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Efficient transformation of scutellar tissue of immature maize embryos

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Abstract An efficient transformation system for maize was established by improving transformation conditions for the particle bombardment of the scutellar tissue of immature embryos. Particle bombardment was carried out using constructs containing the *pat* gene as the selection marker and a PDS 1000/He gun (Biorad). Transformation parameters, such as the amount of gold particles used per bombardment, particle velocity, preculture time of the scutellum prior to bombardment and osmotic treatment of the target tissue before and after bombardment, were analysed. Fertile transgenic regenerants of the maize inbred lines H99, A188 and Pa91 and the crosses A188 × H99 and Pa91 × H99 were selected on Basta-containing medium. The transformation frequency was between 2% and 4%. A total of 29 transgenic plant lines was obtained and verified with Southern blot analysis. All of the transgenic plants were fertile and set seeds. The R1 progeny of single plants was analysed. A Mendelian segregation of the transgenes was observed for all of the transformants tested. For 1 candidate, stable inheritance and stable expression of the transgenes were followed up to the R_4 generation.

Key words Biolistic transformation · Maize · Cereals · Somatic embryogenesis · Immature embryos

This publication is dedicated to Prof. Dr. L. Bergmann on the occasion of his 70th birthday.

Communicated by G. Wenzel

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Introduction

In recent years various approaches for the transformation and regeneration of fertile maize plants have been developed. In addition to a protocol for PEG-mediated protoplast transformation (Golovkin et al. 1993) most of these approaches have been based on the biolistic transformation of suspension cells (Fromm et al. 1990; Gordon-Kamm et al. 1990; Murry et al. 1993; Vain et al. 1993) or long-term type-I callus cultures (Genovesi et al. 1992; Walters et al. 1992).

The fundamental problems of transformation methods based on protoplast or suspension cultures in cereals are the continuous loss of embryogenic capacity of the suspension culture during long-time culture (Jähne et al. 1991), genotype dependence, occurrence of somaclonal variation (Wang et al. 1992) and a relatively high input of labour and energy. These limitations can be overcome by directly targeting primary explants, which can be obtained easily, manipulated in vitro and which can be regenerated to fertile plants. In various cereals several tissues, such as the shoot meristem of zygotic embryos (Ritala et al. 1994), immature inflorescences (Barcelo et al. 1994) and microspores (Jähne et al. 1994) have been used successfully as targets for biolistic transformation. For all cereal species the scutellar tissue of immature embryos is a suitable primary explant from which fertile plants can be regenerated at a high frequency via somatic embryogenesis.

The biolistic transformation method facilitates DNA transfer into intact tissues with high efficiency. Up to now, scutellar tissues of rice (Christou et al. 1991), barley (Wan and Lemaux 1994), wheat (for example: Weeks et al. 1993; Becker et al. 1994) hexaploid triticale (Zimny et al. 1995) and rye (Castillo et al. 1994) have been successfully used to obtain fertile transgenic plants. The usefulness of maize scutellar tissue for transformation has already been demonstrated by

tissue electroporation (D'Halluin et al. 1992) and by biolistic transformation as first reported by Koziel et al. (1993). D'Halluin et al. (1992) demonstrated for the first time that the compact type-I callus, which can be obtained from the scutellum of many maize genotypes, is a suitable target for transformation and that it is not necessary to establish type-II callus cultures.

A number of factors influence the transformation and regeneration of transgenic plants from bombarded tissues (Birch and Franck 1991). One critical point in the transformation via particle bombardment of such compact tissues as the scutellum is the transfer of DNA into many cells competent for somatic embryogenesis, without damaging the tissue which would reduce the capacity for somatic embryogenesis. One attempt to minimise the damage from bombardment is to plasmolyse the target cells by treatment with an osmoticum. Armaleo et al. (1990) obtained higher transformation rates when yeast cells were subcultured for some hours before and after particle bombardment on medium with a high osmolarity. The same effect was demonstrated for maize suspension cells by Vain et al. (1993). In both cases, the beneficial effect of this treatment is believed to come from the reduced turgor pressure so that more cells can be penetrated by particles without osmotic disruption.

In this report, several components of the biolistic transformation process, including osmotic treatment, were analysed for their effect on the scutellar tissue of immature maize embryos. As a result a high-frequency regeneration of fertile transgenic maize plants was achieved.

Materials and methods

Isolation and culture of immature embryos for particle bombardment

Immature maize embryos 1.0–1.5 mm long were isolated under sterile conditions and then cultured with the scutellar side facing upward on MScas medium (Zhong et al. 1992) supplemented with 2 mg/l 2,4-D or on modified N6 medium (D'Halluin et al. 1992) with 1 mg/l 2,4-D, both solidified with 0.8% agarose.

The explants were bombarded 1–6 days after isolation. For the osmotic pretreatment the embryos were placed with the scutellar side facing upward on MSC medium in which a higher osmotic value was achieved with the addition of sucrose. The osmotic treatment was started 4 h before bombardment and was continued for 20–24 h after bombardment. In experiments where the rhizobial part of the embryo was bombarded, the embryo was cut either immediately or 1 day after isolation.

Particle bombardment

Particle acceleration was performed with a PDS 1000/He gun (BioRad, Munich, Germany). Five micrograms plasmid DNA was precipitated onto gold particles with an average size of 0.4–1.2 μm (Heraeus, Karlsruhe, Germany) following a protocol described by BioRad (Munich, Germany) and modified according to Becker et al. (1994). For the stable transformation experiments, the particle-DNA pellet was first resuspended in 240 μl ethanol and then 3.5 μl was spread on the macrocarrier for each bombardment. In the co-transformation experiments 2.5 μg DNA of each plasmid was mixed and precipitated onto the particles.

Different plasmids were used in the transformation experiments. In the optimisation experiments for transient analysis and stable transformation, the pDB1 construct (Becker et al. 1994) was used. This plasmid contains the *uidA* gene under the control of the actin-1 promoter and the *pat* gene under the control of the CaMV 35S promoter. In subsequent experiments, other plasmid constructs were transferred into maize. In all of the experiments a plasmid containing a *pat* gene under the control of a CaMV 35S promoter was used as the selection marker gene. This was either subcloned with the gene of interest or co-transformed on a separate plasmid with a second plasmid containing the gene of interest.

Selection of transgenic plants

For the selection of transgenic plants, two selection schemes were applied. (1) Explants which were cultured on MScas medium before bombardment were cultured for another 2 weeks on MScas medium at 26°C in the dark after bombardment. Selection was started 14 days after bombardment when the explants were subcultured on MS medium (without casamino acids) supplemented with 2 mg/l 2,4-D and 1 mg/l phosphinotricin (PPT), the active compound of the commercial herbicide Basta. For regeneration, embryogenic calli were placed on hormone-free MS medium (without casamino acids) supplemented with 1 mg/l PPT after 2 weeks and cultured at 24°C under light (16 h). (2) Explants which were cultured on N6 medium before bombardment were cultured afterwards on N6 medium with 1 mg/l 2,4-D for another 2 weeks. For selection, the calli were transferred to N6 medium (without casamino acids) with 5 mg/l PPT. After 2 weeks, the calli were cultured for another 2 weeks on MS medium (without casamino acids) supplemented with 1 mg/l 2,4-D and 1 mg/l PPT. For regeneration, embryogenic calli were transferred to MS medium with 1 mg/l PPT without any hormones and cultured at 24°C under light (16 h).

Green shoots were transferred to Magenta boxes containing half-strength MS medium supplemented 1 mg/l PPT and solidified with 0.4% agarose. Plantlets with a height of 6–8 cm which survived selection were transferred into soil after they generated roots.

Small plants were cultured in soil in the greenhouse for 1 week under a plastic or glass lid. Routinely, more than 90% of the regenerants survived the transfer into soil. To confirm the selection, we sprayed the surviving plants once or twice with a Basta solution containing 250–300 mg/l PPT and 0.1% Tween.

Histochemical GUS staining

For the transient expression studies, bombarded scutellar tissues were incubated 2 days after bombardment at 37°C for 20 h in X-gluc staining solution (McCabe et al. 1988). Tissues of stably transformed plants were stained in the same way. After staining, leaf tissues with X-gluc chlorophyll was extracted in ethanol/acetic acid glacial (3:1) for 30–60 min at 65°C and washed once with 70% ethanol. To detect GUS activity in pollen grains, the mature pollen were collected in small centrifuge tubes, X-gluc staining solution (McCabe et al. 1988) was added and the probes were incubated for 30 min at 37°C in a thermomixer at 1000 rpm. The pollen was pelleted by a short centrifugation (30 s/8000 rpm) and washed once with distilled water. After centrifugation (30 s/8000 rpm) the pollen was resuspended in distilled water. Small aliquots were pipetted onto a slide and analysed under a stereo microscope.

DNA isolation and Southern blot hybridisation

Total genomic DNA was isolated from primary transformants and their progeny using the protocol of Dellaporta et al. (1983). Aliquots of DNA (10 or 15 μg), uncut or digested with restriction enzymes, were separated by electrophoresis and transferred to a Hybond N membrane (Amersham/England) or a BiodyneA membrane (Pall/England). Introduced DNA was detected using a modified protocol of the non-radioactive digoxigenin chemiluminescent method (Neuhaus-Url and Neuhaus 1993). Filters were hybridised with a polymerase chain reaction (PCR)-labelled *pat* or *uidA* probe (for details see Becker et al. 1994).

Results

For a stable transformation of the scutellar tissues of maize embryos with biolistic transformation it is necessary to transfer DNA into as many cells as possible while at the same time retaining as much embryogenic capacity as possible. To achieve this end, different transformation parameters were optimised.

Transient expression assays

Different transformation parameters were analysed in the transient expression assays with plasmid pDB1 (Becker et al. 1994). The scutellar tissue of the maize inbred line H99 was bombarded under different conditions, and transient GUS expression of the *uidA* gene fused to the actin 1 promoter (McElroy et al. 1990) was detected 2 days after bombardment (Fig. 2a).

In preliminary experiments, conditions for bombardment with the PDS1000 Hegun (BioRad/Munich) were analysed in transient expression studies. Basically, the same bombardment conditions were used as those described for scutellar tissue of wheat by Becker et al. (1994) (Table 1). Damage to the scutellar tissue was reduced by decreasing the amount of gold particles used per bombardment from 120 μg to 30 μg. The number of transient signals per bombardment was only slightly reduced when using 30 μg (approx. 1500 signals per plate) instead of 120 μg (approx. 1800 signals per plate) and was within the frame of statistical variation between experiments. Higher numbers of transient signals were obtained when the immature embryos were subcultured for 4 h before and 20 h after bombardment on callus induction medium with a higher osmolarity. The osmolarity was increased stepwise from 250 mOsm/kg to 500 mOsm/kg sucrose. In these experiments, the number of transient signals increased from 63 (250 mOsm/kg) to 294 signals (500 mOsm/kg) per embryo (Fig. 1). More than 7000 transient expression signals per plate, each plate containing 25 embryos with lengths between 1.5 mm and 2.0 mm, were obtained.

Table 1 Parameters used for particle bombardment

Distance between	
A: rupture disk and macrocarrier	2.5 cm
B: macrocarrier and stopping screen	0.8 cm
C: stopping screen and target cells	5.5 cm
Gas pressure	900 or 1300 psi
Partial vacuum	27 inch Hg
Particles	gold (size: 0.4–1.2 μm)
Amount of particles	30 μg per shot

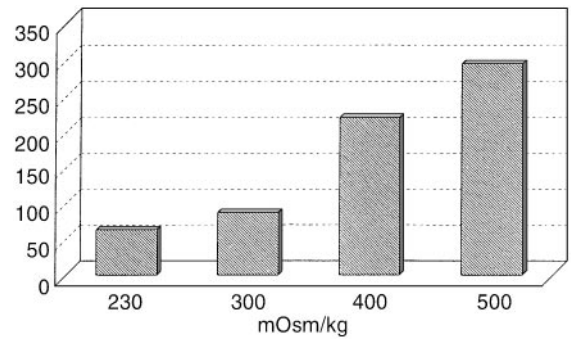

Fig. 1 Effect of osmotic treatment on transient transformation. For each treatment three plates, each with 25 immature H99 embryos, were bombarded with pDB1 containing the *uidA* gene under control of the actin promoter of rice (Becker et al. 1994), 2 days after isolation. For the osmotic treatment the embryos were cultured 4–5 h before and 20–24 h after particle bombardment on MSC medium supplemented with different concentrations of sucrose. Two days after transfer to callus-induction medium, the embryos were incubated for 24 h with the X-gluc solution. Transient GUS signals were counted. The values shown in the diagram are the average signal numbers per scutellum

Somatic embryogenesis in bombarded scutellar tissues

A strong inhibition of somatic embryogenesis was observed when immature embryos were bombarded directly after isolation. A higher frequency of embryogenic callus formation was observed when the embryos were cultured for 2–6 days on callus induction medium before bombardment. The same observation was made when the amount of particles per bombardment was reduced as described earlier. In comparison with non-bombarded control explants, the number of somatic embryos per embryogenic calli increased from 20% to 60% when the amount of particles was reduced from 120 to 30 μg (Table 2). This effect was less pronounced when the immature embryos were precultured longer than 5 days before bombardment. In these latter experiments only a slight reduction of the embryogenic capacity was observed when the bombardment was performed with 120 μg of gold particles.

An enhancement of somatic embryogenesis was achieved when the embryos were precultured 4 h before and 20–24 hours after bombardment on medium with an osmotic value of 500 mOsm/kg (Table 3). In control experiments where we tested sucrose concentrations

Table 2 Effect of the amount of particles used per bombardment on somatic embryogenesis of maize

		Number of calli with somatic embryos[a]			
		−	+	++	+++
Control	(non-bombarded)	2	14	27	57
2 days	120 µg gold	34	42	22	2
	60 µg gold	6	24	31	39
	30 µg gold	2	22	35	41
6 days	120 µg gold	3	24	50	23
	60 µg gold	0	20	44	36
	30 µg gold	1	14	37	48

[a] Immature embryos were bombarded 2 or 6 days after isolation with different amounts of gold particles (0.4–1.2 µm). Two weeks after transformation the resulting calli were evaluated for their capacity to form embryogenic callus. The values given are the number of scutella formed: − = no somatic embryos, + = 1–4 somatic embryos per embryogenic callus, ++ = 5–10 somatic embryos per embryogenic callus, +++ = > 10 somatic embryos per embryogenic callus

Table 3 Effect of osmotic treatment during bombardment on somatic embryogenesis of maize

	Number of calli with somatic embryos[a]			
	−	+	++	+++
Control (non-bombarded)	0	17	21	61
230 mOsm/kg	49	25	23	2
300 mOsm/kg	38	24	35	4
400 mOsm/kg	17	26	38	21
500 mOsm/kg	10	20	34	36

[a] Immature embryos were transferred 2 days after isolation and 4–5 h before particle bombardment onto MScas medium with an increased concentration of sucrose. The embryos were transferred back to the callus-induction medium 20–24 h after bombardment. Two weeks after transformation, the resulting calli were evaluated for their capacity to form embryogenic callus. The values given are the number of scutella formed: − = no somatic embryos, + = 1–4 somatic embryos per embryogenic callus, ++ = 5–10 somatic embryos per embryogenic callus, +++ = > 10 somatic embryos per embryogenic callus

resulting in osmotic values up to 600 mOsm/kg, we never saw any negative effect of increased sucrose concentration on callus formation.

When the improved transformation parameters described previously were combined, the embryogenic capacity of bombarded immature embryos was comparable to that of the non-bombarded controls.

Stable transformation

Parameters and culture conditions established in transient transformation experiments were analysed in stable transformation experiments. In all experiments, a plasmid containing the selectable *pat* gene under the control of the CaMV 35S-promoter was used. This *pat* construct was either fused with a second gene on the same plasmid, i.e. pDB1 *pat*/*uidA* (Becker et al. 1994), or the plasmid DNA containing the CaMV 35S *pat* gene (P. Eckes, unpublished) was mixed with an equal amount of a plasmid carrying a second gene construct. In all experiments, 72 ng of plasmid DNA was used per bombardment.

For callus induction, the immature embryos were placed on solid callus induction medium with the scutellar side facing upward. To increase the transformation rate of cells which were competent for embryogenic callus formation we tested two alternative methods: (1) the embryo was bombarded not only on the scutellar side but also on the side with the embryo axis; in this way the edge of the scutellum was bombarded from both sides; (2) following the report of Dunder et al. (1995), we bombarded preferentially the coleorhizal end of the scutellum; this was achieved by cutting the embryo either immediately or 1 day after isolation along the apical meristem into two halves. The half with the coleorhizal end was placed 4 h before and 20 h after bombardment with its cut side on medium supplemented with 0.5 or 0.6 *M* sucrose so that the coleorhizal end of the scutellum was directed towards the particle accelerator during bombardment.

After transformation, all explants were cultured for 14 days on callus induction medium without PPT. The resulting calli (Fig. 2b) were transferred to callus induction medium with 1–5 mg/l PPT for 2–4 weeks. For regeneration, embryogenic calli were placed on medium without hormones and with 1 mg/l PPT. Green shoots (Fig. 2c) were transferred into Magenta boxes containing half-strength MS and 1 mg/l PPT. The surviving plantlets were potted into soil. As a final screen, 10–14 days after transfer into the greenhouse all regenerants were sprayed once or twice with a Basta solution containing 250 mg/l PPT and 0.1% Tween (Fig. 2d). All plants, surviving this selection procedure were also positive in the Southern blot analysis and contained

Fig. 2a–f Regeneration of transgenic plants from bombarded scutellar tissue and transgene expression in transgenic plants. **a** Transient *uidA* signals of pDB1 in scutellum of H99 embryos bombarded 1 day after isolation. *Bar*: 100 µm, **b** embryogenic callus of a bombarded H99 embryo, 2 weeks on callus induction medium without selection, before subculture on callus-induction medium with 5 mg/l PPT, **c** transgenic regenerant, selected for 4 weeks in the dark on callus-induction medium with 5 mg/l PPT and 3 weeks in the light (16 h) on regeneration medium with 1 mg/l PPT, **d** transgenic regenerants in the greenhouse after being sprayed twice with a Basta solution (250 mg/l PPT, 0.1% Tween), **e** mature pollen grains of transgenic plant 33.1 stained with the X-gluc solution to show expression of pDB1. *Bar*: 100 µm, **f** seed-grown R_4 progeny plants from a homozygous R_3 parental plant (33.1.2.6) together with untransformed H99 plants after being sprayed twice with a Basta-solution (250 mg/l PPT, 0.1% Tween)

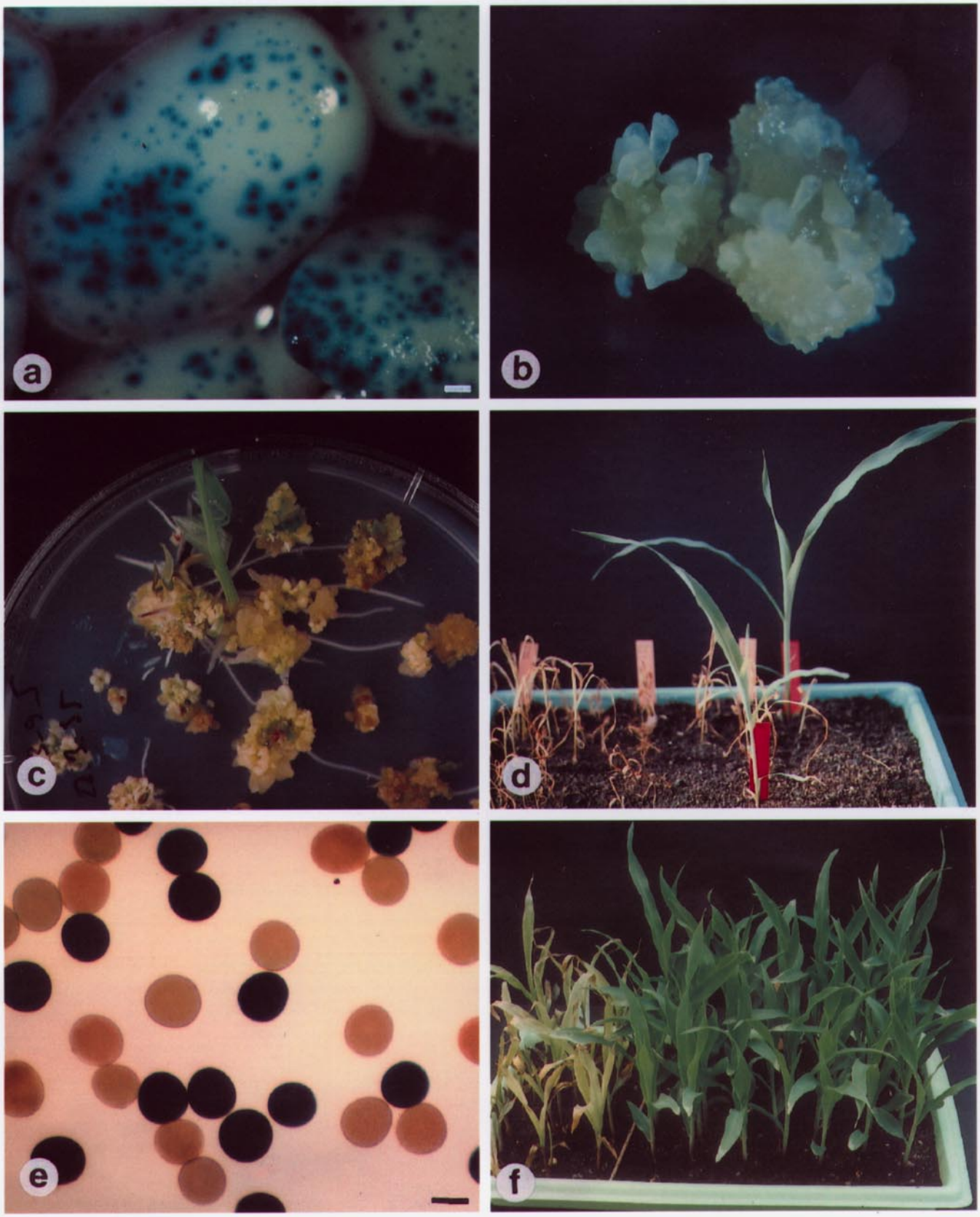
a
b
c
d
e
f

Table 4 Summary of stable transformation experiments

Genotype	Number of explants	Preculture time (days)	Osmotic treatment (mOsm/kg)	Helium pressure (psi)	Orientation for bombardment[a]	Number of transformed plants/transformation events
H99	60	1	–	1100	Sc + EA	–
H99	60	1	–	900	Sc + EA	1/1
H99	80	2	–	900	Sc + EA	–
H99	80	1	–	1300	Sc + EA	–
H99	180	1	–	900	Sc + EA	–
H99	15	4	–	900	Sc + EA	2/1
H99	45	5	–	1300	R	–
A188	50	4	–	1300	R	–
A188	300	2, 5, 7	–	1300	Sc + EA/R	–
H99	158	6	–	900	R	–
H99	525	2, 6	–	900	Sc + EA/R	–
H99	400	2, 6	–	900	Sc + EA	–
H99	325	1	–	900	Sc + EA	–
H99	150	1	–	900	Sc + EA/R	–
H99	225	1	500	900	Sc	–
H99	250	1	500	900	R	–
H99	200	2	500	900	Sc	1/1
H99	60	5	500	900	Sc	–
H99	60	3	500	900	R	–
H99	300	1	500	900	R	–
H99	150	2	500	1300	R	5/2
H99	150	2	500	1300	R	16/3
Pa91 × H99	150	5, 12	500	1300	Sc + EA	4/4
A188 × H99	150	6	600	1300	R	13/7
A188	125	2	600	1300	R	2/2
A188 × H99	175	2	600	1300	R	17/5
H99	100	1	600	1300	R	1/1
Pa91 × H99	300	0	600	1300	Sc + EA/R	–
Pa91	50	2	600	1300	EA + Sc	1/1
Pa91 × H99	150	2	600	1300	EA + Sc	2/1

[a] Sc + EA, Immature embryos were bombarded on both sides – the scutellum side and the side with the embryo axis; Sc, immature embryos were bombarded on the scutellum side; R, immature embryos were cut after isolation, and the coleorhizal part of the embryo was bombarded

one or more copies of the *pat* gene. The results of all transformation experiments are shown in Table 4.

Parameters which had a beneficial effect on embryogenic capacity in bombardment experiments led to higher stable transformation rates. The transformation frequency was greatly increased, to 2%, when the scutellar tissues were precultured on medium supplemented with additional sucrose (500 or 600 mOsm/kg) and bombarded with a helium pressure of 1300 psi (regardless of other parameters). A significantly lower frequency, 0.12%, was obtained when the embryos were pretreated osmotically but bombarded only with 900 psi. A longer preculture time for the immature embryos on callus induction medium before bombardment also increased the transformation rate. No transgenic plant was regenerated from 300 bombarded scutella when the embryos were placed on medium with 0.6 *M* sucrose directly after isolation and bombarded the same day. One transformed plant from 100 bombarded tissues (1%) was regenerated when both the osmotic pretreatment and the biolistic bombardment were performed 1 day after isolation. Higher transformation rates (2%) were obtained when the embryos were precultured on callus induction medium for 2 days after isolation. In two experiments where the embryos were precultured for 6 days before bombardment the transformation frequency was 4%, i.e. 1 transformed line from 25 bombarded embryos.

No clear effect was observed when the embryo was bombarded from both sides or when only the coleorhizal part of the scutellum was bombarded. With both methods, a good transformation rate was obtained (Table 4).

Transgenic plants could be regenerated from all of the tested maize inbred lines and crosses between these inbred lines. Some direct regenerants of the inbred line H99 and A188 showed phenotypic alterations, like reduced height, delayed flowering of the female flower or hybrid flowers. In some cases seeds were obtained only after cross pollination or outcrossing with a seed grown plant from a corresponding inbred line. Regenerants of crosses between the inbred

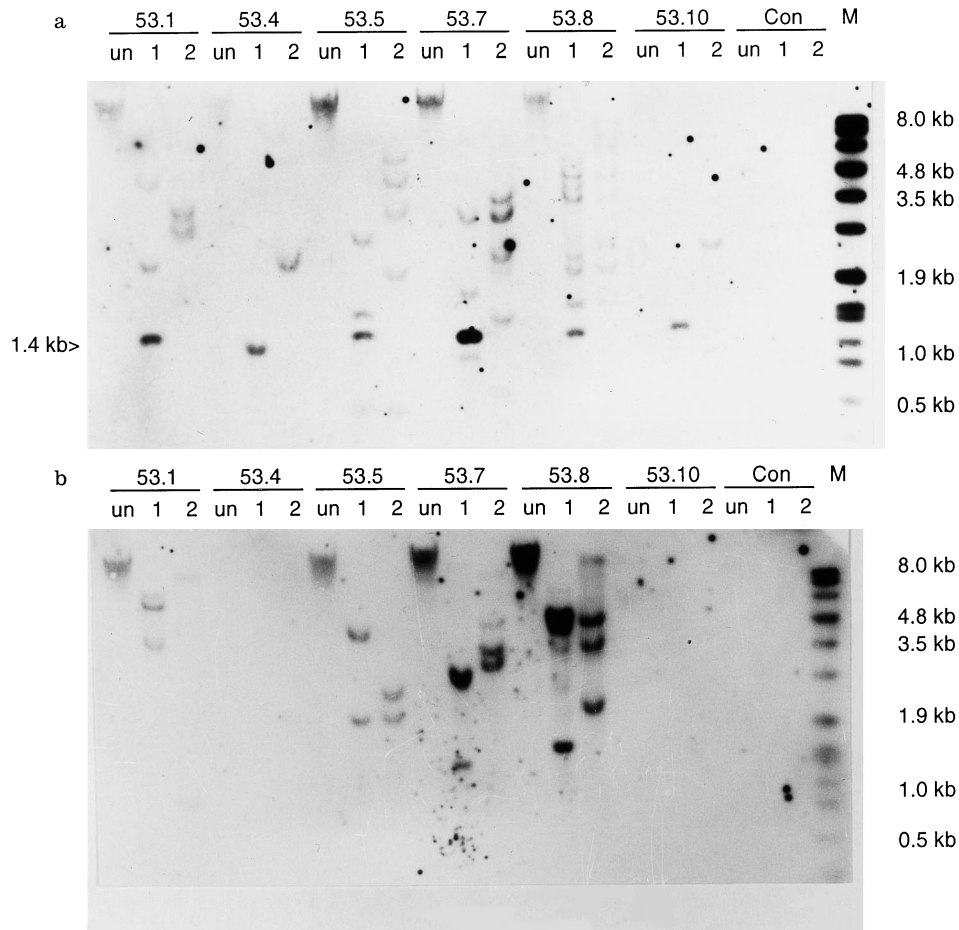

Fig. 3a, b Southern blot analysis of transgenic maize plants from one transformation experiment. Fifteen micrograms of genomic DNA from an untransformed control plant A188 × H99 (*Con*) and 6 transgenic regenerants was digested with restriction enzymes and separated in a 0.8% agarose gel. The genomic DNA was digested with *Eco*RI (*1*) or *Hin*dIII (*2*). Fifteen micrograms of undigested genomic DNA (*un*) from each plant was loaded in the lanes adjacent to those loaded with digested DNA. The filter-bound DNA was hybridised to a DIG-labelled *pat* fragment (*a*). The same filter was hybridised subsequentely to a DIG-labelled *uidA* fragment (*b*). The DIG-labelled MVII marker (Boehringer Mannheim; Germany) was used as size standard (*M*)

lines (A188 × H99, Pa91 × H99) showed mostly a normal phenotype and seeds were obtained by self pollination. In general, we obtained seeds from all transgenic plants.

Transgene integration and expression analysis of transformed plants

In plants which survived the selection procedure, integration of the selectable marker gene into the plant genome was shown with Southern blot analysis. In Fig. 3, Southern blot analysis of six transformed regenerants from transformation experiment 53 is shown. The genomic DNA was either restricted with *Eco*RI or *Hin*dIII or left undigested, and was separated on a 0.8% agarose gel. The DNA was blotted onto a nylon membrane and hybridised to a digoxigenin-labelled *pat* or *uidA* fragment. The *pat* probe hybridised to an *Eco*RI fragment of 1.4 kbp. This size would be expected from the *pat* construct and indicates that all Basta-resistant plants contain at least 1 intact copy of the *pat* gene construct with the CaMV35S promoter, the coding region of the *pat* gene and the CaMV35S polyadenylation signal (Fig. 3a). *Hin*dIII linearises the plasmid containing the *pat* gene construct. The number of bands obtained after *Hin*dIII digestion indicates the number of *pat* gene copies and integration sides in the maize genome. The copy numbers in Fig. 3a varied between 1 and 8 copies or integration sites. The Southern blot analysis showed that plant 53.4 and 53.10 were clonal regenerants of the same transgenic callus. Four of five independently transformed plants shown in Fig. 3 had also the *uidA* gene integrated into the maize genome; it had been co-transformed on a separate plasmid (Fig. 3b).

The co-transformation rate in all experiments where two independent plasmids were used was 70%. In experiments where a second plasmid carrying a third chimeric gene construct was mixed with a plasmid containing two chimeric genes, 6 out of 12 transformation events contained copies of all three transgenes in the plant genome (50%).

In general, the integration pattern varied between 1 copy or 1 integration site only to more than 20 copies or integration sites. In most of the cases the copy number of the co-transformed construct was as high as that of the selectable marker. In some cases, however,

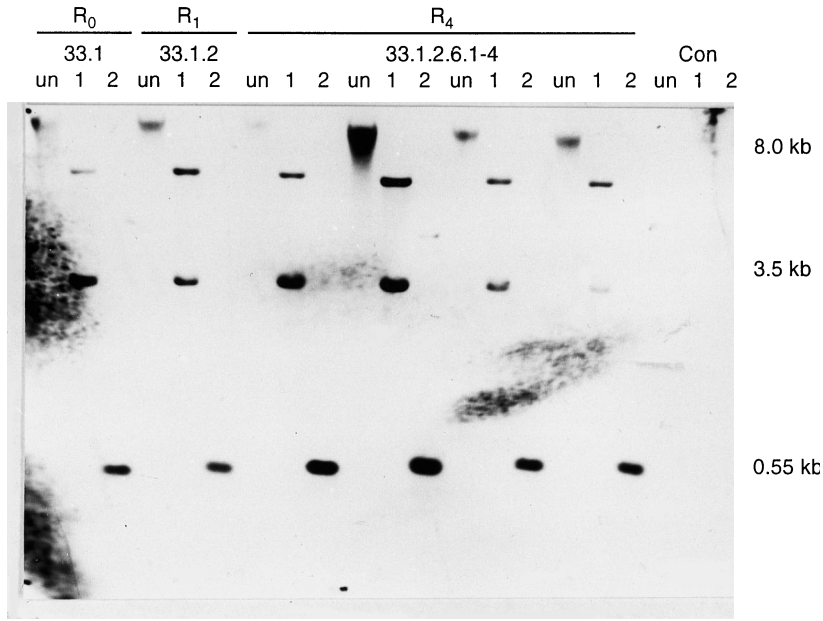

Fig. 4 Southern blot analysis of progeny plants of plant 33.1. Fifteen micrograms of genomic DNA from an untransformed control H99 plant (*Con*), the R_0 plant 33.1, a R_1 progeny plant 33.1.2 and 4 R_4 plants (33.1.2.6.1–4) was digested with restriction enzymes and separated in a 0.8% agarose gel. The genomic DNA was digested with *Nco*I (*1*) or *Sal*I (*2*). Fifteen micrograms of undigested genomic DNA (*un*) from each plant was loaded in the lanes adjacent to those lanes loaded with digested DNA. The filter-bound DNA was hybridised to a DIG-labelled *pat* fragment

the copy number of the second construct was much lower. For example, plant 53.5 contained 4 copies of the *pat* gene but only 2 copies of the *uidA* gene (Fig. 3).

Plants analysed in Southern experiments had been selected during in vitro culture and by two sprayings in the greenhouse with a 250-mg/l PPT solution. This stringent selection scheme might be the reason why we never found any indication for a chimeric transgenic plant. The GUS expression of 6 plants (21.1, 33.1, 39a.1, 39a.2, 39b.3, 39a.4) which were transformed with a constitutively expressed pAct1D*gus* construct (McElroy et al. 1993) was analysed in more detail. The strength of histochemical GUS signals varied between different transformation events. In all candidates the *uidA* gene was expressed in leaves, roots, tassels and pollen (Fig. 2e), indicating that the whole plants were transgenic and non-chimeric.

Inheritance of marker genes

Seeds were obtained from all transgenic plants by selfing or cross pollination. Progeny plants of self-pollinated plant 33.1 transformed with the plasmid pDB1 were analysed in more detail. Histochemical GUS assays of the mature pollen grains of plant 33.1 indicated a monohybrid inheritance of the integrated *uidA* gene copies. The ratio of GUS + : GUS − pollen grains was approximately 1 : 1 (101 blue/95 white pollen grains). These data were supported by Basta spraying for PAT activity as well as histochemical GUS assays of leaves of 16 R1 plants. Of 16 plants which germinated from randomly chosen mature seeds of plant 33/1, 12 plants showed GUS activity and survived two sprayings. The staining of pollen grains of the 12 positive plants showed that they are heterozygous with respect to the transgenes (Fig. 2e).

In the R_2 progeny of 2 self-pollinated heterozygous R_1 plants of line 33.1, 13 plants from a total of 20 plants analysed showed GUS activity and survived sprayings with a Basta solution (250 mg/l PPT). Staining of pollen grains with X-gluc for GUS activity showed that 5 plants were homozygous for the transgenes. From 2 self-pollinated R_2 plants which were homozygous, 10 seeds were germinated and analysed for PAT and GUS activity. All plants survived Basta sprayings and showed GUS activity. This result confirmed that both R_3 parental plants were homozygous for the transgenes and that both genes were inherited as 1 coupled locus.

To show that the transgenes were stably expressed after four generations, we germinated 100 seeds from 2 self-pollinated homozygous R_3 plants. All plants survived two Basta sprayings (250 mg/l PPT, 0.1% Tween) and all of the 25 randomly chosen R_4 plants showed GUS activity (Fig. 2f).

DNA was isolated from single individual plants of the R_1 and R_4 generations which survived the Basta spraying and subsequently, analysed in Southern blot experiments. They showed the same integration pattern of the *pat* gene as the R_0 plant (Fig. 4). These results confirmed that both transgenes were stably inherited and expressed over four generations in this transgenic maize line.

Discussion

The biolistic transformation of maize was optimised by increasing the number of scutellum cells into which the

particles with DNA were transferred. The most convenient method for measuring the efficiency of DNA delivery into intact cells was to determine the number of cells which transiently expressed the *uidA* gene. We found that it was very important to avoid tissue damage from bombardment. Parameters which may influence the transformation frequency were analysed with respect to two criteria: transient expression rates and capacity for somatic embryogenesis. Important factors which affected transient expression rates and somatic embryogenesis in maize scutellum tissue were the preculture time before bombardment, osmotic treatment before and after bombardment and the amount of particles used per bombardment.

The amount of particles used in our experiments was reduced from 120 μg to 30 μg. The effect on the number of transient signals was only minimal, but there was a clear beneficial effect on the capacity for somatic embryogenesis. This effect was more pronounced with scutella of embryos bombarded 2 days after isolation and less pronounced with scutella bombarded 6 days after isolation. The same effect has been described by Becker et al. (1994) for wheat scutellum tissue. Using the same amounts of gold particles, they also found no differences in transient signal numbers but only that lower particle density gave the same rate of somatic embryogenesis as that found in non-bombarded controls.

Instead of reducing the particle amount, a similar effect is probably achieved when a net is placed between the particle accelerator and the plate with explants. Birch and Franks (1991) have reported that by interposing a mesh screen tissue damage was decreased, especially in the central area also called "zone of death". Although some microprojectiles were blocked by the mesh, they found a higher number of transient expression signals because particles were deflected to penetrate cells over a wider area of the target tissue. Dunder et al. (1995) described the same effect using the PDS-1000He gun and 400 μg gold particles per bombardment for scutellum tissue of maize embryos. Placement of a steel mesh (104-μm aperture) between the stopping screen and the target enables a higher transformation frequency and more prolific callus as a result of decreased damage. Nevertheless, they found that in the centre of the plate the response of the callus was reduced. For this reason they recommend placing the immature embryos in concentric circles around the centre of the target plate and bombarding each plate twice. Using 13-times fewer particles without a mesh, we found that the transient signals were more or less evenly distributed over an area with a radius of 2.0–2.5 cm in the centre of the plate. We found no indication that tissue damage was higher in any area.

Tranformation frequency was greatly increased when immature embryos were cultured 4 h before bombardment and 16–20 h after bombardment on callus induction medium with higher osmolarities. The protocol used here was basically the same as that described by Vain et al. (1993) for the stable transformation of maize suspension cells. In contrast to Vain et al. (1993) we increased the sucrose concentration in the callus induction medium to 0.5 *M* or 0.6 *M* instead of using an osmoticum such as mannitol or sorbitol. The osmotic pretreatment is believed to induce plasmolysis of the cells so that fewer cells are disrupted by the penetrating particles (Armaleo et al. 1990; Vain et al. 1993). In our experiments we found a five fold higher number of transient expression signals on scutella that had been subjected to osmotic treatment. Not only was the number of transient signals higher but the signals were also more distinct. With the osmotic treatment we obtained more GUS signals appearing as distinct spots without a smear of blue colour in the neighbouring cells. This indicates that the outer cell layers were less damaged.

Vain et al. (1993) suggested that osmotic conditioning of suspension cells could also be achieved by partial drying of the target tissue. This might explain our observation that without osmotic treatment scutellum tissue precultured for 6 days after isolation could tolerate more particles without losing regeneration capacity than scutellum tissue bombarded 1 or 2 days after isolation. In general, we obtained a higher stable transformation frequency when the scutella were precultured for 2–6 days. In fact, we never got any transformation event when the scutellum was bombarded directly after isolation, irrespective of osmotic treatment. The same was observed by Zimny et al. (1995). With triticale scutellum tissue, a clear reduction of tissue damage was achieved when the explants were precultured for 2–7 days. Vasil et al. (1993) reported for wheat scutellum tissue that the regeneration capacity and, consequently, the transformation frequency were reduced when the explants were bombarded up to 7 days after isolation as compared to experiments in which the explants were bombarded more than 1 week after isolation. A partial drying of the outer cell layers may explain why a high transformation frequency could be obtained without any osmotic treatment using barley embryos after a 2-week preculture time (Wan and Lemaux 1994) and "long-term" callus cultures of maize (Wan et al. 1995).

The parameters described so far all showed a clear effect on transient expression levels or/and on somatic embryogenesis. Our strategy for improving the stable transformation frequency was to enhance the transient signal number by increasing somatic embryogenesis to the level of non-bombarded controls. However, in applying this strategy not all parameters may be improved. We never found significant differences in transient expression assays and in somatic embryogenesis between bombardments performed at 900 or 1300 psi (data not shown). However, in stable transformation experiments we found significant higher transformation rates at 1300 psi than at to 900 psi. This could indicate that cells which are competent for

embryogenesis are located in deeper cell layers and that these are penetrated at a higher helium pressure. A similar observation was made by Dunder et al. (1995) for type-I callus induced from immature maize embryos. They recommended using 1300 psi for type-I callus of maize and 900 psi for type-II callus. Depending on the maize genotypes and the tissue culture conditions we preferentially induce type-I callus. Thus, our results confirm their observation and might indicate that type-I callus originates from deeper cell layers.

For a long time it was controversial whether type-I callus in general was a suitable target for transformation because in several reports a multi-cell origin for somatic embryos of type I-callus was postulated. D'Halluin et al. (1992) and Wan et al. (1995) reported that type-I callus of maize is suitable target for transformation by tissue electroporation as well as by particle bombardment to obtain high numbers of non-chimeric transgenic plants. In our experiments, we never observed chimeric regenerants. This indicates that the somatic embryos which were transformed originated from a single cell or, more likely, when taking into account that cultures were transformed 6 days after the initiation of callus induction, that a multicellular somatic embryo was transformed and a transgenic part of this embryo was selected and multiplied so that afterwards a fully transgenic plant was regenerated. The fact that Wan et al. (1995) were able to regenerate fully transgenic plants from long-term type-I callus cultures supports the second hypothesis.

Whether the orientation of the scutellum is important for bombardment cannot be answered from our experiments. Dunder et al. (1995) found that turning a plate with isolated maize embryos in a way such that the coleorhizal part of the embryo was directed toward the particle accelerator increased the transformation rate. No influence of the orientation of the embryo prior to bombardment was reported by Wan and Lemaux (1994) for immature embryos of barley. They bombarded either the scutellum alone, both sides of the embryo or the cut side of a longitudinally-cut embryo.

The optimisation of the parameters described previously resulted in a comparatively high stable transformation frequency. On average, we obtained 1 transformed plant from 25 to 50 bombarded scutella (3%) or 1 plant from 1–2 bombarded plates with 25 embryos each. For maize scutellum tissue only little data are available. Koziel et al. (1993) obtained a transformation frequency of 1% in their experiments. However, the transformation procedure was not described in detail. A number of reports describe the scutellum from immature embryos of other cereal species as the target tissue for bombardment. For wheat, frequencies of 1 per 500–1000 (Weeks et al. 1993) 1 per 80 (Becker et al. 1994; Nehra et al. 1994) and 1 per 50 (Vasil et al. 1993) were reported. The highest transformation frequencies so far reported have been for rice (1 per 1.3; Li et al. 1993), barley (1 per 23; Wan and Lemaux 1994) and triticale (1 per 30; Zimny et al. 1995).

For maize, long-term suspension cultures or callus cultures were used as targets for biolistic transformation. A comparison between the transformation efficiency obtained with these systems and a scutellum system is difficult and can only be done when comparing the number of transgenic plants regenerated per bombarded target plate. The transformation frequency of type-II callus tissue initiated either from suspension cultures or maintained as callus culture vary from 1 transgenic plant line per 7–10 target plates (Walters et al. 1992) to more than 1 transgenic plant line per target plate (Gordon-Kamm et al. 1990). The disadvantage of using type-II callus is the strong genotype dependence as well as the labour- and time-consuming process of establishing and maintaining a regenerable culture for transformation. In addition, many of the regenerants show phenotypic alterations, especially sterility (Gordon-Kamm et al. 1990; Fromm et al. 1990).

Recently, Wan et al. (1995) reported a high transformation frequency when using 10-month-old type-I cultures initiated from anther cultures. On average, they obtained more than 2 regenerable transgenic callus lines from 1 bombarded target plate. In comparison to type-II cultures type-I callus cultures can be started from a wider range of genotypes and are regenerable over a longer period of time. Furthermore, Wan et al. (1995) argue that using callus as the target for biolistic transformation has the advantage of being independent of greenhouse conditions and seasonal variations. Both factors can influence the quantity and quality of somatic embryogenesis obtained from the scutellum of immature embryos (D'Halluin et al. 1992). We observed that under our greenhouse conditions the embryos needed more time to reach the optimal developmental stage for starting embryogenic cultures in winter and that this developmental phase was more limited than for embryos which developed in the spring or summer. Thus, under normal greenhouse conditions, we are able to start highly embryogenic cultures at any season of the year. In fact, the most successful transformation experiments were performed from November to March in Hamburg/northern Germany.

We obtained transformed plants from three different maize inbreed lines and from crosses between these lines, indicating that the method presented here is more genotype-independent than methods where suspension cultures are used (Gordon-Kamm et al. 1990; Fromm et al. 1990; Walters et al. 1992; Golovkin et al. 1993). We have to concede, however, that this method is limited to genotypes which produce type-I callus. Recently, two reports presented attempts to overcome this genotype limitation by bombarding the shoot meristem of zygotic embryos (Lowe et al. 1995; Zhong et al. 1996). The transgenic part of the resulting chimeric shoots was multiplied in vitro under selection until fully transgenic regenerants were obtained. But for some

genotypes it is still difficult to regenerate plants from shoot cultures (Lowe et al. 1995). The major disadvantages are the low frequency of useful transformation events and the time period of more than 6 months for regenerating a transgenic plant. With our system, the first transgenic regenerants are transferred to the greenhouse 10–12 weeks after transformation.

The molecular analysis of the transgenic regenerants showed an integration pattern typical of that obtained by direct DNA transformation. The number of integrated DNA fragments varied from 1 to more than 20 copies. Under our transformation conditions, the majority of plants contained 3–8 copies of the transgenes. The co-transformation frequency obtained when two unlinked plasmids were mixed in a ratio of 1 : 1 before transformation was 70%. This ratio is slightly higher than the 63% value obtained by Spencer et al. (1992) in non-embryogenic maize callus lines and is in the range of the 77% value reported from Gordon-Kamm et al. (1990) for stable embryogenic maize lines. Wan and Lemaux (1995) obtained a co-transformation rate of 85% in embryogenic callus lines and scutellum tissue of barley when they increased the molar ratio of non-selected to selected genes to 2 : 1. In experiments where three chimeric gene constructs were used for transformation, two constructs linked on 1 plasmid and a third on a separate plasmid, more than 50% of all transformation events carried copies of all three constructs. Recently, Hadi et al. (1996) reported that they were able to transfer 12 different plasmids into the genome of soybean callus cultures during one particle bombardment. These results demonstrate that the biolistic transformation process is a simple method for transforming several gene constructs during 1 transformation event. The reason for this phenomenon and the fact that very often multiple copies of one construct are integrated into the plant genome lies in the fusion of DNA molecules by homologous and non-homologous recombination before integration into the plant genome (Register et al. 1993; Hadi et al. 1996). Many of the multi-copy integration events in transgenic plants produced by biolistic transformation are inherited as a single locus.

Seeds were obtained from all transgenic regenerants. An analysis of the progeny of all transformed plants is now in progress, and only a limited number of R_1 plants from some lines have been analysed. Up to now, we have no indication for a non-Mendelian segregation or gene silencing of the *pat* or *uidA* marker genes in R_1 plants. From 1 transgenic plant containing 2 copies of the *pat* gene and 4 copies of the *uidA* gene, the progeny was analysed in more detail up to the fourth generation. In all of the generations the transgenes were stably expressed and inherited as single locus in a Mendelian fashion.

Recently, the first successful report on *Agrobacterium*-mediated transformation of maize was published by Ishida et al. (1996). In this report, immature embryos of the same developmental stage as those used in this report were infected with an *Agrobacterium* strain containing additional *virB* and *virG* gene copies on the binary Ti-plasmid (Hiei et al. 1994). With this strain, a high transformation frequency (5–30%) was obtained when using embryos of maize inbred line A188. The frequency was lower (0.4–5.0%) when crosses of other inbred lines with A188 were used for bombardment, and no data about the transformation of genotypes other than A188 were shown. This report demonstrates that maize tissues which are competent for regeneration are susceptible for *Agrobacterium* transformation under defined circumstances. But it does appear that this method is limited to maize genotype A188. It would be very promising if other genotypes could be used and if similarly high transformation frequencies could be achieved.

Our results confirm that the scutellar tissue is a suitable target for DNA delivery by particle bombardment to obtain stable transgenic maize plants. We have improved this transformation system so that transgenic regenerants can be obtained at a high frequency after 10–12 weeks in all maize genotypes producing type-I callus. The introduced marker genes are inherited in a Mendelian fashion and are stably expressed. The use of scutellar tissues as primary explants for transformation substantially reduces the time required for the regeneration of transgenic plants by eliminating the need for establishing callus lines or suspension cultures.

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