Herbicide Resistant Turfgrass(Zoysia japonica cv. 'Zenith') Plants by Particle bombardment-mediated Transformation

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ABSTRACTS

Transgenic zoysiagrass (Zoysia japonica cv. Zenith) plants have been obtained by particle bombardment of embryogenic callus with the plasmid pSMABuba, which contains hygromycin resistance (hpt) and bialaphos resistance (bar) genes. Parameters on DNA delivery efficiency of the particle bombardment were partially optimized using transient expression assay of a chimeric β-glucuronidase (gusA) gene driven by the CaMV 35S promoter. Stably transformed zoysiagrass plants were recovered with a selection scheme using hygromycin. Transgenic zoysiagrass plants were confirmed by PCR analysis with specific primer for bar gene. Expression of the transgene in transformed zoysiagrass plants was demonstrated by Reverse transcriptase (RT)-PCR analysis. All the tested transgenic plants showed herbicide BastaR resistance at the field application rate of 0.1%-0.3%.

Key word: Basta, herbicide resistance, particle bombardment, turfgrass transformation

Introduction

By direct gene transfer or by Agrobacterium, more and more genes have been transferred to important cereals and vegetables for various purposes.

Zoysiagrass (Zoysia spp.) is the most widely used and important warm-season turfgrass in Asia and transition zones in USA. Since this turfgrass can grow under severe stresses such as heavy traffic, and low water and fertilizer conditions, there is an increasing demand for new cultivars with desirable traits such as disease and pest resistance. Improvement of such species by genetic transformation is attractive, since there are many difficulties in their breeding using conventional selection procedures. Agrobacterium-mediated transformation has been widely used to transfer foreign genes

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into many dicot plants. However, most gramineous species plants are generally recalcitrant to Agrobacterium-mediated transformation, although good progress has been made on the transformation of rice and maize using Agrobacterium. Among various techniques used for monocot transformation, direct gene transfer into competent protoplasts by electroporation or PEG, and particle bombardment of embryogenic cells have so far been most effective for developing transgenic monocotyledonous species (Fromm et al., 1990; Jähne et al., 1994; Cao et al., 1992; Vasil et al., 1992; Weeks et al., 1993). Most of transgenic turfgrass plants have been obtained by direct gene transfer of Agrostis alba (Asano and Ugaki, 1994), A. palustris (Zhong et al., 1993; Hartman et al., 1994; Asano et al., 1998), Festuca arundinacea (Ha et al., 1992), Lolium multiflorum (Ye et al., 1997; Dalton et al., 1999), and L. perenne (Spangenberg et al., 1995), and Zoysia japonica (Park and Ahn, 1998; Inokuma et al., 1998). Recently, few group reported by Agrobacterium mediated gene transfer to A. palustris, A. tenuis (Chai et al., 2000), and Z. japonica (Chai et al., 2000).

Phosphinothricin (glufosinate), the active principle of the herbicide BastaR or bialaphos, is a glutamic analogue that inhibits glutamate synthase. The inhibition of glutamine synthase by phosphinothricin (PPT) in plants results in an accumulation of ammonium, which is mainly derived from photorespiration. High concentrations of ammonium interfere with the electron-transport systems of both chloroplasts and mitochondria, resulting in the production of free radicals and ultimately leading cell death. The enzyme phosphinothricin acetyltransferase (PAT), encoded by the bar gene, inactivates PPT through acetylation (Thompson et al., 1987).

Several crops have been transformed with the bar gene, including tobacco, tomato, and potato showing tolerance to the common spray of glufosinate used to eradicate weeds (De Greef et al., 1989). The bar gene was introduced and expressed in canola (De block et al., 1987; De Greef et al., 1989), cabbage (Lee et al., 2000), maize (Gordon-kamm et al., 1990; Spencer et al., 1992), and rice (Datta et al., 1992; Cao et al., 1992). Although herbicide resistance has been introduced into other turfgrass species such as creeping bentgrass (Hartman et al., 1994), tall fescue (Wang et al., 1992), zoysiagrass transformation with the bar gene has not yet been reported.

Here we report the transfer of selectable marker gene and herbicide tolerant gene into zoysiagrass, and the regeneration of transgenic, morphologically normal plants that are shown resistant to herbicide BastaR.

Materials and Methods

Plant materials

Commercial seeds of zoysiagrass cultivar 'Zenith' were soaked in 70% ethanol for 30 minutes, surface sterilized in 4% sodium hypochloride solution for 30 minutes and rinsed five times in sterile distilled water. The seeds were placed on callus induction medium (2N6 medium supplemented with 300mg/L casein hydrolysate, 500mg/L glutamine, 500 mg/L L-proline, 2mg/L 2,4-D, 3% sucrose), and were kept in the dark at 28℃. After 4~5 weeks, calli were selected and transferred new medium for transformation.

Transformation, selection, and regeneration

Stable transformation experiments were carried out with plasmid pSMABuba, bearing a chimeric bar gene under control of the maize Ubi-1 promoter. A chimeric gusA gene driven by the 35S promoter (pIG121HM) was used in transient transformation experiments.

The transformation protocol was based on the method described by Hartman et al. (1994). Efficient shoot regeneration from embryogenic callus and as well as the optimization of parameters which affect transformation, such as target distance, and helium pressures, have been investigated.

For direct gene transfer, 5$\mu\ell$ of plasmid DNA (5μg), 50$\mu\ell$ of gold particles (appx. 1mg), 25$\mu\ell$ of 2.5M CaCl2 and 10$\mu\ell$ of 100mM spermidine were throughly mixed, centrifuged, and resuspended in ethanol. The ethanol wash was repeated for a total of 3 times. The final pellet was resuspended in 48$\mu\ell$ ethanol, and 6$\mu\ell$ of DNA solution was used per shot. Bombardment was carried out using the Bio-Rad PDS 1000/He Biolistic Delivery System at NICEM (Seoul, SNU). The calli were transferred 4 days after bombardment to selective medium (callus induction medium containing 50mg/L hygromycin). Subcultures to fresh selective medium were performed every 2 weeks. After 4-6 weeks, calli with resistance to hygromycin were transferred to regeneration medium (2N6 medium supplemented with 300mg/L casein hydrolysate, 500mg/L glutamine, 500mg/L L-proline, 2mg/L kinetin, 0.5mg/L NAA, 50mg/L hygromycin). After 2 weeks, the regenerated green shoots were transferred hormone free MS medium. Transformed plants were washed free of medium, trimmed and transferred to soil and grown to maturity under greenhouse conditions.

PCR analysis and DNA gel blot analysis

PCR amplification was performed in volumes of 25$\mu\ell$ containing 100ng template DNA

(total genomic DNA), 0.2 μM each of dNTP, 50pM primers (5'-GGTCTGCAATCGTCAACC-3' and 5'-TCAGATCTCGGTGACGGGCA-3' for bar), 1.0 unit Taq DNA polymerase (Roche Molecular Biochemicals, Germany), 2.5$\mu\ell$ 10×buffer (Roche Molecular Biochemicals, Germany). Amplification was carried out at the condition of a 2 min denaturation at 94℃, followed by 35 cycles of 94℃ for 1min, 62℃ for 1min, and 72℃ for 2min. PCR products were separated by electrophoresis on 1.2% agarose gels containing ethidium bromide in 0.5×TBE, and visualized under UV light. Southern blotting and hybridization were carried out following the manufacture's manual for ECL system (Amersham Life Science, England). About 2.0 kb EcoRI fragment of pSMABuba, which corresponds to the bar coding sequences, was used as a probe.

RT-PCR analysis and gel blot analysis

Three μg of total RNA was reverse-transcribed in a 20$\mu\ell$ reaction volume containing 50 mM Tris-Cl, pH 8.4, 10 mM dithioerythritol, 100mM NaCl, 2.5mM MgCl2, 40 μM each dNTP, 0.15 μM of oligo(dT)15, 40 units of RNase inhibitors, 20 units of MMLV reverse transcriptase at 42℃ for 60 min. Reaction mixture of first strand cDNA was diluted with 30$\mu\ell$ of water and used for PCR amplification. PCR was carried out with bar specific primers. Amplification and gel blot analysis were carried out following corresponding conditions of DNA gel blot analysis.

Herbicide Spraying

Potted plants were sprayed with 0.3% solution of the commercial herbicide BastaR (Aventis crop science Korea), and evaluated for herbicide resistance 21 days later.

Results and Discussions

Embryogenic callus cultures were established from germinating seedling of Zenith. Media with different concentrations of 2, 4-D were used for callus culture induction (data not shown). Embryogenic callus formation was good with 2mg/L of 2, 4-D. Embryogenic callus features were the same as some other plants that were yellow or yellowish, compact or friable. These calli were used for transformation.

A chimeric gusA gene driven by the CaMV 35S promoter was used to partially optimize delivery parameters of DNA-coated microprojectiles to embryogenic callus of zoysiagrass. GUS gene was strongly expressed at helium pressure 1,100psi and 6~9cm target-bombardment distance (data not shown). The stable integration and expression of the hpt gene results in the ability to phosphorylate hygromycin, thus rendering the plant cells resistance to the antibiotic. Dose-response experiments performed with

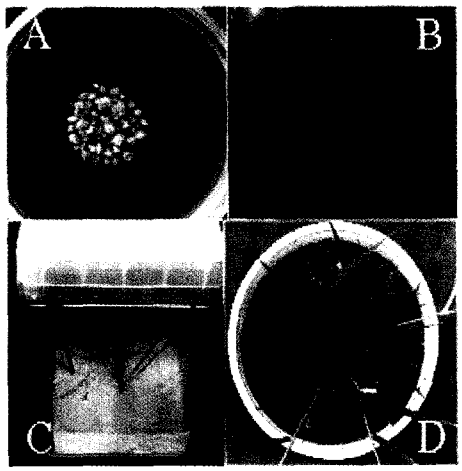

Fig. 1. Transgenic Z. japonica plants obtained via particle bombardment of embryogenic callus.
(A) Embryogenic callus prior to particle bombardment treatment
(B) Shoot differentiation from hygromycin resistance callus
(C) Regenerated plants growing from hormone-free medium
(D) Putative transgenic zoysiagrass plant in soil

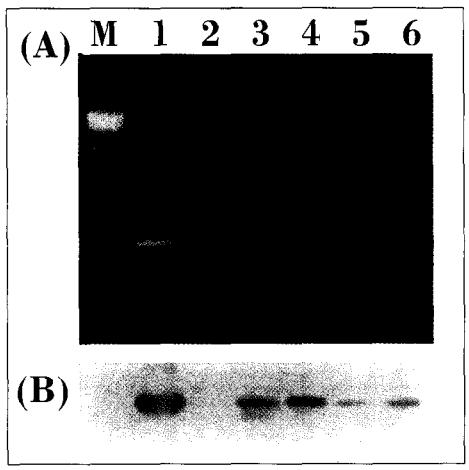

Fig. 2. PCR analysis of genomic DNA from transgenic and untransformed zoysiagrass leaves.
(A) Agarose gel electrophoresis of PCR products amplified with bar-specific primers.
(B) DNA gel blot analysis of the amplified products using the bar coding region as a probe. lanes 1: pSMABuba positive control, 2: untransformed zoysiagrass, 3~6: four independent hygromycin resistance plant.

untransformed embryogenic callus of zoysiagrass plated on media containing 50~400 mg/L hygromycin indicated that 4 weeks selection with 100mg/L hygromycin was lethal. For stable transformation experiments, selection of embryogenic callus was started 4 days after bombardment. After 2~3 subcultures every 2 weeks, putative transformed embryogenic callus transferred to regeneration medium with 50 mg/L hygromycin. After 2~4 weeks later, hygromycin-resistant calli regenerated multiple green shoots, but untransformed calli exhibited brownish and growth reduction. Actively shoot-formed calli transferred hormone-free rooting medium (Fig. 1). On average, 2~4 months after direct gene transfer to embryogenic callus, rooting plant could be transferred to soil and further grown under greenhouse conditions. Four putative transgenic plants obtained from this work were successfully cultivated to maturity in greenhouse condition.

In order to confirm the transgenic nature of the Z. japonica plants recovered from hygromycin resistant calli, PCR analysis using bar-specific primers was performed (Fig. 2A). In all cases tested, four putative transgenic plants showed the presence of a band of the 590bp size of the bar fragment, while no product was detected in the untransformed plants. DNA gel blot analysis of the amplified DNA using the bar coding region as a probe confirmed that the amplified DNA contained bar sequences (Fig. 2B). These results indicate integration

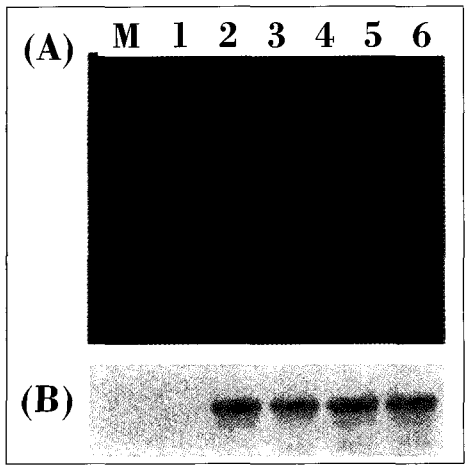

Fig. 3. RT-PCR analysis from transgenic and untransformed zoysiagrass leaves
(A) Agarose gel electrophoresis of RT-PCR products amplified with bar-specific primers.
(B) DNA gel blot analysis of the amplified products using the bar coding region as the probe. lanes 1: untransformed zoysiagrass, 2~5: four independent hygromycin resistance plant.

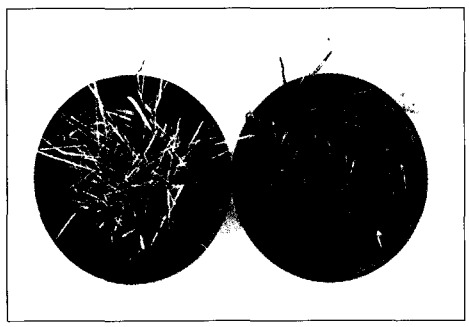

Fig. 4. Response of untransformed (left) and transformed (right) zoysiagrass after BastaR application to leaves

of the bar gene into plant genome by particle bombardment-mediated transformation. Expression of the bar gene in the transgenic zoysiagrass was studied by RT-PCR analysis and DNA gel blot analysis. RT-PCR analysis revealed the expression of bar gene of the expected size in all of the plants examined and its absence in an untransformed plant (Fig. 3A). To confirm amplified DNA fragment including bar gene, we performed DNA gel blot analysis with bar gene. Figure 3B indicates that all transformed plants expressed the bar gene.

The herbicide BastaR was applied to four transgenic zoysiagrasses and untransformed plant. At the field application rate of 0.1%-0.3% (vol/vol), untransformed control plants completely bleached and died within 7~21days. In contrast, four transgenic zoysiagrasses showed highly resistance at 0.3% (vol/vol) (Fig. 4). This indicates the presence of bar-encoded PAT activity in the transgenic plants that inactivates the herbicide Basta^R. These transgenic plants are currently being vernalized to determine their fertility. In the previous report, the herbicide bialaphos is toxic to fungal pathogens such as Rhizoctonia solani, which causes "brown patch", one of the most severe fungal disease of turfgrass (Uchimiya et al., 1993). Therefore, by application of Basta^R or bialaphos, transgenic zoysiagrass plants expressing the bar gene may be possible to simultaneously combat both fungal and weed infection in the field.

In conclusion, we have established a reproducible particle bombardment transformation protocol using embryogenic callus and hpt as a selectable marker gene in Z. japonica, and regenerated transgenic zoysiagrass plants.

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