Plant Cell Reports (1997) 16: 379–384

X. Ye · Z.-Y. Wang · X. Wu · I. Potrykus
G. Spangenberg

Transgenic Italian ryegrass (*Lolium multiflorum*) plants from microprojectile bombardment of embryogenic suspension cells

Received: 22 July 1996 / Revision received: 8 August 1996 / Accepted: 9 August 1996

Abstract Transgenic forage-type Italian ryegrass (*Lolium multiflorum* Lam.) plants have been obtained by microprojectile bombardment of embryogenic suspension cells using a chimeric hygromycin phosphotransferase (*hph*) gene construct driven by rice *Act1* 5′ regulatory sequences. Parameters for the bombardment of embryogenic suspension cultures with the particle inflow gun were partially optimized using transient expression assays of a chimeric β-glucuronidase (*gusA*) gene driven by the maize *Ubi1* promoter. Stably transformed clones were recovered with a selection scheme using hygromycin in liquid medium followed by a plate selection. Plants were regenerated from 33% of the hygromycin-resistant calli. The transgenic nature of the regenerated plants was demonstrated by Southern hybridization analysis. Expression of the transgene in transformed adult Italian ryegrass plants was confirmed by northern analysis and a hygromycin phosphotransferase enzyme assay.

Key words Forage and turf grasses · Italian ryegrass · *Lolium multiflorum* · Microprojectile bombardment · Transgenic plants

Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid · *GUS* -Glucuronidase · *Hm* Hygromycin · *HPH* Hygromycin phosphotransferase · *MS medium* Murashige and Skoog medium · *PCR* Polymerase chain reaction · *X-Gluc* 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Communicated by N. Amrhein

X. Ye · Z-Y Wang[1] · X. Wu · I. Potrykus · G. Spangenberg (✉)[1]
Institute for Plant Sciences, Swiss Federal Institute of Technology, CH-8092 Zürich, Switzerland

Present address:
[1] Plant Sciences and Biotechnology, Agriculture Victoria, La Trobe University, Bundoora, Victoria 3083, Australia

Introduction

The genus *Lolium* L., ryegrass, comprises eight diploid (2n=2x=14 chromosomes) species indigenous to temperate regions of Europe and Asia (Jauhar 1993). Their geographic distribution has been further extended by humans through introductions in the Americas, North Africa, Australia, and New Zealand. Within the genus, two closely related and interfertile species, *L. multiflorum* (Italian or annual ryegrass) and *L. perenne* (English or perennial ryegrass), are key forage grasses. Progress in breeding using conventional selection procedures is slow, since they are highly self-infertile. Biotechnological approaches, therefore, may contribute to the development of improved ryegrass cultivars for forage and turf purposes.

Transgenic pasture and turf grasses have been obtained by direct gene transfer to protoplasts of *Dactylis glomerata* (Horn et al. 1988), *Festuca arundinacea* (Ha et al. 1992; Wang et al. 1992), *F. rubra* (Spangenberg et al. 1994), and *Agrostis alba* (Asano and Ugaki 1994). Using embryogenic cell and callus cultures as targets for microprojectile-bombardment-mediated transformation, transgenic plants have been recovered in *A. palustris* (Zhong et al. 1993; Hartman et al. 1994), *F. arundinacea* and *F. rubra* (Spangenberg et al. 1995a), and *L. perenne* (Spangenberg et al. 1995b). For *L. multiflorum*, however, only stably transformed callus clones have been obtained from protoplasts (Potrykus et al. 1985). As yet, there is no report of the recovery of transgenic Italian ryegrass plants.

Here we describe the reproducible generation of transgenic plants in annual (ssp. *westerwoldicum*) and biennial (ssp. *italicum*) *L. multiflorum* using a chimeric hygromycin phosphotransferase (*hph*) gene construct, and embryogenic suspension cells as direct targets for microprojectile bombardment.

Materials and methods

Establishment and maintenance of suspension cultures

Seeds from Italian ryegrass (*L. multiflorum* Lam. ssp. *westerwoldicum* cv 'Andy' and *L. multiflorum* Lam. ssp. *italicum* cvs 'Axis' and 'Gorka'), kindly provided by B. Boller (FAL-Zürich-Reckenholz, Switzerland) and T. Takamizo (NGRI-Nishinasuno, Tochigi, Japan), were used for the initiation of embryogenic calli. After surface sterilization in 3% (wt/vol) calcium hypochlorite for 50 min, seeds were rinsed (x3) in sterile water and placed on Murashige and Skoog (1962) (MS) medium supplemented with 400 mg/l casein hydrolysate, 5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 3% (wt/vol) sucrose, solidified with 0.8% (wt/vol) agar, and kept in the dark at 25°C. Callus appearing within 4–6 weeks after inoculation of seeds was maintained by subculturing on the same medium every 4 weeks (Wang et al. 1993a). After 1–2 subcultures, friable, yellowish, embryogenic callus derived from single seeds – representing individual genotypes – was individually transferred to 6-cm culture dishes (Greiner 967161, 190 ml, Greiner, Nürtingen, Germany) with 12 ml liquid AAF medium: AA medium (Müller and Grafe 1978) containing 1.5 mg/l 2,4-D, 2% (wt/vol) sucrose and 2% (wt/vol) sorbitol (Wang et al. 1993b). The suspension cultures were kept at 25°C in the dark on a gyratory shaker (60 rpm), and subcultured weekly into fresh culture medium.

Transforming DNA and microprojectile bombardment of embryogenic suspension cells with a particle inflow gun

Stable transformation experiments were carried out with plasmid pAcH1 (5.6 kb), kindly provided by R. Bilang, bearing a chimeric truncated *hph* gene (Bilang et al. 1991) under control of the rice actin 1 (*Act1*) 5′ regulatory signals from pCOR117 (McElroy et al. 1991), kindly made available by R. Wu and D. McElroy. A chimeric *gusA* gene driven by the maize *Ubi-1* promoter (Christensen et al. 1992) was constructed (pUbiGUS, 6.8 kb) and used in transient transformation experiments.

Different bombardment parameters for a particle inflow gun (Finer et al. 1992) were evaluated in single or multifactorial transient transformation experiments. Different bombardment pressures (4–7 bar), bombardment distances (12–17 cm), baffle mesh sizes (100–1000 µm), particles per bombardment (0.1–5 mg), DNA per bombardment (5–100 µg), number of shots per target (1–3 shots), and particle suspension volume per bombardment (5–20 µl) were tested. Gold particles (gold powder, spherical, 1.5–3.0 µm, 99.9%; no. 32.658-5, Aldrich) autoclaved in glycerol (no. 49770; Fluka, Fuchs, Switzerland) were coated with plasmid DNA according to Finer et al. (1992) with modifications. Osmotic prebombardment treatments of cell suspensions for 10–60 min in liquid MSP medium (Spangenberg et al. 1994) containing 30 g/l sucrose and supplemented with equimolar amounts of mannitol and sorbitol to yield 0.1–0.6 M, as well as postbombardment osmotic treatments of suspension cells for 1–4 days on the same media solidified with 1% (wt/vol) agar, were also tested. For routine stable transformation experiments, 20 µl of supercoiled plasmid DNA (20 µg), 10 µl gold particles (1 mg), 25 µl of 2.5 M $CaCl_2$, and 10 µl of 100 mM spermidine were mixed, and incubated at 4°C for 10 min. The supernatant was then removed, the particle pellet rinsed twice in absolute ethanol, air-dried, and resuspended in 20 µl sterile water. Osmotically pretreated suspension cells were distributed as a 2-cm-diameter monolayer of embryogenic cell aggregates on a filter paper disk (LS14, 4 cm diameter, Schleicher & Schüll, Dassel, Germany), placed onto solidified MSP medium in a 5-cm culture dish (Sterilin, Bibby Sterilin, Stone, UK) and bombarded with 10 µl of the suspension of DNA-coated particles.

Histochemical β-glucuronidase (GUS) activity assay

GUS-expressing cells were routinely visualized 48–72 h after microprojectile bombardment by incubating bombarded suspension cells in 100 mM sodium phosphate, pH 7.0, 10 mM Na-EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% (wt/vol) 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) (Biosynth, Staad, Switzerland) and 0.1% (v/v) Triton X-100 at 37°C for 12–24 h (Mendel et al. 1989).

Selection of stably transformed colonies and recovery of transgenic plants

After a 3- to 4-day postbombardment osmotic treatment on solid MSP medium supplemented with 0.25 M sorbitol and 0.25 M mannitol without hygromycin (Hm), filter paper disks supporting bombarded embryogenic suspension cells were transferred into liquid AAF medium with 25 mg/l Hm. Cells selected in Hm-containing liquid AAF medium were kept at 25°C in the dark on a gyratory shaker at 60 rpm. After the first 2 weeks in liquid selection in the presence of 25 mg/l Hm, the culture medium was completely replaced by fresh Medium containing 50 mg/l Hm for a further 2 weeks of culture before plating onto Hm-containing solid MSP medium for proliferation in the presence of 100 mg/l Hm for 3–4 weeks. Resistant calli were transferred onto regeneration medium MSK (MS medium supplemented with 0.2 mg/l kinetin) without Hm. The regenerated shoots were transferred for rooting onto half-strength hormone-free MS medium solidified with 0.3% Gelrite (Duchefa, no. G1101, Haarlem, The Netherlands). All cultures were kept at 25°C in a photoperiod of 16 h fluorescent light (40 µmol m^{-2} s^{-1}). After 4–5 weeks, rooted plantlets were hardened off, transferred to soil, and grown to maturity under greenhouse conditions (16/8 h photoperiod; 23/18°C; fluorescent light 145 µmol m^{-2} s^{-1}).

DNA and RNA isolation, gel electrophoresis, and hybridization experiments

Total cellular DNA was isolated from freeze-dried leaf material from greenhouse-grown plants. Isolation and digestion of genomic DNA was performed according to Lichtenstein and Draper (1985). Total cellular RNA was isolated from leaves of greenhouse-grown plants using the RNA-Clean™ System (SUBAG, Niederrohrdorf, Switzerland) following the manufacturer's instructions. Restriction enzyme analysis, gel electrophoresis, DNA and RNA blotting, and Southern and northern hybridizations were carried out following standard protocols (Sambrook et al. 1989). The hybridization probe (*hph* gene, 1344-bp *Bam*HI-fragment of pAcH1) was [^{32}P]dATP-labeled by random priming (Feinberg and Vogelstein 1983).

Polymerase chain reaction (PCR) experiments

PCR reactions were carried out in 25 µl of commercial buffer (Eurobio, Germany) containing 1 mM $MgCl_2$, 200 µM dATP, dTTP, dCTP, dGTP, Taq polymerase (Eurobiotaq, 1 U/µl), DNA (100 ng genomic or 10 pg plasmid), and oligonucleotide primers (5′-GCTGGGGCGTCGGTTTCCACTATCCG-3′ and 5′-CGCATAACAGCGGTCATTGA CTGGAGC-3′; 25 pmol/25 µl) designed to amplify an internal 375-bp *hph* fragment. DNA was amplified in a Perkin Elmer Cetus thermocycler (denaturation at 94°C, 1 min; annealing at 65°C, 30 s; extension at 72°C, 1 min; 35 cycles). PCR amplification products were analyzed by electrophoresis in 1.5% agarose/ethidium bromide gels.

Hygromycin phosphotransferase (HPH) enzyme assay

HPH activity was detected in extracts from 0.1–0.2 g leaf material of Hm-resistant plants following a protocol by R.D. Shillito (Fütterer et al. 1995).

Results and discussion

Microprojectile-DNA delivery parameters and *gusA* transient expression assays in bombarded suspension cells

A chimeric *gusA* gene driven by maize ubiquitin 1 (*Ubi-1*) 5′ regulatory sequences was used to partially optimize delivery of DNA-coated microprojectiles to cells of single-genotype-derived suspension cultures of Italian ryegrass. Approximately 200 mg suspension cells plated as a 2-cm-diameter monolayer on a filter paper disk (Fig. 1A) was used as a target for microprojectile bombardment. The cost-effective particle inflow gun (Finer et al. 1992) was used as the microprojectile acceleration device. An approximately 50-fold increase in transient transformation events, with a maximum of over 2000 GUS-expressing foci per bombardment (Fig. 1B), was achieved after evaluation of different DNA-particle delivery parameters (e.g., bombardment pressure and distance, baffle mesh size and baffle distance, particle concentration, and DNA concentration) and different target cell treatments (e.g., pre- and post-bombardment osmotic treatments). A 30-min high-osmoticum treatment of the suspension cells prior to the bombardment significantly increased the number of GUS-expressing foci. Osmotic treatments enhancing microprojectile-bombardment-mediated transient and stable transformation of suspension cells have been described for various graminaceous monocots (Vain et al. 1993; Spangenberg et al. 1995a, b). The DNA-particle delivery parameters reproducibly giving the highest number of transient GUS expression events for bombarded Italian ryegrass suspension cells were 500 μm baffle mesh size, 12 cm baffle and 15 cm target-bombardment distances, 6 bar bombardment pressure, one shot with a 10-μl DNA-spermidine-bound particle suspension per target using 0.5 mg gold particles coated with 10 μg plasmid DNA.

Stable transformation and recovery of transgenic Italian ryegrass plants

Embryogenic cell aggregates from individual-genotype-derived cultures established from two cultivars of annual and biennial Italian ryegrass were used for microprojectile-bombardment-mediated stable transformation. Microprojectiles were coated with plasmid DNA (pAcH1) bearing a chimeric *hph* gene under control of the rice *Act1* promoter. The experimental conditions (microprojectile bombardment parameters, treatments of target embryogenic suspension cells) allowing the highest number of GUS-expressing foci in the transient *gusA* gene expression assays were chosen for the stable transformation experiments with the *hph* expression vector. Dose-response experiments performed with nontransformed suspension cells of Italian ryegrass plated on media containing 25–200 mg/l Hm indicated that a 4-week selection with 100 mg/l Hm was lethal. For stable transformation experiments, selection of suspension cells was started 4 days postbombardment. Selection started in liquid culture and involved a stepwise increase of the Hm concentration from 25 mg/l to 50 mg/l Hm after a 2-week subculture, followed by a 3- to 4-week plate selection with 100 mg/l Hm on solidified medium (Fig. 1C, D). Fifteen independent experiments including on average 10 dishes supporting 200 mg fresh weight embryogenic suspension cells were performed. Hm-resistant calli of Italian ryegrass could be clearly identified using the selection scheme described and were recovered in all experiments (Fig. 1D). On average, one Hm-resistant callus was recovered in 59% of the bombarded dishes (93 resistant calli/156 bombarded dishes). These results are in the range of those reported from analogous studies on microprojectile-bombardment-mediated stable transformation of *F. arundinacea*, *F. rubra*, and *L. perenne* (Spangenberg et al. 1995a, b).

When transferred onto regeneration medium, approximately 60% of the Hm-resistant calli were morphogenic and differentiated either green or albino plantlets. Similar regeneration frequencies and green:albino ratios were observed for the corresponding control experiments with nontransformed calli obtained from the same single-genotype-derived suspension cultures of Italian ryegrass. This suggested that the hygromycin selection scheme did not impair morphogenesis. One-third (31/93) of the Hm-resistant calli differentiated multiple green shoots (Fig. 1E, F), which could be established in vitro as rooted plantlets (Fig. 1G), while the remaining regeneratable calli differentiated albino shoots only. All 31 rooted green plantlets survived transfer to soil, and transformed Italian ryegrass plants from microprojectile bombardment established in soil and growing under greenhouse conditions (Fig. 1H) were obtained within 4 months.

Molecular analysis of transformed Italian ryegrass plants

A PCR screening of the 31 Italian ryegrass in vitro plantlets (Fig. 1G) regenerated from Hm-resistant calli was performed with primers designed to amplify a 375-bp internal *hph* fragment. In all cases, analysis of the PCR amplification products revealed a band of the expected size, indicating that the selection scheme used was tight. Representative results of this analysis are shown in Fig. 2A for ten plantlets regenerated from Hm resistant calli. The transgenic nature of the *L. multiflorum* plants was confirmed by Southern hybridization analysis using undigested and digested total cellular DNA samples. Corresponding results are shown for six transgenic Italian ryegrass plants (Fig. 2B). Hybridization signals corresponding to the full-length *hph* gene (an expected 1344-bp band hybridizing to the *hph* probe) and to high-molecular-weight bands were observed in the Southern hybridization analysis using *Bam*HI-digested and undigested DNA samples, respectively (Fig. 2B). This indicated the integration of complete transgene copies in the genome of the transformed Italian ryegrass plants analyzed. Different hybridization patterns including additional *hph*-hybridizing bands were observed

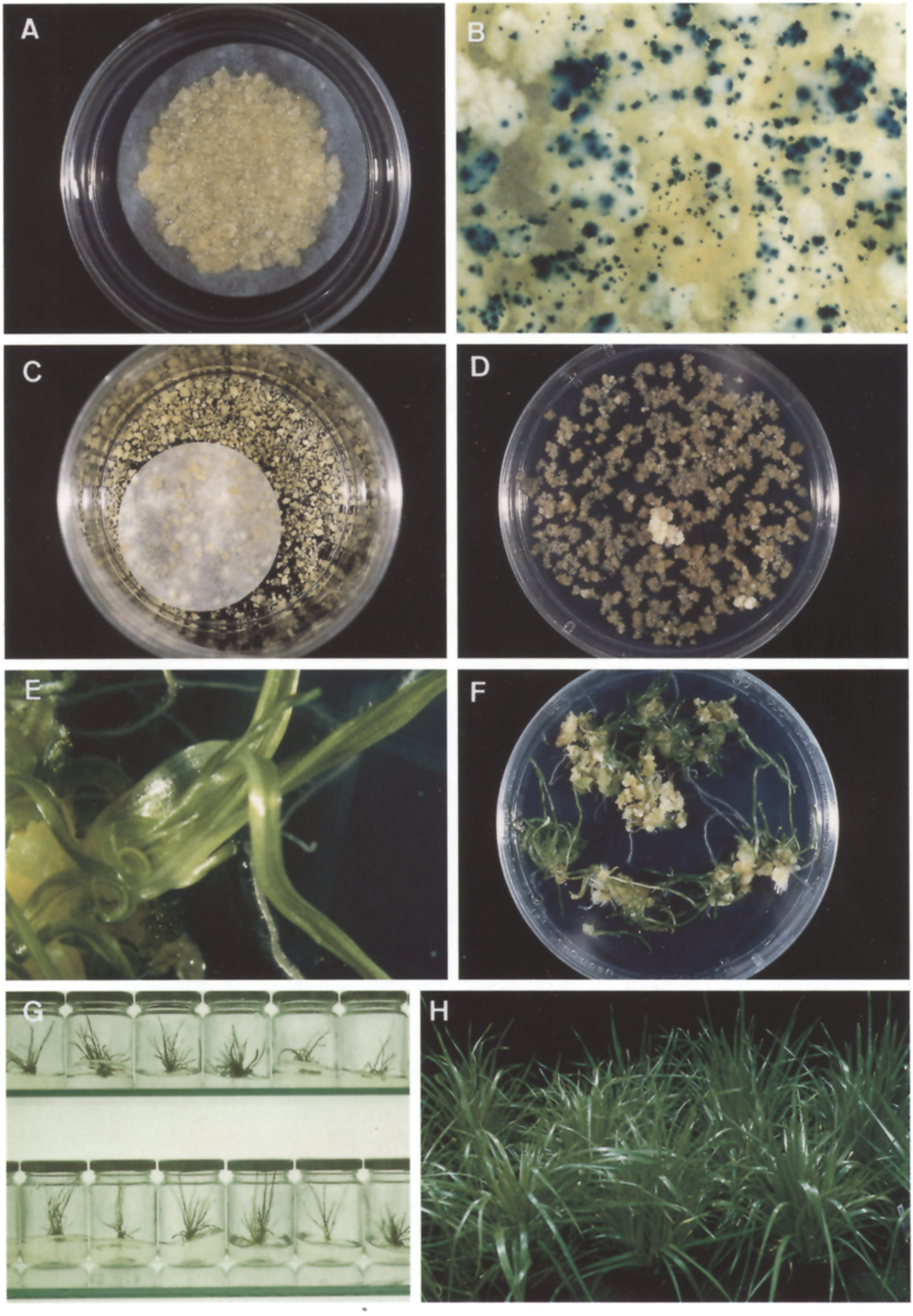
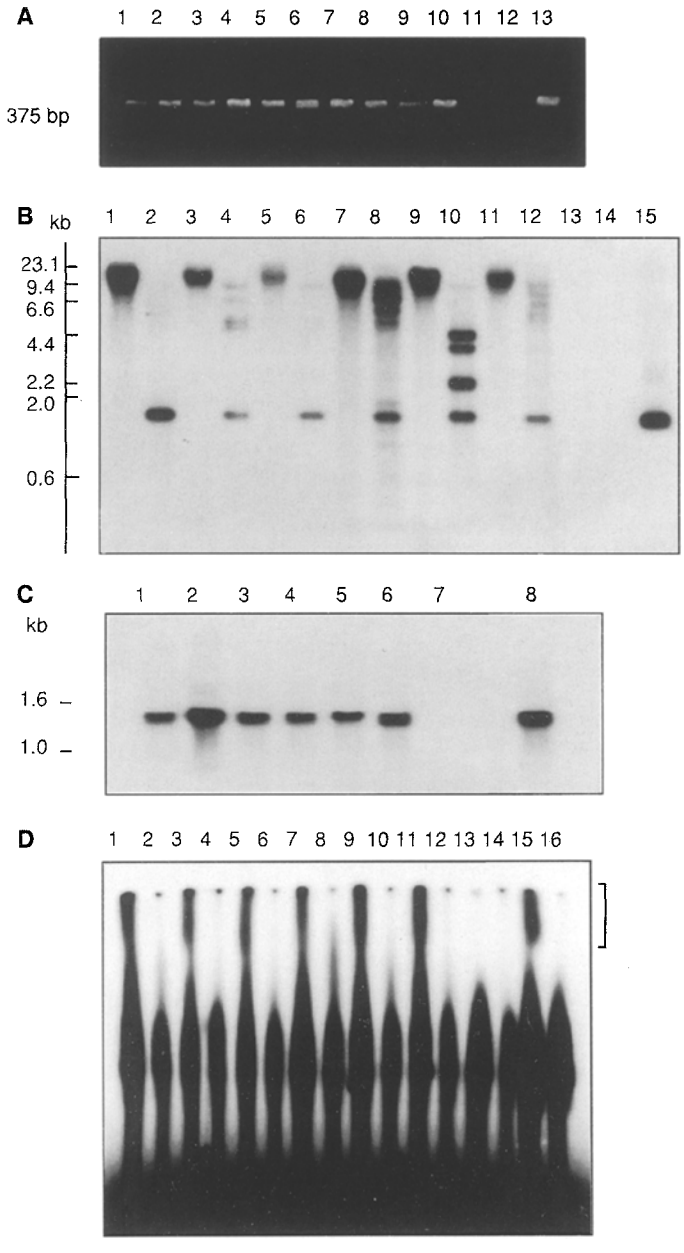

A
B
C
D
E
F
G
H

Fig. 1A–H Transgenic *Lolium multiflorum* plants from particle-inflow-gun-mediated microprojectile bombardment of embryogenic suspension cells. **A** Suspension cells of *L. multiflorum* plated on a filter paper disk prior to microprojectile bombardment. **B** β-Glucuronidase (GUS)-expressing foci evaluated 4 days after bombardment of Italian ryegrass suspension cells with microprojectiles coated with a chimeric *gusA* gene. **C** Embryogenic suspension cells of *L. multiflorum* cultured in liquid AAF medium containing 25 mg/l hygromycin (Hm) 2 weeks after bombardment with pAcH1-coated microprojectiles. **D** Hm-resistant *L. multiflorum* calli obtained from microprojectile-bombarded embryogenic suspension cells using a chimeric *hph* gene (pAcH1) followed by selection on MSP in the presence of 100 mg/l Hm. **E** Shoot differentiation from Hm-resistant callus of *L. multiflorum* 2 weeks after transfer onto medium MSK for regeneration. **F** Regenerating Hm-resistant *L. multiflorum* calli 10 weeks after bombardment of embryogenic suspension cells with pAcH1-coated microprojectiles. **G** Transgenic *L. multiflorum* plantlets growing in vitro 12 weeks after microprojectile bombardment of embryogenic cells. **H** Transgenic soil-grown *L. multiflorum* plants 4 months after bombardment of embryogenic suspension cells



for some *Bam*HI-digested samples (Fig. 2B), indicating that the plants were derived from independent transformation events, and that multiple insertions of rearranged or partial copies of the transgene might have occurred. Similar results have been reported for transgenic plants of creeping bentgrass (Zhong et al. 1993; Hartman et al. 1994), tall and red fescues (Spangenberg et al. 1995a), and perennial ryegrass (Spangenberg et al. 1995b) recovered from microprojectile-bombarded embryogenic cells.

Expression of the transgene in the transformed *L. multiflorum* plants was studied by northern hybridization analysis and in vitro enzyme assay for HPH activity. Northern hybridization analysis performed with total RNA samples isolated from leaf tissue from the transformed Italian ryegrass plants showed accumulation of the expected *hph* transcript. No hybridization signal was detected in the nontransformed control plant. Representative results are shown in Fig. 2C for six independently recovered transgenic Italian ryegrass plants. HPH assays using protein extracts from leaf samples of greenhouse-grown transformed adult Italian ryegrass plants revealed HPH activity above background levels, demonstrating the mitotic stability of the transgene expression (Fig. 2D).

This molecular analysis proved the transgenic nature of the Italian ryegrass plants regenerated from Hm-resistant calli, confirming that the selection scheme chosen was highly efficient. In contrast, false-positive escapes have been reported after direct gene transfer to protoplasts and G418 selection in *L. multiflorum* (Potrykus et al. 1985). Since Italian ryegrass is an obligate outbreeder, representative transgenic *hph*-expressing plants will be brought to flower, and after emasculation and controlled crossings, progeny testing will be attempted.

Fig. 2A–D Molecular analysis of transgenic plants of *L. multiflorum* from microprojectile bombardment of embryogenic suspension cells. **A** Ethidium-bromide stained-agarose gel of PCR amplification products from ten Hm-resistant *L. multiflorum* plantlets growing in vitro. *Lanes 1–10* ten independent Hm-resistant regenerants, *11* nontransformed *L. multiflorum* negative control, *12* negative control for PCR reaction in absence of template DNA, *13* positive control pAcH1. **B** Southern blot hybridization with undigested (odd-numbered lanes) and *Bam*HI-digested (even-numbered lanes) total cellular DNA samples isolated from six Hm-resistant *L. multiflorum* plants hybridized with the *hph* probe. *Lanes 1–12* six independent Hm-resistant pAcH1 transformants, *13–14* nontransformed *L. multiflorum* negative control, *15* 10 pg *Bam*HI-digested pAcH1 as positive control. **C** Northern blot hybridization using total cellular RNA samples isolated from leaves of *L. multiflorum* plants derived from microprojectile bombardment and the *hph* probe. *Lanes 1–6* six independent pAcH1-transformed plants of *L. multiflorum* (same plants as in **B**), *7* nontransformed Italian ryegrass plant (negative control), *8* RNA from transformed Hm-resistant tobacco as positive control. **D** Expression of hygromycin phosphotransferase in transgenic plants of *L. multiflorum* derived from microprojectile bombardment of embryogenic suspension cells. *Lanes 1–12* six independent transgenic *L. multiflorum* plants, *13–14* nontransformed *L. multiflorum* plant as negative control, *15–16* transformed Hm-resistant tobacco plant as positive control. Assays were performed in the presence (odd-numbered lanes) and absence (even-numbered lanes) of Hm

In conclusion, we have established a reproducible particle bombardment transformation protocol using embryogenic suspension cells and *hph* as a selectable marker gene in *L. multiflorum*, and recovered transgenic Italian ryegrass plants for the first time. Previously, only transformed callus clones (Potrykus et al. 1985) could be obtained from direct gene transfer to protoplasts isolated from nonmorphogenic suspension cells.

Acknowledgements Thanks are due to H. Waldner for making the particle inflow gun, and to J. Finer for providing the required information for its construction. P. Frick and K. Konja are gratefully acknowledged for carefully growing plants in the greenhouse. This work was partly funded by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung (SNF grants 5002-37299 and 3100-40315) and Eidgenössische Technische Hochschule (ETH grant 41-2703.5).

References

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

Bilang R, Iida S, Peterhans A, Potrykus I, Paszkowski J (1991) The 3′-terminal region of the hygromycin-B-resistance gene is important for its activity in *Escherichia coli* and *Nicotiana tabacum*. Gene 100:247–250

Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18:675–689

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

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

Fütterer J, Gisel A, Iglesias V, Klöti A, Kost B, Mittelsten Scheid O, Neuhaus G, Neuhaus-Url G, Schrott M, Shillito R, Spangenberg G, Wang ZY (1995) Standard molecular techniques for the analysis of transgenic plants. In: Potrykus I, Spangenberg G (eds) Gene transfer to plants. Springer, Berlin Heidelberg New York, pp 215–262

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

Hartman CL, Lee L, Day PR, Tumer NE (1994) Herbicide resistant turfgrass (*Agrostis palustris* Huds.) by biolistic transformation. Biotechnology 12:919–923

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

Jauhar PP (1993) Cytogenetics of the *Festuca-Lolium* complex. Monogr Theor Appl Genet 18:1–8

Lichtenstein C, Draper J (1985) Genetic engineering of plants. In: Glover DM (ed) DNA cloning, vol 2. IRL, Washington, pp 67–119

McElroy D, Blowers AD, Jenes B, Wu R (1991) Construction of expression vectors based on the rice actin 1 (*Act1*) 5′ region for use in monocot transformation. Mol Gen Genet 231:150–160

Mendel RR, Müller B, Schulze J, Kolesnikov V, Zelenin A (1989) Delivery of foreign genes to intact barley cells by high-velocity microprojectiles. Theor Appl Genet 78:31–34

Müller AJ, Grafe R (1978) Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Mol Gen Genet 161:67–76

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

Potrykus I, Saul MW, Petruska J, Paszkowski J, Shillito RD (1985) Direct gene transfer to cells of a graminaceous monocot. Mol Gen Genet 199:183–188

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning – a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

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

Vain P, McMullen MD, Finer JJ (1993) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep 12:84–88

Wang ZY, Takamizo T, Iglesias 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. Biotechnology 10:691–696

Wang ZY, Nagel J, Potrykus I, Spangenberg G (1993a) Plants from cell suspension-derived protoplasts in *Lolium* species. Plant Sci 94:179–193

Wang ZY, Valles MP, Montavon P, Potrykus I, Spangenberg G (1993b) Fertile plant regeneration from protoplasts of meadow fescue (*Festuca pratensis* Huds.). Plant Cell Rep 12:95–100

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