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Transgenic plants of blue grama grass, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud., from microprojectile bombardment of highly chlorophyllous embryogenic cells

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Abstract For the first time, the production of transgenic plants of the forage grass blue grama, *Bouteloua gracilis* [H.B.K.] Lag. ex Steud., is reported. Transgenic plants containing a *gus* :: *nptll* fusion driven by a double CaMV35S promoter were obtained by microprojectile bombardment of the highly chlorophyllous embryogenic cell line 'TIANSJ98'. Transformed *B. gracilis* cell lines resisted a lethal concentration of 160 mg/l of kanamycin for at least 8 months. Chlorophyll development and growth rate were used as useful criteria for discriminating transformed from non-transformed clones. Stable integration of the transgene in the blue grama genome was demonstrated by PCR and Southern-hybridization analysis. Expression of the NPTll protein in transgenic plants grown under greenhouse conditions was confirmed indirectly by spraying kanamycin (150–250 mg/l) on plant foliage, and directly by ELISA immunological tests. Control plants sprayed with kanamycin showed foliar necrosis and reduced growth (tillering) compared to plants containing the transgene. NPTll was found in transgenic plants in levels ranging between 12.6 and 29.6 ng/mg FW of cells, as determined by ELISA reactions.

Keywords *Bouteloua gracilis* · Forage grass · Turfgrass · Transgenic grass · Genetic transformation · Microprojectile bombardment

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Introduction

Since production of the first transgenic forage grass, orchardgrass (*Dactylis glomerata*; Horn et al. 1988), systems for genetic transformation have been described for eight additional turf/forage grasses, namely: *Agrostis palustris* (Creeping bentgrass: Zhong et al. 1993; Hartman et al. 1994; Lee et al. 1995; Sugiura et al. 1997; Asano et al. 1998), *Agrostis alba* (Redtop: Asano and Ugaki 1994), *Agrostis stolonifera* (Dalton et al. 1998), *Festuca arundinacea* (Tall fescue: Ha et al. 1992; Wang et al. 1992; Dalton et al. 1995; Spangenberg et al. 1995a; Dalton et al. 1998), *Festuca rubra* (Red fescue: Spangenberg et al. 1994, 1995a), *Lolium perenne* (Perennial ryegrass: Spangenberg et al. 1995b; Wang et al. 1997; Dalton et al. 1998), *Lolium multiflorum* (Italian ryegrass: Wang et al. 1997; Ye et al. 1997; Dalton et al. 1998) and *Zoysia japonica* (Korean lawngrass; Inokuma et al. 1998). Compared to the number of tissue, protoplast or cell-culture methods reported for forage and turf grasses (revised by Green 1978; Ahloowalia 1984; Lee 1996; Chai and Sticklen 1998; Aguado-Santacruz et al. 2001a) the species for which transformation systems have been developed is still very low. Among the explanations accounting for this discrepancy are: (1) a slow growth of the target tissue incompatible with genetic transformation systems, (2) loss of the regenerative potential of the totipotent material with time, and (3) technical constraints related to the transformation and/or regeneration systems employed (Green 1978; Chai and Sticklen 1998). In grass transformation, protoplast, embryogenic calli or suspension cultures have been utilized as the target material for direct DNA delivery techniques such as microprojectile bombardment, electroporation, PEG and, more recently, the silicon carbide fiber method.

Blue grama, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud., is a C_4 (Waller and Lewis 1979), drought tolerant (Hoover et al. 1948), facultative apomictic (Gustafsson 1946) and perennial grass spreading throughout the North American grassland, where it yields abundant and high quality forage for domestic livestock and native

Table 1 Estimated transformation efficiency of the 'TIANSJ98' cell line using two osmotic media and three bombardment pressures

Treatment		Filters expressing transient GUS (%)[b]	Number of positive clones[c]	Transformation efficiency[d]
Mannitol (M)[a]	Pressure (PSI)			
0.0	60	10	1	0.1
0.0	80	20	3	0.3
0.0	100	10	2	0.2
0.4	60	40	16	1.6
0.4	80	60	97	9.7
0.4	100	50	50	5.0
1.0	60	10	0	0
1.0	80	20	0	0
1.0	100	20	0	0

[a] Supplied in both pre- and post-bombardment media
[b] Two days after bombardment
[c] Clones growing vigorously and developing a high content of chlorophyll in 150 mg/l of kanamycin
[d] Average number of positive clones per paper filter supporting 2 g of FW cells

fauna (Stubbendieck et al. 1986). *B. gracilis* is considered the most important native grass of the Mexican semiarid grassland (De Alba 1958; Jaramillo 1986; Orozco 1993) and the shortgrass prairie of USA (Gould 1951; Sims et al. 1973; Wilson and Briske 1979). After developing tissue and cell-culture systems for this grass (Aguado-Santacruz et al. 2001a, b) we now describe the production, by microprojectile bombardment, of transgenic plants of blue grama using a *sui generis* system based on the highly chlorophyllous and embryogenic cell line 'TIANSJ98' (Aguado-Santacruz et al. 2001b).

Materials and Methods

'TIANSJ98' cell line establishment and maintenance

The embryogenic, highly chlorophyllous 'TIANSJ98' cell line was obtained from culturing shoot apice-derived green calli in liquid MPC medium as described previously (Fig. 1a; Aguado-Santacruz et al. 2001b). This cell line was subcultured every 20 days, transferring 1 ml of the cell suspension into 24 ml of fresh MPC medium. Although the green embryogenic calli from which these cell cultures were derived could be alternatively used for transformation experiments we preferred to bombard the suspension form. The reasons for utilizing the finely dispersed condition of the embryogenic calli were 1) to synchronize the physiological stage of the target cells, 2) to maximize the distribution of the totipotent material on the paper filters (optimizing the shoot cover of the bombarded plasmids), and 3) to facilitate the identification of independent transformation events (green spots) within the dispersed cell clusters under selection.

Plasmid DNA

We used the plasmid pB1426 (Datla et al. 1991) in our experiments. This plasmid contains the sequences encoding a GUS-NPTII fusion protein under the control of a double 35S Cauliflower Mosaic Virus promoter, and a leader sequence from Alfalfa Mosaic Virus (see Fig. 3).

Microprojectile bombardment of embryogenic cells

The highly chlorophyllous embryogenic cell Iine 'TIANSJ98' was used as the target for the microprojectile delivery experiments. The cells were distributed onto 2.0-cm diameter paper-filter disks (approximately 2 g FW cells). Bombardment mixtures were as follows: 50 µl of M10 tungsten particles (15 mg/ml), 10 µl of DNA (1 µg/ml), 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M espermidine were mixed in sequential order, vortexed during 5 min and then briefly sonicated. The mixture was centrifuged at 10,000 rpm for 10 s. Sixty microliters of the supernatant were removed and the rest dispensed in 5-µl aliquots for individual shoots. Bombardments were performed using the Particle Inflow Gun (Finer et al. 1992). The particle/DNA mixture was placed in the center of the syringe filter unit. Embryogenic cells were covered with a 500-µm baffle, placed at a distance of 10 cm from the screen filter unit containing the particles, and bombarded once in the vacuum chamber at 60 mmHg. Two different osmotic media for pre- and post-bombardment treatments (0.4 and 1 M mannitol supplied in solidified MPC medium) and three bombardment pressures (60, 80 and 100 PSI) were tested. Pre-bombardment treatment was applied 24 h before shooting. After discharge, the paper filters supporting the embryogenic cells were maintained for 3-days more on the same osmotic medium used in the pre-bombardment treatment. Thus, a total of nine treatments were evaluated with ten dishes bombarded per treatment (see Table 1). As a control, filters with suspension material were bombarded using particles without DNA.

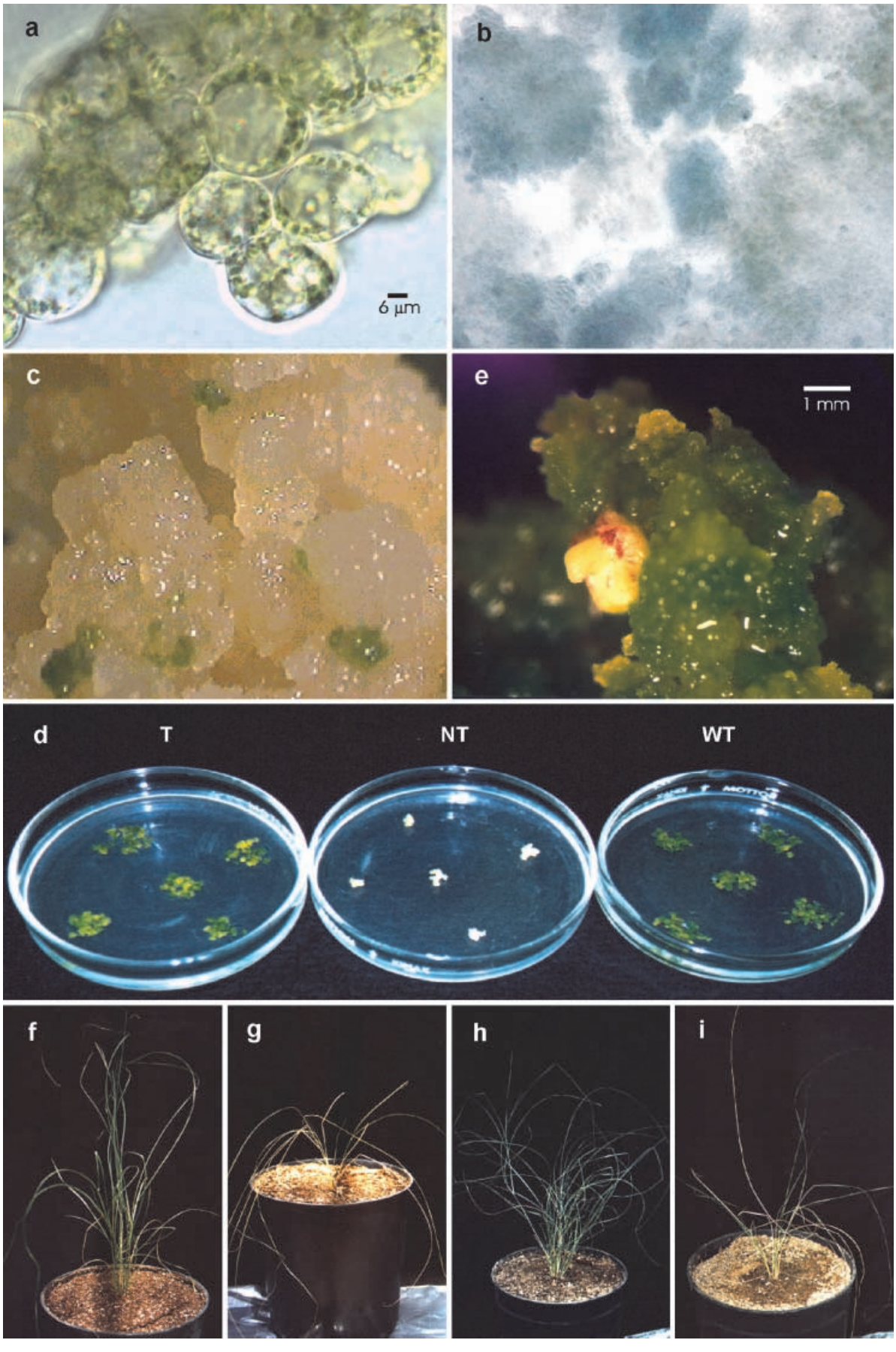
Histochemical GUS assays

GUS-expressing cells were visualized 24–48 h after DNA delivery by incubating 1/4 of the bombarded cell suspensions from each filter in 100 mM of sodium phosphate, pH 7, 10 mM of Na-EDTA, 5 mM of potassium ferricyanide, 5 mM of potassium ferrocyanide, 0.3% X-Gluc and 0.1% Triton X-100 at 37 °C for 24 h (Jefferson et al. 1987). The chlorophyllous cells were cleared with a mixture of methanol-acetone (3:1, v/v).

Selection of stable transformed clones and recovery of plants

After the 3-day post-bombardment osmotic treatment on MPC medium containing 0.4 or 1 M manitol, but lacking antibiotic, the paper filter disks supporting the bombarded cells were transferred onto MPC medium containing 140 mg/l of kanamycin and incubated at 30 ± 1 °C in white light provided by cold fluorescent lamps. The same procedure was followed for cells bombarded but not subjected to osmotic treatment. The kanamycin concentration was raised 2-months later to 150 or 160 mg/l. The cells were sub-

Fig. 1a–i Transformation of *B. gracilis* by microprojectile bombardment of highly chlorophyllous cells: **a** chlorophyllous 'TIANSJ98' cells grown in liquid MPC medium; **b** cell clumps showing transitory GUS expression 2 days after bombardment; **c** transgenic green clusters emerging from non-transformed cell masses; **d** transgenic (*T*) and non-transgenic (*NT*) clones grown on 160 kanamycin, and wild-type cells (*WT*) cultured on maintenance medium (MPC) without antibiotic; **e** germinating transgenic embryo 45 days after transference to regeneration medium; **f** transgenic and **g** non-transgenic plants sprayed during 35 days with 150–200 mg/l of kanamycin; reduced tillering in 65-day old non-transgenic **i**, as compared to transgenic **h**, plants sprayed with kanamycin


a
6 µm
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cultured every 3 weeks and maintained for 8 months in selection. After this period, kanamycin-resistant clones were transferred to regeneration medium containing full-strength MS medium (Murashige and Skoog 1962), 3% sucrose, 2.5% phytagel (Sigma, St. Louis, Missouri) but no antibiotic. The regenerated shoots were transferred for rooting to 1/2 MS containing 3.0 μM (0.56 mg/l) α-naphthaleneacetic acid, 2.5 μM (0.51 mg/l) indole-3-butyric acid and 2.5% phytagel, and incubated at 30 ± 1 °C under continuous fluorescent light. Later, rooted plantlets were transferred to pots, hardened off, and grown to maturity in a greenhouse.

DNA extraction

Total genomic DNA was prepared from kanamycin-resistant and untransformed control plants using a modification of the protocol described by Shure et al. (1983). Approximately 250 mg of cells were collected in 2-ml Eppendorf tubes and ground to a fine powder in liquid N_2 using a glass pestle attached to a homogenizer (Caframo, Stirrer type RZR). Powdered cells were re-suspended with 500 μl of extraction buffer (7.0 M urea, 0.35 M NaCl, 0.05 M Tris-HCl, pH 8.0, 0.02 M EDTA, 1% sarcosine) for at least 45 min. The cell homogenate was extracted with 1 vol of phenol/chloroform. The aqueous phase was separated by centrifugation and then precipitated using an equal volume of isopropyl alcohol. The precipitated DNA was washed once with 70% ethanol and resuspended in TE buffer (0.01 M Tris-HCl, 0.01 M EDTA, pH 8.0).

PCR analysis

For PCR analysis, 100 to 150 ng, and 10 ng were used for genomic and plasmid DNA amplifications, respectively, in 25-μl reactions. Primers 5′ -TATTCGGCTATGACTGGGCA-3′ and 5′ -GCCAAC-GCTATGTCCTGATA-3′ were designed for amplifying an internal 620-bp fragment of the *nptII* gene. PCR reactions were carried out using a Perkin Elmer thermocycler for 30 cycles. Reaction temperatures were denaturation 95 °C (2 min), annealing 56 °C (30 s), and extension 72 °C (30 s). The 25-μl reaction volumes contained: 1 × PCR buffer, 0.25 mM of dNTPs, 2 mM $MgCl_2$, 0.2 μM of primers and 2.5 u of *Taq*. The amplification products were electrophoresed in 1% agarose/ethidium bromide gels.

Southern-blot analysis

Southern blotting and hybridization was carried out following the manufacturer's manual for the DIG system (Boehringer, Mannhein, Germany). Total plant DNA (25 μg $A_{260/280}$) from putative transformants and wild-type plants was digested overnight at 37 °C, using 15 units/μg of *Bam*HI and *Eco*RI, separated in a 0.7% agarose gel and transferred to a nylon membrane (Hybond N+, Amersham, Piscataway, NJ). The filter was pre-hybridized at 65 °C in 7% SDS, 0.5 M $NaPO_4$ and 1% bovine serum albumin (BSA) for 4 h, and hybridized overnight at 65 °C in the same buffer. A digoxigenin-labelled *nptII* probe (620 pb) was prepared by PCR using 15 ng of plasmidic DNA. After cutting the genomic DNA with *Bam*HI and *Eco*RI, the expected size in the hybridization analysis with the digoxigenin-labelled *nptII* probe (620 bp) was approximately 3,050 bp, which includes the complete *gus* :: *nptII* fusion contained in plasmid pB1426 (Fig. 3).

Filters were sequentially washed in 2 × SSC, 0.1% SDS for 5 min at RT, 1 × SSC, 0.1% SDS at 65 °C for 15 min 0.5 × SSC, 0.1% SDS at 65 °C for 5 min. Filters were visualized by autoradiography using X-irradiation (Cronex, DuPont, Wilmington, DE).

Immunological detection of NPTII protein

The expression of NPTII was analyzed in kanamycin-resistant and control clones using a kit for immunological detection of this protein (Pathoscreen kit for Neomycin Phosphotransferase II, Agdia Inc., Elkhart, Ind.) following the manufacturer's indications. ELISA reactions were read in a Titertek Multiskan Plus spectrophotometer (Titerker Inc., Huntsville, Ala.) at three incubation times before stopping reactions with 3 M sulfuric acid.

In planta resistance to kanamycin

Thirty days after transplanting the regenerated transgenic and control plants to pots in a greenhouse, they were sprayed every 2 days with 3 ml of a solution of kanamycin, 150 mg/l plus SDS 0.1%, during 2 weeks. Later, the concentration of kanamycin was raised to 200 mg/l and applied daily for another 7 days. A final daily spraying of 250 mg/l of this antibiotic for the next week was applied to the plants.

Results

Transformation of the chlorophyllous 'TIANSJ98' cell line

For each individual bombardment, approximately 2.0 g of FW cells were collected from 6-month-old green cultures of *B. gracilis* (subcultured every 20 days in fresh liquid MPC medium), distributed onto a paper filter disk and placed 24 h before bombardment on solidified MPC medium containing 0, 0.4 or 1.M mannitol (1-day pre-bombardment treatment). After shooting, 1/4 of each paper filter was utilized for GUS staining and the rest of the material transferred for 3 days to post-bombardment media containing the same concentration of mannitol utilized in the pre-bombardment phase (0, 0.4 or 1 M). Pre-bombardment osmotic treatment with 0.4 M mannitol resulted in a higher number of filters expressing GUS transitorily. A similar percentage of GUS-positive filters was observed for treatments in which no mannitol or 1 M mannitol was added to the maintenance medium (Table 1 and Fig. 1b).

After the 3-day post-bombardment treatment, the osmotic-stressed cells were transferred to maintenance medium containing kanamycin (140 mg/l) as a selective agent. The 3-day post-bombardment treatment in 1.0 M mannitol proved to be lethal for cells transferred to selective media; no stable transgenic clones could be recovered from osmotic media containing 1.0 M mannitol because cell death occurred within 1 week after transference to Km-media (Table 1).

A higher number of filters expressing transient GUS and positive clones was obtained from 80 PSI-bombarded cells subjected to 0.4 M mannitol treatment, while fewer filters showing blue-staining and less-positive clones were produced when bombardment pressures of 60 or 100 PSI were utilized. Thus, the best transformation efficiency (9.7 clones per filter) was obtained when a pre- and post-bombardment treatment with 0.4 M mannitol was applied to the cells 1-day before and for 3-days after shooting at 80 PSI (Table 1). Although the transformation efficiencies obtained in this study ranged from 0.05 to 4.8 clones/g of FW cells (Table 1), values would be expected to be a little higher as part of the bombarded material (1/4 of each filter) was sacrificed for histochemical GUS assays.

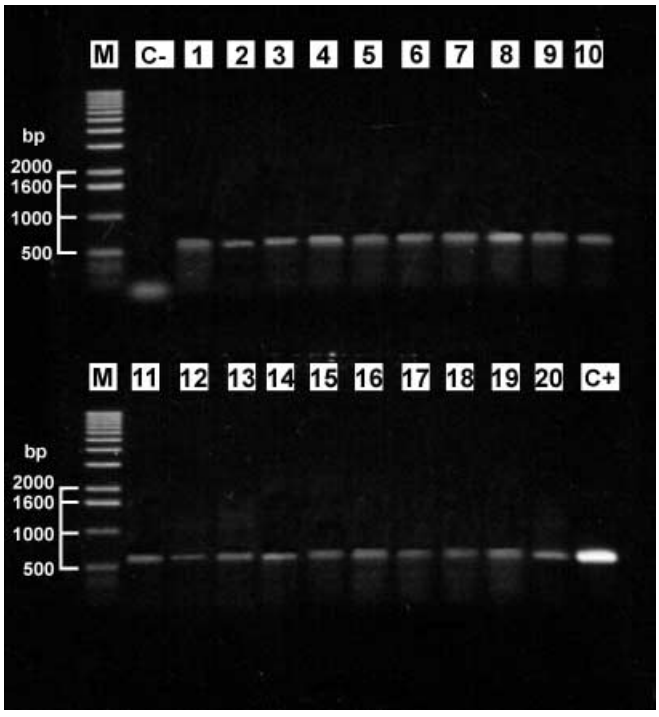

Fig. 2 PCR analysis of 150 mg/l of kanamycin-resistant *B. gracilis* clones. *Lanes M:* 1-kb molecular marker; *lane C–*: untransformed control plant; *lanes numbered 1–10* and *11–20*: putative transformant clones derived from five independently bombarded filters; *lane C+*: positive control (pB1426 DNA)

Dose-response experiments with 'TIANSJ98' cells, cultured in solid and liquid MPC medium containing kanamycin in the concentration range of 10 to 150 mg/l, had shown that selection on solidified MPC medium with 150 mg/l of kanamycin was lethal to untransformed cells, while cell growth in liquid conditions was inhibited from 40–50 mg/l of kanamycin onwards (data not shown). Based on these data, the initial utilized kanamycin concentration of 140 mg/l was raised 2 months later to 150 or 160 mg/l in order to increase the selection strength.

Fast growth and the development of high-chlorophyll content identified positive clones. Positive clones were initially detected as green or green-yellowish points within a yellow, light yellow, yellow-whitish or white cell-masses (Fig. 1c) within 4 days after transference to selective medium containing 140 mg/l of kanamycin. Transgenic clones initially appearing as green yellowish spots turned green with subculturing (between 3–4 months). The green color of Km-resistant clones was similar to that observed for control cells grown on the maintenance medium without antibiotics (Fig. 1d).

Regeneration of transgenic plants

After 8 months in selection with l60 mg/l of kanamycin, cells were transferred to regeneration medium containing full-strength MS medium but no antibiotics. A period of 1.5 to 2 months was required to obtain vigorous shoots. The development of adequately rooted plantlets took another 2 months of growth on rooting media containing $^1/_2$ MS plus α-naphthalene acetic acid and indole-3-butyric acid. The regenerated transgenic and control plantlets were transferred to soil under greenhouse conditions when they developed at least one long (2–3 cm) and thick root, which secured obtaining 100% establishment, independently of the plantlet source.

Analysis of kanamycin-resistant lines

To determine whether kanamycin-resistant clones were indeed transgenic, an initial PCR screening was performed with primers designed to amplify a 620-bp internal *nptll* fragment. Analysis from representative plants (20 individuals) is presented in Fig. 2. The PCR amplified products revealed the presence of the expected band in putative transformants (numbered lanes) and in the positive control (plasmidic DNA, lane C+); this band was absent in control plants (lane C–). Transgenic clones included in the following groups were obtained from different filters: (1) 1 and 2; (2) 3–11 and 18; (3) 12, 17 and 20; (4) 13–16 and 19.

In order to confirm the transgenic status of the same 20 Km-resistant clones previously analyzed by PCR, Southern-blot hybridization was performed. With this aim, total DNA from PCR positive plants and untransformed control plants was digested with *Bam*Hl and *Eco*RI, separated in a 0.7% agarose gel, transferred to a nylon membrane and hybridized against a 620-bp digoxigenin-labelled *nptll* probe. The expected size in the hybridization analysis was approximately 3,050 bp, which includes the complete *gus* :: *nptll* fusion contained in plasmid pB1426. Figure 3 shows representative results for 24 transgenic *B. gracilis* plants. The predicted band is evident in all of the previous PCR positive clones (numbered lanes) and in positive controls (plasmidic DNA, lanes C+), but absent in non-transformed control plants (lanes C–). Besides the expected 3,050-bp signal, a fragment of higher molecular weight (approximately 3,930 bp)was also observed in some clones (lanes numbered 7–12, 15–19 and 21). Particularly for clone #20, the presence of multiple hybridization bands suggests the integration of multiple partial copies of the transgene (Fig. 3)

Assays for immunological detection of the NPTll protein confirmed its presence in transgenic kanamycin-resistance clones. NPTll levels in transgenic clones varied between 12.6 and 29.6 ng/mg of FW cells, with an average background level of 2.2 ng/mg of FW cells in control cells (Fig. 4).

Our selection scheme based on 150 mg/l of kanamycin apparently did not impair somatic embryogenesis (Fig. 1e) since regeneration frequencies from transgenic clones were similar to those observed for control cells grown under non-selective conditions (data not shown). Conversely, non-transformed clones grown under selec-

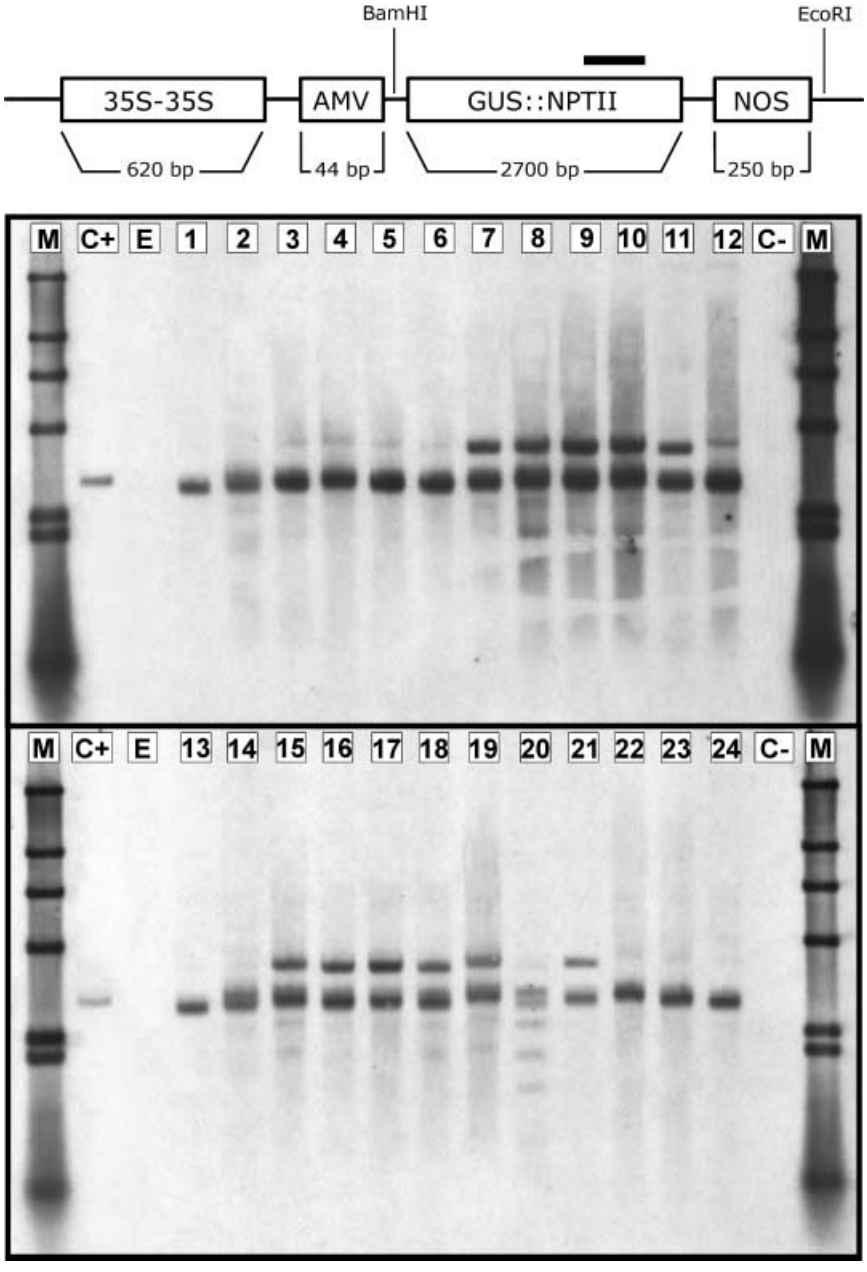

Fig. 3 Southern-blot hybridization analysis of PCR-positive 150 mg/l of kanamycin-resistant *B. gracilis* clones; 25 µg of *B. gracilis* total genomic DNA, and 650 pg of pB1426 vector, were digested with *Bam*HI and *Eco*RI enzymes and hybridized against a 620-bp *nptII* internal fragment. *Lanes M*: λ *Hin*dIII molecular marker; *lanes C+*: positive controls (pB1426); *lanes E*: empty tracks; *lanes C–*: untransformed control plants; *lanes 1–I2* and *13–24*: PCR-positive transformed clones (the first 20 clones are the same individuals shown in the PCR analysis of Fig. 2). A schematic representation of pB1426 is presented indicating unique restriction sites for the construct. The bold line represents the PCR probe used for hybridization analysis

tion (yellow-whitish, light yellow or white clusters) and then transferred to regeneration medium were normally not committed into embryogenesis; but when this occurred only yellow plantlets were produced, which stopped growth 1 month after transference to regeneration medium failing to form roots and dying a few days later.

One month after transference to soil, plants were assayed for resistance to sprayed kanamycin (150 mg/l plus 0.1% SDS) on the foliage. The day after the first application of antibiotic, the non-transformed control plants leant over, while transgenic plants remained standing and turgid (Fig. 1f, g). Later, control plants sprayed with this antibiotic showed necrotic leaves and reduced tillering as compared to transgenic plants; by day 35 after the first application of kanamycin, the control plants had an average of three tillers, while transgenic plants had produced around 14 tillers (Fig. 1h, i).

We found differences in kanamycin resistance among *B. gracilis* transgenic lines, which relates to the level of NPTll present in each clone. As a result, some transgenic clones remained yellow in 160 mg/l of kanamycin but turned green when transferred to 150 or 140 mg/l of kanamycin. When yellow transgenic clones were transferred into regeneration medium, only yellow plantlets were also obtained. Thus, evidence for a chlorophyll-dependent embryogenesis and/or plant regeneration in 'TIANSJ98' cells was noticed with only green clones developing viable green plantlets.

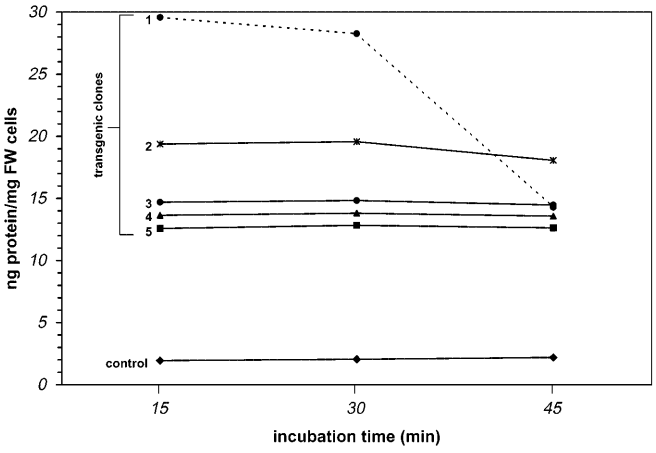

Fig. 4 Production of NPTll protein in five transgenic clones of *B. gracilis* as determined by ELISA immunological assays at three incubation times

Discussion

In this work, transgenic plants of *B. gracilis* were obtained by microprojectile delivery into the highly chlorophyllous 'TIANSJ98' cell line (Aguado-Santacruz et al. 2001b). Although embryogenic cell-suspension systems have been previously used for genetic transformation of grasses (Hartman et al. 1994; Spangenberg et al. 1995a, b; Dalton et al. 1998), this is the first report in which highly chlorophyllous embryogenic cells are used with this aim.

Selection based on aminoglycoside antibiotics, such as kanamycin, neomycin, paromomycin or geneticin G418, has been generally considered of low efficiency in the Poaceae (Hauptmann et al. 1988; Inokuma et al. 1998; Dekeyser et al. 1989), occuring only one report on the utilization of aminoglycosides (G418) for the production of transgenic grasses (Asano and Ugaki 1994). In our case, however, we found kanamycin to be a very efficient selectable agent at moderately high levels for *B. gracilis*. This concentration of kanamycin is comparable to that utilized previously in some dicots such as papaya (Cai et al. 1999) or tobacco (Holmström et al. 2000). Studies with other morphogenic callus lines of *B. gracilis* (Aguado-Santacruz et al. 2001a) have also shown the efficiency of the aminoglycoside antibiotic, paramomycin, as a selective agent, at similar concentrations to those reported in this work for kanamycin (150 mg/l).

Kanamycin and related aminoglycosides act as inhibitors of ribosome-protein synthesis (Davies and Smith 1978). As a consequence, chlorophyll production in non-transformed 'TIANSJ98' cells grown in MPC medium with 150 mg/l of kanamycin was seriously affected. This sensitivity in chlorophyll development to kanamycin makes the 'TIANSJ98' line a very good system for genetic transformation of *B. gracilis* because of the selection of (green) transformant clones. Based on this characteristic, our selection scheme allowed for a tight and effective discrimination of transgenic 'TIANSJ98' clones. In all tested cases the green Km-resistant cells proved to be transgenic by PCR screening and Southern-hybridization analysis.

The highest transformation efficiency (9.7 positive clones/bombarded filter) was obtained using pre-bombardment medium with 0.4 M mannitol and a bombardment pressure of 80 PSI. Under these conditions, all bombarded dishes developed at least five transgenic clones. This efficiency is higher than that reported for other grasses using comparable technical approaches. For example, Spangenberg et al. (1995b) recovered hygromycin-resistant calli of perennial ryegrass (*L. perenne*) in 14 and 26% of the bombarded dishes, depending on two selection schemes used. In tall fescue (*F. arundinacea*) and red fescue (*F. rubra*) 19 to 45% of the bombarded filters contained resistant clones, with 1.2 and 1.4 hygromycin-resistant calli per bombarded dish for tall and red fescue, respectively (Spangenberg et al. 1995a).

The integration patterns of the *gus* :: *nptll* gene fusion in *B. gracilis* were simple and with few gene rearrangements, as seen in other systems (Zhong et al. 1993; Spangenberg et al. 1995a; Dalton et al. 1998). Although the hybridization patterns observed for many of our transgenic clones were quite similar, at least those obtained from different bombarded dishes are considered independent transformation events. As in our case, Inokuma et al. (1998) working with *Zoysia japonica* and Kuai et al. (1999) with *F. arundinacea*, obtained almost identical hybridization patterns using probes for the *gus* and *bar* genes, respectively, in transgenic plants generated by PEG-mediated direct gene transfer. In contrast, most of the recovered transgenic grasses up to now show more-complex integration patterns (Horn et al. 1988; Wang et al. 1992; Hartman et al. 1994; Spangenberg et al. 1994, 1995b).

Transitory GUS expression was observed in transformed cells 1–2 days after bombardment with an apparent relationship to the frequency of stably transformed clones. GUS staining was never detected in control cells. However, we found that GUS could not be a reliable reporter for the stable transformation of *B. gracilis* if technical considerations are not taken into account, because clones expressing GUS lost this capacity within 4–6 months after bombardment. In addition, a strong endogenous β-glucuronidase activity was detected, by means of both histochemical and quantitative fluorimetric GUS enzymatic assays (data not shown), in wild-type plants of *B. gracilis* (at least 15 different genotypes tested), especially in zones metabolically and mitotically more active such as the collar region and stem nodes, where Zhong et al. (1993) also refer to the more intense GUS staining in trangenic *A. palustris*.

Problems associated with GUS expression have been reported previously in transgenic grasses. Dalton et al. (1998) detected transitory GUS expression in 20–40% of colonies in cell suspensions of *L. multiflorum*, *L. perenne* and *F. arundinacea*, which were co-transformed with the *hph* and *gus* genes contained in two different plasmids. According to the authors, however, this activity could

not be found in regenerated plants due to unstable co-transformation.

That 'TIANSJ98' cells containing a NPTII :: GUS fusion protein failed to express GUS 4–6 months after bombardment seems rather intriguing, since these cells were under selective conditions (kanamycin 140–160 mg/l) when they finally failed to show blue staining. Because a complete methylation or inactivation of the transgene would lead to cell death, a proteolitic activity being developed in the ageing process of the cultured cells against the GUS protein might explain this finding. Accordingly, Kuai and Morris (1996) have found that the age and stage of the growth cycle are important factors affecting GUS expression in cell cultures of *F. arundinacea*. Unknown inhibitors of the GUS reaction have been postulated before in rice (Meijer et al. 1991) where, additionally, the GUS background can present a technical problem (Kosugi et al. 1990).

We report here the first protocol for genetic transformation of the forage grass *B. gracilis*. As such, different parameters affecting the efficiency of transformation should be optimized. For example, testing for alternative osmotic treatments would be important in the future. In our first trials, we achieved lower transformation efficiencies without the application of the 0.4 M mannitol pre-bombardment or the 3-day post-bombardment osmotic treatment, so we are routinely using these treatments as part of our protocol for the genetic transformation of *B. gracilis*. In this work a construct containing a *gus* :: *nptll* fusion, under control of a double 35S Cauliflower Mosaic Virus promoter and a leader sequence from Alfalfa Mosaic Virus, was introduced into the chlorophyllous 'TIANSJ98' cells. Expression of foreign genes under the control of monocot-specific promoters, such as the Act1 5′ regulatory sequence (Zhang et al. 1991), should also be tested. In addition, the kanamycin sensitivity shown by the 'TIANSJ98' cell line would permit testing constructs originally designed for dicots based on NPTll and driven by constitutive promoters such as 35S.

Further refinements of our transformation protocol could also include a preliminary screening of clones putatively transgenic to *nptll* by spectophotometric determination of chlorophyll or/by using the chlorophyll fluorescence for the kanamycin resistance assay originally proposed by Eu et al. (1998) for discriminating transgenic plants carrying the *nptll* gene as a selectable marker.

This work opens the possibility of evaluating genes with capabilities for altering the structure and/or physiology of higher plants to solve an old and well-documented problem in *B. gracilis*, namely the establishment of seedlings under natural conditions. (Hyder et al. 1971). Also important would be the improvement of the blue grama forage quality, a topic gaining relevance in forage plants (Tabe et al. 1995; Heath et al. 1998). Because of the special nature of the highly chlorophyllous 'TIANS98' cell line, very important information can also be generated by studying cellular and plant processes related to photosynthesis and osmotic stress (Aguado-Santacruz et al. 2001b). Finally, applied and basic knowledge related to the chloroplast transformation technology (Maliga et al. 1993) is another issue which could be focused in graminaceous plants with the 'TIANSJ98' chlorophyllous cell line.

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References

Aguado-Santacruz GA, Cabrera-Ponce JL, Olalde-Portugal V, Sánchez-González MR, Márquez-Guzmán J, Herrera-Estrella L (2001a) Tissue culture and plant regeneration of blue grama grass, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud. In Vitro Cell Dev Biol-Plant (in press)

Aguado-Santacruz GA, Cabrera-Ponce JL, Ramírez-Chávez E, León-Ramírez CG, Rascón-Cruz Q, Herrera-Estrella L, Olalde-Portugal V (2001b) Establishment, characterization and plant regeneration from highly chlorophyllous embryogenic cell cultures of blue grama grass, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud. Plant Cell Rep 20:131–136

Ahloowalia BS (1984) Cereals. Forage grasses. In: Ammirato PV, Evans DA, Sharp WR, Yamada Y (eds) Handbook of plant cell culture. Mcmilliam Publishers, New York, Vol 3, pp 91–125

Asano Y, Ugaki M (1994) Transgenic plants of *Agrostis alba* obtained by electroporation-mediated direct gene transfer into protoplasts. Plant Cell Rep 13:243–246

Asano Y, Ito Y, Fukami M, Sugiura K, Fujiie A (1998) Herbicide-resistant transgenic creeping bentgrass plants obtained by electroporation using an altered buffer. Plant Cell Rep 17:963–967

Cai W, Gonsalves C, Tennant P, Fermin G, Souza M Jr, Sarindu N, Jan F-J, Zhu H-Y, Gonsalves D (1999) A protocol for efficient transformation and regeneration of *Carica papaya* L. In Vitro Cell Dev Biol-Plant 35:61–69

Chai B, Sticklen MB (1998) Applications of biotechnology in turfgrass genetic improvement. Crop Sci 38:1320–1338

Dalton SJ, Bettany AJE, Timms E, Morris P (1995) The effect of selection pressure on transformation frequency and copy number in transgenic plants of tall fescue (*Festuca arundinacea* Schreb.). Plant Sci 108:63–70

Dalton SJ, Bettany AJE, Timms E, Morris P (1998) Transgenic plants of *Lolium multiflorum*, *Lolium perenne*, *Festuca arundinacea* and *Agrostis stolonifera* by silicon carbide fibre-mediated transformation of cell suspension cultures. Plant Sci 132:31–43

Datla RSS, Hammerlindl JK, Pelcher LE, Crosby WL, Selvaraj G (1991) A bifunctional fusion between β-glucuronidase and neomycin phosphotransferase: a broad-spectrum marker enzyme for plants. Gene 101:239–246

Davies J, Smith DI (1978) Plasmid-determined resistance to antimicrobial agents. Ann Rev Microbiol 32:469–518

De Alba J (1958) Alimentación del Ganado en América Latina. La Prensa Médica Mexicana, México, D. F.

Dekeyser R, Claes B, Marichal M, Van Montagu M, Caplan A (1989) Evaluation of selectable markers for rice transformation. Plant Physiol 90:217–223

Eu YJ, Lee MH, Chang HS, Rhew TH, Lee HY, Lee CH (1998) Chlorophyll fluorescence assay for kanamycin resistance screening in transgenic plants. Plant Cell Rep 17:189–194

Finer JJ, Vain P, Jones MW, McMullen MD (1992) Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep 11:323–328

Green CE (1978) In vitro regeneration of cereals and grasses. In: Thorpe TA (ed) Frontiers of plant tissue culture. University of Calgary Press, Alberta, Canada, pp 411–418

Gould FW (1951) Grasses of southwestern United States. The University of Arizona Press, Tuscon, Arizona

Gustafsson A (1946) Apomixis in higher plants. Otto Harrassowitz, Leipzig

Ha SB, Wu F-S, Thorne TK (1992) Transgenic turf-type tall fescue (*Festuca arundinacea* Schreb.) plants regenerated from protoplasts. Plant Cell Rep 11:601–604

Hartman CL, Lee I, Day PR, Tumer NE (1994) Herbicide-resistant turfgrass (*Agrostis palustris* Huds.) by biolistic transformation. Bio/Technology 12:919–923

Hauptmann RM, Vasil V, Ozias-Akins P, Tabaeizadeh Z, Rogers SG, Fraley RT, Horsch RB, Vasil IK (1988) Evaluation of selectable markers for obtaining stable transformants in the Gramineae. Plant Physiol 86:602–606

Heath R, Huxley H, Stone B, Spangenberg G (1998) cDNA cloning and differential expression of three caffeic acid o-methyltransferase homologues from perennial ryegrass (*Lolium perenne*). J Plant Physiol 153:649–657

Holmström K-O, Somersalo S, Mandal A, Palva TE, Welin B (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. J Exp Bot 51: 177–185

Hoover MM, Hein MA, Dayton WA. Erlanson CO (1948) The main grasses for farm and home. In: Stefferud A (ed) Grass. Yearbook of Agriculture. USA Dept Agric, pp 312–339

Horn ME, Shillito RD, Conger BV, Harns CT (1988) Transgenic plants of Orchardgrass (*Dactylis glomerata*) from protoplasts. Plant Cell Rep 7:469–472

Hyder DN, Everson AC, Bement RE (1971) Seedling morphology and seeding failures with blue grama. J Range Manag 24: 287–292

lnokuma C, Sugiura K, lmaizumi N, Cho C (1998) Transgenic Japanese lawngrass (*Zoysia japonica* Steud.) plants regenerated from protoplasts. Plant Cell Rep 17:334–338

Jaramillo VV (1986) La importancia de los coeficientes de agostadero y de las gramíneas en el manejo de los agostaderos del país. In: Gutiérrez CJ (ed) Memorias del Segundo Congreso Nacional de Manejo de Pastizales. Saltillo, Coahuila, México, pp 8–15

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kosugi S, Ohashi Y, Nakajima K, Arai Y (1990) An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci 70:133–140

Kuai B, Morris P (1996) Screening for stable transformants and stability of β-glucuronidase gene expression in suspension cultured cells of tall fescue (*Festuca arundinacea*). Plant Cell Rep 15:804–808

Kuai B, Dalton SJ, Bettany AJE, Morris P (1999) Regeneration of fertile transgenic tall fescue plants with a stable highly expressed foreign gene. Plant Cell Tissue Org Cult 58:149–154

Lee L (1996) Turfgrass biotechnology. Plant Sci 115:1–8

Lee L, Hartman C, Laramore C, Tumer N, Day P (1995) Herbicide-resistant creeping bentgrass. USGA Green Section Record 33: 16–18

Maliga P, Carrer H, Kanevski I, Staub J, Svab, Z (1993) Plastid engineering in land plants: a conservative genome is open to change. Phil Trans R Soc Lond B 342:203–208

Meijer EGM, Schilperoort RA, Rueb S, van Os-Ruygrok EP, Hensgens LAM (1991) Transgenic rice cell lines and plants: expression of transferred chimeric genes. Plant Mol Biol 16: 807–820

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Orozco AMS (1993) Efecto de la profundidad de siembra y la fertilización en el establecimiento de tres zacates forrajeros. MSc thesis, Colegio de Postgraduados, Texcoco, México

Shure M, Wessler S, Fedoroff N (1983) Molecular identification and isolation of the waxy locus in maize. Cell 35:225–233

Sims PL, Lang'at RK, Hyder DN (1973) Developmental morphology of blue grama and sand bluestem. J Range Manag 26:340–344

Spangenberg G, Wang ZY, Nagel J, Potrykus I (1994) Protoplast culture and generation of transgenic plants in red fescue (*Festuca rubra* L.). Plant Sci 97:83–94

Spangenberg G, Wang ZY, Wu XL, Nagel J, lglesias VA, Potrykus I (1995a) Transgenic tall fescue (*Festuca arundinacea*) and red fescue (*F. rubra*) plants from microprojectile bombardment of embryogenic suspension cells. J Plant Physiol 145: 693–701

Spangenberg G, Wang ZY, Nagel J, Wu XL, Potrykus I (1995b) Transgenic perennial ryegrass (*Lolium perenne*) plants from microprojectile bombardment of embryogenic suspension cells. Plant Sci 108:209–217

Stubbendieck J, Hatch SL, Hirsch KJ (1986) North American range plants, 3rd edn. University of Nebraska Press, Canada

Sugiura K, lnokuma C, lmaizumi N, Cho C (1997) Transgenic creeping bentgrass (*Agrostis palustris* Huds.) plants regenerated from protoplasts. J Turf Manag 2:43–53

Tabe LM, Wardley-Richardson T, Ceriotti A, Aryan A, McNabb W, Moore A, Higgins TJV (1995) A biotechnological approach to improving the nutritive value of alfalfa. J Anim Sci 73:2752–2759

Waller SS, Lewis JK (1979) Occurrence of C_3 and C_4 photosynthetic pathways in North American grasses. J Range Manag 32:12–28

Wang GR, Binding H, Posselt UK (1997) Fertile transgenic plants from direct gene transfer to protoplasts of *Lolium perenne* L. and *Lolium multiflorum* Lam. J Plant Physiol 151:83–90

Wang ZY, Takamizo T, lglesias VA, Osusky M, Nagel J, Potrykus I, Spangenberg G (1992) Transgenic plants of tall fescue (*Festuca arundinacea* Schreb.) obtained by direct gene transfer to protoplasts. Bio/Technology 10:691–696

Wilson AM, Briske DD (1979) Seminal and adventitious root growth of blue grama seedlings on the Central Plains. J Range Manag 32:209–213

Ye X, Wang ZY, Wu X, Potrykus I, Spangenberg G (1997) Transgenic Italian ryegrass (*Lolium multiflorum* plants from microprojectile bombardment of embryogenic suspension cells. Plant Cell Rep 16:379–384

Zhang W, McElroy D, Wu R (1991) Analysis of the rice *Act1* 5′ region activity in transgenic rice plants. Plant Cell 3: 1155–1165

Zhong H, Bolyard MG, Srinivasan C, Sticklen MB(1993) Transgenic plants of turfgrass (*Agrostis palustris* Huds.) from microprojectile bombardment of embryogenic callus. Plant Cell Rep 13:1–6