*I digests, ranges from one to five events (Fig. 4, Panel A, lanes D, G, K, N, Q).

Only four of the analysed plants contain intact *uid*A fragments (Fig. 4, Panel B, lanes C, F, J, M, P). With plant II a hybridisation signal of about 2.8 kb has been detected. This plant does not exhibit GUS activity as assayed by either the histochemical or the fluorometric techniques. Although plants III and IV contain intact copies of the *uid*A gene, neither of them displays enzyme activity. The number of integration sites per genome varies from one to five copies (Fig. 4, Panel B, lanes B, E, H, L, O). Integration of additional rearranged copies into the genome was evident for both genes.

Hybridisation of undigested genomic DNA of each tested plant resulted in signals in the high-molecular-weight range indicating the integration of the two marker genes into the genomic DNA (Fig. 4, Panel A, lanes B, E, H, L, O and Panel B, lanes D, G, K, Q). However, only weak signals have been observed for the *bar* gene.

Phenotype of R_0 plants

All transformed plants showed normal morphology and were self-fertile (Fig. 3 D). Most plants displayed a full seed set and only two plants showed a reduction by 50%.

Microspores of all transformants have been stained histochemically for the presence of GUS activity. All pollen grains of plants without any detectable GUS activity in leaves were completely GUS negative. GUS-positive R_0 plants produced pollen grains which expressed the *uid*A gene exclusively indicating the homozygous state of the transformants (Fig. 3 E).

Analysis of progeny

In order to analyse R_1 plants as early as possible immature embryos (approximately 1.5–3 mm) of two plants have been dissected from young caryopses and cultured on hormone-free regeneration medium containing 3 mg/l of PPT. Two-hundred-and-ten embryos derived from plant V (PAT+/GUS+) and 189 embryos dissected from plant III (PAT+/GUS–) have been germinated and transferred to soil. All R_1 plants were phenotypically normal.

The activity of the *uid*A gene has been assayed histochemically by staining leaf pieces. For one plant varying staining intensities were detected. From 210 analysed progeny plants only three showed no histochemically-detectable activity, leaf pieces of six plants gave weak GUS signals, leaf pieces of 52 plants showed medium staining intensities and in 149 plants a dark blue colour was exhibited in the whole leaf. In none of the tested progeny of plant III could GUS activity be detected.

All progeny of plants III and V have been sprayed with an aqueous solution of the herbicide BASTA. They did not show any necrotic lesions and proved to be completely resistant (Fig. 3 F).

Progeny of plants III and V have been analysed by Southern hybridisation. R_1 plants of the parental line V have been hybridised to a *uid*A probe. In all tested R_1 plants, hybridisation signals corresponding to the 1.8-kb *uid*A fragment have been visualized (Fig. 5, lanes B, D, F, H, K). Furthermore, the integration pattern of the parental line is conserved in the progeny plants (Fig. 5, lanes C, E, G, J, L). Progeny of plant III have been analysed using a *bar* probe. All R_1 plants contain intact copies of the *bar* gene with an identical integration pattern to that of in the parental line.

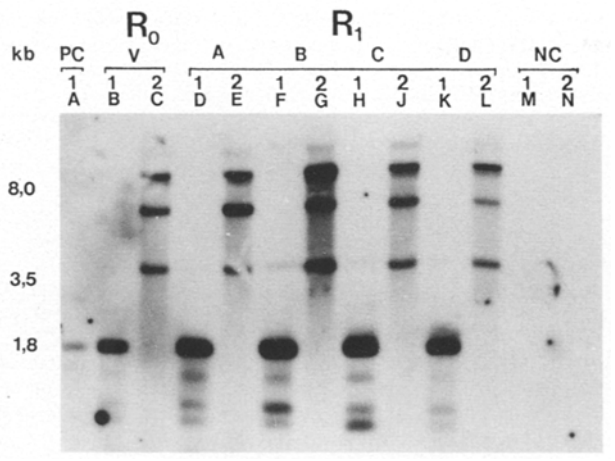

Fig. 5 Southern-blot analysis for R_1 progeny recovered from plant V which are GUS and PAT positive. Genomic DNA (20 µg/lane) from the primary regenerant (*V*), a wheat positive control (*PC*), a barley negative control (*NC*) and four progeny (*A–D*) were digested with *Bam*HI/*Sac*I (lanes labelled with "*1*") to excise the *uid*A gene, and with *Nco*I (lanes labelled with "*2*") to determine the number of integration sites per genome. Hybridization was carried out using a DIG-11-dUTP-labelled *uid*A probe

Discussion

The aim of the present study was to develop a simple and efficient procedure for the biolistic transformation of barley microspores. Barley microspores cultured in liquid medium can be regenerated at high frequencies (Hoekstra et al. 1993), but for particle bombardment it is necessary to use rather dry target tissues. Therefore, different plating methods have been compared. Only on media containing 0.2% GelRite did microspores divide reproducibly and regenerate with a high efficiency. The effect of different gelling agents on anther-culture response has been demonstrated for barley (Sorvari 1986; Kuhlmann and Foroughi-Wehr 1989) and wheat (Simonson and Baenziger 1992). However, in our experiments we could not detect any difference in barley anther-culture response for agarose- or GelRite-solidified media. This demonstrates that microspores respond much more sensitively to the composition of media than do anthers.

The effect of media composition on barley anther and microspore culture has been studied in detail (Olsen 1987; Hoekstra 1992; Morhorst and Lörz 1993). These reports underline the importance of glutamine for the culture and regeneration of microspores. Our results show that glutamine has a negative effect on the development of microspore-derived aggregates. Similar observations have been made for the culture of immature wheat embryos (Becker, unpublished results). However, the regeneration frequency of microspore cultures varied over a wide range indicating the importance of constant growth conditions for donor plants.

The aim of optimization of the parameters for the particle bombardment process was to enhance transient transformation by minimizing tissue damage. The viability of microspores was slightly reduced when helium gas pressures higher than 1800 PSI and particle densities of 116 µg per bombardment were used.

Microspores are very promising explants for biolistic transformation not only because they are unicellular but also as the time required for the production of homozygous plants is extremely low. Indeed, the first regenerants are already of homozygous nature. This is an important advantage of the microspore system because transformed plants which have integrated foreign DNA in coding regions necessary for plant metabolism or development will not be selected. The dihaploid state of the regenerants excludes the recovery of plants carrying lethal mutants. In our experiments we were able to transfer selected plants to soil 7–8 weeks after bombardment. The risk of somaclonal variation and albinism increases with the length of time in culture (Kott and Kasha 1984; Jähne et al. 1991 a). In our experiments we have never observed albinism or morphological abnormalities as reported for maize (Gordon-Kamm et al. 1990), wheat (Vasil and Vasil 1992), oat (Somers et al. 1992) and barley (Wan and Lemaux 1994). Obviously primary explants are more suitable for the generation of normal transgenic plants than suspension or callus cultures because the time in culture is significantly reduced compared to embryogenic callus or suspension cultures.

The transformation efficiency was strongly influenced by the regeneration capacity of the actual microspore preparation. In all experiments where the morphogenetic competence of the microspore cultures was low it was not possible to select putative transformants. In total 28 experiments were performed but only in nine of them did the bombardment of microspores lead to the recovery of transgenic plants originating from independent transformation events.

The regeneration of transgenic plants using PPT as a selective agent has proven to be very successful. A concentration of 3 mg/l of PPT completely inhibited the growth of control calli. Using this or higher concentrations we have only selected plants which have later been confirmed as transformants. These results underline the efficiency of this selection system.

DNA analysis of the transformants confirmed the introduction of both marker genes. All plants contain intact copies of the *bar* gene, which is also expressed and confers BASTA resistance. Only 4 out of 12 regenerants express the *uid*A gene. In Southern-blot analysis a fragment larger than the 1.8-kb coding region of *uid*A has been found in one GUS-negative plant. However, some plants containing intact copies of the *uid*A gene also showed no detectable GUS activity. This phenomenon may be due to methylation or mutations in the promoter or coding region and needs further detailed analysis.

In addition, other larger or smaller fragments have been observed in Southern analysis, suggesting that deletions, rearrangements and/or methylations at one or both restriction sites have occurred. Furthermore, multiple integration sites have been observed. This seems to be a general feature of microprojectile bombardment (Birch and Franks

1991). One plant containing a single-copy integration of *bar* and *uid*A has been identified, but this plant expressed the *bar* gene only.

The introduced marker genes were inherited in all the progeny showing an integration pattern identical to the parental line, which indicates the homozygous nature of R_0 plants.

The biolistic transformation of barley microspores has proven to be an interesting method for the transfer of genes of agronomic interest. Homozygous transgenic plants can be regenerated in a very short time. However, the transformation efficiency is not very high due to the varying regeneration frequencies of the microspores. A recent publication reports that it is possible to minimise fluctuations in the composition of the microspore population by growing donor plants in a defined, conditioned environment (Hoekstra 1993). Therefore, it will most probably be possible to increase the transformation efficiency significantly when reliable microspore preparations are used as the target tissue for particle bombardment.

Acknowledgements The authors thank Beatrix Petzold for technical assistance and Marion Gohra for assistance in the greenhouse, Drs. A. Mordhorst and M. Jäger-Gussen for helpful discussions and Dr. K. Düring for critically reading the manuscript. The financial support of Saaten-Union GmbH (Hannover, Germany) is gratefully acknowledged.

References

Barcelo P, Hagel C, Becker D, Martin A, Lörz H (1994) Transgenic cereal (Tritordeum) plants obtained at high efficiency by microprojectile bombardment of inflorescence tissue. The Plant J 5(4): 583–592

Becker D, Brettschneider R, Lörz H (1994) Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. The Plant J 5(2):299–307

Birch RG, Franks T (1991) Development and optimisation of microprojectile systems for plant genetic transformation. Aust J Plant Physiol 18:453–469

Bolik M, Koop HU (1991) Identification of embryogenic microspores of barley (*Hordeum vulgare* L.) by individual selection and culture, and their potential for transformation by microinjection. Protoplasma 162:61–68

Bower R, Birch RG (1992) Transgenic sugarcane plants via microprojectile bombardment. The Plant J 2:409–416

Cao J, Duan X, McElroy D, Wu R (1992) Regeneration of herbicide-resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep 11:586–591

Christou P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically-important indica and japonica varieties via electrical discharge particle-acceleration of exogenous DNA into immature zygotic embryos. Bio/Technol 9: 957–962

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 4:19–21

D'Halluin K, Bonne E, Bossut M, De Beuckeleer M, Leemans J (1993) Transgenic maize plants by tissue electroporation. Plant Cell 4:1495–1505

Fennell A, Hauptmann R (1992) Electroporation and PEG delivery of DNA into maize microspores. Plant Cell Rp. 11:567–570

Funatsuki H, Lörz H, Lazzeri PA (1992) Use of feeder cells to improve barley protoplast culture and regeneration. Plant Sci 85: 179–187

Golds TJ, Babczinsky J, Mordhorst AP, Koop HU (1994) Protoplast preparation without centrifugation: plant regeneration from barley (*Hordeum vulgare* L.). Plant Cell Rep 13:188–192

Gordon Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines R J, Start WG, O'Brian JV, Chambers SA, Adams JWR, Willetts NG, Rice TB, Mackey CJ, Krueger W, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618

Hoekstra S, van Zijderveld MH, Louwerse JD, Heidekamp F, van der Mark F (1992) Anther and microspore culture of *Hordeum vulgare* L. cv Igri. Plant Sci 86:89–96

Hoekstra S, van Zijderveld MH, Heidekamp F, van der Mark F (1993) Microspore culture of *Hordeum vulgare* L.: the influence of density and osmolality. Plant Cell Rep 12:661–665

Huang C, Yan H, Yan Q, Zhu M, Yuan M, Xu A (1993) Establishment and characterization of embryogenic cell-suspension cultures from immature and mature embryos of barley (*Hordeum vulgare* L.). Plant Cell Tissue Org Cult 32:19–25

Jähne A, Lazzeri PA, Jäger-Gussen M (1991 a) Plant regeneration from embryogenic cell suspensions derived from anther cultures of barley (*Hordeum vulgare* L.). Theor Appl Genet 82:74–80

Jähne A, Lazzeri PA, Lörz H (1991 b) Regeneration of fertile plants from protoplasts derived from embryogenic cell suspensions of barley (*Hordeum vulgare* L.). Plant Cell Rep 10:1–6

Jefferson RA (1987) Assaying chimeric genes in plants by the *GUS* fusion system. Plant Mol Biol Rep 5:387–405

Kott LS, Kasha KJ (1984) Initiation and morphological development of somatic embryoids from barley cell cultures. Can J Bot 62: 1245–1249

Koziel GM, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E, Rhodes R, Warren GW, Wright M, Evola SV (1993) Field performance of elite transgenic maize plants expressing an insecticidial protein derived from *Bacillus thuringiensis*. Bio/Technol 11:194–200

Kuhlmann U, Foroughi-Wehr B (1989) Production of doubled haploid lines in frequencies sufficient for barley breeding programs. Plant Cell Rep 8:78–81

Kuhlmann U, Foroughi-Wehr B, Graner A, Wenzel G (1991) Improved culture system for microspores of barley to become a target for DNA uptake. Plant Breed 107:165–168

Lazzeri PA, Brettschneider R, Lührs R, Lörz H (1991) Stable transformation of barley via PEG-induced direct DNA uptake into protoplasts. Theor Appl Genet 81:437–444

McCabe DE, Swain WF, Martinell BJ, Christou P (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. Bio/Technol 6:923–926

Mordhorst AP, Lörz H (1992) Electrostimulated regeneration of plantlets from protoplasts derived from cell suspensions of barley (*Hordeum vulgare* L.). Physiol Plant 85:289–294

Mordhorst AP, Lörz H (1993) Embryogenesis and development of isolated barley (*Hordeum vulgare* L.). Microspores are influenced by the amount and composition of nitrogen sources in the culture media. J Plant Physiol 142:485–492

Neuhaus-Url G, Neuhaus G (1993) The use of the nonradioactive digoxigenin chemiluminescent technology for plant genomic Southern-blot hybridization: a comparison with radioactivity. Transgenic Res 2:115–120

Olsen FL (1987) Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*. The effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. Carlsberg Res Commun 5:393–404

Olsen FL (1991) Isolation and cultivation of embryogenic microspores from barley (*Hordeum vulgare* L.). Hereditas 115:255–266

Sanford J C (1990) Biolistic plant transformation. Physiol Plant 79: 206–209

Simonson RL, Baenziger P S (1992) The effect of gelling agents on wheat anther and immature embryo culture. Plant Breed 109: 211–217

Somers DA, Rines HW, Gu W, Kaeppler HF, Bushnell WR (1992) Fertile transgenic oat plants. Bio/Technol 10:1589–1594

Sorvari S (1986) Comparison of anther cultures of barley cultivars in barley-starch and agar-gelatinized media. Ann Agric Fenn 25: 249–254

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 503–517

Vasil IK, Vasil V (1992) Advances in cereal protoplast research. Physiol Plant 85:279–283

Vasil V, Castillo AM, Fromm ME, Vasil IK (1992) Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technol 10:667–674

Wan Y, Lemaux PG (1994) Generation of large numbers of independently transformed fertile barley plants. Plant Physiol 104:37–48

Weeks JT, Anderson OD, Blechl AE (1993) Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). Plant Physiol 102:1077–1084

Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining the viability of cultured plant cells. Stain Technol 47:189–194

Ziauddin A, Marsolais A, Simion D, Kasha KJ (1992) Improved plant regeneration from wheat and barley microspore culture using phenylacetic acid (PAA). Plant Cell Rep 11:489–498