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Transgenic sorghum plants via microprojectile bombardment

(*Sorghum bicolor*/gene transfer/particle gun)

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ABSTRACT **Transgenic sorghum plants have been obtained after microprojectile bombardment of immature zygotic embryos of a drought-resistant sorghum cultivar, P898012. DNA delivery parameters were optimized based on transient expression of *R* and *C1* maize anthocyanin regulatory elements in scutellar cells. The protocol for obtaining transgenic plants consists of the delivery of the *bar* gene to immature zygotic embryos and the imposition of bialaphos selection pressure at various stages during culture, from induction of somatic embryogenesis to rooting of regenerated plantlets. One in about every 350 embryos produced embryogenic tissues that survived bialaphos treatment; six transformed callus lines were obtained from three of the eight sorghum cultivars used in this research. Transgenic (T_0) plants were obtained from cultivar P898012 (two independent transformation events). The presence of the *bar* and *uidA* genes in the T_0 plants was confirmed by Southern blot analysis of genomic DNA. Phosphinothricin acetyltransferase activity was detected in extracts of the T_0 plants. These plants were resistant to local application of the herbicide Ignite/Basta, and the resistance was inherited in T_1 plants as a single dominant locus.**

Sorghum (*Sorghum bicolor* L. Moench) is an important grain and forage crop that is uniquely adapted to semiarid environments. It is typically the cereal grown in areas where the extremes of high temperature and low soil moisture are unsuitable for maize. In 1991 sorghum was fifth in production amongst all cereals with 58 million metric tons harvested on 45 million hectares of land (1). It is a primary staple in the semiarid tropics of Africa and Asia for over 300 million people. These are evidentiary statistics of the significance of this crop in these regions, which are predominated by subsistence agriculture. In the western hemisphere sorghum is used primarily as livestock feed (2). The development of hybrid varieties of sorghum in the 1950s contributed substantially to the increase in production in the United States. Presently, sorghum is third amongst cereals in U.S. production and is the preferred crop in areas of low water availability because of its yield stability under drought conditions.

Advances in biotechnology are now beginning to be used to augment traditional approaches for crop improvement. Restriction fragment length polymorphism (RFLP) linkage maps are being constructed that should greatly facilitate plant breeding efforts for marker-assisted backcross programs (3, 4) and, in the near future, may be used to clone agriculturally important genes through the use of map-based cloning strategies (5). To this date, however, there are no programs in sorghum to access the pool of genes that are available as the result of genetic engineering research, because of the lack of a transformation system. Transformation of protoplasts by electroporation (6) or cell suspensions by microprojectile bombardment (7) has resulted in stable expression of transferred genes; however, transgenic plants were not obtained.

Microprojectile bombardment as a method to introduce DNA into cells circumvents two major constraints of cereal transformation. These are the lack of an available natural vector such as *Agrobacterium tumefaciens* and the difficulty to regenerate plants when protoplasts are used for transformation. Particle bombardment can target cells within tissues or organs that have high morphogenic potential. Immature or mature zygotic embryos (8–10), immature inflorescences (11, 12), and shoot tips (13) of sorghum exhibit embryogenic competence.

In this paper, we describe a procedure to produce transgenic sorghum plants. To date, transformation systems have been described for cereals of major agricultural importance including maize (14, 15), oat (16), rice (17, 18), and wheat (19). Notable exceptions among important cereals in the world are barley and sorghum. Our research has defined parameters for the optimization of DNA delivery to immature zygotic embryos by microprojectile bombardment, identified an appropriate selection agent, bialaphos, and established a selection protocol to recover transformed embryogenic callus and transgenic (T_0) plants. Herbicide resistance was transferred to the T_1 progeny.

MATERIALS AND METHODS

Plant Material and Tissue Culture. Sorghum genotypes representing a range of genetic backgrounds and a variety of agronomic types were used in this research. These included a high-tannin (IS4225), three food grain (CS3541, M91051 and Tx430), and two drought-resistant (P898012, P954035) cultivars, as well as a *Striga*-resistant (SRN39) and a Kaoliang sorghum (Shanqui red) cultivar.

For isolation and culture of immature embryos, the procedure of T. Cai and L.G.B. (unpublished data) was utilized. Immature zygotic embryos (12–15 days after pollination) were isolated and cultured onto a basal medium containing Murashige and Skoog (20) salts, modified B5 vitamins (ref. 21; without the addition of calcium panthotenate) and agar (8 mg/ml; Taiyo bacteriological grade) supplemented with asparagine (150 μg/ml), 10% coconut water, 2,4-dichlorophenoxyacetic acid (2 μg/ml), and sucrose (30 mg/ml) (I_6 medium) for induction of embryogenesis and initiation of embryogenic callus. Procedures for selection and maintenance of embryogenic tissue and shoot and root formation from organized structures were as described (12). Media contained the basal constituents supplemented with 2,4-dichlorophenoxyacetic acid (2 μg/ml), kinetin (0.5 μg/ml), sucrose (30 mg/ml) for maintenance of embryogenic tissue or indole-3-

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Abbreviations: GUS, β-glucuronidase; PAT, phosphinothricin acetyltransferase.

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acetic acid (1 μg/ml), kinetin (0.5 μg/ml), and sucrose (20 mg/ml) to facilitate shoot development. Plantlets (about 2 cm in height) were transferred to medium containing Murashige and Skoog salts (all at 0.5 × concentration), 1-naphthaleneacetic acid (0.5 μg/ml), indole-3-butyric acid (0.5 μg/ml), sucrose (20 mg/ml), and agar (8 mg/ml) to allow root development. Bialaphos and indole-3-acetic acid were filter (0.2-μm pore diameter) sterilized and added to cooled media (40–50°C). Cultures of immature embryos and embryogenic tissue were grown in darkness and recultured every 2 weeks and were maintained at 26°C. Tissues on shoot regeneration medium were subcultured every 4 weeks and grown at 26°C under a 16-hr photoperiod (1000–2000 lx from fluorescent, cool white light).

Plasmids. The plasmids used in this research, pPHP620 (8.537 kb) and pPHP687 (9.056 kb), were provided by Pioneer Hi-Bred International. In these plasmids (pUC18) all marker genes were driven by a double cauliflower mosaic virus 35S promoter (22), with the ΩRNA leader sequence (23) and the first intron of the maize alcohol dehydrogenase gene (24). The plasmid pPHP620 contains the reporter gene *uidA* (25), encoding β-glucuronidase (GUS), and the selectable marker gene *bar* (26), encoding phosphinothricin acetyltransferase (PAT). pPHP687 contains the maize anthocyanin regulatory elements *R* (27) and *C1* (28).

Microprojectile Bombardment. All experiments were conducted with the Biolistics PDS 1000/He system (29) using tungsten (M-25, 1.7 μm in diameter, DuPont no. 75056) or gold (1.5–3.0 μm in diameter, Aldrich no. 32,658-5) microprojectiles. Gold (3 mg) or tungsten (0.75 mg) particles (previously washed in ethanol) in aqueous suspension (50 μl) were coated with 5–10 μg of plasmid DNA, as described by the manufacturer (Bio-Rad). The particles were finely dispersed with an ultrasonic cleaner (Sonicor Instrument Corporation, Copiague, NY) before bombardment. Bombardment pressures and distances from the launching plate were experimentally determined and were as indicated in the text. The plasmids were mixed in a ratio of 5 μg of pPHP620 to 1 μg of pPHP687—i.e., 1 μg/0.2 μg—per bombardment, or pPHP620 was used alone at 2 μg per bombardment.

Immature zygotic embryos, 10–15 per plastic Petri dish (15 × 60 mm, Falcon no. 1007), were bombarded between 24 and 72 hr after culture onto I_6 medium. The embryos were transferred onto filter papers (4.5 cm in diameter; Baxter glass fiber no. 391 overlayed onto Whatman no. 1) that were premoistened but not saturated with liquid I_6 medium. The filter papers functioned to absorb the water from the surface of the embryos, and the embryos were left for 2–3 hr on the papers prior to bombardment. Immediately after bombardment, the immature embryos were removed from the papers and transferred to semisolid I_6 medium.

Selection Agent. The herbicide bialaphos (Meiji Seika, Yokohama, Japan) was used as the selection agent in these experiments (30). Bialaphos was dissolved in water (0.1 g/ml) and purified by filtration through a C_{18} reverse-phase column (Baker no. 7020-13). The filtrate was diluted to a final bialaphos concentration of 1 mg/ml.

Analysis of Transgenic Tissues and Plants. Transient expression was evaluated 48 hr after bombardment. First, anthocyanin accumulation (31) was determined. GUS activity was then evaluated by histochemical assay (25). The number of red (anthocyanin accumulation) or blue (GUS activity) spots was determined using a stereo microscope (×50 magnification). Each distinct spot was counted as one expression event and the results are expressed as mean number of spots per embryo ± SE. Control embryos were bombarded with particles without DNA and no anthocyanin or GUS activity was detected. Samples for histology were prepared as described by Kononowicz *et al.* (32).

For DNA blot analysis, 15 μg of genomic DNA was isolated according to Dellaporta *et al.* (33), reacted overnight with restriction endonucleases, separated in 0.8% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P labeled probe (34). The *bar* gene was detected with a 0.8-kb fragment from pPHP620 that contains the entire *bar* coding region and the proteinase inhibitor II (PinII) terminator, whereas the *uidA* gene was visualized with a 2.2-kb fragment corresponding to the GUS coding region and the nopaline synthase terminator.

PAT activity was evaluated in callus and leaf extracts according to DeBlock *et al.* (30). The effects of the herbicide were assessed 2 and 4 days after local application of a 0.6% aqueous solution of Ignite/Basta (Hoechst), containing 0.1% Tween 20 onto the surface of young leaves. This herbicide contains glufosinate (200 mg/ml), the ammonium salt of phosphinothricin. T_1 seedlings (two- or three-leaf stage) were sprayed with the herbicide and survival was evaluated 5 days later.

RESULTS

Optimization of DNA Delivery Parameters by Transient Expression. The maize *R* and *C1* transcriptional activators appeared to function in cells of immature zygotic sorghum embryos (Fig. 1*A*). Since anthocyanin accumulation is cell autonomous and its visualization is nondestructive, it was chosen as the marker to optimize DNA delivery to individual cells in the scutellum of immature embryos. Little difference was detected based on the type of microprojectile used. A substantially more uniform microprojectile bombardment pattern and greater reporter gene expression was achieved when the microcarriers were dispersed with an ultrasonic cleaner prior to loading onto the flying disk (Fig. 1 *A* and *B*). GUS expression was detected 4 and 15 days after bombardment indicating that division and growth of the cells to which DNA was delivered had occurred (Fig. 1 *C* and *D*).

Cultured sorghum cells release phenolics into the culture medium (Fig. 2*A*), and the oxidized products inhibited morphogenesis and growth (8) and interfered with the evaluation

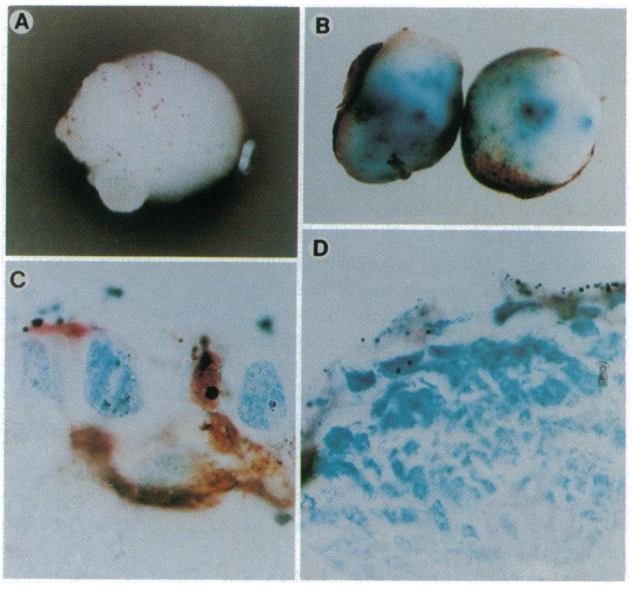

FIG. 1. Transient gene expression in the scutellum of immature zygotic sorghum embryos after DNA delivery by particle bombardment. Anthocyanin accumulation (*A*) and GUS activity (*B*) are detected 48 hr after bombardment. A transverse section of the scutellum (*C*) shows GUS activity 4 days after bombardment. GUS activity is seen in callus derived from the scutellum, 15 days after bombardment (*D*). (*A* and *B*, ×10; *C*, ×300; *D*, ×150.)

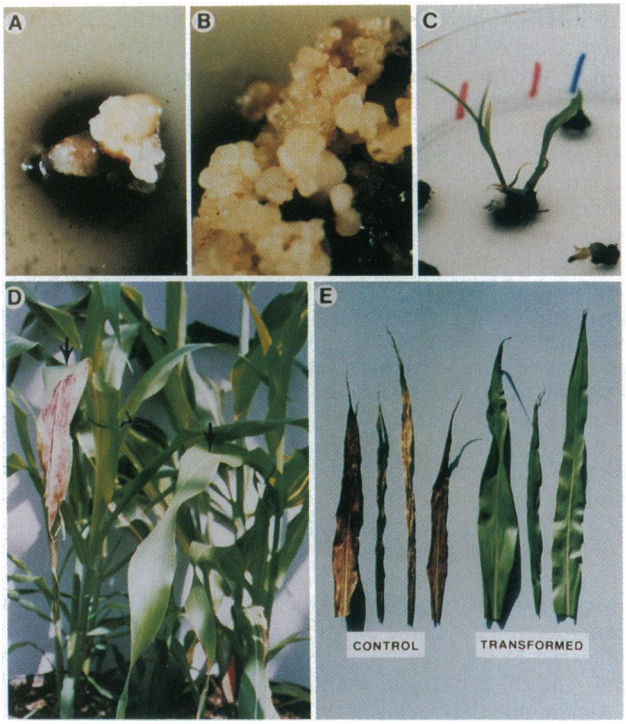

FIG. 2. Selection of transformed sorghum (cultivar P898012) callus, plant regeneration, and herbicide resistance of transgenic plants. (*A*) Immature zygotic embryo on induction medium containing bialaphos at 1 μg/ml. (*B*) Embryogenic callus on maintenance medium with bialaphos at 3 μg/ml. (*C*) Plant regeneration on medium with bialaphos at 3 μg/ml. (*D*) Untransformed (left) and transformed (right) regenerated plants 48 hr after local application of herbicide to the leaves (arrows). (*E*) Leaves from four untransformed control plants (from the left, nos. 1 and 2 were seed derived, and nos. 3 and 4 were regenerated) and three transformed plants, 4 days after treatment with the herbicide.

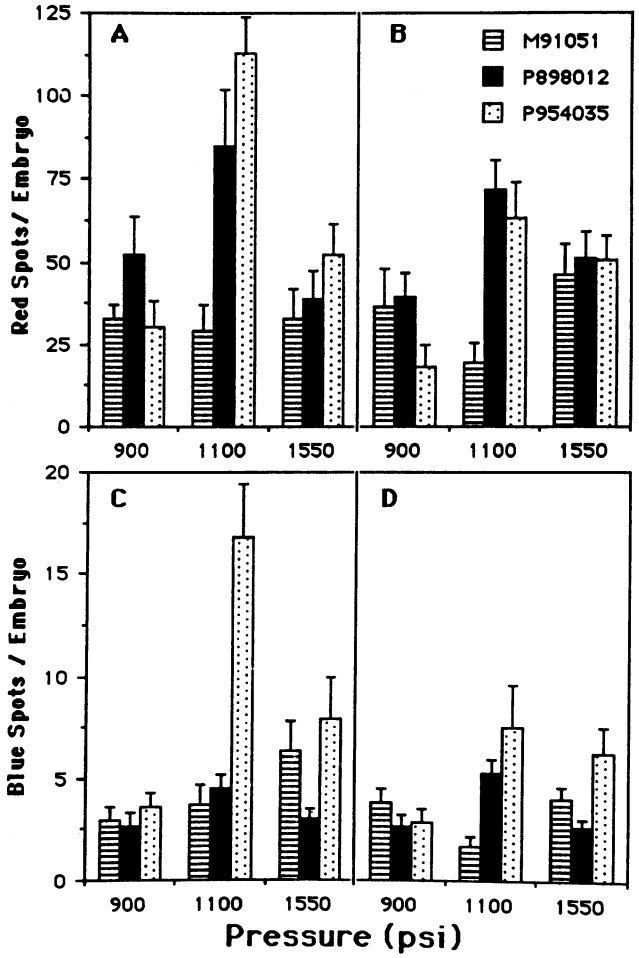

FIG. 3. Transient expression of *R* and *C1* transcriptional activators and the *uidA* gene in immature zygotic sorghum embryos. Anthocyanin accumulation (*A* and *B*) and GUS activity (*C* and *D*) were evaluated in three cultivars (M91051, P898012, and P954035), at three bombardment pressures (pressure) and at two distances between the embryos and the particle launch site (*A* and *C*, 6.0 cm; *B* and *D*, 9.2 cm).

of *R* and *C1* expression. The moistened glass fiber filter and filter paper support onto which embryos were placed for bombardment absorbed the surface moisture from tissues. This presumably facilitated increased particle penetration and higher transformation frequencies. It was repeatedly observed that bombardments conducted when embryos were on the support resulted in a higher percentage of embryos expressing the introduced genes (data not shown). Further, there was less phenolic pigment production during the subsequent culture period.

DNA delivery was estimated by transient expression of the regulatory elements *R* and *C1*, but these genes are in a different plasmid than *uidA* and *bar* (Fig. 1 *A* and *B*). Although differences in the absolute expression of *R/C1* and *uidA* genes were detected, similar patterns of relative expression as a function of genotype, bombardment pressure, and distance were observed (Fig. 3). The levels of expression of both reporter genes detected in sorghum tissues were lower than those detected in maize type I callus (data not shown). The greatest effect on reporter gene expression was attributable to variation amongst genotypes; however, there also appeared to be a genotype by bombardment pressure interaction (Fig. 3).

Selection of Transformed Callus and Plant Regeneration. Sorghum tissues were very sensitive to bialaphos. Concentrations greater than 3 μg/ml for a 4-week exposure period were toxic. Different selection strategies were imposed on immature embryos during the induction stage to accommodate the possibility that immediate exposure to bialaphos would be lethal to the transformed cells. Embryos were grown on induction medium without selection for 2 weeks and then transferred to maintenance medium with bialaphos at 3 μg/ml, or selection was started on induction medium by transferring the embryo immediately after bombardment or 3 or 7 days later to bialaphos at 1 μg/ml. Once that embryogenic callus had developed, tissues were transferred to maintenance medium with the herbicide at 3 μg/ml. Although substantial cell death occurred on induction medium supplemented with bialaphos (Fig. 2*A*), no clear difference could be attributed to the different selection strategies. However, all the material that was transferred directly to high bialaphos eventually died. Transfer of calli to maintenance medium with the herbicide at 3 μg/ml imposed much higher selection pressure on the tissues. Since GUS activity could not be detected in sorghum tissues later than 3 weeks after bombardment, growth on bialaphos at a rate similar to untransformed tissue on medium without bialaphos was used as an initial phenotypic indicator of transformed callus (Fig. 2*B*).

Embryogenic calli that had survived for 3 months on medium with bialaphos at 3 μg/ml were transferred to regeneration medium containing the same level of bialaphos and grown under light. A high proportion of this callus remained viable, whereas callus from embryos not bombarded with the *bar*-containing plasmid died within 2 weeks. Plantlets (Fig. 2*C*) were maintained on regeneration medium for 2–3 months and then rooted on medium with bialaphos at 1 μg/ml. The regenerated plants were transferred to soil,

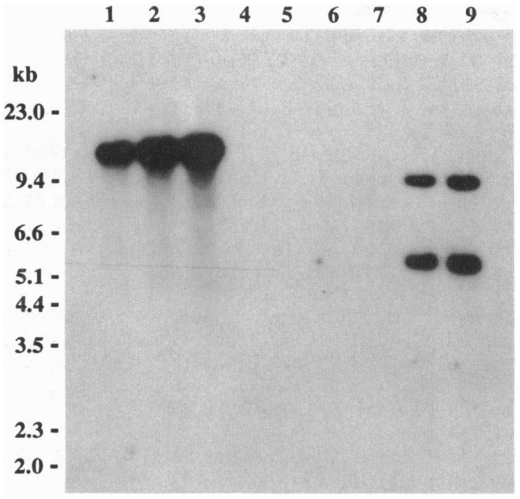

FIG. 4. Southern blot analysis of genomic DNA from transgenic sorghum plants (cultivar P898012). Plant genomic DNA (15 μg) was digested with *Eco*RI and probed with the *bar* insert. DNA was from plants regenerated from transformed callus line 1119 (lanes 1–3), untransformed seed-derived (lane 4) and regenerated (lane 5) plants of cultivar P898012, untransformed seed-derived (lane 6) and regenerated (lane 7) plants of cultivar P954035, and plants regenerated from transformed callus line 1409 (lanes 8 and 9).

acclimated to low humidity in a growth chamber, and eventually moved to the greenhouse. The total period from initiation of the cultures to acclimation of plants in the greenhouse was 7 months.

Most of the putative transformed calli that survived bialaphos treatment on maintenance medium died when transferred to bialaphos-containing regeneration medium. Of the surviving calli only a few exhibited shoot development. Exposure to light accelerates the development of phytotoxic activity of the herbicide. These results suggest that the calli might not be uniformly transformed and that only the cell sectors expressing the *bar* gene survive and grow after being transferred to the light.

Only three of the eight genotypes (cultivars P898012, P954035, and Tx430) produced embryogenic calli that survived the bialaphos selection strategies imposed in this study. Approximately 1 out of 350 embryos (6 out of 2150 total) that were bombarded produced calli that survived bialaphos. Calli of only the cultivar P898012 regenerated plants after being subjected to bialaphos selection pressure on the regeneration and root-formation media. The lack of success in recovering embryogenic callus of cultivars CS3541, M91051, SRN39, and Shanqui red that survives bialaphos selection may be partially attributable to their low morphogenic response from

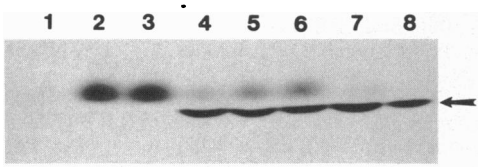

FIG. 5. PAT activity in leaves of transgenic sorghum (cultivar P898012) plants regenerated from callus lines 1119 and 1409. The reaction mixture contained 30 μg of protein in 24 μl of a buffer solution (50 mM Tris·HCl, pH 7.5/2 mM EDTA) plus 4 μl of 1 mM phosphinothricin and 4 μl of acetyl-^{14}C-CoA (sp. act. 57.9 mCi/mmol; NEN). Lanes 1, no plant extract; lanes 2 and 3, extracts from untransformed seed-derived and regenerated plants, respectively; lanes 4–6, extracts from three plants from line 1119; lane 7, extract from regenerated plant from line 1409; lane 8, extract from transgenic rice plant (35). Sixteen microliters (lanes 1–7) or 8 μl (lane 8) was applied to the chromatogram. Acetylated phosphinothricin is identified with the arrow.

immature embryos. These genotype-related differences might be diminished by using different explants (immature inflorescences or shoot tips; unpublished data) as targets for transformation.

Evaluation of Transgenic Material. Embryogenic calli from two immature embryos of cultivar P898012 (1119 and 1409) survived bialaphos selection and produced plants. Genomic DNA hybridization with the *bar* probe indicated the presence of one and two genes in plants regenerated from lines 1119 and 1409, respectively (Fig. 4). The restriction enzyme used linearized the plasmid pPHP620 in a region flanking the *bar*–PinII probe. The presence of distinctive bands required digestion of the plant DNA, demonstrating integration of the gene in the plant genome. Analogous results were obtained with the *uidA* probe (data not shown).

The plants regenerated from the two callus lines were morphologically similar to seed-derived sorghum plants. Plants derived from callus line 1119 flowered and all of them were fully fertile. These plants were resistant to bialaphos (Fig. 2 *D* and *E*), and this resistance was due to the expression of the *bar* gene (Fig. 5). One plant regenerated from the 1409 line has been evaluated for PAT activity and the result was positive (Fig. 5). The leaves of control plants (seed-derived or regenerated from tissues bombarded without DNA) treated with Ignite/Basta suffered substantial necrosis within 48 hr and eventually died. In many cases, the symptoms spread to the surrounding leaves, leading to the death of the entire tiller.

Analysis of the T_1 progeny showed segregation for herbicide resistance with 97 of 129, 145 of 182, and 52 out of 69 seedlings (derived from three plants regenerated from callus line 1119) surviving after treatment with the herbicide, whereas all control seedlings died. These results agree with a 3:1 Mendelian segregation for a single dominant locus ($P > 0.05$).

DISCUSSION

Transgenic sorghum plants have been obtained following microprojectile bombardment of immature zygotic embryos. Delivering DNA directly to the primary explant results in transformation of the embryogenically competent cells in the scutellum prior to the initiation of embryogenesis (10). Regardless of whether the somatic embryos arise directly from competent cells or are derived from a callus intermediary (36), DNA is delivered at a stage when the one (or few) cell progenitor of each organized structure exists. This should reduce or eliminate chimerism (the mixture of untransformed and transformed cells) in the organized structure or regenerated plants.

Transgenic plants were obtained from only 2 of 600 embryos of cultivar P898012 that were initially subjected to microprojectile bombardment. Although the reason for this is not clear at this point, the low frequency may be attributable to a number of different factors that affect the transformation process. It is generally assumed that transient expression is an indicator of the potential for DNA integration and stable expression. Although the DNA delivery parameters were defined as a part of this research, it is possible that an optimal protocol has yet to be established. The transformation vectors were designed for use with maize, and perhaps these constructs are not optimal for expression in sorghum cells. Transient expression in sorghum is lower than in maize. It is also possible that inherent characteristics of the sorghum scutellar cells make them somewhat unresponsive for transient expression. Osmotic pretreatments have been shown to increase transient expression and enhance integration of transferred DNA, and perhaps these would increase the responsiveness of sorghum cells (37, 38).

Evidence also indicates that the transferred gene(s) is not expressed after sustained periods of culture. GUS activity,

which was high in transient assays, could not be detected in callus that had been maintained for prolonged periods on bialaphos selection pressure, despite the fact that Southern analysis indicated the presence of the *uidA* gene. This suggests that DNA methylation occurs in sorghum cells that inactivates the expression of transferred genes (39, 40).

Although *bar* has proven to be a reliable selectable marker gene, conditions have not been established to optimize the application of bialaphos selection pressure for the efficient recovery of transgenic plants. The impact of selection pressure at various stages of embryogenic differentiation and shoot and root development needs to be established. Assuming some efficient capacity to screen for transgenic plants, it is not certain that applying selection pressure to the extent that all nontransformed cells or organized structures are eliminated is necessarily the most effective way to obtain transgenic plants.

The sorghum cultivar transformed, P898012, is well adapted in Niger and Sudan and is known to have both preflowering and postflowering drought resistance. The availability of a gene transformation and regeneration system in sorghum opens up new opportunities to improve protein nutritional quality and other traits of a high-yield, drought-resistant sorghum cultivar which serves as a staple food for millions of people in sub-Saharan Africa.

We have now demonstrated the usefulness of a selectable marker gene to optimize a sorghum transformation system which will eventually allow the introduction of agronomically important traits to sorghum by genetic transformation.

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1. Food and Agriculture Organization of The United Nations (1992) *FAO Production Yearbook 1991* (FAO, Rome), Vol. 45.
2. Rooney, L. W. & Serna-Saldivar, S. O. (1991) in *Handbook of Cereal Science and Technology*, eds. Lorenz, K. J. & Kulp, K. (Marcel Decker, New York), pp. 233–267.
3. Whitkus, R., Doebley, J. & Lee, M. (1992) *Genetics* **132,** 1119–1130.
4. Melake Berhan, A., Hulbert, S. H., Butler, L. G. & Bennetzen, J. L. (1993) *Theor. Appl. Genet.* **86,** 598–604.
5. Martin, G. B., de Vicente, M. C. & Tanksley, S. D. (1993) *Mol. Plant-Microbe Interact.* **6,** 26–34.
6. Battraw, M. & Hall, T. C. (1991) *Theor. Appl. Genet.* **82,** 161–168.
7. Hagio, T., Blowers, A. D. & Earle, E. D. (1991) *Plant Cell Rep.* **10,** 260–264.
8. Cai, T., Daly, B. & Butler, L. (1987) *Plant Cell Tissue Organ Cult.* **9,** 245–252.
9. Dunstan, D. I., Short, K. C., Dhaliwal, H. & Thomas, E. (1979) *Protoplasma* **101,** 355–361.
10. Dunstan, D. I., Short, K. C. & Thomas, E. (1978) *Protoplasma* **97,** 251–260.
11. Brettell, R. I. S., Wernicke, W. & Thomas, E. (1980) *Protoplasma* **104,** 141–148.
12. Cai, T. & Butler, L. (1990) *Plant Cell Tissue Organ Cult.* **20,** 101–110.
13. Bhaskaran, S. & Smith, R. H. (1988) *In Vitro Cell. Dev. Biol.* **24,** 65–70.
14. Fromm, M. E., Morrish, F., Armstrong, C., Williams, R., Thomas, J. & Klein, T. M. (1990) *Bio/Technology* **8,** 833–839.
15. Gordon-Kamm, W. J., Spencer, T. M., Mangano, M. L., Adams, T. R., Daines, R. J., Start, W. G., O'Brien, J. V., Chambers, S. A., Adams, W. R., Jr., Willetts, N. G., Rice, T. B., Mackey, C. J., Krueger, R. W., Kausch, A. P. & Lemaux, P. G. (1990) *Plant Cell* **2,** 603–618.
16. Somers, D. A., Rines, H. W., Gu, W., Kaeppler, H. F. & Bushnell, W. R. (1992) *Bio/Technology* **10,** 1589–1594.
17. Cao, J., Duan, X., McElroy, D. & Wu, R. (1992) *Plant Cell Rep.* **11,** 586–591.
18. Christou, P., Ford, T. L. & Kofron, M. (1991) *Bio/Technology* **9,** 957–962.
19. Vasil, V., Castillo, A. M., Fromm, M. E. & Vasil, I. K. (1992) *Bio/Technology* **10,** 667–674.
20. Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* **15,** 473–497.
21. Gamborg, O. L., Shyluk, J. P., Brar, D. S. & Constabel, F. (1977) *Plant Sci. Lett.* **10,** 67–74.
22. Kay, R., Chan, A., Daly, M. & McPherson, J. (1987) *Science* **236,** 1299–1302.
23. Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. & Wilson, T. M. A. (1987) *Nucleic Acids Res.* **15,** 3257–3272.
24. Callis, J., Fromm, M. & Walbot, V. (1987) *Genes Dev.* **1,** 1183–1200.
25. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. (1987) *EMBO J.* **6,** 3901–3907.
26. Thompson, C. J., Movva, N. R., Tizard, R., Crameri, R., Davies, J. E., Lauwereys, M. & Botterman, J. (1987) *EMBO J.* **6,** 2519–2523.
27. Ludwig, S. R., Habera, L. F., Dellaporta, S. L. & Wessler, S. R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7092–7096.
28. Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P. A. & Saedler, H. (1987) *EMBO J.* **6,** 3553–3558.
29. Sanford, J. C., Devit, M. J., Russell, J. A., Smith, F. D., Harpending, P. R., Roy, M. K. & Johnston, S. A. (1991) *Technique J. Methods Cell Mol. Biol.* **3,** 3–16.
30. DeBlock, M. E., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gossele, V., Movva, N. R., Thompson, C., Van Montagu, M. & Leemans, J. (1987) *EMBO J.* **6,** 2513–2518.
31. Ludwig, S. E., Bowen, B., Beach, L. & Wessler, S. R. (1990) *Science* **247,** 449–450.
32. Kononowicz, A. K., Nelson, D. E., Singh, N. K., Hasegawa, P. M. & Bressan, R. A. (1992) *Plant Cell* **4,** 513–524.
33. Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983) *Plant Mol. Biol. Rep.* **1,** 19–21.
34. Sambrook, J., Fritsch, E. F. & Maniatis, T., eds. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
35. Rathore, K. S., Chowdhury, V. K. & Hodges, T. K. (1993) *Plant Mol. Biol.* **21,** 871–884.
36. Vasil, V., Lu, C.-Y. & Vasil, I. K. (1985) *Protoplasma* **127,** 1–8.
37. Russell, J. A., Roy, M. K. & Stanford, J. C. (1992) *In Vitro Cell. Dev. Biol.* **28P,** 97–105.
38. Vain, P., McMullen, M. D. & Finer, J. J. (1993) *Plant Cell Rep.* **12,** 84–88.
39. Klein, T. M., Kornstein, L. & Fromm, M. E. (1990) in *Gene Manipulation in Plant Improvement II*, ed. Gustafson, J. P. (Plenum, New York), pp. 265–288.
40. Bochardt, A., Hodal, L., Palmgren, G., Mattsson, O. & Okkels, F. T. (1992) *Plant Physiol.* **99,** 409–414.