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Rapid and reproducible *Agrobacterium*-mediated transformation of sorghum

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Abstract A rapid and reproducible *Agrobacterium*-mediated transformation protocol for sorghum has been developed. The protocol uses the *nptII* selectable marker gene with either of the aminoglycosides geneticin or paromomycin. A screen of various *A. tumefaciens* strains revealed that a novel C58 nopaline chromosomal background carrying the chrysanthopine disarmed Ti plasmid pTiKPSF$_2$, designated NTL$_4$/Chry5, was most efficient for gene transfer to sorghum immature embryos. A NTL$_4$/Chry5 transconjugant harboring the pPTN290 binary plasmid, which carries *nptII* and GUS*Plus*^{TM} expression cassettes, was used in a series of stable transformation experiments with Tx430 and C2-97 sorghum genotypes and approximately 80% of these transformation experiments resulted in the recovery of at least one transgenic event. The transformation frequencies among the successful experiments ranged from 0.3 to 4.5%, with the average transformation frequency being approximately 1% for both genotypes. Over 97% of the transgenic events were successfully established in the greenhouse and were fully fertile. Co-expression of GUS*Plus*^{TM} occurred in 89% of the transgenic T$_0$ events. Seed set for the primary transgenic plants ranged from 145 to 1400 seed/plant. Analysis of T$_1$ progeny demonstrated transmission of the transgenes in a simple Mendelian fashion in the majority of events.

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Introduction

Sorghum (*Sorghum bicolor* L. Moench) is a widely grown grain and forage crop, and is more closely related than rice to the major crops of tropical origin such as maize, sugarcane, and pearl millet. Sorghum ranks fifth worldwide in production among cereal crops, and is an important model for tropical grasses of worldwide importance. It is unique among major cereals because it adapts well to environmental extremes, notably drought and heat. These attributes make sorghum the logical grain to support human and animal populations in areas with extreme heat and minimal precipitation. Even in the absence of drought, water availability is an emerging problem that will affect at least six billion people worldwide by 2025. Increased demand for limited fresh water, coupled with global climate trends, and expanding populations, will increase the attractiveness of dryland crops such as sorghum. Moreover, it is second only to maize within the US as a feedstock for ethanol production.

The sorghum genome contains ca. 750 Mb of DNA, which is somewhat larger than that of rice (430 Mb) but 3–4-fold smaller than that of maize (Arumuganathan and Earle 1991). The sorghum scientific community has a wealth of genomics tools at its disposal including a detailed genetic map, genetically-anchored sequence-ready physical map, large repository of ESTs, and a draft genome sequence (Bedell et al. 2005). These genomics resources, along with the ability to anchor the sorghum map to those of rice and maize, permit comparative genomics studies across important cereal crops. These genomics tools offer great potential to improve sorghum genetics. Additionally, the ability to introduce, express, and modulate genes in transgenic plants represents a very powerful tool to directly test gene function and provide a means to broaden the germplasm of crops for plant improvement.

Several laboratories have reported successful transformation of sorghum utilizing particle bombardment (Able et al. 2001; Casas et al. 1993) or *Agrobacterium*-mediated transformation with the *bar* gene (Zhao et al. 2000) or particle bombardment with the *nptII* gene (Tadesse et al. 2003) as selectable markers. More recently, an *Agrobacterium*-based system was coupled with a visual marker gene selection strategy to identify sorghum transformants (Gao et al. 2005). The microprojectile systems previously reported are hampered by reproducibility and relatively low efficiencies. On the other hand, *Agrobacterium*-mediated transformation is relatively efficient for sorghum using the 'super binary' vector system (Ishida et al. 1996), short subculture intervals, and the addition of PVPP to tissue culture media (Zhao et al. 2000). These latter two steps help block the negative impacts of associated phenolic production from sorghum tissue. The recent demonstration of standard binary plasmids being suitable for *Agrobacterium*-mediated transformation of sorghum (Gao et al. 2005) indicates the extra copies of the virulence genes *virG*, *virC*, and *virB* associated with the 'super binary' system are not essential for practical transformation efficiencies.

To date, there have been no reports utilizing *Agrobacterium*-mediated transformation implementing *nptII* as a selectable marker coupled with standard binary plasmids. The development of an efficient sorghum transformation system with a non-herbicide resistance marker gene is particularly important for sorghum because of its known ability to outcross with 'weedy' relatives such as shattercane (*Sorghum bicolor* L. Moench) or Johnsongrass (*Sorghum halepense*). Moreover, having an effective marker selection system for the crop alleviates labor requirements associated with monitoring for transgenic clusters among proliferating populations of non-transformed cells, which is necessitated when using a visual marker gene such as green fluorescent protein (gfp). We report here a reproducible, rapid, and efficient *Agrobacterium*-mediated transformation protocol using *nptII* as the selectable marker for sorghum that does not rely on a 'super binary' plasmid system. The reproducibility, efficiency, and quick turn around time of this system allows it to be a useful tool for functional genomics programs and serve as a means to broaden the germplasm of this important agricultural crop.

Material and methods

Plant material

Two sorghum lines were used in this study: a public line (Tx430) and an elite line (C2-97) (Sato et al. 2004) that was developed in the sorghum and millet breeding and genetics program at the University of Nebraska, Lincoln. Sorghum stock plants were grown under greenhouse conditions utilizing a 10/14 h photoperiod with day/night temperatures of 29°C/24°C. Plants were grown in Metromix® 200 supplemented with 1 lb of Micromax® micronutrients per cubic yard. Plants were fertilized bi-weekly with a 1x solution of Peter's 20-20-20. Immature embryos, 1.5–2.5 mm in length, were aseptically isolated from freshly harvested sorghum heads or sorghum heads stored at room temperature no longer than 2 days prior to isolation. Heads were surface sterilized by submerging each head in a 500 ml graduated cylinder containing 50% Ultra Clorox® plus 2 drops Tween®-20 with constant stirring for 20 min. The heads were subsequently rinsed three times with sterile distilled water and allowed to air dry in a laminar flow hood. Individual seeds were picked from the heads and subsequently sterilized in 70% ethanol for 1 min followed by one rinse with sterile distilled water and 20 min in 30% Ultra Clorox® followed by three rinses with sterile distilled water. The seeds were then placed in an open petri plate and allowed to briefly air dry in a laminar flow hood.

Agrobacterium preparation

The *Agrobacterium tumefaciens* strain NTL_4 (Luo et al. 2001) harboring the disarmed Chry5 Ti plasmid (Palanichelvam et al. 2000) designated $pTiKPSF_2$, (NTL_4/Chry5), containing the plasmid pPTN290 (Fig. 1) was streaked on LB medium containing 100 mg l^{-1} erythromycin, spectinomycin, and streptomycin from frozen glycerol stocks and grown for 2 days at 28°C.

The vector pPTN290 is a derivative of pPZP212 (Hajdukiewicz et al. 1994) that carries a GUS*Plus*™ cassette under the control of the maize ubiquitin 1 promoter coupled with its first intron. The GUS*Plus*™ (CAMBIA) open reading frame (ORF) was engineered by PCR to introduce an *Nco* I and *Xba* I site at the 5′ and

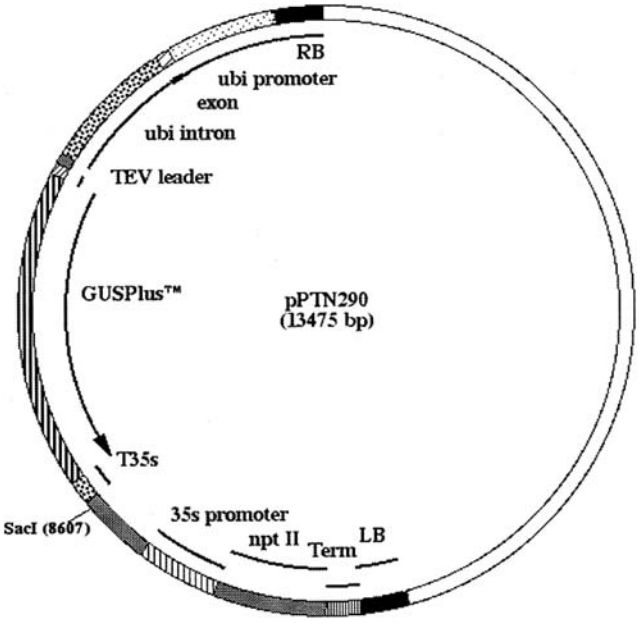

Fig. 1 Binary Plasmid pPTN290. *RB* and *LB* refer to right border and left border elements, respectively. Position of the only *Sac I* (used in Southern blot analysis see M & M) site within the vector is shown

3′ end, respectively, using the plasmid pCAMBIA1301 (CAMBIA) as template DNA in the reaction. The derived PCR product was subsequently fused to the tobacco etch translational enhancer element (TEV) (Carrington and Freed 1990) and subcloned between the maize ubiquitin 1 promoter and the 3′ UTR of the CaMV 35S transcript. The GUS*Plus*™ ORF in pPTN290 is interrupted with the rice catalase intron (CAMBIA) to prevent expression in the bacterium.

One day prior to setting up an inoculation, 50 ml of liquid LB with the same antibiotics was inoculated with a loop of the *Agrobacterium* and grown for 8 h under continuous shaking at 28°C. The bacteria were centrifuged and the bacterial pellet subsequently suspended in AB minimal medium (Chilton et al. 1974) to a final $O.D._{650}$=0.2 and grown overnight under continuous shaking at 28°C. The following morning, the bacteria were centrifuged and the pellet suspended in co-culture medium (see below) supplemented with 200 μM acetosyringone to a final $O.D._{650}$ ranging between 0.3 and 0.5. The inoculum was kept on ice until ready for use.

Embryo isolation and transformation

Immature embryos were aseptically isolated and prepared for inoculation one of two ways. For method one, 50 immature embryos were isolated directly into a 35 mm×10 mm petri plate containing 1–2 ml liquid co-culture medium which consisted of PHI-T medium (Zhao et al. 2000) except 1/2MS salts were substituted for full strength salts, no ascorbic acid or gelling agent was added and the pH was adjusted to 5.2 prior to autoclaving. The co-cultivation medium was supplemented with acetosyringone to a final concentration of 300 μM after autoclaving. In method one, the embryos remained in the liquid co-cultivation medium until all the embryos had been isolated for the experiment (up to 6 h). For method two, the immature embryos were isolated and placed scutellum-side up onto four sterile filters (Whatman Cat # 1001 070) contained in a 100×20 mm petri plate and saturated with 4.2 ml M11 medium (Elkonin and Pakhomova 2000) supplemented with 1.5 mg l^{-1} 2, 4-dichlorophenoxyacetic acid (2,4-D) with the pH adjusted to 5.7 prior to autoclaving. Embryos were pre-cultured on the modified M11 medium at 28°C in the dark for 1 day. Prior to inoculation, the pre-cultured embryos were briefly rinsed with co-culture medium in 35×10 mm petri plates. From this point on, the embryos from each isolation protocol were treated the same throughout the rest of the experiment.

For inoculation, co-culture medium was removed and replaced with *Agrobacterium* inoculum for 5 min. The *Agrobacterium* inoculum was then removed and the embryos were placed scutellum-side up onto four sterile filters (Whatman Cat # 1001 070) placed within a 100×20 mm petri plate saturated with 4.2 ml of co-culture medium supplemented with 300 μM acetosyringone. The embryos were co-cultured in the dark at 24°C for 2 days.

Transient gene expression assays

GUS*Plus*™ transient gene expression assays were used to screen *Agrobacterium* strains and sorghum genotypes for use in stable transformation experiments. Freshly isolated C2-97 immature embryos (method 1 isolation) were used in the *Agrobacterium* strain comparisons and inoculated as described above. Embryos were monitored for transient GUS*Plus*™ expression using histochemical analysis for ß-glucuronidase (Jefferson 1987) immediately following the co-cultivation period. *Agrobacterium* strains C58C1/pMP90 (Koncz and Schell 1986), LBA4404/pAL4404 (Hoekema et al. 1983), EHA101/pTiEHA101 (Hood et al. 1986), and cured Chry5 chromosomal strain carrying pTiEHA105 (Palanichelvam et al. 2000), C58/pAL4404 (MOG101) (Hood et al. 1993) and NTL_4/Chry5 were evaluated. NTL_4/Chry5 containing the plasmid pPTN290 was the *Agrobacterium* which gave elevated and the most consistent number of GUS expressing foci across all the experiments (Fig. 2C and D). On the basis of this criterion we selected NTL_4/Chry5/pPTN290 for use in evaluation of two sorghum genotypes, Tx430 and C2-97, for stable transformation.

Stable transformation and regeneration of sorghum

Freshly isolated immature embryos or immature embryos that had been precultured for 1 day were used in stable transformation experiments. The embryos were isolated and inoculated as described above. At the end of the 2 day co-cultivation period, the top filter with embryos was placed on three new filters saturated with 4.2 ml of M11 medium (Elkonin and Pakhomova 2000) supplemented with 1.5 mg l^{-1} 2, 4-D and 200 mg l^{-1} carbenicillin with the pH adjusted to 5.7 prior to autoclaving, in a 100×20 mm petri plate. The embryos were cultured on this "delay" medium in the dark at 28°C for 4 days. At any point during the delay or selection period, visible coleoptiles were removed.

For selection, M11 medium used during the delay period, was supplemented with 10 mg l^{-1} geneticin solidified with 2 g l^{-1} Phytagel or 20 mg l^{-1} paromomycin solidified with 7 g l^{-1} Phytagar. The embryos were transferred to fresh selection every 2–3 weeks and remained on selection medium for 6–9 weeks. At the end of the selection period, the embryos were transferred to regeneration medium which consisted of MS medium (Murashige and Skoog 1962) supplemented with 3 mM MES, 0.5 mg l^{-1} kinetin, 1 mg l^{-1} IAA, 100 mg l^{-1} carbenicillin, 10 mg l^{-1} geneticin, solidified with 2 g l^{-1} Phytagel, pH 5.7 prior to autoclaving. The callus was cultured on this medium for 2 weeks under a 16/8 h photoperiod at a temperature of 28°C. For shoot maturation, regenerating callus was transferred to MSO medium, regeneration medium with growth regulators and carbenicillin removed, pH 5.7 prior to autoclaving and solidified with 2 g l^{-1} Phytagel. After 2 weeks on this medium, shoots with at least one root were transferred to three parts Metromix® 360 and one part fine sand in jiffy pots and placed in Magenta® GA7 (Sigma Cat # V8505)

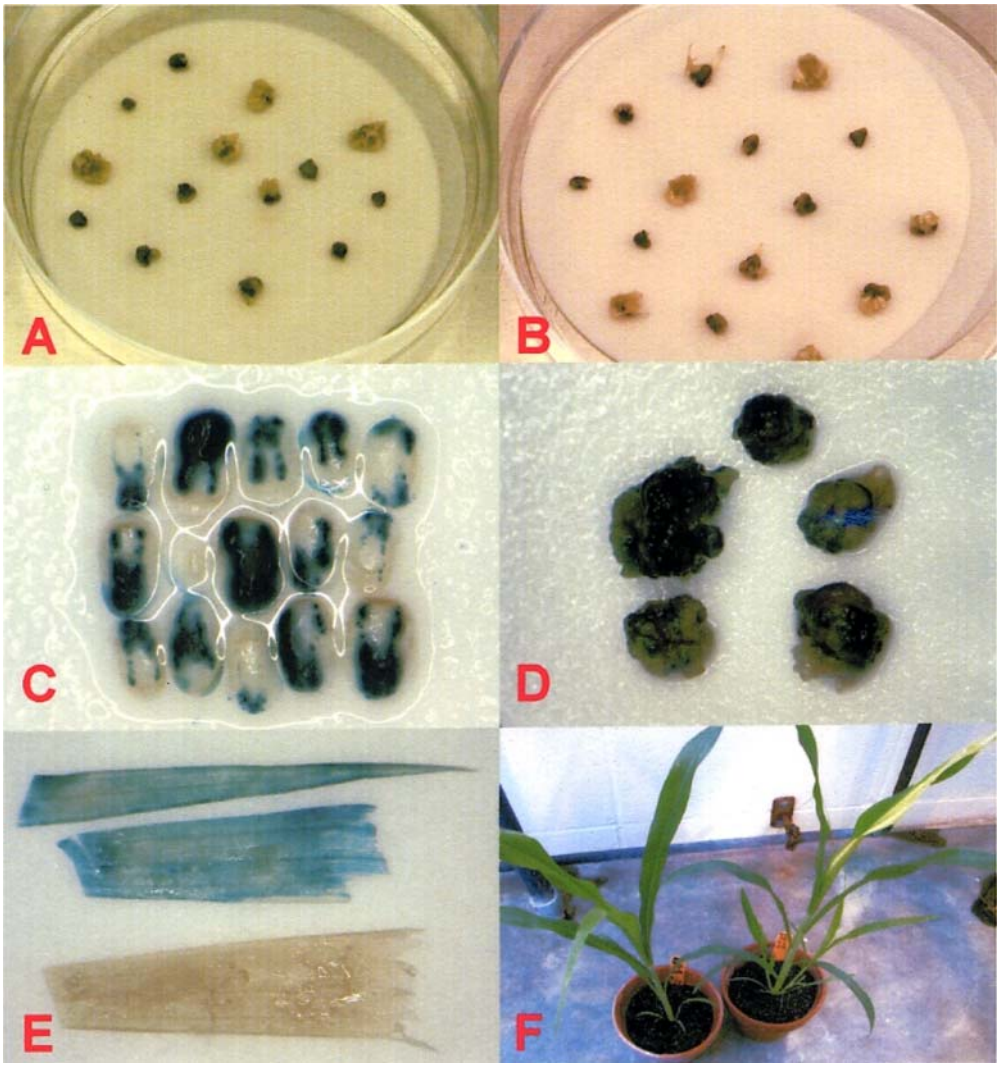

Fig. 2 Stages of *Agrobacterium*-mediated sorghum transformation. C2-97 embryogenic clusters 8 days on 20 mg l^{-1} paromomycin selection (**A**); C2-97 embryogenic clusters 8 days on 10 mg l^{-1} geneticin selection (**B**); Transient GUS*Plus*™ expression of immature Tx430 sorghum embryos inoculated with NTL_4/Chry5/pPTN290 (**C**); Stable GUS*Plus*™ expression in embryogenic Tx430 sorghum callus identified following 3 weeks on 20 mg l^{-1} paromomycin (**D**); GUS*Plus*™ expression in leaf tissue of primary transformant (*Top*), and control non-transgenic sorghum leaf tissue (*Bottom*) (**E**); For the *in planta nptII* assay, a solution of kanamycin and paromomycin was dropped into the whorl of the transgenic primary transformant (*Left*) and control non-transgenic sorghum plant (*Right*) 5 days earlier (**F**)

containers for hardening off under growth chamber conditions with a 16/8 h photoperiod at 24°C. Five to 7 days after transplanting, the plantlets were moved to the greenhouse and were grown under the same conditions as the stock plants.

Analyses for GUSPlus and nptII Expression

Co-expression of GUS*Plus*™ was monitored utilizing the histochemical assay for the enzyme following the protocol described by Jefferson (Jefferson 1987). Leaf tissue from rooted putative transgenic sorghum plants were incubated in the GUS*Plus*™ substrate solution at 37°C for 12 h. The tissues were subsequently cleared in 100% ethanol and scored for the presence or absence of blue color.

Expression of *nptII* was monitored by either an npt ELISA kit (Agdia® Cat # PSP 73000/0480) and/or *in planta* whole plant assay (Howe and Feng 2003). The former was conducted following the manufacturer's recommended protocol. The *in planta* assay was carried out by preparing a solution of 1.0 g l^{-1} kanamycin and 1.0 g l^{-1} paromomycin supplemented with 0.06% Silwet® L77. Twenty microliters of the aminoglycoside solution was applied directly into the whorl of a V2 stage or larger sorghum plant. Three to 5 days post application, the sorghum plants were scored for *nptII* expression indicated by no visible damage to the sorghum plant, or absence of *nptII* expression indicated by distinct bleaching and necrosis on the application area of the sorghum plant.

Southern blot analysis

For southern blot analysis, total genomic DNA was isolated from young sorghum leaves following a modified protocol described by Dellaporta et al. (1983). Ten micrograms of genomic DNA was digested with the restriction enzyme *Sst*I and separated on a TAE-0.8% agarose gel run at 20 V overnight. After transfer to a nylon membrane, the blot was prehybridized and hybridized in 1 mM EDTA, pH 8.0, 0.5 M Na_2HPO_4, pH 7.2, 7% SDS and 1% w/v BSA at 65°C. Blots were hybridized with a 326 bp *Sca* I fragment of GUS*Plus*™. The probe was labeled with ^{32}P by random primer synthesis using Stratagene's Prime-It Kit (Cat #

300385). The blot was exposed to Fuji Super RX X-ray film for 3 days.

Results and discussion

NTL_4/Chry5 strain for use in sorghum transformation

Early reports on *Agrobacterium*-mediated gene transfer to monocotyledonous plants suggested that extra copies of *A. tumefaciens vir* genes positively impact T-DNA transfer (Hiei et al. 1994; Park et al. 2000). Indeed, the first report on the successful use of *A. tumefaciens* as a vehicle to deliver transgenes into sorghum (Zhao et al. 2000) relied on the use of the so-called 'super binary' system (Ishida et al. 1996). The 'super binary' vector carries an additional 15 kb fragment of the virulence region of pTiBo542 (Hood et al. 1986), which harbors the virulence region encompassing genes *virC*, *virB*, and *virG*. The 'super binary' vector coupled with the disarmed octopine *A. tumefaciens* strain LBA4404 (Hoekema et al. 1983) was more consistent on rice, with respect to T-DNA transfer, than combining the 'super binary' in the EHA101 strain (Hood et al. 1986). *A. tumefaciens* strain EHA101 is a novel combination of a nopaline chromosome (A136) and a hypervirulent disarmed Ti plasmid, from which the extra *vir* genes for the 'super binary' were derived. These data reveal the influence the chromosome/virulence region may have on *Agrobacterium*-mediated transformation of plant cells.

Transient GUS*Plus*™ expression analysis was used as an indicator to monitor for T-DNA transfer to scutellar cells of immature sorghum embryos from a diverse set of *A. tumefaciens* strains (data not shown). Our screen of novel chromosome/Ti-plasmid combinations conducted on immature embryos of sorghum revealed a highly efficient gene transfer for the combination of a nopaline chromosome in strain NTL_4 (Luo et al. 2001), coupled with the Ti-plasmid pKPSF2 (Palanichelvam et al. 2000), a disarmed derivative found in the supervirulent strain isolated from chrysanthemum (Bush and Pueppke 1991; Kovacs and Pueppke 1993). Figure 2C displays transient GUS*Plus*™ expression in Tx430 immature embryos inoculated with NTL_4/Chry5/pPTN290 after a 2 day co-cultivation period. The enhanced virulence observed on sorghum with the NTL_4/Chry5 strain is a necessary component for a reliable *Agrobacterium*-mediated transformation system for sorghum. The other components are the ability to efficiently identify transgenic cells, and subsequently regenerate these into fertile plants.

Selection of transgenic embryogenic sorghum clusters and conversion to whole plants

Freshly isolated immature embryos or 1 day precultured immature embryos were inoculated with NTL_4/Chry5/pPTN290 in 20 separate experiments designed for whole plant recovery. The immature embryos and subsequent callus derived from the immature embryos were selected on 10 mg l^{-1} geneticin or 20 mg l^{-1} paromomycin for 6–9 weeks. Geneticin and paromomycin supplemented media were equally effective in selection of transgenic callus clusters (data not shown). However, embryo isolation method 2 (one day preculture) tended to increase the number of escape plants with genotype C2-97, under both selection regimes, while no apparent difference with respect to number of escape plants, was observed with genotype Tx430 across the two embryo isolation methods. Hence, the embryo isolation method did not directly affect transformation frequency *per se*.

The selection pressure imparted by the aminoglycosides during the callus induction phase was very effective in early identification of putative transgenic embryogenic clusters of sorghum (Fig. 2A and B). Although the overall transformation frequency with this system is relatively low (Table 1), the selection pressure implemented during the callus induction step permitted a significant amount of tissue to be discarded early in the protocol, which in turn drastically reduced the labor requirement associated with subculturing the callus.

One problem associated with *in vitro* culturing of sorghum is the substantial amount of phenolic compounds that are secreted from the immature embryo explants. This has a negative impact on tissue growth and differentiation. Earlier reports on sorghum transformation have dealt with this issue by shortening the subculturing period and by addition of polyvinylpolypyrrolidone (PVPP) to the medium (Gao et al. 2005; Zhao et al. 2000). While this approach can reduce the negative effects of the phenolic compounds, shortening the subculturing period adds labor and materials, hence cost, and PVPP can impact the effective concentration of growth regulators, thereby influencing the *in vitro*

Table 1 Sorghum transformation frequencies

Exp. No.	Explant. No.	No. of Events	Transformation Frequency (%)	Genotype
AH44	155	2	1.3	C2-97
AH50	615	3	0.5	C2-97
AH55	373	3	0.8	C2-97
AH57	396	2	0.5	C2-97
AH59	284	2	0.7	C2-97
AH61	89	4[a]	4.5	C2-97
AH62	233	2	0.9	C2-97
AH43	329	3	0.9	Tx430
AH45	256	1	0.4	Tx430
AH46	134	1	0.7	Tx430
AH47	150	2	1.3	Tx430
AH48	240	2	0.8	Tx430
AH52	310	1	0.3	Tx430
AH53	311	6[a]	1.9	Tx430
AH56	272	1	0.4	Tx430
AH58	170	1	0.6	Tx430
AH60	194	1	0.5	Tx430

[a]Implies two out of four events and four out of six events co-expressed GUS*Plus*™, respectively. No. of events column refers to the total number of transgenic independent sorghum events established in the greenhouse

response of the tissue. In a search for media that reduced this problem, Elkonin and Pakhomova (2000) evaluated various media compositions for their affects on *in vitro* culture of sorghum. One of these compositions designated M11 (Elkonin and Pakhomova 2000) was shown to significantly reduce phenolic production, while providing high embryogenic competence in sorghum (Sato et al. 2004). By combining the positive attributes of the M11 medium with the efficient gene transfer of the NTL_4/Chry5 combination, and coupling it with aminoglycoside selection, we were effectively able to identify putative transgenic embryogenic clusters, in a relatively short time period, without the compounding effect of phenolic secretion (Fig. 2).

Seventeen out of 20 experiments initiated, produced at least one transgenic event that regenerated into a fertile transgenic plant. The highest transformation frequency obtained in these initial experiments, based on the number of independent transgenic events that produced at least one fertile transgenic sorghum plant established in soil divided by the number of starting immature embryos, was 4.5% (Table 1). The average transformation frequency of the successful experiments for both genotypes was approximately 1.0%. Thirty-seven out of 38 independent events that produced *nptII* and/or GUS*Plus*^{TM} positive plants survived in soil. Seed set for the primary transgenic plants ranged from 145–1 400 seeds/plant. Typical primary transformants generated with this protocol are shown in Fig. 3. The total time from inoculation of the explants to a plant in the greenhouse was approximately 15 weeks.

Another critical step for selection of transgenic sorghum plants in this protocol occurs at the regeneration phase of the system. Here embryogenic tissue selected during the callus induction selection phase was transferred to regeneration medium containing 10 mg l^{-1} geneticin for 2 weeks. Callus tissue which appeared healthy at the end of the first regeneration step was transferred to MSO medium for shoot and root maturation. This 2-week regeneration selection provides a rather stringent screen for transgenic sorghum events, as the frequency of recovering non-transgenic sorghum was very low, as described below.

Characterization of transgenic sorghum events

Putative primary (T_0) transgenic sorghum plants were confirmed to be transgenic by GUS histochemical assay (Jefferson 1987) (Fig. 2E), *nptII* ELISA or an *in planta* whole plant assay (Fig. 2F). This method provides a convenient, non-destructive assay that readily identifies transgenic plants or their progeny (Howe and Feng 2003). Co-expression of the GUS*Plus*^{TM} gene was 89% with 17/19 independent Tx430 events and 16/18 independent C2-97 events expressing both *nptII* and GUS*Plus*^{TM}, while the remaining four events expressed only *nptII*. Progeny from 10 independent C2-97 events and 10 independent Tx430 events were analyzed by GUS histochemical assays or *in planta nptII* whole plant assays to determine goodness-of-fit to the expected 3:1 Mendelian ratio for self pollinations. These tests demonstrated that the introduced genes were inherited as a single Mendelian locus in 9 out of 10 C2-97 events tested and 9 out of 10 Tx430 events tested (Table 2).

Southern blot analysis was performed on T_1 progeny selected from four independent C2-97 events to confirm that the introduced transgenes were stably integrated and trans-

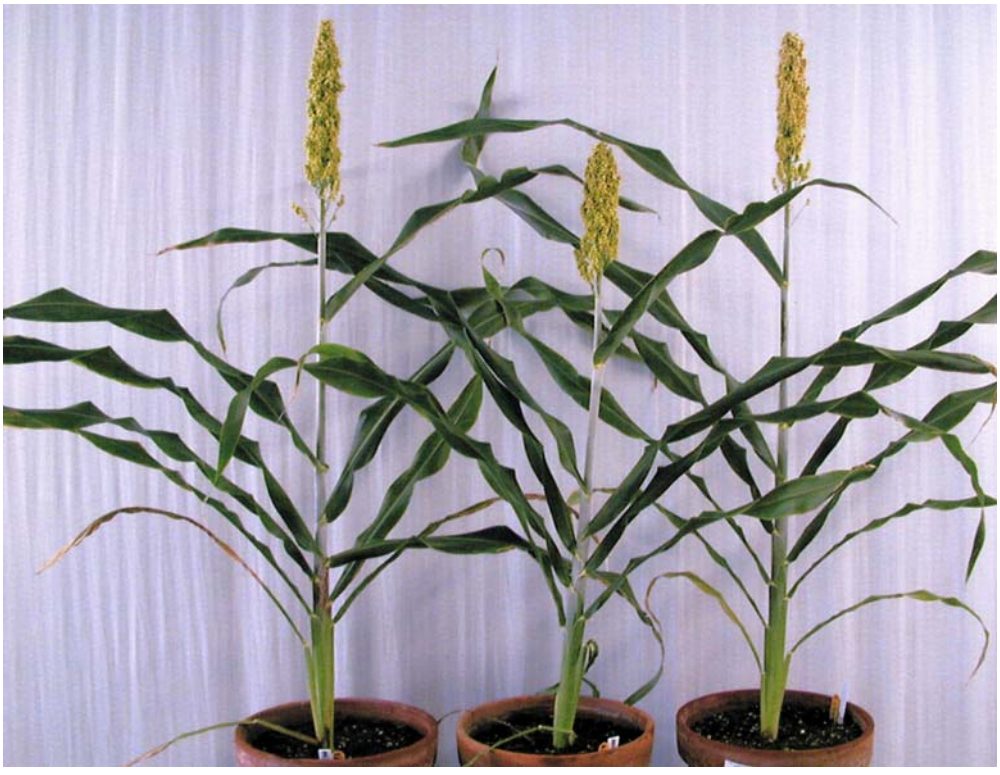

Fig. 3 Primary transformants of genotype C2-97

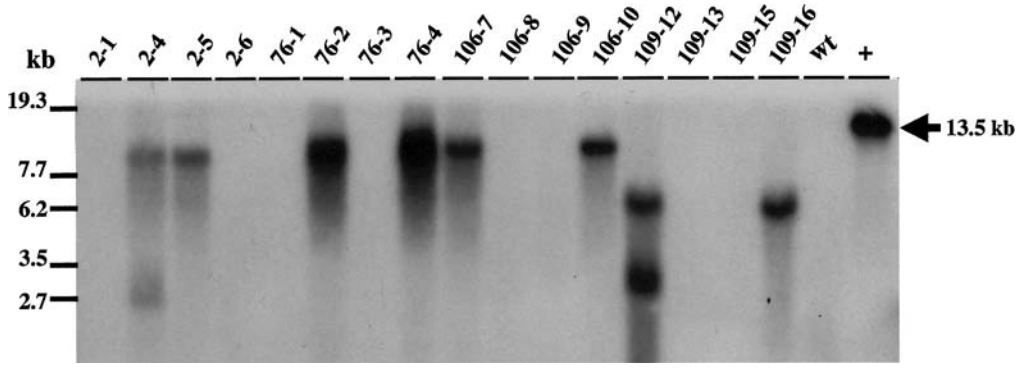

Fig. 4 Southern blot analysis on T_1 segregating individuals. Four individuals derived from four transgenic events designated 2, 76, 106 and 109. Total genomic DNA was digested with *Sac* I. Blot was hybridized with GUS*Plus*™ probe (see M & M). *wt* lane refers to control sorghum DNA. (+) lane represents 50 pg of pPTN290 digested with *Sac* I. Hybridization signal across the lanes correlated with *nptII*/GUS expression data among the T_1 individuals screened

mitted to the progeny (Fig. 4). A set of GUS*Plus*™ negative and GUS*Plus*™ positive plants were selected from events which were segregating 3:1 on the basis of GUS and *nptII* assays. Transgenic lines harbored from 1 to 2 loci with estimated 2–3 copies per locus (Fig. 4).

Comparison to previous sorghum transformation systems

Successful transformation of sorghum was first reported in 1993 (Casas et al. 1993). This initial work relied on microprojectile bombardment coupled with the herbicide resistance gene *bar* (Thompson et al. 1987) as the selectable marker. This protocol had a relatively low transformation frequency of 0.2%, a protracted time in culture of 7 months, and used a sorghum genotype, PI898012, with poor agronomic traits (Casas et al. 1993). A more recent effort used microprojectile-mediated gene transfer to establish embryogenic callus and the *bar* gene as the selectable marker to recover fertile transgenic sorghum events (Able et al. 2001). The non-herbicide marker gene *nptII* was successfully implemented in a microprojectile-mediated gene transfer system for sorghum (Tadesse et al. 2003), but like the other microprojectile-mediated transformation protocols, required an extended *in vitro* culture time and only a few transgenic lines were reported.

The reports on *Agrobacterium*-mediated gene transfer protocols for sorghum revealed improved transformation frequencies, increased reliability as reflected by the number of transgenic events characterized, and shortened *in vitro* culture time (Gao et al. 2005; Zhao et al. 2000). The published *Agrobacterium*-mediated sorghum transformation protocols have relied on hypervirulent *A. tumefaciens* strain EHA101 (Gao et al. 2005) or the 'super binary' system coupled with octopine strain LBA4404 (Zhao et al. 2000). Our protocol outlined herein draws upon the identified hypervirulent nature of the NTL_4/Chry5 strain, a medium salt composition that alleviates *in vitro* culture problems associated with phenolic secretion and a tight selection regime that utilizes a non-herbicidal marker gene, *nptII*. These attributes provide for a reliable, low labor transformation protocol that is effective for elite sorghum genotypes. This transformation system can be exploited as a tool for functional genomics programs and as a means to broaden the germplasm of this important cereal crop.

Table 2 Segregation analyses on progeny derived from 10 independent C2-97 and Tx430 events

Event. No.	T_1 Segregation (positive: negative)[a]	Chi-square value[b]	Genotype
002	15:5	0.07	C2-97
003	18:2	1.67	C2-97
072	13:7	0.60	C2-97
073	20:0	5.40[c]	C2-97
076	15:5	0.07	C2-97
106	18:2	1.67	C2-97
109	15:5	0.07	C2-97
137	16:4	0.07	C2-97
138	16:4	0.07	C2-97
149	14:6	0.07	C2-97
009	13:7	0.60	Tx430
026	17:3	0.60	Tx430
028	15:5	0.07	Tx430
029	17:3	0.60	Tx430
033	14:6	0.07	Tx430
038	15:5	0.07	Tx430
049	9:11	8.07[c]	Tx430
056	14:6	0.07	Tx430
067	16:4	0.07	Tx430
094	19:1	3.27	Tx430

[a] T_1 segregation data was based on GUS expression and/or npt II expression

[b] Chi-square values are based on 3:1 ratio (p=0.05)

[c] Segregation ratio deviates from 3:1

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