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Agrobacterium-mediated sorghum transformation

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Abstract

Agrobacterium tumefaciens was used to genetically transform sorghum. Immature embryos of a public (P898012) and a commercial line (PHI391) of sorghum were used as the target explants. The *Agrobacterium* strain used was LBA4404 carrying a 'Super-binary' vector with *a bar* gene as a selectable marker for herbicide resistance in the plant cells. A series of parameter tests was used to establish a baseline for conditions to be used in stable transformation experiments. A number of different transformation conditions were tested and a total of 131 stably transformed events were produced from 6175 embryos in these two sorghum lines. Statistical analysis showed that the source of the embryos had a very significant impact on transformation efficiency, with field-grown embryos producing a higher transformation frequency than greenhouse-grown embryos. Southern blot analysis of DNA from leaf tissues of T_0 plants confirmed the integration of the T-DNA into the sorghum genome. Mendelian segregation in the T_1 generation was confirmed by herbicide resistance screening. This is the first report of successful use of *Agrobacterium* for production of stably transformed sorghum plants. The *Agrobacterium* method we used yields a higher frequency of stable transformation that other methods reported previously.

Introduction

Sorghum (*Sorghum bicolor* L.) was first domesticated in East Africa several thousand years ago and it ranks as the sixth most planted crop in the world, behind wheat, rice, maize, soybean and barley. In 1999, it was grown on over 40 million hectares worldwide. Sorghum is essentially grown on marginal lands mostly distributed in the developing areas of the world, such as Africa and Asia. Sorghum is plagued by diseases, especially in higher-yielding environments. Until recently, the improvement of sorghum for agronomic and quality traits, such as pest and disease resistance and grain protein quality, has been carried out by traditional plant breeding methods and improved culture management practices. Availability of a routine genetic transformation technology would facilitate improvement of sorghum. However, sorghum has been categorized as one of the more difficult plant species to manipulate for tissue culture and transformation (Zhu *et al.*, 1998). Sorghum tissue culture and plant generation have been successful with certain explants (Masteller and Holden, 1970; Ma and Liang, 1987; Cai *et al.*, 1987; Cai and Butler, 1990; Kaeppler and Pedersen 1997). Although work on transformation of sorghum began about a decade ago, much less success has been achieved than with other crops. The first report of genetic transformation of sorghum described the introduction of DNA into protoplasts by electroporation and selection of transformed cells, without achieving plant regeneration (Battraw and Hall, 1991). Bombardment was applied to sorghum transformation by Hagio *et al.* (1991) and a non-regenerable cell suspension was transformed. Casas *et al.* (1993) obtained the first transgenic sorghum plants with bombardment of immature embryos and later on they obtained transgenic plants using immature inflorescences (Casas *et al.*, 1997). Zhu *et al.* (1998) reported success in

sorghum transformation with biolistic bombardment. Godwin and Chikwamba (1994) reported inoculation of sorghum meristem tissue with *Agrobacterium*.

Recently, *Agrobacterium* has been successfully used in monocot transformation for species such as rice (Chan *et al.*, 1992, 1993; Hiei *et al.*, 1994), maize (Ishida *et al.*, 1996; Zhao *et al.*, 1998), wheat (Cheng *et al.*, 1997) and barley (Tingay *et al.*, 1997). In this present study, a public line (P898012) and a commercial elite line (PHI391) of sorghum were transformed genetically. To our knowledge, this is the first report to verify that *Agrobacterium* can be used to genetically transform sorghum and produce transgenic plants that can transmit the introduced gene to progeny in a Mendelian fashion.

Materials and methods

Plant material and transformation process

Two sorghum lines were used in this study: a public line, P898012 (provided by Dr John Axtell, Purdue University), and an elite line, PHI391, used in Pioneer Hi-Bred's commercial breeding program. All sorghum plants used for this work were grown either in a greenhouse located in Johnston, Iowa, or in the field in Taft, Texas, during the summer. Immature panicles were harvested 9–12 days after pollination. The panicles grown in Texas were shipped to Johnston, Iowa, in a cooler with blue ice packs by overnight shipping. The kernels were sterilized with 50% bleach and 0.1% Tween-20 for 30 min with vacuum. The kernels were rinsed with sterile water three times. Immature zygotic embryos (1.0–1.5 mm in length) were isolated from these kernels. The transformation process can be divided into 5 sequential steps: agroinfection, co-cultivation, resting, selection, and plant regeneration. The compositions of all media used in this study are shown in Table 1. The freshly isolated embryos were immersed in an *Agrobacterium* suspension at either 1×10^9 cfu/ml (OD = 0.7 at 550 nm) or 0.5×10^9 cfu/ml in PHI-I medium for 5 min for the agroinfection process. For all subsequent culture steps, the embryos were cultured with the scutellar side up and facing away from the medium. The immature embryos were cultured on PHI-T medium in the dark at 25 °C for 3 or 7 days for the co-cultivation step. For the resting step the embryos were cultured on PHI-T medium (without acetosyringone) plus 100 mg/l carbenicillin for 0 or 4 days. Next, the embryos were moved to PHI-U medium for 2 weeks to initiate the selection step then moved to PHI-V medium for the remainder of the selection process. The resting step and the whole selection process were maintained at 28 °C in the dark. The subculture interval was typically 2 weeks; however, if the sorghum tissue produced more phenolic pigment, the subculture interval was reduced to 5–7 days (Cai and Butler, 1990). For plant regeneration, the herbicide-resistant callus was cultured at 28 °C in the dark on PHI-W medium for 2–3 weeks to increase the putative transformed callus and to develop somatic embryos, and then cultured on PHI-X medium for 2–3 weeks to develop shoots. When shoots started to appear, cultures were moved to a room under conditions of 16 h light (270 μE m^{-2} s^{-1}) and 8 h dark at 250 °C. Shoots (about 3–5 cm tall) were moved to plastic boxes (10 cm × 9 cm × 10 cm) containing either PHI-Z medium if the shoots had good roots; or PHI-FA medium if the shoots had no or poor roots under the same light and temperature conditions. Each box contained shoots derived from a single embryo. When the plantlets were about 8–10 cm tall with healthy roots, they were transferred to pots with Universal Mix (Strong-Lite, Seneca, IL) in a greenhouse. A transgenic event in this study was defined as a T_0 plant(s) derived from a single embryo showing resistance to 1% Ignite by leaf painting (Casas *et al.*, 1993) and/or T-DNA integration into the sorghum genome by Southern blotting.

Eight protocols, Treatments A–H (Table 2), were tested in stable transformation experiments on P898012. Treatment A was used as the basic or standard protocol. One or more of the conditions in Treatment A was modified for each of the other Treatments B–H. Treatment A vs. Treatment B and Treatment C vs. Treatment D were used for comparing *Agrobacterium* concentrations. Treatment A vs. Treatment C and Treatment B vs. Treatment D were used for comparing the effect of a resting step on transformation efficiency. To increase T-DNA delivery, double agroinfection/co-cultivation (Treatment E) and wounding of sorghum tissue by bombardment prior to agroinfection (Treatment G) (Bidney *et al.*, 1992) were tested. For double agroinfection/co-cultivation, fresh embryos were infected with *Agrobacterium* (0.1×10^9 cfu/ml), co-cultivated for 3 days and then infected a second time with *Agrobacterium* (0.5 or 1.0×10^9 cfu/ml), followed by co-cultivation for another 4 days under the same conditions described above. The embryos were then cultured on selection medium. For wounding by bombardment, fresh em-

Table 1. Composition of media used.

Media	Composition
pHI-I	4.3 g/l MS salts (GIBCO BRL catalog no. 11117-874), 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 1 mg/l thiamine HCl, 0.1 g/l myo-inositol, 1 g/l vitamin assay casamino acids, 1.5 mg/l 2,4-D, 68.5 g/l sucrose, 36 g/l glucose, pH 5.2. Add 100 μM acetosyringone before using.
PHI-T	PHI-I with reducing sucrose to 20 g/l and glucose to 10 g/l, increasing 2,4-D to 2 mg/l, adding 0.5 g/l MES buffer, 0.7 g/l L-proline, 10 mg/l ascorbic acid, 100 μM acetosyringone and 8 g/l agar, pH 5.8.
PHI-R	PHI-T with 30 g/l sucrose, 150 mg/l asparagine, 100 ml/l coconut water and 5.3 g/l Sea Kem agarose; without glucose, proline, acetosyringone and agar.
PHI-U	PHI-T with 1.5 mg/l 2,4-D, 100 mg/l carbenicillin, 5 mg/l PPT, without glucose and acetosyringone.
PHI-V	PHI-U with 10 mg/l PPT.
PHI-W	PHI-U with 0.5 mg/l kinetin.
PHI-X	4.3 g/l MS salts, 0.1 g/l myo-inositol, 5.0 ml MS vitamins stock* solution, 0.5 mg/l zeatin, 700 mg/l proline, 60 g/l sucrose, 1 mg/l IAA, 0.1 μM ABA, 0.1 mg/l thidiazuron, 5 mg/l PPT, 100 mg/l carbenicillin, 8 g/l agar, pH 5.6.
PHI-FA	2.15 g/l MS, 0.05 g/l myo-inositol, 2.5 ml MS vitamins stock solution, 20 g/l sucrose 0.5 mg/l NAA, 0.5 mg/l IBA, 7 g/l agar, pH 5.6.
PH-Z	PHI-FA without NAA and IBA.

*MS vitamins stock contains 0.1 g/l nicotinic acid, 0.1 g/l pyridoxine HCl, 0.02 g/l thiamine HCl and 0.4 g/l glycine.

bryos were cultured on PHI-R medium for 5 or 7 days and then bombarded with tungsten particles (Casas *et al.*, 1993) with no DNA attached. The bombarded tissue was immediately infected with *Agrobacterium* suspension and co-cultivated as in Treatment A. To test the transformation capability of cultured embryos, fresh embryos were cultured on PHI-R for 5 or 7 days prior to agroinfection (Treatment F). To test the impact of PVPP (polyvinylpolypyrrolidone) for sorghum transformation, 1% PVPP was added to PHI-T for co-cultivation (Treatment H). Treatments B and D (plus 1% PVPP in PHI-T medium) were used in stable transformation experiments on PHI391.

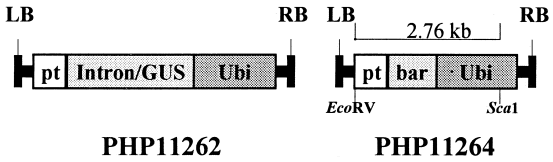

Figure 1. Maps of the T-DNA regions used in sorghum transformation. PHP11262 containing the intron/GUS plant expression cassette and PHP11264 containing the *bar* plant expression cassette inserted between the T-DNA borders in pSB11. Those two restriction sites used in DNA blots and the fragment (2.76 kb) generated with these two restriction enzymes were illustrated for PHP11264. RB, right border; LB, left border; Ubi, maize ubiquitin promoter; pt, pinII terminator; bar, *bar* coding region; Intron/GUS, GUS coding region with intron insertion.

Agrobacterium *strain and vector*

A. tumefaciens LBA4404 and vectors pSB1 and pSB11 (Komari, 1990; Komari *et al.*, 1996) were obtained from Japan Tobacco Inc. A DNA fragment containing the maize ubiquitin (Ubi) promoter (Christensen *et al.*, 1992) driving a *bar* gene (Thompson *et al.*, 1987) was inserted between the T-DNA borders in pSB11. The *pin*-II (An *et al.*, 1989) terminator was ligated downstream of the *bar* coding sequence to construct the transformation vector PHP11264. In another vector, PHP11262, the *bar* gene was replaced with an intron-GUS gene (Zhao *et al.*, 1998; Ohta *et al.*, 1990). Intron-GUS is a GUS gene in which the second intron from the potato *ST LSI* gene (Vancanneyt *et al.*, 1990) has been inserted into the coding region to eliminate expression of this marker gene in *Agrobacterium*. The T-DNA regions of these two vectors are shown in Figure 1. To verify GUS expression, histochemical stain of the sorghum tissue with X-gluc was used (McCabe *et al.*, 1988).

Table 2. Conditions tested in stable transformation experiments.

Treatment	Conditions
A	Fresh embryos with agroinfection at 1×10^9 cfu/ml, co-cultivation 7 days, no resting and selection with PHI-U and PHI-V
B	Same conditions as Treatment A except for agroinfection at 0.5×10^9 cfu/ml
C	Same conditions as Treatment A except for co-cultivation 3 days and resting 4 days
D	Same conditions as Treatment C except for agroinfection at 0.5×10^9 cfu/ml
E	Fresh embryos, first agroinfection and co-cultivation 3 days and then second agroinfection and co-cultivation another 4 days and same conditions as Treatment A for other steps
F	Fresh embryos cultured with PHI-R for 5 to 7 days and then agroinfection and other steps with same conditions as Treatment A
G	Fresh embryos cultured with PHI-R for 5 or 7 days and wounded by bombarment, then agroinfection and other steps with same conditions as Treatment A
H	Same conditions as Treatment A except for addition of 1% PVPP in PHI-T medium

DNA blot hybridization

T_0 plants from 67 stably transformed events (51 events in P898012 and 16 events in PHI391) randomly selected from the 131 events in total obtained in this study were used for DNA blot analysis. Sorghum genomic DNA was isolated from leaves of T_0 plants by the method described (Richards, 1997) and was quantitated fluorometrically (Labarca and Paigen, 1980). The DNA was digested with *Hpa*I for *bar* integration analysis and was digested with *Sca*I/*Eco*RV for analysis of the T-DNA insert. A fragment (567 bp) of the *bar* gene sequence generated by PCR (Kramer and Coen, 1997) was used as the probe for Southern blotting. The probe was labeled with P32-CTP (Feinberg and Vogelstein, 1983, 1984). A standard procedure (Johnson *et al.*, 1984) for DNA blotting was used and the DNA bands were visualized with a Molecular Dynamics Storm Phosphor Imaging System. The images were electronically captured with Molecular Dynamics ImageQuant software.

Statistical analysis

The P898012 stable transformation data were analyzed as a one-way completely randomized design with the SAS statistical software Mixed Procedure (Montgomery, 1997). The measured outcome used in all analyses was the proportion of transformants obtained from each replicate. Treatments were labeled A–H including both greenhouse and field data. To stabilize the variance in the analysis, the transformation $\sin^{-1}(\sqrt{Y})$ was used. Each treatment mean was compared to the same standard (Treatment A) using Dunnett's test (SAS/STAT software menu, 1996). Additional treatment mean comparisons were done using contrast statements. The significance level used for all statistical tests was 0.05.

Results

Tests of transformation parameters in P898012

Sorghum line P898012 is capable of producing good quality callus (Kaeppler and Pedersen, 1997; Casas *et al.*, 1993). In this study, prior to initiating experiments on stable transformation, we performed preliminary experiments using P898012 as a model line in order to test a number of parameters involved in *Agrobacterium*-mediated transformation of sorghum. These parameters included the effect of media on callus formation and maintenance, the effect of antibiotics on sorghum cultures, the effect of *Agrobacterium* concentration and co-cultivation time on delivery of T-DNA into embryos, and the effect of agroinfection on callus response. The results of these initial tests enabled us to determine more rapidly optimal conditions and provided a baseline for the conditions used in subsequent experiments on stable transformation of sorghum.

One of the most critical factors in making our transformation protocol successful is the efficient formation and maintenance of good-quality callus from the embryos. To this end, we tested the effect of different media on callus formation using control (no agroinfection) immature embryos. In our initial tests comparing basic MS-based (Murashige and Skoog,

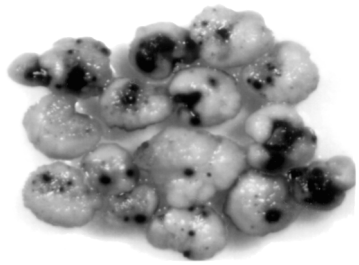

Figure 2. GUS gene transient expression in sorghum embryos of P898012. Sorghum embryos were agroinfected (1×10^9 cfu/ml) with LBA4404(PHP11262), co-cultivated for 7 days, and then stained with X-gluc overnight.

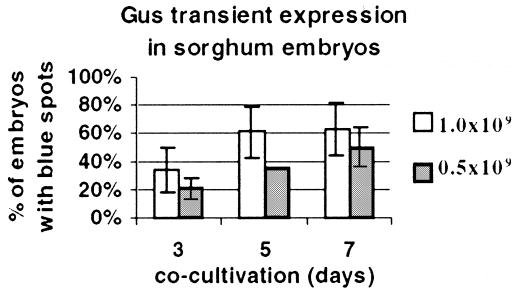

Figure 3. The percentage of sorghum embryos with blue spots stained with X-gluc after agroinfection with 1.0×10^9 and 0.5×10^9 cfu/ml of LBA4404(PHP11262) and 3, 5 and 7 days co-cultivation in P898012.

1962) and N6-based (Chu *et al.*, 1975) media, we found that N6 not only decreased callus response from embryos (76% with MS vs. 20% with N6) but also increased the production of phenolic pigments, so MS salts were used in all media throughout the rest of the study. A series of media was then created based on MS (Table 1) designed for specific steps in *Agrobacterium* transformation. PHI-R, PHI-T, and PHI-U (without PPT) were tested individually for callus response with the control immature embryos and each was shown to be capable of supporting callus response from 95–100% of the embryos tested.

Carbenicillin is commonly used in *Agrobacterium*-mediated plant transformation to eliminate *Agrobacterium* upon inoculation (Shackelford and Chlan, 1996). Antibiotics can also have a detrimental effect on plant tissue cultures (Pollock *et al.*, 1983; Lin *et al.*, 1995; Holford and Newbury, 1992) so we evaluated the effect of carbenicillin on sorghum cultures. PHI-R medium and PHI-T (without acetosyringone) medium each with or without 100 mg/l carbenicillin were used. Uninfected control embryos were cultured on these media for 1 month and callus initiation frequency, callus quality and callus growth rate were compared. The results showed there was no obvious difference among these media (callus response greater than 95% with or without carbenicillin), and no negative effect of carbenicillin was observed. Carbenicillin at 100 mg/l was used in all of the subsequent stable transformation experiments in this study.

Transient GUS gene expression was used to monitor T-DNA delivery into sorghum embryos after *Agrobacterium* infection and co-cultivation of immature embryos (Figure 2). LBA4404 (PHP11262) at either 1×10^9 or 0.5×10^9 cfu/ml and three different co-cultivation times (3, 5 or 7 days) were used. GUS gene expression in embryos ranged from 21 to 63% by X-gluc staining after *Agrobacterium* inoculation for either concentration (Figure 3), while no GUS expression was observed in the uninoculated control embryos. The percentage of embryos with visible blue spots increased with the higher concentration (1×10^9 cfu/ml) of *Agrobacterium*. The percentage of embryos with blue spots also increased, and the intensity of the blue spots was greater, with longer co-cultivation times at both *Agrobacterium* concentrations. The results showed that the conditions tested here for agroinfection and co-cultivation were effective for T-DNA delivery and expression in sorghum embryos. To test the relationship of GUS transient expression to the efficiency of stable transformation in this sorghum line, these two *Agrobacterium* concentrations and two co-cultivation times (3 and 7 days) were used in stable transformation experiments.

Plant tissue damage caused by agroinfection has been reported (Pu and Goodman, 1992; Hansen, 2000). It is one of the major obstacles for *Agrobacterium*-mediated plant transformation. After agroinfection and co-cultivation, the embryos were cultured on resting medium for about 2 weeks and the resulting cultures compared to cultures from uninfected embryos to assess potential damage due to *Agrobacterium*. After agroinfection of P898012, the production of pigment was increased significantly compared to non-agroinfected control cultures. Some of the callus showed necrosis, but the majority of the embryos still could initiate good-quality callus. However, in the continued presence of high concentrations of phenolics, the callus fails to propagate efficiently. To reduce the toxicity of the phenolic pigment to the plant cells (Cai *et al.*, 1987), short subculture intervals (5–7 days) and the addition of 1% PVPP into the culture medium were tested and both showed promotion of fresh callus growth. Therefore, these two condi-

tions were incorporated into our stable transformation experiments. Short subculture intervals were incorporated into all transformation treatments and PVPP was tested in transformation experiments.

Based on these results and our previous experience with *Agrobacterium*-mediated transformation of maize (Zhao *et al.*, 1998) and the experience of sorghum tissue culture (Cai *et al.*, 1987; Cai and Butler, 1990), we designed several different protocols, Treatments A–H, for evaluation in stable transformation experiments (Table 2). Prior to initiating those experiments, we tested the effect of certain combinations of media and conditions on callus response with uninfected control embryos (Table 3). Combination 1 was designed to test the basic media and conditions used in Treatment A, B, E and H; combination 2 to test the basic media and conditions used in Treatments C and D; combination 3 to test the basic media and conditions used in Treatment F; and combination 4 to test the effect of wounding by bombardment used in Treatment G. The results in Table 3 indicate that the first three combinations were highly effective for callus induction and maintenance in P898012. Combination 4 which included wounding by bombardment showed a somewhat reduced efficiency of callus response (74%) vs. the same treatment without wounding (91% for combination 3).

Stable transformation in P898012

We then tested a variety of conditions in stable transformation experiments, including *Agrobacterium* concentration, length of co-cultivation and resting periods, single or double agroinfection/co-cultivation, freshly isolated embryos or cultured embryos, wounding by bombardment prior to agroinfection, adding 1% PVPP in co-cultivation medium, and the source of embryos. All of the initial experiments to determine optimal conditions for stable transformation were performed with embryos from greenhouse-grown plants. After plant regeneration, herbicide painting of leaf tissue of the T_0 plants and DNA blot analysis (Figure 4) of some of the plants, the stable transformation of sorghum line P898012 was confirmed. The observed transformation frequencies varied with different conditions (Table 4). For Treatments A, B, C, D, F and H, we were able to recover transformants in the range of 0.95–2.34%. We saw no statistical difference among these six treatments. These six treatments were also compared to Treatments E and G which had given transformation frequencies of 0.17% and 0.25%. All six treatments had higher observed transformation frequencies than E and G and were statistically significant. Stably transformed events were obtained from both infected fresh embryos (all treatments except Treatments F and G) and infected cultured embryos (Treatment F). No transformed callus was recovered from 296 uninfected control embryos.

We found that the source of the embryos had a significant effect on transformation frequency. Both field-grown and greenhouse-grown embryos were evaluated in Treatments B, C and D. The following transformation frequencies were obtained: Treatment B, 9.7% for field-grown vs. 1.1% for greenhouse-grown; Treatment C, 10.1% for field-grown vs. 2.17% for greenhouse-grown; Treatment D, 7.4% for field-grown vs. 0.95% for greenhouse-grown. The statistical analysis showed the transformation frequencies with field-grown embryos were significantly higher than those with greenhouse-grown embryos in all three of these treatments (Table 5).

In summary, 115 transgenic events were obtained in P898012 from all of the treatments (5890 infected embryos) used in this entire study.

Stable transformation in PHI391

PHI391 is a sorghum line used currently in Pioneer Hi-Bred's commercial breeding program. Prior to performing stable transformation experiments on PHI391, we performed similar preliminary experiments to those described above for P898012. Under the same conditions as used for P898012, PHI391 clearly showed lower GUS transient expression and less tolerance to *Agrobacterium* damage than did P898012. Based on the results from these preliminary tests on PHI391 and from the stable transformation experiments on P898012, we chose the following conditions to use for stable transformation of PHI391: (1) Treatments B or D plus 1% PVPP in PHI-T medium; and (2) field-grown embryos. From 80 agroinfected embryos with Treatment B (plus 1% PVPP in PHI-T medium), 5 stably transformed events were recovered, giving a transformation frequency of 6.3%. From 205 agroinfected embryos with Treatment D (plus 1% PVPP in PHI-T medium), 11 stable events were recovered, resulting in a transformation frequency 5.4%. In summary, a total of 16 transgenic events were obtained from 285 agroinfected embryos on PHI391.

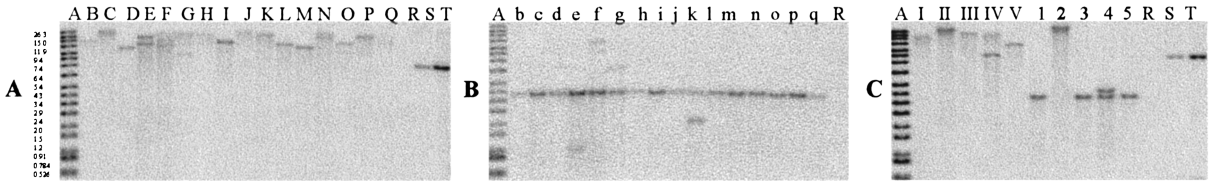

Figure 4. DNA blots of genomic DNA from transgenic sorghum T_0 plants probed with the *bar* gene. A. A T-DNA integration blot of P898012; lane A, RFLP Ladder System (Life Technologies, catalog 10283-018); lanes B–Q, DNA from T_0 plants derived from 16 individual embryos digested with *Hpa*I (no *Hpa*I site within the T-DNA borders of PHP11264); lane R, DNA from an untransformed sorghum plant; lanes S and T, 1 copy and 5 copies of a linear plasmid DNA (6244 bp) containing the *bar* gene. B. A T-DNA insert blot of P898012; lanes b–q, DNA from the same plants as lanes B–Q in blot A were digested with *Sca*I/*Eco*RV; other lanes the same as in blot A. C. A T-DNA integration and T-DNA insert blot of PHI391; lanes I–V, DNA from T_0 plants derived from 5 individual embryos of PHI391 digested with *Hpa*I for T-DNA integration assay; lanes 1–5, DNA from the same 5 T_0 plants digested with *Sca*I/*Eco*RV for T-DNA insert assay, other lanes the same as in blot A.

Table 3. Tests of combined media and associated conditions in P898012.

Combination	Media and associated conditions	Response embryo/ total embryo (%)
1	1 week with PHI-T and 3 weeks with PHI-U without PPT	182/184 (99)
2	3 days with PHI-T, 4 days with PHI-T (without acetosyringone) plus 100 mg/l carbenicillin and 3 weeks with PHI-U without PPT	34/35 (97)
3	1 week with PHI-R, 1 week with PHI-T and 2 weeks with PHI-U without PPT	234/256 (91)
4	1 week with PHI-R, wounded by bombardment, 1 week with PHI-T and 2 weeks with PHI-U without PPT	31/42 (74)

Table 4. Stable transformation with greenhouse-grown embryos in P898012.

Replicate	*bar*$^+$ events/embryos inoculated (%)							
	Treatment							
	A	B	C	D	E	F	G	H
1	1/88 (1.1)	1/93 (1.1)	2/155 (1.3)	1/27 (0.8)	0/76 (0.0)	1/48 (2.1)	0/119 (0.0)	4/103 (3.9)
2	1/104 (1.0)		1/28 (0.8)	1/91 (1.1)	0.84 (0.0)	1/70 (1.4)	0/120 (0.0)	3/117 (2.6)
3	2/90 (2.2)		1/23 (0.8)		0.100 (0.0)	1/92 (1.1)	1/103 (1.0)	3/195 (2.2)
4	1/95 (1.1)		2/123 (1.6)		1/84 (1.2)	3/110 (2.7)	0/100 (0.0)	1/96 (1.0)
5	1/33 (0.8)		1/162 (0.6)		0/85 (0.0)	1/227 (0.4)		1/50 (2.2)
6	2/47 (4.3)		1/148 (0.7)		0/93 (0.0)	1/94 (1.1)		
7	1/53 (1/9)		21/22 (1.6)		0/100 (0.0)			
8	1/117 (0.9)		1/30 (0.8)					
9	2/108 (1.9)		5/76 (6.6)					
10	2/118 (1.7)		2/51 (3.9)					
11	2/229 (0.9)		1/144 (0.7)					
12	2/121 (1.7)		2/30 (6.7)					
13	1/96 (1.0)							
$\bar{x}$	1.58%	1.1%	2.17%	0.95%	0.17%	1.47%	0.25%	2.34%
P*	NA	1.0000	0.9984	0.9980	0.0002	1.0000	0.0081	0.9035

*P=Dunnett's *P*-value, all treatments compared to Treatment A.

Table 5. Stable transformation with field-grown embryos in P898012

Treatment	Replicate	*bar*+ events/embryos inoculated (%)	*P* value*
B	1	6/62 (9.7)	0.0026
C	1	18/179 (10.1)	0.0004
D	1	17/177 (9.6)	0.0002
	2	4/83 (4.8)	
	3	5/63 (7.9)	

*Comparing the same treatments in Table 4 and Table 5 for statistical analysis.

Analysis of T_0 and T_1 plants

All of the regenerated T_0 plants were grown in the greenhouse for maturation. All of these plants were painted with 1% of Ignite (Casas *et al.*, 1993) to verify *bar* expression and all of them showed resistance to Ignite painting while untransformed controls showed necrosis upon painting. DNA blot analysis was performed with the leaf samples collected from 67 T_0 plants representing 51 embryo-derived events on P898012 and 16 embryo-derived events on PHI391. All of these T_0 plants showed the integration of the *bar* gene into the sorghum genome (Figure 4). Based on the integration pattern and number of insertions, these T_0 plants can be divided into two groups. Group 1 (48 events representing 72% of the analyzed events) showed a single insertion site with no apparent rearrangement. Group 2 (19 events or 28% of the analyzed events) showed multiple insertions or a single insertion with rearrangement. About 100 T_1 seeds from 5 independent events were planted and 1% Ignite was applied to the T_1 plants to identify *bar* gene segregation in T_1 generation. These T_1's showed typical Mendelian segregation ratio, i.e. 3 Ignite-resistant to 1 Ignite-susceptible plants in the T_1 generation derived from self-pollinated T_0 plants.

Discussion

Genetic transformation of sorghum has been achieved through *Agrobacterium*-mediated DNA delivery. A total of 131 stably transformed events, each derived from a single embryo, were obtained from 6175 agroinfected embryos in two sorghum genotypes, P8980112 and PHI391. The overall transformation frequency was about 2.1%, but because this is an average frequency across all treatments, including evaluation of different protocols and conditions, it is likely that a higher transformation frequency could be obtained routinely under a more optimal set of conditions. DNA blots and genetic analysis confirmed that the T-DNA was integrated into the sorghum genome and transmitted to the next generation. The transformation efficiency reported here for *Agrobacterium* is much higher than what has been reported thus far for bombardment-mediated transformation of sorghum (Casas *et al.*, 1993; Zhu *et al.*, 1998). In addition, the majority (72%) of the DNA blot analyzed events showed a single T-DNA insertion in the sorghum genome with no rearrangement. This pattern is very similar to the T-DNA insertion pattern documented in *Agrobacterium*-mediated transformation of maize (Ishida *et al.*, 1996; Zhao *et al.*, 1998).

Prior to our initiating stable transformation experiments, we first used preliminary assays to determine the best components and conditions to use for stable transformation. These assays focused mainly on four parameters: media, antibiotics, T-DNA delivery, and effect of agroinfection on callus response. We found these preliminary tests to be particularly useful in rapidly identifying factors that have either a positive or negative effect without needing to go through the more time-consuming process of stable transformation. Transient gene expression in plant tissues is a very useful tool for verifying that T-DNA delivery has occurred to plant cells and for monitoring conditions for agroinfection. However, we have found that the level of transient gene expression (e.g., GUS) resulting from agroinfection of sorghum embryos is not necessarily directly correlated with the frequency of stable transformation. Higher concentrations of *Agrobacterium* or longer co-cultivation result in a high percentage of GUS-expressing embryos, but in stable transformation experiments of P898012, statistical analysis showed that these conditions did not alter transformation frequency significantly. For example, transformation frequency with Treatment C (2.17%) was about twice higher than Treatment D (0.95%); statistical analysis showed this difference was not significant. However, we did observe that a higher concentration (1×10^9 cfu/ml) of *Agrobacterium* resulted in serious damage of the embryo tissue upon *Agrobacterium* inoculation on PHI391. A lower concentration of *Agrobacterium*, such as 0.5×10^9 cfu/ml, was more suitable for stable transformation in this line. Perhaps the most critical parameter is callus response to agroinfection. *Agrobacterium* is a plant pathogen and is capable of inducing plant necrosis (Pu and

Goodman, 1992; Hansen, 2000), but little is known about the mechanism of plant responses on transformation of plant cells (Aronen, 1997). Studies in other plant species (Perl *et al.*, 1996) have described the use of antioxidants with *Agrobacterium*-mediated transformation. Our preliminary assays have enabled us to identify a negative effect of agroinfection, and a protective effect of the antioxidant PVPP on sorghum callus response, although statistical analysis indicated that adding 1% PVPP in the co-cultivation medium did not increase transformation frequency significantly on PH898012. For further studies, with more replicates per treatment, and with the number of replicates being more balanced across treatments, it may be possible to identify specific conditions that improve the stable transformation frequency. The source (greenhouse vs. field) of embryos had a large impact on transformation efficiency. We also observed this phenomenon in our work on other crops such as maize. It is not currently understood how the physiological state of the plant and/or embryo affects the transformation process.

The public sorghum cultivar P898012 is well adapted in Niger and Sudan because it has both pre-flowering and post-flowering drought resistance (Casas *et al.*, 1993). The sorghum inbred PHI391 is currently used in a commercial breeding program in the US. We have demonstrated that both lines are capable of being transformed routinely with *Agrobacterium*. Although even higher transformation efficiencies can probably be obtained with further development of the technology, we believe the current frequencies are already sufficient to enable the genetic improvement of sorghum with agronomically useful genes by *Agrobacterium*-mediated transformation. The present study has provided a useful basis for further improvements in transformation of sorghum by *Agrobacterium.*

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