Transformation and Regeneration of Creeping Bentgrass (*Agrostis palustris* Huds.) Protoplasts

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ABSTRACT

An efficient plant regeneration system from protoplasts of creeping bentgrass (*Agrostis palustris* Huds.) cultures is essential for gene transfer to these grasses through direct DNA uptake into protoplasts. A simple and efficient plant regeneration system for protoplasts isolated from embryogenic suspension cultures of seven creeping bentgrass cultivars was established. Suspension cultures were derived from embryogenic callus cultures established from surface-sterilized mature seeds. Four creeping bentgrass cultivars were tested to determine their effect as feeders on plating efficiencies, callus development, and regeneration of protoplasts. Embryogenic suspension cultures from all four cultivars worked as feeders with plating efficiencies ranging from 0.05 to 0.32%. Protoplast derived calli formed within 3 wk after isolation and were regenerated with, or without, the addition of cytokinin to the regeneration medium. Some cultivars required the use of a particular feeder to regenerate plants. Plants were regenerated from all the cultivars tested. The *bar* gene, which confers resistance to the herbicide bialaphos [2-amino-4-(hydroxymethylphosphinyl)butanoic acid], was transformed into protoplasts by means of either PEG or electroporation. Bialaphos-resistant colonies were obtained from five creeping bentgrass cultivars. Resistant colonies of the cultivar Cobra were regenerated into plants. One hundred fifty-three Cobra transformants analyzed were resistant to five times the field rate of commercial formulation of the herbicide. Molecular characterization of the transformants revealed the stable integration of the *bar* gene into the genome and expression of transcripts corresponding to the *bar* gene.

CREEPING BENTGRASS is a widely used cool-season grass for fine-textured, closely cut lawns, high quality putting greens for golf courses, and bowling clubs. Although creeping bentgrass has been improved through traditional breeding, disease resistance is still a concern. Creeping bentgrass is susceptible to diseases such as brown patch (*Rhizoctonia solani* Kühn), Pythium blight (*Pythium graminicola* Subramanian), dollar spot (*Sclerotinia homoeocarpa* F.T. Bennett), and take-all patch [*Gaeumannomyces graminis* (Sacc.) Arx. & D. Olivier var. *avenae* (E.M. Turner) Dennis] (Beard, 1973, p. 71–78), and is a potential target for improvement through genetic engineering. Biolistic transformation has been used both to produce creeping bentgrass that is herbicide resistant (Hartman et al., 1994) or which expresses the β-glucuronidase gene (Zhong et al., 1993). Direct gene transfer to protoplasts was used to obtain transgenic tall fescue (*Festuca arundinacea* Schreb.) by Wang et al. (1992) and Ha et al. (1992). In this paper we report a simple reproducible protoplast regeneration system for creeping bentgrass and production of herbicide resistant transgenic plants.

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Although plant regeneration from protoplasts isolated from embryogenic suspension cultures of creeping bentgrass was reported by Terakawa et al. (1992), the method was complex and used *conditioned medium* and agarose beads for the formation of protoplast colonies with the cultivar Penncross. In this study, we used a feeder system to simplify protoplast regeneration, and regenerated plants from protoplasts isolated from seven different cultivars of creeping bentgrass. The establishment of the protoplast regeneration system led to successful transformation of creeping bentgrass protoplasts with a chimeric *bar* gene, and the regeneration of morphologically normal plants resistant to bialaphos.

MATERIALS AND METHODS

Plant Materials and Culture Initiation

Embryogenic callus cultures were initiated from surface sterilized seeds of seven creeping bentgrass cultivars: Cobra, Emerald, PennLinks, Providence, Putter, Southshore, and SR1020. Callus initiation media were MS (Murashige and Skoog, 1962) basal medium and MS vitamins, supplemented with 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, and either 150 mg L^{-1} asparagine and 2 mg L^{-1} 2,4-D for MSA2D, or 500 mg L^{-1} casein hydrolysate, 6.6 mg L^{-1} dicamba, and 0.5 mg L^{-1} 6-BA for MMS (Zhong et al., 1991). Media were solidified with 0.2% Phytagel (Sigma Chemical Co., St. Louis). After 4 to 6 wk in the dark at 25°C, embryogenic callus lines were selected and transferred to fresh medium. Suspensions were established from embryogenic callus cultures by adding 1 to 2 g callus to 250 mL flasks with 50 mL liquid media, incubated in the dark at 25°C with shaking (G10 Gyrotory Shaker, New Brunswick Scientific Co. Inc., Edison, NJ) at 120 rpm and subcultured twice a week.

Protoplast Isolation

Protoplast isolation was performed 4 d after subculture. Cells were incubated with filter-sterilized enzyme solution containing 1% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Co. LTD, Tokyo), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co. LTD, Tokyo), and 0.1% MES (2-[*N*-morpholino]ethane-sulfonic acid) (Sigma Chemical Co.) in culture media (MSA2D or MMS with 5% mannitol) for 4 h at 28°C with shaking at 50 rpm. Protoplasts were filtered through Miracloth (Calbiochem, La Jolla, CA) and washed twice with culture medium containing 5% mannitol. Mannitol was used as an osmotic stabilizing agent.

Protoplast Culture and Regeneration

Protoplasts were cultured using a feeder layer system (Rhodes et al., 1988). The washed, filtered protoplasts were

Abbreviations: MS, Murashige and Skoog; MMS, MS + dicamba + 6-BA + casein hydrolysate; 6-BA, 6-benzyl adenine; PEG, polyethylene glycol; AI, active ingredient; PAT, phosphinothricin acetyltransferase; PPT, phosphinothricin; CaMV, Cauliflower mosaic virus; TMV, tobacco mosaic virus; *adh*, alcohol dehydrogenase; GUS, β-glucuronidase; *hph*, hygromycin phosphotransferase; *npt* II, neomycin phosphotransferase II.

pipetted onto black nitrocellulose membranes (Lee et al., 1989) placed over a feeder layer of suspension cells which had been spread on 5% mannitol MSA2D culture medium. One week later, the membranes with protoplasts were transferred to a fresh feeder layer on MSA2D culture medium with 3% mannitol. The membranes with protoplasts were removed from the feeder layer 2 wk after isolation. Plating efficiency was determined by dividing the number of visible colonies 3 wk after isolation by the total number of protoplasts plated. Plants were regenerated by placing protoplast-derived calli on MS medium without hormones or with 1 mg L^{-1} of 6-BA or kinetin. After 4 to 5 wk, shoots were transferred to a Phytatray II (Sigma Chemical Co.) with MS medium containing no hormones for rooting.

Direct DNA Transfer to Protoplasts

Protoplasts were transformed using either PEG following the protocol of Negrutiu et al. (1987), or electroporation at 170 V cm^{-1} with a Gene-Pulser (Bio-Rad Laboratories, Richmond, CA). In PEG experiments, freshly isolated protoplasts were resuspended at a density of 1×10^7 protoplasts per ml in 5% mannitol containing 15 m*M* $MgCl_2$ and 0.1% MES. Approximately 0.3 ml of protoplasts were incubated with 20 to 40 μg plasmid DNA and 13% PEG for 10 to 15 min, diluted stepwise and resuspended in culture medium with 5% mannitol (pH 5.8) after centrifugation. In electroporation experiments, protoplasts were resuspended at a density of 5×10^6 protoplasts per mL in cold filter-sterilized electroporation buffer containing 5.2 g L^{-1} KCl, 0.835 g L^{-1} $CaCl_2$, 0.976 g L^{-1} MES, and 5% mannitol at pH 5.8. Protoplasts (0.8 mL) were mixed with 20 μg DNA by inversion, electroporated at 170 V cm^{-1} and placed on ice for 15 min., then diluted to a total of 3 mL with culture medium containing 5% mannitol.

Two different vectors containing the *bar* gene (Hartman et al., 1994) were used. In one plasmid vector, pSLJ02011 (Jones et al., 1992), the *bar* gene was driven by the CaMV 35S promoter and the 5′ untranslated leader of TMV (omega fragment), and is terminated by the octopine synthase 3′ end. The other plasmid vector, pBARGUS (Fromm et al., 1990), contained the *bar* gene downstream of the CaMV 35S promoter and the maize *adh*1 intron, with the nopaline synthase 3′ end. It also contains the GUS gene downstream of the *adh*1 promoter and the *adh*1 intron.

Selection and Herbicide Tests

Selection with 4 mg L^{-1} of bialaphos was initiated 16 d after protoplast isolation and transformation. Resistant colonies were visible 3 to 4 wk after selection. Bialaphos was not included in regeneration medium for shoot or root formation. Plants were regenerated by placing resistant colonies on MS medium without hormone, with 6-BA or kinetin as described above. Shoots were transferred to Phytatray II for rooting.

A commercial formulation of bialaphos under the trade name Herbiace (Meiji Seika Kaishya, LTD., Tokyo) was used in greenhouse herbicide tests. Herbicide rates for Herbiace were established with control plants, and were based on the commercial rate of 0.75 lb AI/acre (1× the field rate). The herbicide was applied to all the tillers above ground with an artist's paint brush at the rate of 120 mL per flat. The area of the flat is 0.1431 m^2 and it holds 96 or 24 plants. Initial test was performed with 96 plants; a second test was further performed with 24 plants.

Northern Analysis

Total RNA was isolated from 1 g of leaf tissue of transgenic and control greenhouse grown plants with Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH), which includes phenol and guanidine thiocyanate in a mono-phase solution and analyzed by electrophoresis on a 1.3% agarose gel containing formaldehyde (Tumer et al., 1986). The RNA was transferred to Genescreen Plus (DuPont Biotechnology Systems, Willmington, DE) and hybridized with a ^{32}P-labeled random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN) 0.6-kb *Xho*I and *Bam*HI fragment of pSLJ02011(Hartman et al., 1994) containing the coding sequence of the *bar* gene.

Southern Analysis

Total genomic DNA was isolated from 1 g of leaf tissue of transgenic and control greenhouse grown plants with a mortar and pestle with liquid nitrogen following the protocol of Dellaporta et al. (1983). The sample was then further purified with phenol-chloroform-isoamylalcohol extraction, ethanol precipitated, and dissolved in water. Southern analyses were performed according to standard procedures (Sambrook et al., 1989) and hybridized with the same 0.6-kb probe used in the Northern analysis.

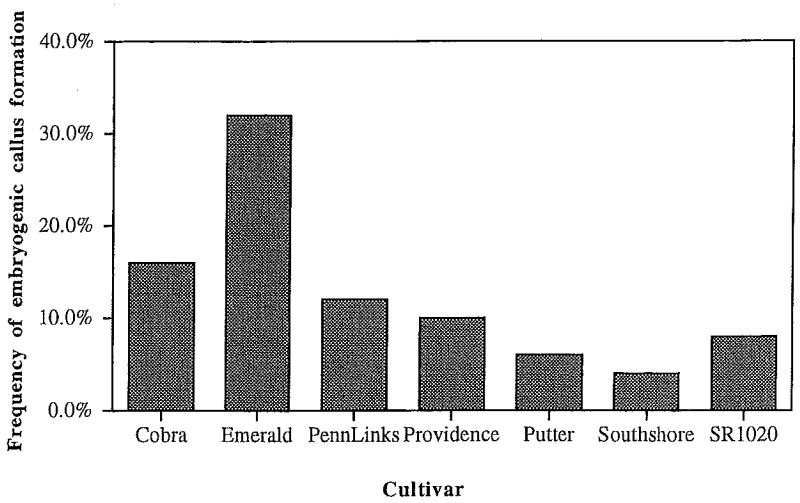

Fig. 1 Frequency of embryogenic callus formation of seven cultivars of creeping bentgrass about 60 d after surface sterilized seeds were placed on MMS medium.

RESULTS AND DISCUSSION

Embryogenic Callus Culture Establishment

Embryogenic callus cultures were obtained from seven cultivars of creeping bentgrass using either MSA2D or MMS medium. From 5 to 30% of seeds produced embryogenic callus cultures (Fig. 1). Embryogenic callus lines possess high regeneration potential upon transfer to MS medium without hormone. About 200 to 400 plants were obtained from 1 g fresh weight of callus and regenerability of some cultures did not decrease with culture age; even 2-yr-old cultures were as regenerable as younger cultures (data not shown).

Embryogenic suspension cultures were initiated from callus lines. Fast growing, fine suspension cultures were established in 4 to 6 wk. Up to 6-mo-old suspension cultures produced regenerants from protoplasts. However, as in other monocot species such as rice (*Oryza sativa* L.) and maize (*Zea mays* L.), some cultivars were more amenable to the establishment of suspensions, and were regenerable for a longer time, than other cultivars.

Protoplast Isolation and Culture

Protoplasts were isolated from embryogenic suspensions of seven cultivars of creeping bentgrass. The yields ranged from 5×10^6 to 42×10^6 protoplasts per gram fresh weight with 3- to 24-wk-old suspension cultures (Table 1). Protoplasts were from 10 to 20 μm in diameter. Protoplast-derived calli were obtained from all seven cultivars at various suspension culture ages ranging from 4 to 24 wk. Plants were regenerated from cultivars SR1020, Southshore, PennLinks, Putter, Emerald, and Cobra. Feeder cells were necessary for the formation of protoplast-derived callus. This is similar to the importance of conditioned medium (Terakawa et al., 1992) for protoplast colony formation and growth of Penncross creeping bentgrass. In our system, protoplast-derived calli formed within 2 to 3 wk for all seven cultivars of creeping bentgrass.

Figure 2 shows the effect of feeder cells from four cultivars on the plating efficiencies of protoplasts isolated from suspension cultures of the same four cultivars. Plating efficiencies of the cultivars as protoplasts varied from 0.32% for Cobra to 0.05% for PennLinks and were similar to the results reported by Terakawa et al. (1992) for Penncross creeping bentgrass. Feeder layers from all four cultivars supported regeneration; however, those from Cobra and Emerald were most effective. Similar results were obtained from indica rice suspension cultures (Lee et al., 1989) where some suspension cell lines were more effective than others as feeder cells. Cobra protoplasts grew equally well with all four feeders while PennLinks grew least well with most feeders. These results demonstrate that the suspension source,

Table 1. Plant regeneration from protoplast derived callus of embryogenic suspension cultures of seven creeping bentgrass cultivars.

Cultivar	Culture age†	Yield	Callus‡	Plants‡
	wk	$\times 10^6$ g^{-1}		
SR1020	3	5	–	–
	19	5	+	+
	24	22	+	+
Southshore	11	42	+	+
	12	35	+	+
	13	18	+	+
	15	15	+	+
	16	26	+	+
PennLinks	4	22	+	+
	8	40	+	+
	9	45	+	+
Providence	19	3.5	+	–
Putter	5	6.6	+	+
Emerald	8	30	+	+
	9	16	+	+
	10	16	+	+
	11	15	+	+
Cobra	8	20	+	+
	9	25	+	+
	10	34	+	+
	11	35	+	+

† Culture age refers to the amount of time embryogenic callus has been grown in liquid media.
‡ "+" means the formation of callus or plants and "–" means no formation of callus or plants.

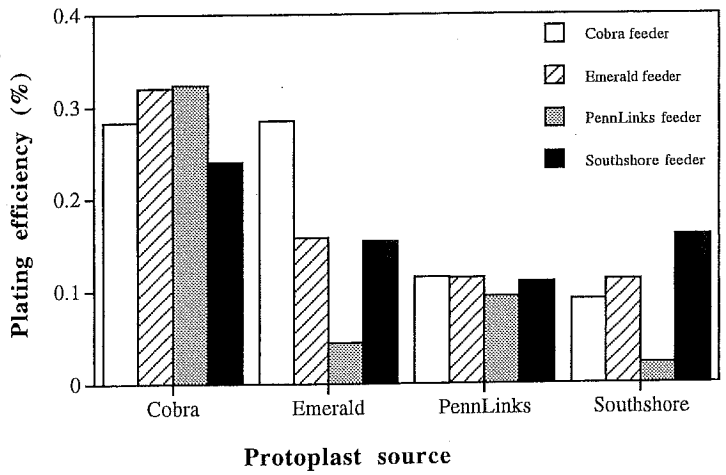

Fig. 2 Effect of feeders on the plating efficiencies of protoplasts isolated from four suspension cultures of creeping bentgrass.

(cultivars as feeders or protoplasts), may have a marked effect on protoplast development.

Plant Regeneration

Plants were regenerated from protoplast-derived calli on MS medium without hormones. After transfer of protoplast callus colonies to regeneration medium, with or without cytokinins, regeneration frequencies up to 100% were obtained (Table 2). Ten protoplast callus colonies derived from each of four suspension cultures, chosen at random, were transferred to regeneration medium containing either no hormone, BA, or kinetin (Table 2). The number of calli that produced plants (regeneration frequency) were scored, as well as the total number of plants produced by the ten protoplast callus colonies from each suspension.

Emerald had the highest regeneration frequency (90–100%), and produced the most plants, averaging from three to six plants per colony. Although Cobra had the highest plating efficiencies (Fig. 2), regeneration frequencies were lower, between 20 to 70%, and only one to three plants regenerated per callus. In general, regeneration frequencies were higher than the 30% obtained previously (Terakawa et al., 1992).

It is interesting to observe that the feeder layer not only affected plating efficiency but also regeneration. In our study, Emerald and Cobra suspension cultures were more effective as feeders in supporting callus development from protoplasts, and plant regeneration.

Cytokinin had no marked effect on regeneration, although in a few cases, such as Cobra protoplasts on Cobra feeder cells, PennLinks protoplasts on Southshore feeder cells, and Southshore protoplasts on Southshore feeder cells, both BA and kinetin increased regeneration frequencies. Terakawa et al. (1992) reported BA was required for plant regeneration from protoplast derived callus cultures.

Transformation, Selection, and Herbicide Tests

Two different vectors containing the *bar* gene (Hartman et al., 1994) were used to transform embryogenic creeping bentgrass protoplasts isolated from suspension cultures. The *bar* gene, isolated from *Streptomyces hygroscopicus*, encodes PAT, an enzyme that inactivates the herbicidal compound PPT by acetylation (Murakami et al., 1986; Thompson et al., 1987). PPT inhibits glutamine synthetase (Tachibana et al., 1986a), causing rapid accumulation of ammonia and cell death (Tachibana et al., 1986b).

The vectors containing the *bar* genes were introduced into protoplasts by electroporation or PEG treatment. Selection (on bialaphos) was initiated at an early stage of colony formation to minimize establishment of non-transformed escapes. Sixteen days after isolation was determined to be the optimum time for the exposure to bialaphos. Cells were selected on MSA2D medium with 4 mg L^{-1} bialaphos. Although untreated protoplast controls occasionally formed microcolonies on this medium, growth was stopped by bialaphos selection.

Resistant colonies of Cobra, Emerald, Southshore, SR1020, and PennLinks were obtained from four protoplast transformation experiments (Table 3) with transformation frequencies ranging from 1×10^{-6} to 16×10^{-6}. Wang et al. (1992) and Ha et al. (1992) obtained similar transformation frequencies using *bar* and *hph* or *hph* alone in tall fescue. Likewise, similar transformation frequencies were obtained using *npt*II in redtop (*Agrostis alba* L.) (Asano and Ugaki, 1994).

In reports describing tall fescue transformation, selection was accomplished with phosphinothricin for the *bar* gene and hygromycin for the *hph* gene (Wang et al., 1992; Ma et al., 1992). However, because an enriched amino acid medium was used for protoplast regrowth, selection was not optimal. In our system, bialaphos added to MSA2D culture medium containing asparagine, gave effective selection. However, on MMS medium containing casein hydrolysate, as for tall fescue protoplast transformation, selection was ineffective.

Table 2. Effect of four embryogenic suspensions as feeders on protoplast regeneration. Ten protoplast callus colonies, chosen at random, were tested in each regeneration.

Cultivar	Feeder	Regeneration frequency (%)			Total number of regenerants		
		MS0†	MSBA†	MSKIN†	MS0†	MSBA†	MSKIN†
Cobra	Cobra	0	20	30	0	3	3
	Emerald	30	70	30	7	11	1
	PennLinks	20	20	20	3	2	6
	Southshore	0	20	10	0	5	3
Emerald	Cobra	90	100	100	24	65	60
	Emerald	100	100	100	59	60	85
	PennLinks	90	100	100	44	81	71
	Southshore	100	100	100	56	65	58
PennLinks	Cobra	10	50	40	1	10	9
	Emerald	0	0	0	0	0	0
	PennLinks	0	0	0	0	0	0
	Southshore	0	0	10	0	0	1
Southshore	Cobra	0	0	0	0	0	0
	Emerald	10	40	0	2	12	0
	PennLinks	0	0	0	0	0	0
	Southshore	0	0	10	0	0	2

† MS0, MS medium with no hormones; MSBA, MS medium with 1 mg L^{-1} 6-BA; MSKIN, MS medium with 1 mg L^{-1} kinetin.

Table 3. Summary of protoplast transformation experiments.†

Exp.	Cultivar	Vector	Resistant colonies
1	Emerald	pBARGUS	3
2	Emerald	pBARGUS	2
	Southshore		2
	PennLinks		1
3	Cobra	pSLJ02011	1
		pBARGUS	1
4	SR1020	pBARGUS	4
	Southshore		16

† Protoplasts were isolated from embryogenic suspension cultures of Cobra, Emerald, PennLinks, Southshore, and SR1020 creeping bentgrass and treated with 13% PEG (Exp. 1-3) or electroporation (Exp. 4) using plasmids pBARGUS or pSLJ02011.

A total of 153 Cobra regenerants from exp. 3 (Table 3) were transferred to soil, and tested in the greenhouse. All putative transgenic plants survived Herbiace at 2 mg mL^{-1}. After herbicide application, control plants were completely killed within 7 to 10 d, and resistant transgenic plants continued to grow and were green and healthy (Fig. 3).

The incorporation of the *bar* gene in the plant genome was confirmed by Southern blot hybridization (Fig. 4). Total genomic DNA was isolated from bialaphos-resistant Cobra plants transformed with either pSLJ02011 or pBARGUS, digested with a combination of *Eco*RI and *Bam*HI and analyzed by Southern hybridization probing with a 600-bp *Xho*I and *Bam*HI fragment of pSLJ02011 corresponding to the *bar* coding sequence. No signal was detected in non-transformed control plants undigested and digested (Fig. 4, Lanes 3 and 4). Transgenic plants transformed with pSLJ02011 and subjected to a double digest with *Eco*RI and *Bam*HI yielded a band at 3.5 kb (Fig. 4, Lanes 6 and 8). Transformants with pBARGUS showed a band of 2.1 kb when digested with *Eco*RI and *Bam*HI (Fig. 4, Lanes 10 and 12). Although we did not detect bands corresponding to the expected size in transgenic plants (pSLJ02011, *Eco*RI and *Bam*HI double digest yields a 2.1-kb fragment;

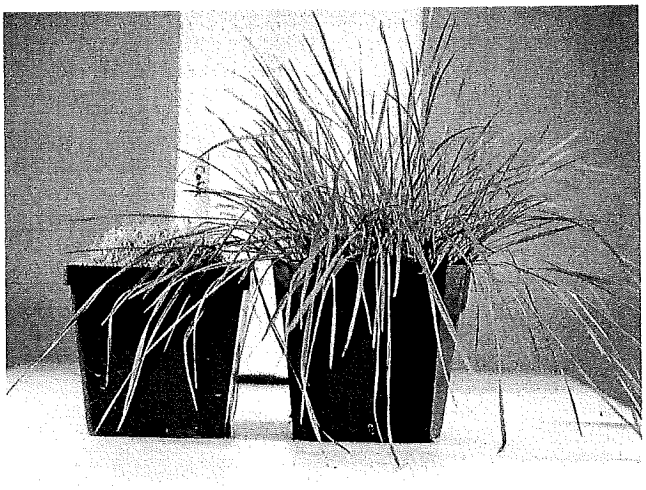

Fig. 3 Herbicide resistance of transgenic plant (right) and control (left) at the 2 mg ml^{-1} rate of Herbiace® 2 wk after herbicide application. Transgenic plants remained green and healthy, and control turned brown and died.

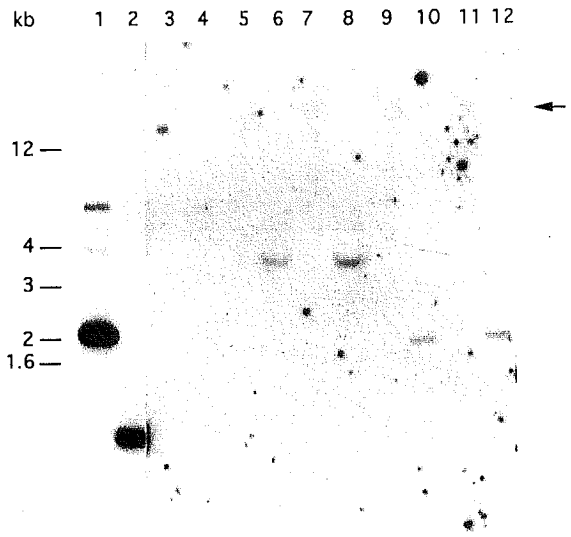

Fig. 4 Southern blot hybridization analysis of transformed creeping bentgrass. Total genomic DNA isolated from leaf tissues was undigested (Lanes 3, 5, 7, 9 and 11) or digested with *Eco*RI and *Bam*HI (Lanes 1, 2, 4, 6, 8, 10 and 12) and hybridized with the 600-bp fragment of pSLJ02011. Lane 1: pSLJ02011, Lane 2: pBARGUS, Lanes 3 and 4: nontransformed control plant, Lanes 5-8: plants transformed with pSLJ02011, and Lanes 9-12: plants transformed with pBARGUS.

pBARGUS, *Eco*RI and *Bam*HI double digestion yields a 1.3-kb fragment), we detected fragments larger than the *bar* gene. A high molecular weight hybridizing band was seen in undigested genomic DNA from the four transformants (Fig. 4, Lanes 5, 7, 9, and 11). Since Southern analysis showed a unique band that was not present in control plants this indicated that the *bar* gene had integrated into the genome of the transgenic plants.

Northern blot analyses of transgenic plants were performed on total RNA isolated from plants containing either pSLJ02011 or pBARGUS, and probed with the same 600-bp fragment as used for the Southern blot. Fig. 5 shows that bialaphos-resistant plants containing pSLJ02011 (Lanes 3 and 5) accumulated transcripts of the expected size of approximately 600 bp and bialaphos-resistant plants containing pBARGUS (Lanes 4, 6, and 7) accumulated transcripts of the expected size of around 550 bp and a larger transcript of about 800 bp which might be due to readthrough of the nopaline synthase terminator in the vector (Tumer et al., 1987). In contrast, no signal was detected in non-transformed control plants (Lane 1, Southshore; Lane 2, Cobra).

In summary, we have established a simple plant regeneration system for protoplasts isolated from creeping bentgrass using a feeder cell method. Our system is applicable to all seven cultivars of creeping bentgrass tested and provides a useful basis for gene transfer by direct DNA uptake into protoplasts. Using PEG to enhance DNA uptake into protoplasts, we obtained transgenic creeping bentgrass plants resistant to five times the field rate of Herbiace. We believe that herbicide-resistant turfgrass has considerable potential for the management of golf course greens and fine lawns. The method described may also be used to transfer other agronomically important genes into creeping bentgrass.

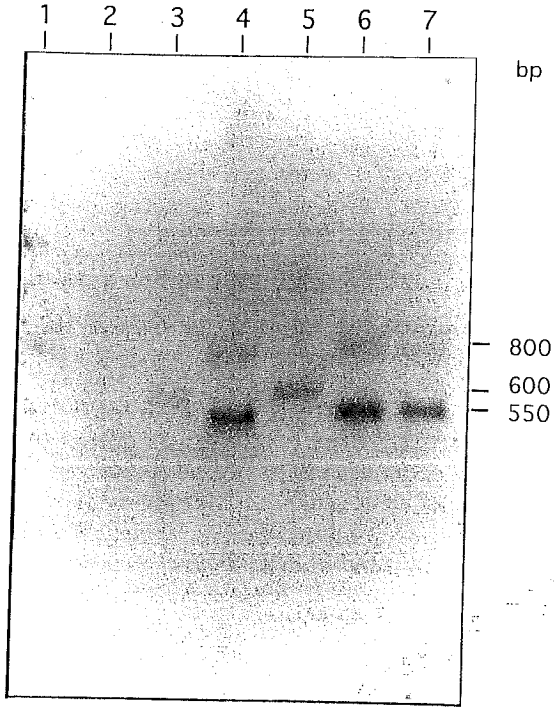

Fig. 5 Northern blot hybridization analysis of transformed creeping bentgrass. Total RNA was isolated from leaf tissues, separated on a 1.3% formaldehyde-agarose gel, transferred to nylon membrane and probed with the 600-bp *bar* fragment. Lane 1: Emerald control; Lane 2: Cobra control; Lanes 3 and 5: transgenic Cobra lines containing pSLJ02011; Lanes 4, 6, and 7: transgenic Cobra lines containing pBARGUS.

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