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Stable transformation of *Sorghum bicolor* protoplasts with chimeric neomycin phosphotransferase II and β-glucuronidase genes

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Summary. Parameters influencing the stable transformation of *Sorghum bicolor* protoplasts with a chimeric neomycin phosphotransferase II (*NPT II*) gene by electroporation were investigated. The mean number of kanamycin-resistant calli produced increased in direct proportion to the concentration of DNA used for transformation. Linearization of the plasmid doubled the mean number of kanamycin-resistant calli produced, while the addition of carrier DNA had no effect. The copy number (1–4) of integrated genes was low compared with that frequently reported for PEG-mediated transformation. Two strategies for transforming protoplasts with a nonselectable, β-glucuronidase (*GUS*) gene were compared. One utilized a plasmid containing a CaMV 35S-*NPT II* gene covalently linked to a CaMV 35S-*GUS* gene, and the other strategy utilized the two genes on separate plasmids. DNA from all 77 kanamycin-resistant calli analyzed contained restriction fragments hybridizing to the *NPT II* probe; approximately 70% of the clones from all transformation treatments contained a 1.7-kb *Eco*RI/*Hin*dIII restriction fragment corresponding to the full-length gene. Of the kanamycin-resistant calli, 38–63% (depending on the transformation treatment) contained *GUS*-hybridizing fragments, and 8–19% contained the full-length gene. The addition of *NPT II* and *GUS* genes on a single plasmid or on separate plasmids did not appear to lead to an appreciable difference in the frequency of cointegration of these genes, although an increased proportion of the plasmid bearing the nonselectable (*GUS*) gene appeared to favor its cointegration.

Key words: β-Glucuronidase – Gene copy number – neomycin phosphotransferase – Protoplasts – *Sorghum bicolor*

Introduction

Of the numerous methods used to transfer DNA into plant cells (reviewed by Potrykus 1990), direct gene transfer is the only one that has unequivocally produced stable transformed cereal (Poaceae, Monocotyledonae) tissues. With the very recent exception of projectile-mediated transformation of maize (Gordon-Kamm et al. 1990), successful procedures have been based on electroporation or polyethylene glycol (PEG) treatment of protoplasts, to induce uptake of a chimeric gene encoding antibiotic resistance. Upon integration and expression, this confers antibiotic resistance to the cells (Potrykus et al. 1985; Fromm et al. 1986; Uchimiya et al. 1986a; Callis et al. 1987; Rhodes et al. 1988; Lyznik et al. 1989), permitting selective growth of the transformed cells.

The low transformation frequencies typically obtained with a nonselectable gene alone rarely yield successful transformation of cereal protoplasts, because analysis of thousands of clones is required to obtain a single transformant. Two strategies have been applied for direct DNA transformation of protoplasts with genes for which there is no direct selection. For example, Schocher et al. (1986) used two plasmids for the transformation of tobacco protoplasts, one containing a selectable gene and the other a nonselectable gene; the cotransformants produced contained a gene from each of the plasmids. In contrast, Uchimiya et al. (1986b) utilized a single plasmid bearing both genes to obtain tobacco transformants containing the nonselectable gene. Concomitant trans-

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formation of protoplasts with an antibiotic resistance gene and a nonselectable gene has also been applied to the cereals. Rice (Shimamoto et al. 1989) and maize (Lyznik et al. 1989) protoplasts have been cotransformed with separate plasmids bearing β-glucuronidase (*GUS*) and antibiotic resistance genes. Utilizing a single plasmid containing an antibiotic resistance gene and a nonselectable gene, Callis et al. (1987) transformed maize protoplasts with the *Adh1-S* gene and we (Battraw and Hall 1990) transformed rice protoplasts with a CaMV 35S-*GUS* gene.

Although approaches using independent plasmids, or a single plasmid containing both selectable and nonselectable genes, have both proven successful in transforming protoplasts, we are unaware of any previous comparison of these approaches for cereal protoplasts. Additionally, we have investigated parameters affecting the transformation frequency of protoplasts from *Sorghum bicolor,* a cereal species not previously stably transformed.

Materials and methods

Protoplast isolation and transformation

Protoplasts were isolated from a *Sorghum bicolor* cultivar NK300 cell suspension culture, essentially as described by Chourey and Sharpe (1985), and resuspended in a solution for electroporation containing 10 m*M* HEPES, 150 m*M* NaCl, 5 m*M* $CaCl_2$, and 0.2 *M* mannitol, pH 7.2 (Fromm et al. 1985), at a final concentration of 1×10^6/ml. After adding up to 50 μg of plasmid DNA (1 mg/ml), 1-ml samples of protoplasts were placed in foil-lined cuvettes (Potter et al. 1984) and incubated for 10 min at 0 °C before discharging a 300 μF capacitor, charged to 250 V, across the electroporation cuvette. Protoplasts were then kept at 0 °C for 10 min, at room temperature for 10 min, and then transferred to a 9-cm plastic petri plate containing 7.5 ml of protoplast culture medium (PCM) (Chourey and Zurawski 1981). After 14 days of incubation at 27 °C, cells were first resuspended in 10 ml of PCM, and then 50 ml of PCM (45 °C) containing 1.4% (w/v) low-melting-point agarose (BRL) and kanamycin sulphate (140 μg/ml; Sigma) was added immediately prior to plating 11.5-ml volumes in 9-cm plastic petri dishes. Sixty to seventy days after electroporation, calli larger than 2 mm were counted as kanamycin-resistant and transferred individually to PCM containing 0.4% (w/v) type 1 agarose and kanamycin sulphate (100 μg/ml; Sigma). Calli were subcultured monthly.

Plasmid constructions

Plasmid DNA used for electroporation was prepared by alkaline lysis and then purified by centrifuging cesium chloride-ethidium bromide gradients to equilibrium, as described by Maniatis et al. (1982). pCaMVNEO (Fromm et al. 1986) contains the neomycin phosphotransferase II (*NPT II*) gene coding sequence downstream from the cauliflower mosaic virus 35S (CaMV 35S) promoter and employs the nopaline synthase (nos) polyadenylation region. This plasmid was used to transform maize protoplasts (Fromm et al. 1986); the gene product provides cells with resistance to the antibiotic kanamycin. pBI221.1 (Jefferson 1987) contains the *GUS* gene coding sequence and also utilizes the CaMV 35S promoter and nos polyadenylation region. A plasmid containing both chimeric genes was produced by inserting the 1.7-kb HindIII fragment of pCaMVNEO, which contains the chimeric *NPT II* gene, into the HindIII site of pBI221.1. A clone in which the promoters were adjacent, pNEOGUS15, was selected. Linearized pCAMVNEO was prepared by digesting with EcoRI to completion, extracting with phenol chloroform, precipitating, and redissolving 1 mg of DNA per milliliter of water. Carrier DNA was prepared by dissolving calf thymus DNA (type 1, Sigma) in 1 m*M* EDTA, 10 m*M* TRIS-Cl (pH 7.4), and filtering it through a 45-μm pore-size polysulfone membrane (Gelman).

Neomycin phosphotransferase II assay

Sorghum calli were assayed for *NPT II* activity based on the procedure of Reiss et al. (1984). Calli were crushed in an equal volume of extraction buffer containing 1% (v/v) β-mercaptoethanol and 50 m*M* TRIS-Cl (pH 6.8), and the homogenate was kept at 0 °C. The protein of the cleared homogenate (5 min at $12{,}000 \times g$) was fractionated by nondenaturing polyacrylamide gel electrophoresis (resolving gel: 10% T, 5% C; stacking gel: 3.5% T, 3% C; running buffer: 40 m*M* glycine, 5 m*M* TRIS; apparatus: Hoefer TSE200 minigel). After equilibration of the gels in a reaction buffer containing 42 m*M* $MgCl_2$, 400 m*M* NH_4Cl, and 67 m*M* Tris-maleate, pH 7.1 (Reiss et al. 1984), 9 ml of reaction buffer containing 1% (w/v) agarose, kanamycin sulphate (100 μg/ml), and 50 μCi γ-^{32}P ATP (3,000 Ci/mmol), at 45 °C, was pipetted directly on the gel. A sheet of P81 paper (Whatman) was placed on top of the gelled agarose and incubated for 30 min at room temperature. Then three sheets of 3MM paper (Whatman), a 2-cm stack of paper towels, a small glass plate and weight (200 g), in that order, were placed on top of the P81 paper for 3 h. To reduce the signal from slower migrating bands, presumably protein kinases, the P81 paper was incubated in a solution containing proteinase K (1 mg/ml) and 1% (w/v) SDS at 60 °C for 30 min (Schreier et al. 1985) before washing three times, 5 min each time, in 50 m*M* sodium phosphate (pH 7.0), at 80 °C, and exposing to X-ray film.

Gel blot hybridization

Total DNA was isolated from calli by precipitation with cetyltrimethylammonium bromide, as described by Taylor and Powell (1982). A 1C value, the DNA content of the unreplicated haploid chromosome content, of 0.8 pg (Laurie and Bennett 1985) was used for copy number reconstruction. DNA concentrations were determined by measuring the enhancement of fluorescence seen when bisbenzimide (Hoechst 33258) binds to DNA (Labarca and Paigen 1980). Restriction endonucleases were used according to the manufacturer's instructions. High-molecular DNA markers and a 1-kb DNA ladder (BRL) were used for size standards. After fractionation of DNA (1.0 μg per lane) by electrophoresis in a 1% agarose gel, the DNA was transferred to nylon membrane (GeneScreen Plus) by alkaline blotting using the rapid transfer protocol, with depurination, of Reed and Mann (1985). Membranes were hybridized to the ^{32}P-labeled BamHI-SstI 1.9-kb fragment of pBI221.1 (Jefferson 1987), the *GUS* gene coding sequence probe, or to the BamHI 1.0-kb fragment of pCaMVNEO (Fromm et al. 1986), the *NPT II* gene coding sequence probe, as described by Reed (1988).

Analysis of β-glucuronidase activity

β-Glucuronidase specific activity was determined by measuring the rate of hydrolysis of the substrate 4-methylumbelliferyl-β-D-glucuronide to form the fluorescent product 4-methylumbelliferone (4-MU), based on the methods of Jefferson et al. (1987) as

described by Battraw and Hall (1990). Histochemical localization of *GUS* activity was performed by incubating calli with the substrate 5-bromo-4-chloro-3-indolyl glucuronide (x-gluc) (Research Organics), as described by Jefferson (1987). Initial assays were performed within 3–4 months of the electroporation date, using calli that were actively growing 2 weeks after subculture to fresh media.

Results

The effect of supplied DNA concentration on the transformation frequency of sorghum protoplasts was determined using four concentrations (3, 10, 25 and 50 μg/ml) of pCaMVNEO (Fromm et al. 1986) in four sets of electroporations. Cells transformed with the chimeric *NPT II* gene in this plasmid are resistant to the antibiotic kanamycin (Fromm et al. 1986). A sorghum cell suspension (Chourey and Sharpe 1985) was used to produce protoplasts for the transformation experiments. Within 7 days after electroporation, protoplasts had formed cell walls and begun to divide. Fourteen days after electroporation, the cells were embedded in fresh culture media containing kanamycin sulphate. After 60 days, kanamycin-resistant calli were produced from protoplasts that were electroporated with pCaMVNEO (Fig. 1 B), but no kanamycin-resistant calli were produced from protoplasts that were electroporated without DNA (Fig. 1 A). The mean number of kanamycin-resistant calli produced from the electroporated protoplasts increased linearly from the lowest concentration, 3 μg/ml, to the highest concentration of pCaMVNEO tested, 50 μg/ml (Fig. 2).

When pCaMVNEO was linearied at the *Eco*RI site, downstream of the polyadenylation signal, and used to transform protoplasts, the mean number of kanamycin-resistant calli produced was greater than when an equal concentration of circular form pCaMVNEO was used for transformation (Fig. 3). The mean number of kanamycin-resistant calli produced using circular form pCaMVNEO with carrier DNA, 25 or 50 μg/ml, was nearly the same as that produced by using circular form pCaMVNEO alone (Fig. 3). No kanamycin-resistant calli were obtained within 60 days after electroporation in the absence of DNA or with carrier DNA, nor were any obtained when 25 μg pCaMVNEO per milliliter was added without electroporation.

Two approaches were used to transform sorghum protoplasts with a nonselectable chimeric *GUS* gene. In

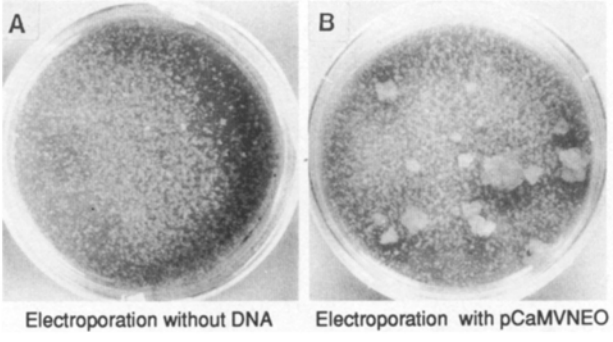

Fig. 1 A and B. Selection of kanamycin-resistant sorghum calli. After 2 weeks of culture, electroporated protoplasts were plated in media containing kanamycin sulphate (100 μg/ml) and photographed after an additional 46 days of culture. **A** No kanamycin-resistant calli grew from protoplasts electroporated without DNA. **B** Kanamycin-resistant calli that developed from protoplasts after electroporation with pCaMVNEO DNA

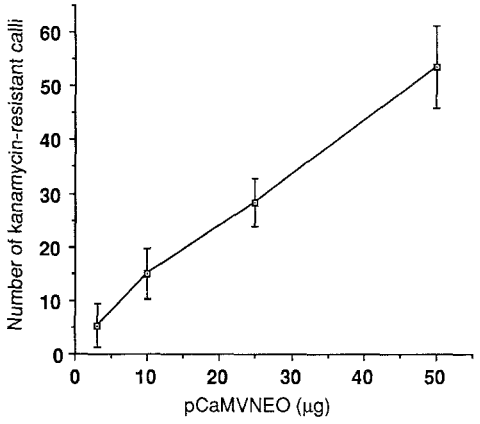

Fig. 2. Effect of DNA concentration on the stable transformation of sorghum protoplasts. Each 1-ml sample contained 1×10^6 protoplasts and 3, 10, 25, or 50 μg pCaMVNEO DNA, circular form. Electroporation and selection of resistant calli are as described in 'Materials and methods'. Each data point is the mean value of four electroporation treatments and vertical bars represent the standard deviation

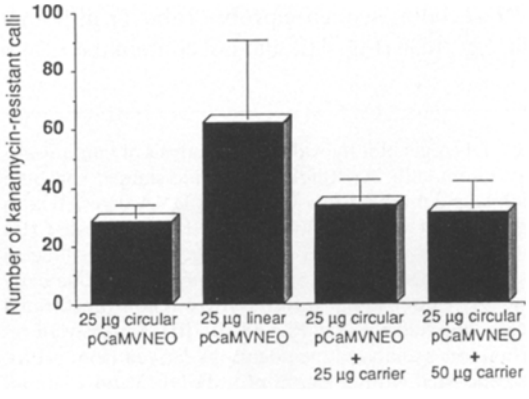

Fig. 3. Effect of plasmid DNA form and carrier DNA on the stable transformation of sorghum protoplasts. Each 1-ml sample of protoplasts contained 1×10^6 protoplasts and 25 μg of pCaMVNEO DNA, circular or linear form. In addition, calf thymus carrier DNA (25 or 50 μg) was included in two of the electroporation treatments. Electroporation and selection of resistant calli are as described in 'Materials and methods'. Bars represent the mean value of three or four electroporation treatments (±standard deviation)

the first approach protoplasts were electroporated with pBI221.1 and pCaMVNEO, plasmids that contain a chimeric *GUS* and *NPT II* gene, respectively. Some of the regenerated calli selected for resistance to kanamycin, presumably due to the expression of an integrated chimeric *NPT II* gene, would also be expected to contain the chimeric *GUS* gene. Two combinations of plasmid concentrations were used for these transformations: 25 μg of each plasmid per milliliter, or 12.5 μg pCaMVNEO and 37.5 μg pBI221.1 per milliliter. The second approach to transforming sorghum protoplasts with a chimeric *GUS* gene employed electroporation with pNEOGUS15, a plasmid that contains the chimeric *NPT II* gene of pCaMVNEO and the chimeric *GUS* gene of pBI221.1, and used the same kanamycin selection scheme as the first approach. Resistant calli were produced from protoplasts after electroporation with pNEOGUS15, or pCaMVNEO and pBI221.1, and were maintained on culture medium containing kanamycin.

Of the resistant calli that developed from protoplasts electroporated with pNEOGUS15 or pCaMVNEO and pBI221.1, 77 were analyzed by DNA gel blot hybridization to determine if they were transformed with the chimeric *NPT II* and *GUS* genes. Total DNA isolated from each callus clone was digested with EcoRI and HindIII, StuI, or was left undigested, and then electrophoresed through an agarose gel. A gel containing DNA from 10 of the 77 clones analyzed is shown in Fig. 4A. EcoRI/HindIII digestion of pCaMVNEO or pNEOGUS15 produces a 1.7-kb full-length chimeric *NPT II* gene, and EcoRI/HindIII digestion of pBI221.1 or pNEOGUS15 produces a 3.0-kb full-length chimeric *GUS* gene. However, none of these plasmids are cut by StuI. The EcoRI/HindIII-digested DNA from all resistant calli analyzed contained fragments that hybridized to the *NPT II* coding sequence probe (Table 1), although some calli, e.g., 1c36 (Fig. 4B), did not contain the 1.7-kb

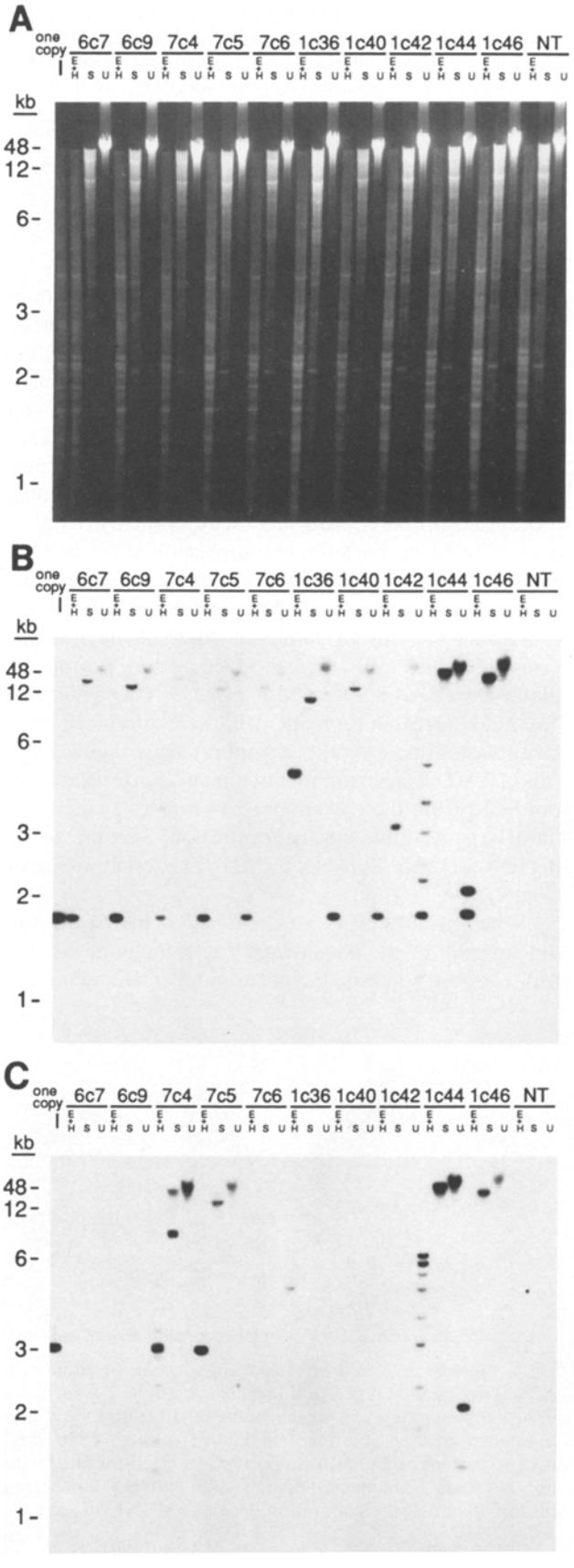

Fig. 4A–C. DNA gel blot hybridization analysis of kanamycin-resistant sorghum calli. **A** Ethidium-bromide-stained sorghum DNA. Each lane contains 1 μg of sorghum DNA digested with EcoRI and HindIII (E+H), StuI (S), or left undigested (U) before size fractionation on a 1% agarose gel. The lane labeled *one copy* contains pNEOGUS15 DNA equivalent to one copy per genome (2C-value), along with DNA from nontransformed sorghum calli, digested with EcoRI and HindIII. Kanamycin-resistant callus clones analyzed include those derived from protoplasts electroporated with 25 μg/ml pCaMVNEO and 25 μg/ml pBI221.1 (6c7 and 6c9), 12.5 μg/ml pCaMVNEO and 37.5 μg/ml pBI221.1 (7c4, 7c5 and 7c6), or 50 μg/ml pNEOGUS15 (1c36, 1c40, 1c42, 1c44, and 1c46). Lanes labeled *NT* contain DNA from a nontransformed callus. Ticks adjacent to numbers in the kb lane mark location of DNA size standards. **B** Autoradiogram of gel blot hybridized with the 1.0-kb BamH1 fragment of the *NPT II* gene from pCaMVNEO. **C** Autoradiogram of gel blot hybridized to the 1.9-kb BamH1-Sst1 *GUS* gene fragment from pBI221.1

restriction fragment characteristic of the full-length chimeric *NPT II* gene. Some clones, e.g., 1c44 and 1c46 (Fig. 4B), contained other hybridizing fragments in addition to the 1.7-kb fragment. EcoRI/HindIII-digested DNA from nontransformed calli did not contain fragments hybridizing to the *NPT II* probe.

The DNA gel blot hybridization analysis of EcoRI/HindIII-digested total DNA from resistant calli derived from protoplasts electroporated with pNEOGUS15 revealed that 50% of the clones contained only a 1.7-kb fragment that hybridized to the *NPT II* probe (Table 1), 18% contained a 1.7-kb fragment plus other hybridizing fragments of other sizes, and 32% contained only fragments of other sizes. Of calli resulting from electroporation with 12.5 μg pCaMVNEO and 37.5 μg pBI221.1 per milliliter, 38% contained only a 1.7-kb fragment, 35% contained a 1.7-kb fragment and other-sized fragments, and 27% contained only other sized fragments. Of calli resulting from electroporation with equal concentrations of the two plasmids, 54% contained only a 1.7-kb fragment, 15% contained a 1.7-kb fragment and other-sized fragments, and 31% contained only other-sized fragments. It appears that cointegration of the full-length, nonselectable (*GUS*) gene is favored when the sequence containing this gene represents a high proportion of the plasmid mixture used for transformation. All of the resistant calli analyzed by DNA gel blot hybridization in Fig. 4 expressed *NPT II* activity, while nontransformed calli did not (Fig. 5). In addition to clone 1c36 (Fig. 5), four other resistant calli whose EcoRI/HindIII-digested DNA yielded only *NPT II* gene-hybridizing fragments of sizes other than 1.7 kb expressed *NPT II* activity (data not shown).

DNA from resistant calli, when digested with StuI, yielded restriction fragments that hybridized to the *NPT II* gene probe which were larger than the 1.7-kb full-length chimeric *NPT II* gene (Fig. 4B). In general, the StuI fragments hybridizing to the *NPT II* probe were larger than 12 kb, but smaller in size than the uncut DNA (which also hybridized to the *NPT II* probe). However, in some cases the StuI-hybridizing fragments were only slightly larger than the full-length chimeric *NPT II* gene, e.g., the 3.2-kb StuI fragment of 1c42 (Fig. 4B). No hybridization band was present in any of the lanes containing DNA, digested or undigested, from nontransformed calli (Fig. 4B).

All transformation treatments produced kanamycin-resistant callus clones whose DNA, upon digestion with EcoRI and HindIII, contained fragments that hybridized to the *NPT II* gene probe (Table 1), although not all calli contained *GUS* gene hybridizing sequences. DNA gel blot hybridization analysis of EcoRI/HindIII-digested total DNA from resistant calli derived from protoplasts electroporated with pNEOGUS15 revealed that 63% of the clones contained fragments hybridizing to the *GUS*

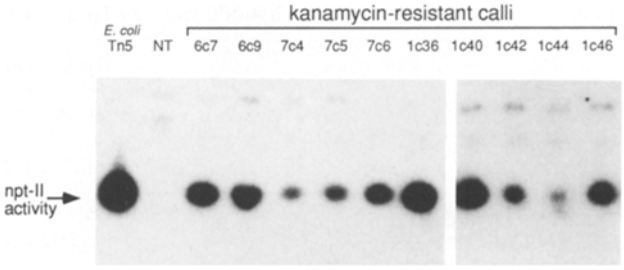

Fig. 5. *NPT II* assay of kanamycin-resistant sorghum calli. Individual kanamycin-resistant callus clones produced from protoplasts electroporated with 25 μg/ml pCaMVNEO and 25 μg/ml pBI221.1 (6c7 and 6c9), 12.5 μg/ml pCaMVNEO and 37.5 μg/ml pBI221.1 (7c4, 7c5 and 7c6), or 50 μg/ml pNEOGUS15 (1c36, 1c40, 1c42, 1c44, 1c46), non-transformed calli (*NT*), and an extract from a *NPT II*-producing strain of *E. coli* were analyzed for *NPT II* activity as described in 'Materials and methods'

gene probe, whereas only 13% contained a 3.0-kb fragment characteristic of the full-length chimeric *GUS* gene (Table 1). Of the calli resulting from electroporation with 12.5 μg pCaMVNEO and 37.5 μg pBI221.1/ml, 42% contained hybridizing fragments, but only 19% contained a 3.0-kb fragment. Of calli resulting from electroporation with equal concentrations of the two plasmids, 38% contained hybridizing fragments, yet only 8% contained a 3.0-kb hybridizing fragment. Of those calli containing EcoRI-HindIII *GUS*-hybridizing fragments, most contained only fragments other than the 3.0-kb fragment representing the full-length gene in the plasmids used for transformation, e.g., 1c44 and 1c46 in Fig. 4C. Fewer calli contained the 3.0-kb fragment and other-sized fragments, e.g., 7c4 in Fig. 4C, while others contained the 3.0-kb fragment exclusively, e.g., 7c5 in Fig. 4C. Only the DNA from calli containing EcoRI-HindIII fragments that hybridized to the GUS gene probe also hybridized after digestion with StuI or when left undigested. The StuI fragments that hybridized to the *GUS* probe often produced a hybridizing band of the same size when hybridized to the *NPT II* probe. Again, no hybridization band was present in any of the lanes containing DNA, digested or undigested, from nontransformed calli.

Those calli containing a 3.0-kb EcoRI-HindIII DNA fragment that hybridized to the *GUS* probe had, on average, 3.5 times greater *GUS*-specific activity (3.0 pmol 4-MU[1] μg protein^{-1} min^{-1}) than did those kanamycin-resistant calli that did not contain a *GUS*-hybridizing fragment (mean = 0.881 pmol 4-MU[1] μg protein^{-1} min^{-1}; standard deviation = 0.339; $n = 31$), when assayed using the substrate MUG, although many calli that contained the 3.0-kb-hybridizing fragment had levels of GUS-specific activity no greater than the nontransformed calli or calli transformed with just the *NPT II* gene. When calli containing the 3.0-kb EcoRI-HindIII *GUS*-hybridizing

Table 1. Results of DNA gel blot hybridization analysis of kanamycin-resistant sorghum calli

Characterization of kan^r clones	Transformation treatment		
	pCaMVNEO 25 μg/ml + pBI221.1 25 μg/ml	pCaMVNEO 12.5 μg/ml + pBI221.1 37.5 μg/ml	pNEOGUS15 50 μg/ml
Number analyzed	13	26	38
Number with *NPT-II* sequence	13 (100%)	26 (100%)	38 (100%)
Number with full-length *NPT-II* gene	9 (69%)	19 (73%)	26 (68%)
Number with full-length *NPT-II* gene exclusively	7 (54%)	10 (38%)	19 (50%)
Number with *GUS* sequence	5 (38%)	11 (42%)	24 (63%)
Number with full-length *GUS* gene	1 (8%)	5 (19%)	5 (13%)
Number with full-length *GUS* gene exclusively	0	1 (4%)	3 (8%)

fragment were incubated in the histochemical substrate x-gluc, some contained small clusters of cells that stained an intense blue, but the majority of cells in the calli did not stain. Not all callus clones containing the 3.0-kb fragment stained blue upon incubation with the substrate x-gluc. However, nontransformed calli or calli transformed with just the *NPT II* gene never stained blue after the same treatment.

Discussion

The increase in the number of resistant calli produced after electroporation with circular form pCaMVNEO (Fig. 2) is similar to the trend seen by Shillito et al. (1985) with tobacco protoplasts, in which the number of resistant calli produced increased with increasing transforming DNA concentration. The curve seen in Fig. 2 suggests that plasmid concentrations greater than the maximum of 50 μg/ml used in this experiment would provide even greater numbers of resistant calli per electroporation treatment, although sufficient numbers of transformants were produced for this work using 50 μg/ml or less.

Although carrier DNA has been used in protoplast transformations using PEG treatment (Krens et al. 1982; Paszkowski et al. 1984; Potrykus et al. 1985), no significant difference in the mean number of resistant calli produced was seen when carrier DNA was added to electroporation treatments (Fig. 3). This differs from the results of Shillito et al. (1985), in which inclusion of calf thymus carrier DNA (50 μg/ml) with the transforming plasmid DNA increased the number of resistant tobacco calli produced. This discrepancy may be due to differences in the transformation procedure of Shillito et al. (1985), which utilizes linearized transforming plasmid DNA and a PEG and electroporation treatment.

The mean number of resistant calli produced from protoplasts after electroporation with pCaMVNEO linearized near the 3′ end of the chimeric *NPT II* gene was approximately two times greater than that produced from circular form pCaMVNEO (Fig. 3). Since a single location was used to determine the effect on transformation efficiency of linearizing the transforming DNA, the effect of the particular site of linearization outside of the chimeric gene cannot be determined. A disadvantage of using linearized DNA in the transformation procedure is the additional expense and effort required to prepare it compared to the circular form plasmid DNA.

The production of kanamycin-resistant sorghum calli after electroporation of sorghum protoplasts with pCaMVNEO (Fig. 1), or pNEOGUS15 containing the same chimeric gene, matches the results obtained by Fromm et al. (1986) after electroporation of maize protoplasts with pCaMVNEO. For every transformation treatment, all kanamycin-resistant calli analyzed by DNA gel blot hybridization contained restriction fragments hybridizing to the *NPT II* gene, with approximately 70% of the clones containing a restriction fragment corresponding to the full-length gene and the remaining 30% containing other-sized hybridizing fragments. All of the resistant calli analyzed that contained only partial genes expressed *NPT II* activity, while nontransformed calli were sensitive to kanamycin and never expressed *NPT II* activity. This provides evidence that resistant callus clones containing restriction fragments differing in size from the 1.7-kb full-length gene still contained functional chimeric *NPT II* genes. Presumably, the loss of sequences not critical to gene expression near the terminal restriction sites of the chimeric gene is responsible for the presence of functional, aberrant-sized restriction fragments. Alternatively, larger deletions may have been complemented by the insertion of a nonfunctional gene adjacent to plant gene regulatory elements that caused expression of the *NPT II* coding sequence. A more detailed mapping of the integrated DNA using probes specific to the ends of the *NPT II* chimeric gene could determine the degree of any deletions.

In addition to the frequency of transformation of calli with a nonselectable gene, the number of copies and the pattern of integration are important factors in evaluating the results of direct DNA transformation. If the objective of obtaining transformants is to determine the consequence of inserting a DNA sequence into the plant genome, then the simplest pattern of integration, in which only the full-length sequence is present, would be the desired outcome. The results of gene expression from transformants containing multiple sizes and copies of a gene cannot be interpreted clearly. The analysis by Schocher et al. (1986) of 12 kanamycin-resistant callus clones obtained from tobacco protoplasts transformed by PEG-mediated uptake of equal concentrations of two plasmids, one containing a chimeric *NPT II* gene and one containing a zein gene, showed that 75% contained zein sequence and 25% contained the full-length gene. The pattern of integration was typically complex, with multiple copies and variable size inserts. The analysis of Lyznik et al. (1989) of 51 kanamycin-resistant callus clones obtained from the PEG-mediated uptake of two plasmids, one containing a chimeric *NPT II* gene and one containing a chimeric *GUS* gene, showed that 61% of the clones contained sequences hybridizing to a *GUS* probe. In this work, calli containing sequences hybridizing to the second nonselectable gene ranged from 38 to 63%, and those containing the full-length sequence ranged from 8 to 19% (depending upon the transformation treatment). These frequencies are somewhat lower than those seen by Schocher et al. (1986) and Lyznik et al. (1989), but the number of copies integrated is generally low and there are fewer variable-sized inserts. Perhaps this difference is due to the use of electroporation instead of PEG-mediated transfer of DNA to protoplasts. If the goal of the transformation procedure is to introduce high copy numbers of a nonselectable gene and the introduction of variable-sized inserts is not important, then the PEG-mediated transformation procedures utilized by Schocher et al. (1986) and Lyznik et al. (1989) may be suitable. However, if the simplest pattern of integration is desired, then an electroporation-mediated transformation procedure like the one utilized in this investigation may be more appropriate.

The results of this investigation unequivocally confirm the stable transformation of sorghum protoplasts after electroporation-mediated uptake of chimeric *NPT II* and *GUS* genes. There appears to be no appreciable difference in the frequency of kanamycin-resistant calli produced that contain a nonselectable *GUS* gene, when sorghum protoplasts are electroporated with either a plasmid containing a chimeric *NPT II* gene covalently linked to a chimeric *GUS* gene or with separate plasmids containing the same genes.

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