Original Paper

Plant Biotechnology, 19 (4), 241-249 (2002)

Production of Transgenic Italian Ryegrass (*Lolium multiflorum* Lam.) via Microprojectile Bombardment of Embryogenic Calli

Wataru TAKAHASHI[1], Hideki OISHI[1], Masumi EBINA[2], Tadashi TAKAMIZO[2]* and Toshinori KOMATSU[2]

[1] *Japan Grassland Farming Forage Seed Association, Forage Crop Research Institute, 388-5 Higashiakada, Nishinasuno, Tochigi 329-2742, Japan*

[2] *National Institute of Livestock and Grassland Science, 768 Senbonmatsu, Nishinasuno, Tochigi 329-2793, Japan*

**Corresponding author* E-mail address: takamizo@affrc.go.jp

Received 5 April 2002; accepted 6 July 2002

Abstract

We established a microprojectile-bombardment-mediated transformation system in embryogenic calli of Italian ryegrass (*Lolium multiflorum* Lam.). Bombardment conditions with a PDS-1000/He helium-driven biolistic device were optimized by transient expression assays of a chimeric β-glucuronidase (GUS) gene construct. The highest number of transient GUS expression events was observed with 24 h of high osmotic pretreatment and a bombardment pressure of 7584 kPa. Diploid and tetraploid Italian ryegrass calli were transformed under optimal conditions with two plasmids harboring the gene for antisense expression of cinnamyl alcohol dehydrogenase (CAD) or hygromycin phosphotransferase (HPT). Transformed calli were obtained via microprojectile bombardment followed by selection of transformants on media containing hygromycin at 50 mg l^{-1} for 1 week and then 100 mg l^{-1} for 5 weeks. A total of 33 transgenic plants were produced. The transformation frequencies in cultivars Waseaoba (diploid) and Meritra (tetraploid) were 3.8% and 2.3%, respectively. The total cointegration frequencies of the HPT and antiCAD genes (antisense orientation of CAD gene) were 55.6% in Waseaoba and 73.3% in Meritra. The expression of the antiCAD gene in the co transformed plants was confirmed by RT-PCR.

Key words: Callus, *Lolium multiflorum* Lam., Microprojectile bombardment, Transgenic plants.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; CAD, cinnamyl alcohol dehydrogenase; CTAB, cetyltrimethylammonium bromide; DIG, digoxygenin; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase; MS medium, Murashige and Skoog medium; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.

Introduction

Italian ryegrass (*Lolium multiflorum* Lam.) originated in the Mediterranean region and is one of the most important forage grasses in the temperate zones of Europe and Asia. It is an outcrossing crop and is thus self-infertile, and there are many genotypes within a cultivar; Conventional breeding procedures are therefore time consuming.

Transformation methods might lead to significant progress in breeding Italian ryegrass. Transgenic Italian ryegrass plants have been obtained by the use of polyethylene-glycol-mediated DNA uptake (Wang *et al.*, 1997), microprojectile bombardment (Ye *et al.*, 1997, 2001; Dalton *et al.*, 1999), and silicon-carbide-fiber-mediated transformation (Dalton *et al.*, 1998). These transformations were performed with embryogenic suspension cells or protoplasts isolated from suspension cells as targets. In most plant transformation systems, plant regeneration from a target tissue such as callus is indispensable. Because the regenerability of Italian ryegrass depends strongly on the genotype (Jackson and Dale, 1988; Wang *et al.*, 1993), screening for regenerable genotypes is of great significance for efficient transformation. However, regenerable genotypes are rare in Italian ryegrass, and plant regeneration from suspension cultures frequently produces mutants such as albinos, or the cultures lose their regenerability relatively quickly (Creem-

ers-Molenaar *et al.*, 1992; Wang *et al.*, 1993). Thus, screening for a callus-regenerable Italian ryegrass should be more fruitful than screening for a suspension culture one. Although there have been two reports of the production of transgenic *Lolium* plants via callus culture (Bhalla *et al.*, 1999, *L. rigidum* L.; and Altpeter *et al.*, 2000, *L. perenne* L.), the optimization of bombardment conditions has not been described in detail, and there is no work on the production of Italian ryegrass via callus culture. We report here the establishment of a transformation system with embryogenic callus induced from three genotypes among diploid and tetraploid cultivars by microprojectile bombardment and foreign gene expression in Italian ryegrass.

Materials and methods

Establishment of callus culture

We used two Italian ryegrass (*L. multiflorum* Lam.) cultivars: Waseaoba (diploid) and Meritra (tetraploid). All media used were based on MS medium (Murashige and Skoog, 1962) and are shown in **Table 1**. One thousand mature seeds from each cultivar were surface-sterilized in 20% (v v^{-1}) sodium hypochlorite solution (5% available chlorine) for 20 min, and rinsed in sterile distilled water three times. The seeds were then placed onto callus induction medium (**Table 1**) for 1 month to induce embryogenic calli. After a further month of subculturing, vigorously growing calli were transferred to regeneration medium (**Table 1**). The genotypes that arose were maintained and propagated aseptically for meristem culture to induce regenerable calli for gene transfer experiments. The meristem culture was performed as for callus induction from mature seeds. Calli were subjected to gene transfer experiments 3 months after their initiation. *In vitro* cultures were incubated in the dark at 25 ℃ for callus culture, under continuous fluorescent light (40 μmol m^{-2} s^{-1}) at 25 ℃ for plant regeneration, and under short-day conditions (8 h light / 16 h dark; 20 ℃ /18 ℃) for plant propagation.

Plasmid DNA

The plasmid pFFantiCAD contains the antisense orientation of the cinnamyl alcohol dehydrogenase (CAD) gene isolated from *Aralia cordata* (Hibino *et al.*, 1993). The gene was expressed in a tandem repeat of the CaMV35S promoter on pFF plasmid (Timmermans *et al.*, 1990). The CAD gene is known to be involved in lignin biosynthesis (Hibino *et al.*, 1993). We used the antiCAD gene to investigate the efficiency of cotransformation and coexpression with a selectable marker gene. The constructs pAct1-F and pAcH1 were used to evaluate the gene transfer efficiency by transient GUS expression and to select transformed hygromycin-resistant cells, respectively. pAct1-F was constructed by McElroy *et al.* (1991) and harbors the β-glucuronidase (GUS) gene driven by a rice *actin1* gene promoter. pAcH1 bears a hygromycin phosphotransferase (HPT) gene under the control of a rice *actin1* gene promoter (Spangenberg *et al.*, 1995). The HPT gene, originating from *Klebsiella* (Gritz and Davies, 1983), confers resistance to the antibiotic hygromycin in plant cells. All plasmids were amplified in *Escherichia coli* strain JM109 for isolation with a QIAfilter™ Maxi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Bombardment

All experiments were performed with a PDS-1000/He helium-driven biolistic device (BioRad, Hercules, USA). As target tissue, 40 pieces of callus (4 mm in diameter) were placed onto solid pretreatment medium (**Table 1**) on a Petri dish to form a circle 4 cm in diameter (**Fig. 1A**). To prepare gold particles, we mixed 100 μl of gold suspension containing 6 mg of gold particles (1.5-3.0 μm in diameter; Aldrich, Milwaukee, USA), 20 μg of each plasmid, 100 μl of 2.5 M $CaCl_2$, and 40 μl of 0.1 M spermidine in a 1.5-ml microtube and incubated the mixture for 30 min at room temperature. Then the mixture was centrifuged briefly, and the pellet was washed with 70% ethanol and resuspended in 100 μl of 99.5% ethanol. Ten microliters of DNA-

Table 1 Culture media used for transformation of Italian ryegrass

Callus induction medium	5 mg l^{-1} 2,4-D + 500 mg l^{-1} casein hydrolysate + 3% (w v^{-1}) sucrose
Regeneration medium	0.2 mg l^{-1} kinetin + 3% (w v^{-1}) sucrose
Pretreatment medium	Callus induction medium + 0.25 M sorbitol + 0.25 M mannitol
First selection medium	Callus induction medium + 50 mg l^{-1} Hygromycin
Second selection medium	Callus induction medium + 100 mg l^{-1} Hygromycin

All media used were based on MS medium adjusted to pH 5.8 and solidified with 0.25% (w v^{-1}) Gelrite (Wako, Osaka, Japan).

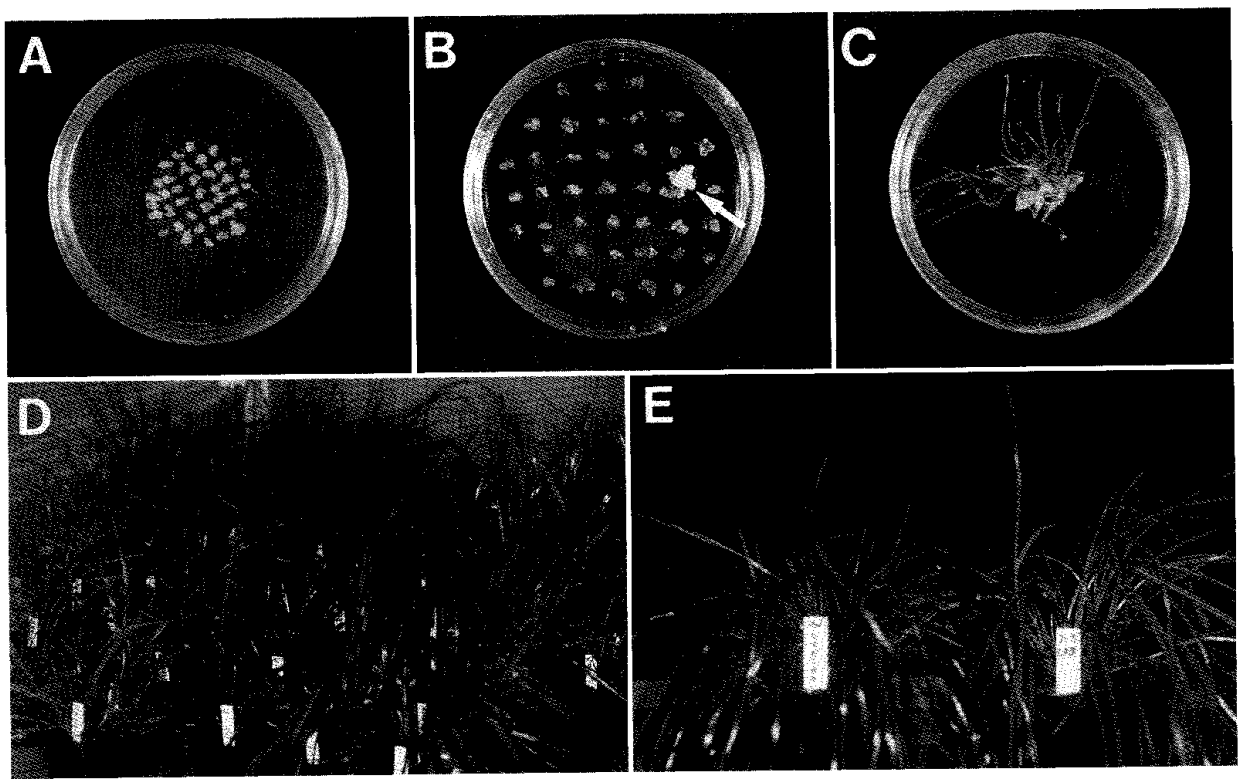

Fig. 1 Production of transgenic Italian ryegrass (*Lolium multiflorum* Lam.) via microprojectile bombardment of embryogenic calli.
(A) High-osmotic pretreatment of embryogenic calli.
(B) Hygromycin-resistant callus growing vigorously on the second selection medium (arrow).
(C) Plant regeneration from hygromycin-resistant callus.
(D) Transgenic plants in glasshouse.
(E) Comparison of phenotypes of transgenic (right) and nontransgenic (left) plants. Both plants were derived from the same genotype.

Table 2 Standard bombardment parameters

Apparatus	PDS-1000/He (BioRad)
Size of gold particle	Aldrich 1.5-3.0 μm
Gold load per bombardment	0.6 mg
DNA load per bombardment	2 μg
Bombardment pressure	7584 kPa
Bombardment distance:	
Gap distance	2.5cm
Stopping screen aperture	10mm
Target distance	9.5cm
Vacuum pressure	95kPa
Number of bombardments	2

coated gold suspension was loaded onto a microcarrier. The target tissues were placed about 9.5 cm from the stop screen and bombarded twice in a vacuum. The bombarded tissues were then incubated in the dark at 25 ℃ on pretreatment medium. For stable transformation, pAcH1 was coated onto the gold particles with pFFantiCAD at a weight ratio of 1:1. **Table 2** lists the standard bombardment parameters.

Transient GUS expression assay

To optimize the bombardment conditions, we assessed the parameters (pretreatment time: 0-48 hours, bombardment pressure: 6205-9308 kPa) by transient GUS assay (Jefferson *et al.*, 1987) under other standard bombardment parameters (**Table 2**). Two days after the bombardment, gene transfer efficiency was evaluated as the number of blue spots resulting from transient expression of the GUS gene. The bombarded tissues were treated with X-Gluc solution [1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt (X-Gluc), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 100 mM phosphate buffer (pH 7), 20% methanol, and 0.3% Triton X-100] and kept in the dark at 37 ℃ overnight to develop the GUS-expressing cells. All experiments were repeated three times with the genotype Waseaoba No. 3. Gene transfer efficiencies are expressed as mean ± SD (standard deviation) of the number of blue spots per Petri dish.

Antibiotic selection and recovery of transgenic plants

We used a two-stage selection scheme. Five days after the bombardment, the calli were transferred to the first selection medium (**Table 1**) and cultured for 1 week. They were then placed onto the second selection medium (**Table 1**) and subcultured every 2 weeks. Vigorously growing hygromycin-resistant calli were cultured until their diameter reached 5 mm, and were then transferred to regeneration medium without hygromycin (**Table 1**) for plant regeneration. One tiller was isolated from each resistant callus and subcultured every month on the same medium under short-day conditions for rooting and propagation. Rooted plants were established in soil in a glasshouse at 18 ℃ .

PCR (polymerase chain reaction) analysis of transgenic plants

The total DNA was extracted from 200 mg of green leaves by the simplified cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich, 1985). The PCR reaction mixture (50 μl) contained 0.1 μg of template DNA, 5 μl of 10x PCR buffer [containing 500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris·HCl (pH 8.3)], 1 μl of 20 μM primer, 4 μl of dNTP mixture (each dNTP was 2.5 mM), 0.5 μl of Taq polymerase (5 units μl^{-1}; TaKaRa Shuzo, Tokyo, Japan), and 37.5 μl of distilled water. The primer sets were as follows: 5'-GCTGGGGCGTCGGTTTCCACTATCCG-3' and 5'-CGCATAACAGCGGTCATTGACTGGAGC-3' to amplify a 375-bp HPT gene fragment (Ye *et al.*, 1997), and 5'-TTGATCAAGTTTGCTGCCGG-3' and 5'-TACACTCTCAGGGAAACAGG-3' to detect an internal 996-bp antiCAD gene fragment. The primer pair for the antiCAD gene was designed by the computer program Oligo ver. 5.0 (Molecular Biology Insights, Cascade, USA). The reaction was carried out in a Gene Amp® PCR system 9700 (Perkin Elmer, Foster City, USA; 35 cycles of denaturation at 94 ℃ , 1 min; annealing at 60 ℃ , 30 s; and extension at 72 ℃ , 1 min). The amplified fragments were electrophoresed on a 1.5% agarose gel at 100 V for 30 min and detected by ethidium bromide staining.

Southern hybridization analysis of transgenic plants

Southern blot analysis was performed as described in the DIG application manual (Roche, Mannheim, Germany). The genomic DNA of a transgenic plant derived from Waseaoba No. 3 where the antiCAD gene had been detected by PCR analysis was extracted by using the CTAB method (Murray and Thompson, 1980). To confirm the integration of the whole genes, the restriction enzymes *Eco*RI and *Bam*HI- which release the 1151-bp antiCAD gene and the 1344-bp HPT gene from pFFantiCAD and pAcH1, respectively-were used for DNA digestion. To estimate the copy number of foreign genes, *Sac*I and *Sph*I were used independently for recognizing the unique site of pFFantiCAD. Five micrograms of digested DNA was electrophoresed on a 0.8% agarose gel at 30 V for 7 h. The isolated DNA was blotted onto a positively charged nylon membrane and UV crosslinked. Each probe was labeled by digoxygenin-11-dUTP (DIG-dUTP) in a Gene Amp® PCR system 9700 with the same primers as above, according to the manufacturer's instructions. The labeled probe was hybridized with DNA-blotted membrane in hybridization buffer.

Expression analysis of transgenic plants by RT (reverse transcription)-PCR

The total RNA was extracted from 0.1 g of transgenic plant leaves with TRIzol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

RT-PCR was performed with Ready-To-Go™ RT-PCR beads (Amersham Pharmacia, Buckinghamshire, UK) in a Gene Amp® PCR system 9700. The RT reaction was carried out at 42 ℃ for 30 min. The RT primer was designed from an internal sequence of the antiCAD gene. After inactivation of the reverse transcriptase at 95 ℃ for 5 min, the first PCR (35 cycles of denaturation at 94 ℃ , 1 min; annealing at 57 ℃ , 30 s; and extension at 72 ℃ , 1 min) was carried out. One microliter of the first PCR mixture was subjected to nested PCR (35 cycles of denaturation at 94 ℃ , 1 min; annealing at 57 ℃ , 30 s; and extension at 72 ℃ , 1 min) to amplify a 573-bp antiCAD gene fragment resulting from the expression of the antisense orientation of the CAD gene. The amplified fragment was electrophoresed on a 1.5% agarose gel at 100 V for 30 min, and made visible by ethidium bromide staining. The primers, designed by Oligo ver. 5.0, were as follows: 5'-ATCAGATGTGACCAAGTTCAAG-3' (for RT reaction and first PCR as a reverse primer), 5'-AGCGGTGTTTATGTAATCCAT-3' (for first PCR as a forward primer), 5'-ATCTAGCATTTCTTCCGTCTCT-3' (for nested PCR as a forward primer), and 5'-AATGATGTCTACACCGATGG-3' (for nested PCR as a reverse primer).

Results and discussion

Establishment of callus culture

One thousand mature seeds from each cultivar

were subjected to callus induction. Callus formation frequencies were investigated after 1 month of the callus culture: they were 18.1% and 13.0% for Waseaoba and Meritra cultivars, respectively. Of these induced calli, 37 and 22 vigorously growing friable calli were selected from Waseaoba and Meritra, respectively, to investigate their regenerability. As a consequence, we obtained 14 and 11 regenerated genotypes from Waseaoba and Meritra, respectively. These genotypes were maintained and propagated aseptically for meristem culture to induce regenerable calli. In addition, as a preliminary experiment we attempted to establish a suspension culture system using these regenerable genotypes. Although most calli induced from these genotypes could be maintained and propagated in liquid suspension culture, no green shoot was regenerated from the suspension culture (data not shown). From these results, we concluded that the establishment of a regenerable suspension culture system is very difficult and inefficient. However, because the callus culture system has been easily established from many genotypes, the system is thought to be more advantageous and applicable to transformation systems for wide genotypes than the suspension culture system. Thus, we decided to establish a transformation system using calli. The regenerable genotypes used were Waseaoba No. 3, Meritra No. 1, and Meritra No. 8. These genotypes were highly regenerable in the callus condition.

Optimization of parameters for bombardment

We examined two bombardment parameters: osmotic treatment times before bombardment and bombardment pressures used for particle acceleration. The gene transfer efficiencies were evaluated as the number of blue spots resulting from transient GUS expression in the target tissues.

Fig. 2 shows the effect of pretreatment time on calli on high-osmotic pretreatment medium containing 0.25 M sorbitol and 0.25 M mannitol, which is the same condition for *L. perenne* (Spangenberg *et al.*, 1995). After 4-48 hours of pretreatment, the number of blue spots was obviously greater than with no pretreatment. Twenty-four hours of pretreatment was more effective than any other length of time (**Fig. 2**). The exposure of target tissue to high-osmotic pretreatment before bombardment causes plasmolysis in the target cells, which buffers the cells from direct shock when they are bombarded (Vain *et al.*, 1993; Kemper *et al.*, 1996). We hoped that high-osmotic pretreatment would also work on Italian ryegrass calli and increase the gene transfer efficiency as well as it did in previous reports of the transformation of Italian ryegrass by particle bombardment (Ye *et al.*, 1997, 2001; Dalton *et al.*, 1999).

The efficiency of gene transfer probably depends on DNA replication and cell stages (Gharti-Chhetri *et al.*, 1990; Kartzke *et al.*, 1990). Higher efficiency of gene transfer to protoplasts was observed during the S phase than during unsynchronized growth (Kartzke *et al.*, 1990). Accordingly, vigorous cell division possibly increases gene transfer efficiency. In a preliminary experiment, we found that prolonged high-osmotic treatment of Italian ryegrass calli reduced their growth (data not shown). Vain *et al.* (1993) also observed that excessively long high-osmotic pretreatment was detrimental to cell proliferation, and it reduced the transient expression frequency of GUS genes in maize cells. This might explain why 24 h of pretreatment was more effective than 48 h of pretreatment in the transient expression of GUS genes.

In transformation experiments with suspension cultures of Italian ryegrass (Ye *et al.*, 1997; Dalton *et al.*, 1998), 30 min of high-osmotic pretreatment was most effective with liquid pretreatment medium containing 0.25 M sorbitol and 0.25 M mannitol. In our preliminary experiment, calli of Waseaoba No. 3 were immersed in liquid pretreatment medium (Ye *et al.*, 1997), but this had no effect on the transient expression of GUS genes (data not shown). We consider that this is because the liquid pretreatment medium did not soak into the target callus sufficiently. Thus, increasing the efficiency of gene transfer by osmotic treatment requires specific pretreatment conditions.

We analyzed the effects of bombardment pressures of 6205, 7584, and 9308 kPa. A bombardment pressure of 7584 kPa was significantly (p=0.03) more effective (as measured by transient GUS expression) than 6205 kPa, and more effective than

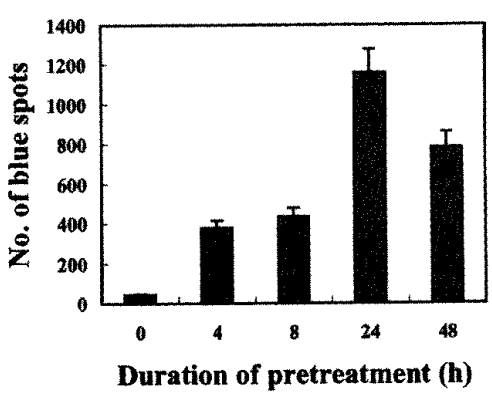

Fig. 2 Effect of high-osmotic pretreatment time on gene transfer efficiencies. Data were evaluated as the number of blue spots resulting from transient expression of the GUS gene. Bars indicate SD from three independent experiments.

9308 kPa, but not significantly. Thus, we selected 7584 kPa as the optimum bombardment pressure. Kemper *et al.* (1996) histologically observed the penetration of bombarded particles into immature maize embryos, and found that the bombardment pressure significantly influenced the degree of penetration: lower pressure (8274 kPa) permitted transformations of cells at the surface of the target tissue, but did not promote deep-cell transformation; whereas although higher pressure (12411 kPa) allowed particles to reach deeper layers and transform that region, it caused excessive cell damage. These observations are consistent with our results. Namely, although the bombardment distance may affect the penetration of gold particles into target tissue, 6205 kPa of bombardment pressure was not sufficient to efficiently penetrate the cell wall of Italian ryegrass calli, and 9308 kPa caused cell damage at the bombardment distance shown in **Table 2.**

Previous studies of the production of transgenic Italian ryegrass by particle bombardment used a particle inflow gun according to the methods of Finer *et al.* (1992), and a bombardment pressure of 600 kPa (Ye *et al.*, 1997, 2001; Dalton *et al.*, 1999), which is much lower than the pressures we used. Therefore, each bombardment parameter has to be optimized for each kind of equipment and target tissue. In this study, the optimum bombardment conditions for Italian ryegrass callus were 24 h of high-osmotic pretreatment and a bombardment pressure of 7584 kPa in the PDS-1000/He helium-driven biolistic device.

Antibiotic selection and recovery of transgenic plants

After 5 days, bombarded calli were transferred and cultured on the first selection medium (**Table 1**) for 1 week in the dark at 25 ℃. They were then placed onto the second selection medium (**Table 1**) and subcultured every 2 weeks. After 6 weeks of selective culture, vigorously growing hygromycin-resistant calli were observed (**Fig. 1B**). The hygromycin-resistant calli were transferred onto regeneration medium without hygromycin (**Table 1**) and cultured for 1 month to the formation of green shoots (**Fig. 1C**). For rooting and plant propagation, the green shoots regenerated from hygromycin-resistant calli were subcultured on the same medium every month.

Table 3 summarizes the transformation experiments. The transgenic plants were initially confirmed by PCR using primers specifically amplifying a 375-bp fragment of the HPT gene (data not shown). Thirty-eight (7.9%), 17 (5.3%), and 11 (3.4%) bombarded calli produced hygromycin-resistant calli in the Waseaoba No. 3, Meritra No. 1, and Meritra No. 8 genotypes, respectively; and 20 (52.6%), 11 (64.7%), and 7 (63.6%) of these hygromycin-resistant calli were regenerated to green plants. Thirty-three out of the 38 regenerated plants exhibited the 375-bp amplified fragment of the HPT gene (**Table 3**). The transgenic plants were established and grew to normal morphology in the glasshouse (**Fig. 1D, E**). The final transformation frequencies of each genotype were 3.8% for Waseaoba No. 3, 2.5% for Meritra No. 1, and 2.2% for Meritra No. 8 (Meritra total: 2.3%). Cotransformation frequencies were 55.6% for Waseaoba No. 3, 62.5% for Meritra No. 1, and 85.7%

Table 3 Summary of transformation experiments using two Italian ryegrass cultivars

Variety	Genotype no.	Number of bombarded calli	Number of hygromycin-resistant calli	Number of calli with albino shoots	Number of calli with green shoots	Number of transgenic plants[1]		Cotransformation frequency (%)	Transformation efficiency (%)[2]
						HPT	antiCAD		
Waseaoba (2)[3]	3	480	38(7.9%)[4]	18(4)[5](47.4%)[6]	20(52.6%)[6]	18	10	55.6	3.8
Meritra (4)	1	320	17(5.3%)	0(0%)	11(64.7%)	8	5	62.5	2.5
	8	320	11(3.4%)	4(1)(36.4%)	7(63.6%)	7	6	85.7	2.2
Total		1120	66(5.9%)	22(5)(33.3%)	38(57.6%)	33	21	63.6	2.9

[1] Foreign gene integration was confirmed by PCR analysis.

[2] Percentage of bombarded calli that regenerated independent transgenic plants transformed with the HPT gene.

[3] Number in parentheses indicates the ploidy of plants used in this study.

[4] Number in parentheses indicates percentage of bombarded calli that produced hygromycin-resistant calli.

[5] Number in parentheses indicates number of calli forming both albino and green shoots.

[6] Number in parentheses indicates percentage of hygromycin-resistant calli with albino or green shoots.

for Meritra No. 8 (Meritra total: 73.3%).

According to Dalton *et al.* (1999), it is harder to produce transgenic plants from diploid cultivars of *Lolium* than from tetraploids; this results from the higher frequency of albino shoot formation or lower regenerability during tissue culture in diploids. In addition, Ye *et al.* (1997) reported a high frequency (2/3) of albino shoot formation on hygromycin-resistant calli from suspension culture in diploid cultivars. Therefore, at least in *Lolium*, albino shoot formation in *in vitro* culture might be a consistent limiting factor for transformation efficiency. However, although we observed a higher frequency of albino shoot formation in the diploid cultivar Waseaoba (47.4%) than in the tetraploid cultivar Meritra (14.3%), the former showed a higher transformation efficiency than did the latter. This indicates that screening for an elite diploid genotype, such as Waseaoba No. 3, can improve transformation efficiency, despite a high frequency of albino shoot formation (**Table 3**).

Molecular analysis of transgenic plants

We performed Southern hybridization analysis of the cotransformed plants with the HPT and antiCAD gene probes. Typical results with the enzymes *Eco*RI for antiCAD gene detection and *Bam*HI for HPT gene detection are shown in **Fig. 3A** and **B**. Representative transgenic plants examined showed the hybridization signal to be of the predicted molecular size, indicating the existence of the full length of each foreign gene in the genome. However, unpredictable hybridization signals were also observed at higher or lower molecular-weight regions. This may be due to the occurrence of rearrangement or partial integration of the transgene sequence into the recipient genome during transformation. This result corresponds to the results of previous reports of Italian ryegrass transformation via biolistic transformation (shotgun technique) (Ye *et al.*, 1997, 2001; Dalton *et al.*, 1999).

The copy number of antiCAD genes was estimated with the enzymes *Sac*I and *Sph*I. Typical results are shown in **Fig. 3C**. Different hybridization patterns for each transformant indicate that each transgenic plant arose from independent transformations. Because the number of hybridization signals reflects the copy number of foreign genes, the results shown in **Fig. 3C** indicate that multiple copies of each foreign gene used were integrated into the plant genome.

We also investigated the expression of the antiCAD gene by RT-PCR analysis (**Fig. 4**). Except in lane 5, a predicted PCR product of 573 bp was

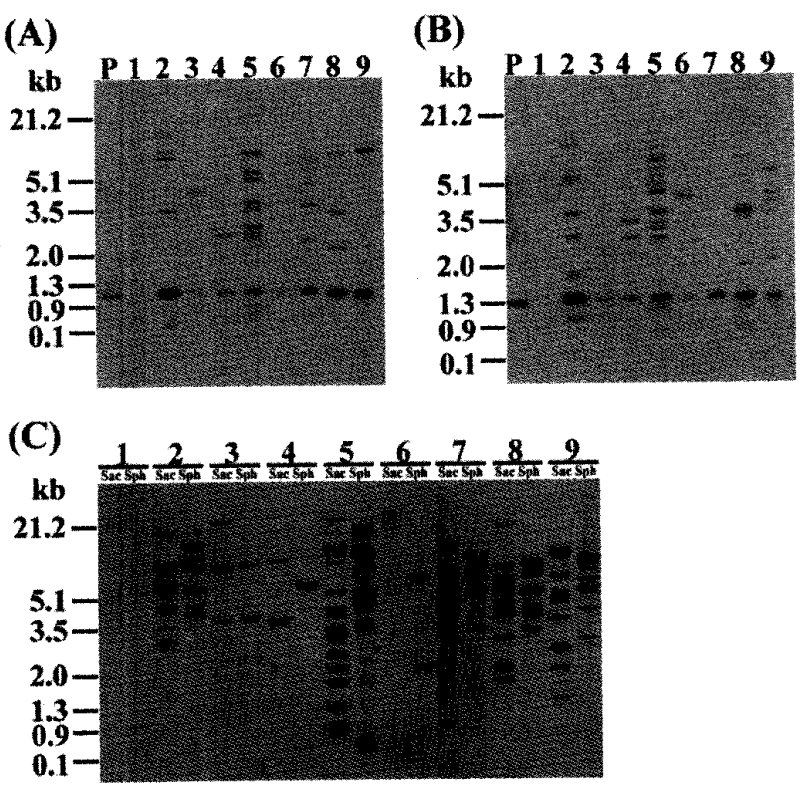

Fig. 3 Southern blot analysis of cotransformed plants hybridized with antiCAD gene probe (A, C) or HPT gene probe (B). Five micrograms of genomic DNA was digested with *Eco*RI (A), *Bam*HI (B), or *Sac*I or *Sph*I (C).
P: pFFantiCAD (A) or pAcH1 (B) (positive control). 1: nontransgenic plant (negative control). 2-9: transgenic plants. Lane numbers (1-9) correspond to each result.

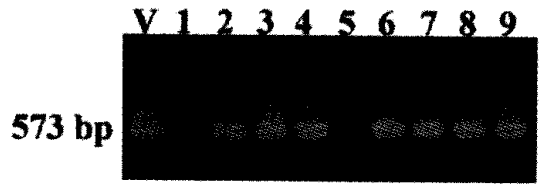

Fig. 4 Expression analysis of antiCAD gene with RT-PCR. The antiCAD gene was detected by RT-PCR as described in the Materials and methods section. V: pFFantiCAD (vector control). 1: nontransgenic plant (negative control). 2-9: transgenic plants. Lane numbers (1-9) correspond to results of Southern blot analysis shown in **Fig. 3**.

detected as a single band. The possibility of DNA contamination was checked by PCR without RT reaction (data not shown). The results proved the expression of the antisense orientation of the CAD gene derived from *Aralia cordata* in transgenic Italian ryegrass plants (**Fig. 4**). The presence of multiple transgene copies often increases transgene silencing (De Wilde *et al.*, 2000). Because the plant corresponding to lane 5 in **Fig. 4** received a large number of antiCAD genes (**Fig. 3C**, lane 5), transgene silencing might explain why the RT-PCR product was not detected in lane 5 of **Fig. 4**.

In conclusion, we established a transformation system using embryogenic calli of Italian ryegrass by microprojectile bombardment and produced independently transformed plants. The expression of a foreign antiCAD gene was confirmed by RT-PCR. Since the PDS-1000/He helium-driven biolistic device is commercially available, our method is more widely applicable than previous methods that have employed the particle inflow gun developed by Finer *et al.* (1992). In addition, previous reports and the present study both suggest that it is very difficult to screen for genotypes that keep their regenerability while cultured in liquid medium as a suspension culture. Thus, our method is more advantageous for the production of transgenic Italian ryegrass than a suspension culture, in that it is easier to establish and more rapid. We are currently evaluating the transgenic plants obtained in this study.

Acknowledgements

We thank Dr. R. Wu (Cornell University, USA), Dr. G. Spangenberg (La Trobe University, Australia), and Dr. M. Ugaki (National Institute of Agrobiological Resources, Japan) for kindly providing the plasmids pAct1-F, pAcH1, and pFF19G, respectively. We also thank Dr. Hibino (Oji Paper, Japan) for providing the cinnamyl alcohol dehydrogenase (CAD) gene, and Dr. T. Sasaki (Japan Grassland Farming Forage Seed Association), Dr. M. Fujimori (National Institute of Livestock and Grassland Science, Japan), and Dr. B. E. Ubi (STA fellowship at National Institute of Livestock and Grassland Science, Japan) for their support. This work was funded by a research grant from the Japan Racing Association.

References

Altpeter, F., Xu, J., Ahmed, S., 2000. Generation of large numbers of independently transformed fertile perennial ryegrass (*Lolium perenne* L.) plants of forage- and turf-type cultivars. Mol. Breed., **6**: 519-528.

Bhalla, P. L., Swoboda, I., Singh, M. B., 1999. Antisense-mediated silencing of a gene encoding a major ryegrass pollen allergen. Proc. Natl. Acad. Sci. U. S. A., **96**: 11676-11680.

Creemers-Molenaar, J., Loeffen, J. P. M., van Rossum, M., Colijn-Hooymans, C. M., 1992. The effect of genotype, cold storage and ploidy level on the morphogenic response of perennial ryegrass (*Lolium perenne* L.) suspension cultures. Plant Sci., **83**: 87-94.

Dalton, S. J., Bettany, A. J. E., 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.

Dalton, S. J., Bettany, A. J. E., Timms, E., Morris, P., 1999. Co-transformed, diploid *Lolium perenne* (perennial ryegrass), *Lolium multiflorum* (Italian ryegrass) and *Lolium temulentum* (darnel) plants produced by microprojectile bombardment. Plant Cell Rep., **18**: 721-726.

De Wilde, C., Van Houdt, H., De Buck, S., Angenon, G., De Jaeger, G., Depicker, A., 2000. Plants as bioreactors for protein production: avoiding the problem of transgene silencing. Plant Mol. Biol., **43**: 347-359.

Finer, J. J., Vain, P., Jones, M. W., McMullen, M. D., 1992. Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep., **11**: 323-328.

Gharti-Chhetri, G. B., Cherdshewasart, W., Dewulf, J., Paszkowski, J., Jacobs, M., Negrutiu, I., 1990. Hybrid genes in the analysis of transformation conditions. 3. Temporal/spatial fate of NPT II gene integration, its inheritance and factors affecting these processes in *Nicotiana plumbaginifolia*. Plant Mol. Biol., **14**: 687-696.

Gritz, L., Davies, J., 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene, **25**: 179-188.

Hibino, T., Shibata, D., Chen, J.-Q., Higuchi, T., 1993. Cinnamyl alcohol dehydrogenase from *Aralia cordata*: cloning of the cDNA and expression of the gene in lignified tissues. Plant Cell Physiol., **34**: 659-665.

Jackson, J. A., Dale, P. J., 1988. Callus induction, plant regeneration and an assessment of cytological variation in regenerated plants of *Lolium multiflorum* L. J. Plant Physiol., **132**: 351-355.

Jefferson, R. A., Kavanagh, T. A., Bevan, M. W., 1987.

GUS fusions: β - glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., **6**: 3901-3907.

Kartzke, S., Saedler, H., Meyer, P., 1990. Molecular analysis of transgenic plants derived from transformations of protoplasts at various stages of the cell cycle. Plant Sci., **67**: 63-72.

Kemper, E. L., da Silva, M. J., Arruda, P., 1996. Effect of microprojectile bombardment parameters and osmotic treatment on particle penetration and tissue damage in transiently transformed cultured immature maize (*Zea mays* L.) embryos. Plant Sci., **121**: 85-93.

McElroy, D., Blowers, A. D., 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.

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

Murray, M. G., Thompson, W. F., 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res., **8**: 4321-4325.

Rogers, S. O., Bendich, A. J., 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol., **5**: 69-76.

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

Timmermans, M. C. P., Maliga, P., Vieira, J., Messing, J., 1990. The pFF plasmids: cassettes utilising CaMV sequences for expression of foreign genes in plants. J. Biotechnol., **14**: 333-344.

Vain, P., McMullen, M. D., Finer, J. J., 1993. Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep., **12**: 84-88.

Wang, G. R., Binding, H., Posselt, U. K., 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, Z. Y., Nagel, J., Potrykus, I., Spangenberg, G., 1993. Plants from cell suspension-derived protoplasts in *Lolium* species. Plant Sci., **94**: 179-193.

Ye, X., Wang, Z.-Y., 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.

Ye, X. D., Wu, X. L., Zhao, H., Frehner, M., Nosberger, J., Potrykus, I., Spangenberg, G., 2001. Altered fructan accumulation in transgenic *Lolium multiflorum* plants expressing a *Bacillus subtilis* sacB gene. Plant Cell Rep., **20**: 205-212.