AGROBACTERIUM-MEDIATED TRANSFORMATION OF SORGHUM

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INTRODUCTION

The first reports on *Agrobacterium*-mediated transformation of monocotyledonous were controversial because they provided neither convincing evidence of a stable integration of the transgenes into the plant genome nor of Mendelian segregation of transgenes in the progeny (Vasil, 1994). Recently researchers from Japan Tobacco Inc. reported the first irrefutable proof that *Agrobacterium* could be used to transform rice (Hiei et al. 1994). They produced a large number of transgenic plants and showed stable integration of the transgenes. Since then many other rice genotypes have been transformed (Aldemita and Hodges 1996, Rashid et al. 1996, Toki 1997, Wang et al. 1997) and the technique has been extended to maize (Ishida et al. 1996), barley (Tingay et al. 1997) and wheat (Cheng et al. 1997).

There are three reports that describe stable transformation of sorghum plants (Casas et al. 1993, 1997; Zhu et al. 1998) by particle bombardment using immature embryos or inflorescence. Although transgenic plants were produced the transformation efficiency was low. The selectable marker used during the transformation procedure was the *bar* gene, a gene known to confer resistance to the commercial herbicide Basta. The use of the *bar* gene presents a potential problem because transformed sorghum plants could cross with some wild relatives and transfer the herbicide resistance trait to weeds. On the other hand, there is no report of *Agrobacterium*-mediated transformation of sorghum.

Agrobacterium-mediated transformation has some significant advantages over other methods of transformation. These include the transfer of relatively large segments of DNA with defined ends, integration of a lower number of gene copies into plant chromosomes, and the absence of a requirement for protoplast culture (reviewed by Vasil, 1994). Furthermore, *Agrobacterium*-mediated transformation is much more efficient in simultaneously producing events with lower gene copy number and simpler insertion patterns than genes inserted by particle bombardment (Zuo-yo Zhao, personal communication, Pioneer HiBred International).

Work reported here demonstrates that *Agrobacterium*-mediated transformation of sorghum is feasible. Several factors that influenced transformation efficiency, such as sensitivity of sorghum explants to *Agrobacterium* infection, explant type, inoculation method and co-cultivation media, are discussed. The sensitivity of immature embryos to the *Agrobacterium* infection was considered the limiting step to increasing the transformation efficiency. GUS expression was evaluated as a tool for monitoring the transformation events and is shown to be expressed only in specific tissues in sorghum under the conditions of this study.

MATERIAL AND METHODS

Plant materials*:* Among several sorghum lines tested for tissue culture ability and *Agrobacterium* infection response, P898012 was selected for transformation. Immature embryos at milk stage of endosperm, usually 1.2 to 2.5 mm long, were used for inoculation. Pre-cultured immature embryos (1 to 5 days), immature inflorescence (0.5 to 5 cm) and callus derived from immature embryos and inflorescence were also tested as explants for transformation.

Bacterial strain and plasmid**:** *Agrobacterium tumefaciens*, strain LBA4404 (pTOK233) obtained from Japan Tobacco Inc. (Hiei et al. 1994) was used in all experiments. The "super virulent" binary vector pTOK233 contains the virB, virC and virG genes, a hygromycin-resistance gene (*hpt*), a kanamycin-resistance gene (*npt*) and a gene for β -glucuronidase (*gus*), which has an intron in the N-terminal region and is driven by the 35S promoter of cauliflower mosaic virus. This intron-*gus* allows expression only in plant cells, not in *Agrobacterium tumefaciens* cells. *Hpt* was used as a selectable marker.

Transformation. LBA4404 (pTOK233) was grown at 28°C for 3 days in YP medium (10g/L yeast extract, 5g/L NaCl, 5g/L peptone, 8g/L agar, and pH 6.8) supplemented with 50g/L hygromycin. The culture was then collected with a scoop and suspended at $O.D._{600}$ = 0.70 (Spectronic 21D, Milton Roy) in inoculation medium (IC), and used for inoculation within 30 minutes. Pluronic F-68 (0.03%) was added to IC medium in most of the experiments. For inoculation immature embryos were soaked in bacterial suspension for 30 min, blotted dry with filter paper to remove excess bacteria, plated on co-cultivation medium with acetosyringone (I6-As, Table 1), and left for 5 days at 25$_o$C for co-cultivation. In some experiments embryos in the inoculation medium were sonicated for 20 s. After co-cultivation the embryos were transferred to the I8+C medium for callus proliferation for 10 days. Calli were cut into small pieces of 1 to 3 mm and transferred to the first selection medium with 15 mg/L hygromycin (I8+15H+C) for two weeks in dark. Calli growing on selection were transferred to a second selection medium containing 25 mg/L hygromycin (S10+25H+C). Calli were subcultured every 2 weeks on S10+25H+C for approximately 4 months. After selection the hygromycin resistant calli were cultured in pre-regeneration medium (PR) medium for two weeks, and transferred to regeneration medium (R8) in Petri dishes until plants reached 2cm. Some plantlets required to be transferred to rooting medium (RT). Plants were then transferred to baby jars with R6 medium for further growth and transplanted to small pots in greenhouse.

Transgene expression: GUS activity was detected histochemically using the protocol described by Hiei et al (1994), and fluorogenically according to Jefferson (1987). GUS expression was evaluated in immature embryos and callus immediately after co-cultivation, and in roots, stem, leaves and inflorescence of transgenic plants.

Inheritance of HygR plants and gus in R0 and R1 progenies. R0 plants were self-pollinated, and 20 days after pollination seeds were harvested, sterilized, and the immature embryos extracted and plated in germination medium (R8), at 28$_o$C for 3-4 days. Germinated embryos were then transferred to R8+25H medium. Resistant and susceptible seedlings were scored 7 days after transferring. Presence of *gus* and *hpt* was detected in mature plants by Southern hybridization.

Table 1. Media used for tissue culture and inoculation.

Medium	Composition
IC	MS salts, N6 vitamins, 68.5 g/L sucrose, glucose 36g/L, 2 mg/L 2,4-D, 100µM acetosyringone, 0.03% Pluronic F-68, pH5.6.
I6As	MS salts, N6 vitamins, 30g/L sucrose, 10 g/L glucose, 2 mg/L 2,4-D, 10% coconut water, 8.0 g/L agar, 100µM acetosyringone, pH 5.4.
I8	MS salts. B5 vitamins, 30 g/L sucrose, 3.0 g/L proline, 2.0 g/L asparagine, 2 mg/L 2,4-D, 5.0 g/L agarose, pH 5.7
I8+C	I8 medium plus 250 mg/L cefotaxime.
I8+C+15H	I8 medium plus 250 mg/L cefotaxime and 15 mg/L hygromycin.
S10+C+25H	MS salts, B5 vitamins, 30 g/L sucrose, 3.0 g/L proline, 2.0 g/L asparagine, 0.5 mg/L kinetin, 2.0 mg/L 2,4-D, 5.0 g/L agarose, pH5.7.
PR	MS salts, B5 vitamins, 30 g/L sucrose, 0.25 mg/L kinetin, 0.5 mg/L IAA, 400 mg/L proline, 200 mg/L asparagine, 2.5 g/L gelrite, pH 5.7.
R6	MS salts, B5 vitamins, 20 g/L sucrose, 0.5 mg/L kinetin, 1.0 mg/L IAA, 400 mg/L proline, 200 mg/L asparagine, 2.5 g/L gelrite, pH 5.7.
R8+25H	MS salts, 15 g/L sucrose, 25 mg/L hygromycin, 2.5 g/L gelrite, pH 5.7.
RT	MS salts, 15 g/L sucrose, ½ B5 vitamins, 0.5 mg/L NAA, 0.5 mg/L IBA, 2.5 g/L gelrite, pH 5.7.

RESULTS

Four transgenic sorghum plants were regenerated from an experiment where 120 immature embryos were co-cultivated with *Agrobacterium*. Three plants grew up to maturity as normal fertile plants (Figure 1). Southern blot analysis and segregation ratio of T_0 progeny (15:1) showed that the all transformed plants originated from the same transformation event and had two copies of the transgene.

Effect of donor plant growing conditions

Immature embryo response depended on donor plant conditions. For example, immature embryos isolated from donor plants growing under sub-optimal conditions such as water stress and low temperature, did not grow well and often died after co-cultivation. On the other hand, immature embryos isolated from donor plants growing under good environmental conditions responded better on tissue culture and *Agrobacterium* infection treatments. This kind of embryo had better chance of survival after co-cultivation, and showed higher transient GUS expression.

Effect of explant

The choice of the explant to be transformed was of fundamental importance for transformation. We tested freshly isolated immature embryos, pre-cultured immature embryos, immature inflorescence and callus. Although inflorescences of many genotypes were very responsive to tissue culture they could not be infected with *Agrobacterium*. Immature inflorescence of most sorghum genotypes tested produced toxic compounds that impaired bacterial growth. Usually a halo without bacterial

growth was observed around the inflorescence.

Pre-cultured immature embryos and callus from immature embryos were a good target for transformation. Both explants recovered well after co-cultivation. However, transient GUS expression in pre-cultured immature embryos and callus was usually much lower than in freshly isolated immature embryos. For this reason pre-cultured immature embryos and callus were not used for further experiments. On the other hand, freshly isolated immature embryos usually showed higher transient expression and allowed better monitoring of the transformation events.

Effect of the method of inoculation and co-cultivation medium

The use of 0.03% Pluronic F-68 in the inoculation medium, and sonication of the immature embryos with *Agrobacterium* dramatically increased transient GUS expression in immature embryos observed after co-cultivation. However, most of the embryos with high GUS expression died after co-cultivation.

Embryo survival after co-cultivation was a limiting step for transformation, and it was strongly influenced by co-cultivation medium. Co-cultivation media was also very important for transient GUS expression. The use of 1/10 MS salts increased transient expression in freshly isolated immature embryos after co-cultivation but reduced embryo survival and callus formation. High levels of proline and asparagine promoted bacterial growth during co-cultivation, and decreased embryo survival and GUS expression. On the other hand, the addition of coconut water to the co-cultivation medium increased immature embryo growth and callus growth during the co-cultivation period (Table 2). Only immature embryos that had some growth during the co-cultivation time were able to form callus. The combination of coconut water in the co-cultivation medium and actively growing immature embryos was a key factor for embryo survival and callus formation after co-cultivation.

Table 2. Effect of co-cultivation media on GUS expression of immature embryos, *Agrobacterium* growth, embryo growth, and on the percentage of embryos that formed callus after co-cultivation.

Media composition	Embryos with blue spots* (%)	*Agrobacterium* growth during co-cultivation** (0 - 5)	Embryo growth during co-cultivation (0 - 5)	Embryos that formed callus after co-cultivation
1)Basic medium	19.6±18.7	3	2	0-10%
2)1/10 MS salts	46.2±35.9	1	0	0-5%
3)+ Proline and asparagine	0.0	5	3	0-5%
4)+ Coconut water	37.4±22.9	4	4	0-60%

Percentage of immature embryos with at least one blue spot. Average of five experiments 0 for no Agrobacterium or embryo growth, 5 to maximum growth. **Observations taken from several experiments. 1) Basic medium: MS salts, N6 vitamins, 30g/l sucrose, 5g/l glucose, 2 mg/l 2,4-D, 200 uM Acetosyringone, 8 g/l agar, pH 5.6. 2) MS salts were replaced by 1/10 MS salts. 3) Proline (3 g/l) and asparagine (2 g/l) were added to the basic medium. 4) Coconut water (100 ml/l) was added to the basic medium.

GUS expression

Transient GUS expression in freshly isolated immature embryos was not consistently

observed between experiments under the same conditions. Apparently the growth conditions in greenhouse and in the field affected transformation efficiency and/or GUS expression. The patterns of expression in freshly isolated immature embryos varied from single spots to large blue patches on the scutelum and edge of embryos. In transgenic plants GUS activity showed a large variation depending on the tissue that was analyzed. Inflorescence, particularly the young ones, showed levels of GUS activity almost 30 to 60-fold higher than leaves, stem and roots (Figure 1). This variation was also indicated by the histochemical assays. Young leaves still at rolled stage usually produced some blue color along the veins or close to the margins. Inflorescence always produced strong staining. The staining ranged from strong blue color in inflorescences smaller than 5 cm up to patches of blue color on the nodes of older inflorescences . Other floral tissue such as palea, gluma and ovary also showed GUS staining. In mature leaves, stem and roots GUS activity was only detected by the fluorogenic assay. No GUS activity was detected in non-transformed sorghum tissues.

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