ISSN 1009 - 3095 *Journal of Zhejiang University SCIENCE* V.4, No.3, P.346 - 351, May - June, 2003
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Agrobacterium-mediated transformation of herbicide resistance in creeping bentgrass and colonial bentgrass

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Received June 6, 2002; revision accepted Aug. 8, 2002

Abstract: Embryogenic calli were induced from the seeds of creeping bentgrass (*Agrostis palustris* Huds.) cv. Regent and colonial bentgrass (*Agrostis Tenuis* Sibth. Fl. Oxen.) cv. Tiger. The embryogenic calli were precultured on fresh medium for 4 - 7 days and then co-cultivated with *Agrobacterium tumefaciens*, LBA4404, which contains plasmid vector-pSBGM harboring bar coding region, synthetic green fluorescent protein (sGFP) coding region and matrix attachment region (MAR). After 3 days of co-cultivation, the calli were washed thoroughly and transferred to MS medium containing 2 mg/L of 2, 4-D, 12 - 15 mg/L phosphinothricin (PPT) and 250 mg/L of cefotaxime. After 2 - 3 months of selection, the actively growing calli of 'Regent' and 'Tiger' were transferred to MS medium with 12 - 15 mg/L PPT and 250 mg/L cefotaxime for regeneration. The putative transformants were maintained on MS medium with 3 mg/L PPT for long period but control died within 1 month. After establishing in greenhouse, the transformants also showed strong resistance to 0.4% of herbicide Basta but control plants died within 2 weeks. Under confocal microscope, both young leaves and roots showed significant GFP expression. PCR analysis revealed the presence of a DNA fragment of GFP gene at the expected size (380 bp) in the transformants and its absence in a randomly selected control plant.

Key words: *Agrostis palustris*, *Agrostis tenuis*, *Agrobacterium*, Herbicide resistance, sGFP(synthetic green fluorescent protein)
Document code: A **CLC number:** S603.6

INTRODUCTION

Creeping bentgrass (*Agrostis palustris* Huds.) is an outstanding cool-season species available for use on golf course putting greens, tees, and closely mowed fairways. Although colonial bentgrass (*Agrostis tenuis* Sibth. Fl. Oxen.) is not well adapted to the very low mowing heights used on creeping bentgrass, it is better adapted to mowing height of 0.5 inch and is better suited to golf course fairway than golf green. Unfortunately, both of them are susceptible to a wide range of diseases, such as dollar spot (*Sclerotina homeocarpa*), brown patch (*Rhizoctonia salani*), Pythium blight (*Pythium graminicola*) and also prone to herbicide injury. For the above reasons, they are attractive targets for biotechnology.

Success in regeneration of creeping bentgrass from embryogenic callus has paved the way for its genetic manipulation (Krans *et al.*, 1982; Blanch *et al.*, 1986; Zhong *et al.*, 1991; Terakawa *et al.*, 1992). Two types of transformation systems had been tested successfully for developing creeping bentgrass transgenic plants: direct DNA uptake using protoplasts mediated by polyethylene glycol (PEG) or electroporation (Lee *et al.*, 1996), and direct DNA delivery to embryogenic callus via microprojectile bombardment (Zhong *et al.*, 1993; Hartman *et al.*, 1994; Warkentin *et al.*, 1997; Zhong *et al.*, 1997; Liu *et al.*, 1998).

Compared with microprojectile bombardment or DNA uptake mediated transformation, the advantages of *Agrobacterium*-mediated gene transfer are relatively lower cost, easier and higher efficiency of transformation. *Agrobacaterium*-mediated transformation can transform intact, regenerative plant tissues and organs (Horsch *et al.*, 1985). Gene integration patterns are more pre-

dictable in *Agrobacterium*-mediated transformation than other gene transfer methods (Birch and Frank, 1991; Smith and Hood, 1995). Transformants from *Agrobacterium*-mediated method often contain genomic insertions by exact and single or low-copy number of transgene cassettes (Deblock, 1993). So it is a good alternative method for transformation in turfgrasses.

Using hygromycin phosphotransferase (HPT) gene as a selectable marker, we obtained stable transformants in creeping bentgrass by *Agrobacterium*-mediated gene transfer (Chai *et al.*, 2000), which was also reported by Yu *et al.* (2000). In this study, we produced herbicide resistant creeping bentgrass 'Regent' and colonial bentgrass 'Tiger' by *Agrobacterium*-mediated gene transfer system using GFP as a reporter.

MATERIALS AND METHODS

Induction of embryogenic callus

Embryogenic calli were initiated from commercial seeds of creeping bentgrass (*Agrostis palustris*) cv. Regent (International Seeds Inc., USA), and colonial bentgrass (*Agrostis tenuis*) cv. Tiger (International Seeds Inc., USA) according to our established procedure (Chai and Kim, 1998). The seeds were disinfected by treating with 70% (v/v) ethanol for 1 minute followed by treatment with 0.1% $HgCl_2$ for 20 minutes. After rinsing thrice with sterile water, the seeds were placed on the surface of the media described below. MS medium (Murashige and Skoog, 1962) with 1.14 g/L proline and 2 mg/L of 2, 4-D was used for callus induction and maintenance. pH of the media was adjusted to 5.8 with 1 mol/L NaOH or 1 mol/L HCl. Phytagel (Sigma P-8169) at 0.3% (w/v) was used as solidifying agent. The medium was sterilized by autoclaving under standard conditions for 15 minutes at 121℃.

Embryogenic callus was separated after 6 weeks of incubation in the dark at 25 ± 2℃ according to the method established in our previous experiment (Chai and Kim, 1998). Both species contained 3 disposable petri-dishes (9 cm in diameter) and each petri-dish was inoculated 80 seeds. Embryogenic calli that proliferated in two subcultures (about two months) on the same kind of the medium for callus induction were used for the following experiments.

Determination of PPT selection concentrations

For determining suitable concentrations of PPT for callus selection during transformation, the rate of increase in callus weight [(final weight of callus - initial weight of callus) / (initial weight of callus)] of 'Regent' and 'Tiger' were checked after 7 weeks on MS + 2 mg/L 2, 4-D with 0, 3, 6, 9, 12, or 15 mg/L of PPT. Each treatment contained 4 disposable petri-dishes, each petri-dish had 0.34 ± 0.03 g about sixty 1 – 2 mm diameter pieces of callus dispersed evenly in the initial phase of the experiment.

Transformation

Agrobacterium tumefaciens strain LBA4404 containing plasmid vector-pSBGM, constructed by Prof. Kim Jukon, Myongji University, Korea, was used in this experiment (Fig.1).

Embryogenic calli induced on above were transferred to fresh MS + 2.0 mg/L 2, 4-D medium for 4 – 7 d pre-culture in darkness at 25 ± 2℃. The *Agrobacterium* was grown for 3 days on solid AB medium (Chilton *et al.*, 1974) supplemented with 50 mg/L spectinomycin and 10 mg/L tetracycline, and then collected with a small spoon, suspended in 30 ml AAM medium containing 100μmol/L acetosyringone for each petri-dish (9 cm in diameter) of the *Agrobacterium* (Hiei *et al.*, 1994) for 1 hour. Pre-cultured calli described above were immersed in this suspension culture for 10 – 15 min in darkness, blotted on filter paper and then transferred onto MS + 2 mg/L 2, 4-D + 100 μmol/L acetosyringone for 3 days co-cultivation in darkness at 25℃. After co-cultivation, the calli were rinsed thoroughly with distilled water, two times with 500 mg /L cefotaxime, and then placed onto callus selection medium: MS + 2 mg/L 2, 4-D + 12 – 15 mg/L PPT + 250 mg/L cefotaxime. During transformation, two experiments were involved. In Expt.1, callus was selected from the callus selection medium after 2 months, and then moved to regeneration selection medium for 4 weeks until shoot formation. In Expt.2, callus was kept for selection 3 months (subculture one time on the same medium), and then moved to regeneration selection medium for 4 weeks until shoot formation. Regeneration selection medium was MS medium with 12 – 15 mg/L PPT and 250 mg/L cefotaxime. All the regenerated small

shoots were moved to MS medium for growing and rooting for 2 weeks, and then maintained on MS + 3 mg/L PPT for long time selection until transplantation.

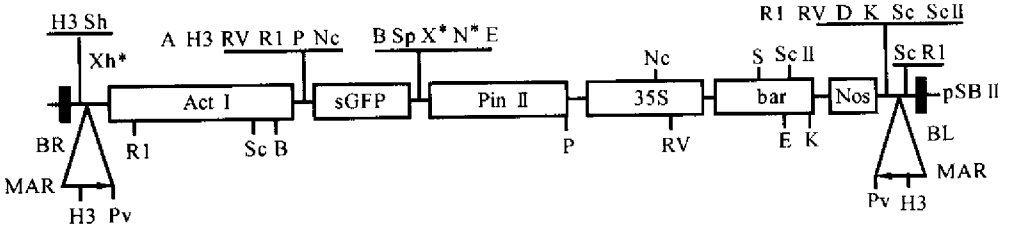

Fig.1 T-DNA regions of plasmid pSBGM

MAR: Matrix attachment region; Act1: Rice Act1 promoter; sGFP: Synthetic GFP coding region; PinII: Potato proteinase inhibitor II gene; 35S: 35S promoter; bar: bar coding region; Nos: Nopaline synthase gene; BR: Right border; BL: Left border; A: Acc1; B: BamH1; D: Dra11; E: Eag1; H3: HindIII; K: Kpn1; N: Nct1; Nc: Nco1; P: Pst1; Pv: PvuII; R1: EcoR1; RV: EcoRV; S: Sal1; Sc: Sac1; ScII: SacII; Sp: Spe1; X: Xba1; Xh: Xho1

Herbicide test

A commercial formulation of PPT under the trade name Basta was used in greenhouse herbicide tests. Herbicide rate for Basta was established according to its recommended rate for grasses. Five month-old potted putative transformants and controls were sprayed 0.4% herbicide Basta, and observed after two weeks.

GFP detection by using confocal laser scanning microscope

GFP expression of the leaves and roots of herbicide resistant plants and control were observed using a Carl Zeiss LSM 410 CLSM (confocal laser scanning microscope) with a standard filter at the Korea Basic Science Institute.

DNA extraction and PCR analysis

DNA was extracted from the shoots and leaves of transformants and control plants of 'Regent' and 'Tiger' according to the cetyltrimethyl-ammonium bromide method (Marray and Thompson, 1980). PCR to detect introgressed GFP gene was performed with 0.8 unit of Ex-taq DNA polymerase (Takara, Japan) and 50 ng of DNA samples. Two 22-nucleotide primers, upstream primer, 5'-CCT GAA GTT CAT CTG CAC CAC C-3'; downstream primer, 5'-CTT GAA GTT CAC CTT GAT GCC G-3' ends of a 380 bp fragment of the GFP gene were used. Samples for enzymatic amplification fragments were subjected to 40 cycles of PCR amplification for 30 sec at 94℃, 40 sec at 55℃, and 1 minute at 72℃. Amplification fragments generated by PCR were separated on 1.5% agarose gel, stained with ethidium bromide and observed under ultraviolet light (312 nm).

RESULTS AND DISSICUSSION

Induction of embryogenic callus

Callus could be induced from all the germinated seeds. Callus appeared to be produced directly from the growing shoot apex immediately following germination. Embryogenic callus was yellow to yellowish ('Regent'), and white opaque ('Tiger'). Usually 'Regent' was nodular and compact, but 'Tiger' was friable. The rates of resulting embryogenic calli were 33.8% and 36.3% for 'Regent' and 'Tiger' respectively.

Determination of PPT selection concentrations

In the initial two weeks, all the calli of 'Regent' and 'Tiger' cultured on medium with different concentrations of PPT showed no significant differences among treatments. This phenomenon agreed with the fact that although PPT is an irreversible inhibitor of glutamine synthase, the only enzyme that detoxifies ammonia produced during the nitrate reduction, photorespiration and amino acid degradation in plant cell (D'Halluin *et al*., 1992), ammonia accumulation to a phytotoxic concentration needs time. After 7 weeks of selection on the medium containing 12 or 15 mg/L PPT, not only callus growth was greatly inhibited (Fig.2); and the color of the calli changed into dark brown. So in the following experiment, 12 or 15 mg/L of PPT was used for callus selection and regeneration. Our result also agreed with the result reported by

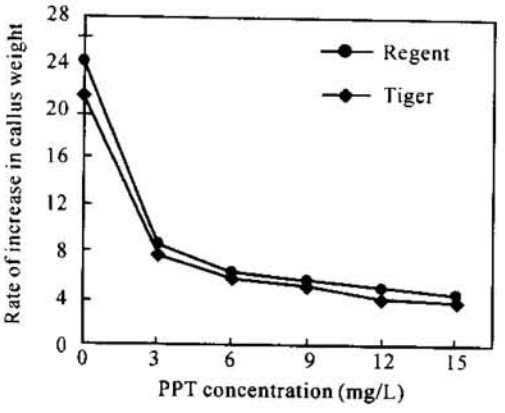

Fig.2 PPT kill curve of in vitro cultured callus of creeping bentgrass (cv. Regent) and colonial bentgrass (cv. Tiger) after 7 weeks of selection culture

Zhong *et al*. (1997). They found that the callus induction medium containing 5 mg/L bialaphos or 15 mg/L PPT for 12 weeks at 4-week intervals was suitable for selection of bombarded callus from allotetraploid creeping bentgrass.

Transformation

Expt. 1 involved keeping the calli in the same callus selection medium for about 2 months, and then moving to regeneration selection medium for 4 weeks until formation of shoot clusters (Table 1, Expt. 1). Expt. 2 involved keeping calli on callus selection medium for 3 months, and then moving to regeneration selection medium for 4 weeks until shoot formation.

Table 1 PPT resistant calli selection and plant regeneration*

Experiment	Number of calli on regeneration selection medium	Number of regenerated calli	Percentage of regeneration
Expt.1			
(1) PPT 12©-PPT 12®	115	37	32.17
(2) PPT 12©-PPT 15®	218	9	4.12
(3) PPT 15©-PPT 12®	113	12	10.61
(4) PPT 15©-PPT 15®	98	13	13.26
Expt.2			
(1) PPT 12©-PPT 15©-PPT 15®	74	1	1.35
(2) PPT 15©-PPT 12©-PPT 15®	203	23	11.33
(3) PPT 15©-PPT 15©-PPT 15®	265	25	9.43

* The concentration unit of PPT is mg/L; ©: callus selection medium; ®: regeneration selection medium

The small shoot clusters from regenerated calli were moved to MS medium until their growth into plants. When the plants were moved to MS + 3 mg/L PPT medium for long period, all the plants from the above experiments kept growing but control plants (regenerated from untreated callus) died within a month (Fig. 3).

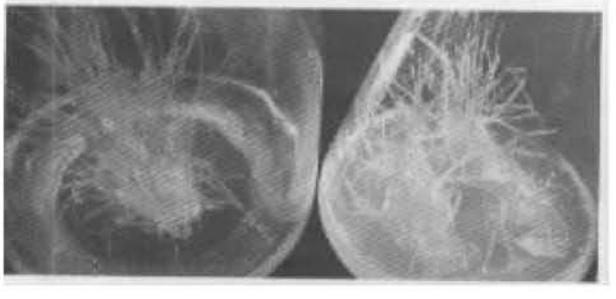

Fig.3 The response of putative transformants to MS medium containing 3 mg/L of PPT after one month culture (left: putative transformants from 'Regent'; right: control plantlets of 'Regent' from untreated calli)

This means that all the selection methods are effective. For decreasing somaclonal variations derived from long period of culture in vitro and for increasing transformation efficiency, the procedure in Expt.1 (1) is the best.

Herbicide test

A total of 120 'Regent' regenerants (Table 1) and 23 'Tiger' regenerants were transferred to soil, and tested in greenhouse. All putative transgenic plants survived Basta at 0.4%. After herbicide application, control plants were completely killed within 5 to 10 days, and resistant transgenic plants continued to grow and were green and healthy (Fig.4).

GFP detection by using confocal microscope

In transformants, fluorescence emanating from GFP was detected in both young leaves and roots in the green channel (Fig.5), whereas no fluorescence was detected in control materials (data not shown).

Fig. 4 Herbicide resistance of transgenic plants (right) and control (left) at the 0.4% rate of Basta 2 weeks after herbicide application

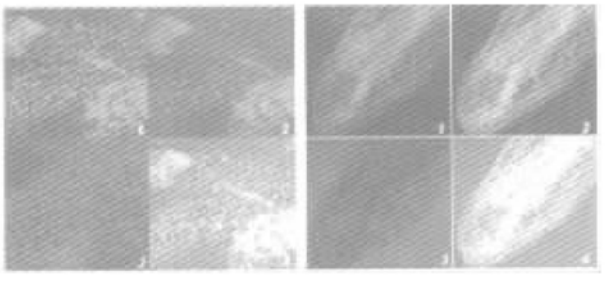

Fig.5 Images of GFP in young leave (left) tissues and root (right) of transgenic bentgrass cv. Regent by confocal microscope. 1: red channel (543 nm), 2: green channel (488 nm), 3: transmission channel, 4: merged channel

Green fluorescent protein (GFP) is rapidly being used as a standard reporter in many biological systems. Compared with the widely used reporters beta-glucuronidase (GUS) and luciferase (LUC), the most notable advantage of GFP is that it allows for convenient non-invasive in vivo analysis without requirement of an added substrate to monitor reporter activity (Niedz *et al*., 1995). In the sGFP (S65T), the serine residue at position 65 was replaced with a threonine to enhance and stabilize the fluorescent signal in the plant cells. The amino acid replacement in the GFP brought about a 100-fold brighter fluorescent signal (Hashizume *et al*., 1999). In creeping bentgrass transformants, the green fluorescence was expressed in young shoots, young leaves, roots, especially the root tips and in new roots developed from nodes (Yu *et al*., 2000). Our results also demonstrated that sGFP is a good reporter for both creeping bentgrass and colonial bentgrass during transformation.

PCR analysis of transformants

PCR analysis revealed the presence of a DNA fragment of the expected size in all of the transgenic plants examined and its absence in a randomly selected control plant (Fig.6), indicating that the introduced sGFP gene was present in the herbicide resistant plants.

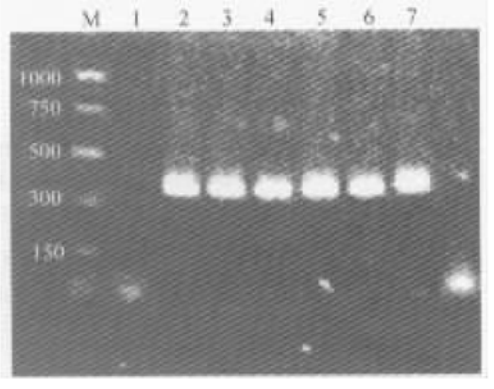

Fig.6 PCR analysis of transgenic plants
M: Marker DNA; 1: control; 2 – 5: Transformants from 'Regent'; 6 and 7: Transformants from 'Tiger'

In summary, we have established a stable transformation system mediated by *Agrobacterium* in creeping bentgrass and colonial bentgrass using sGFP as a reporter gene and BAR gene as a selectable marker. Although no Southern analysis was done in this study, the clear and strong GFP expression in the transgenic plants under confocal microscope, and the stable higher resistance of the transgenic plants to herbicide after 3 generations of vegetative propagation (data not shown) demonstrated the reliability of our experiment. The method described here has also been used to transfer other agronomically important genes, such disease resistant genes and drought tolerant genes into bentgrass and zoysiagrass in our laboratory.

ACKNOWLEDGEMENTS

We sincerely thank Prof. Kim Jukon (Myongji University, Korea) for providing the *Agrobacterium* used in this work, also Prof. Duncan R. R. (University of Georgia, USA) for checking most parts of this manuscript.

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