Development of Seashore Paspalum Turfgrass with Herbicide Resistance

Kyung-Moon Kim*, In Ja Song**, Hyo-Yeon Lee**, Paul Raymer***, Beom Seok Kim****[†], and Wook Kim****[†]

*Kumho Life and Environmental Science Lab, GIST, Gwangju 500-712, Korea
(Current address: BioControl Center, Jeonnam Bioindustry Foundation, Jeonnam 516-942, Korea)
**Subtropical Horticulture Research Center, Cheju National University, Jeju 690-756, Korea
***Department of Crop and Soil Sciences, University of Georgia, Griffin Campus, Griffin, GA30223, USA.
****Division of Life Sciences, Korea University, Seoul 136-701, Korea

ABSTRACT   Seashore Paspalum (*Paspalum vaginatum* Swartz) is a warm season grass and indigenous to tropical and subtropical regions of coastal areas worldwide. The species is used as feed for cattle and horses and has been very successful for golf courses worldwide. One of the most outstanding characteristics of seashore paspalum is its tolerance to saline soils compared to other warm season turfgrasses. The development of new seashore paspalum cultivars with improved traits could be facilitated through the application of biotechnological strategies. The purpose of this study was to product for herbicide resistant seashore paspalum using *Arobacterium*-mediated transformation and this study is the first report on transformation and herbicide-resistant transgenic plants in seashore paspalum. Embryogenic calli were induced from the seeded variety of pseashore paspalum. Embryogenic calli were transformed with *Agrobacterium tumefaciens* strain EHA105 carrying the binary vector pCAMBIA3301 with two genes encoding *gusA* and *bar*. Transformed calli and plants were selected on medium containing 3 mg/l PPT. PCR detected the presence of the *gusA* and *bar* gene, indicating both genes are integrated into the genome of seashore paspalum. A chlorophenol red assay was used to confirm that the *bar* gene was expressed. By application of herbicide BASTA, the herbicide resistance in the transgenic seashore paspalum plants was confirmed.

Keywords : *BAR*, BASTA, CPR, herbicide-resistant, seashore paspalum, transformation, turfgrass

Seashore paspalum (*Paspalum vaginatum* Sw.) is a warm season perennial grass and indigenous to tropical and subtropical regions of coastal areas worldwide. The species is used as forage food for cattle and horses and fit is used in commercial and residential landscaping. This species has been very successful for golf courses worldwide.

In morphology, seashore pasplaum is similar to common bermudagrass (*Cynodon* spp.). Both species are propagated by rhizomes and stolons. The stolons and leaves of seashore paspalum are slightly coarser, thicker, and darker than those of common bermudagrass. Compared to bermudagrass, seashore paspalum can form a higher quality turf in shaded or reduced light condition (Jiang *et al.*, 2004). Diversity within seashore pasaplum is significant: both coarse-textured (used for roadsides) and fine-textured ecotypes (used for golf course and other landscapes) exist (Carrow, 2005).

The most outstanding characteristics of seashore paspalum is its tolerance to saline soils compared to other warm season turfgrasses which do not provide acceptable turf quality when irrigated with non-portable water (Lee *et al.*, 2004). Seashore paspalum grows on soil with a range of pH 3.6 to 10.2 and along the edge of the water that often contained over 4,000 ppm salts. Thus, its salt tolerance appeared to be great (Brosnan & Deputy, 2008). In addition, seashore paspalum has other important uses such as erosion control, wetland restorations, and site reclamation on soil and gas well sites (http://www.plantaxa.com/paspalum_vaginatum_seashore_paspalum.xhtml).

Weed control in turfgrass fields is one of the most important management practice. Genetic manipulation turfgrasses has been widely used, in special, for herbicide resistance trait. Seashore paspalum it not resistant to commercially available herbicides, thus, its weed management is very difficult task. There, development resistant to herbicide will reduce weed

[†]Corresponding author: (Phone) +82-2-3290-3047,
                                         +82-2-3290-3046
(E-mail) biskim@korea.ac.kr,
            kwook@korea.ac.kr <Received November 2, 2009>

management problems for seashore paspalum turfgrass.

Glufosinate ammonium is a widely used for broad-spectrum weed control. In many crop species Glufosinate (commercial name BASTA®, Liverty®, Ignite®) resistant crop species including cotton (*Gossupium hirsutum* L.), corn (*Zea mays* L.), canola (*Brassica napus* L.), rice (*Orysa sativa* L.) barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), sweetpotato (*Ipomoea batatas* Lam L.), etc., have been developed. In addition, this herbicide-resistant turfgrasses have been reported in several species including Zoysiagrass (*Zoysia japonica* steud.), (Toyama *et al.*, 2003), creeping bentgrass (*Agrostis stolonifera* L.) (Kim *et al.*, 2007), bermudagrass (*Cynodon dactylon* L. Pers.) (Li & Qu, 2003; Li *et al.*, 2005), Kenturky bluegrass (*Poa pratensis* L.) (Gao *et al.*, 2006), bahiagrass (*Paspalum notatum* Fkugge) (Sandhu *et al.*, 2007), *etc*. Weed control also is the most important in management of seashore paspalum fields like other turf fields. Thus herbicide resistance traits should be introduced into seashore paspalum.

The objective of this study is to produce herbicide resistant seashore paspalum. Up to date, transformation and herbicide resistance was not reported in seashore paspalum. This is, therefore, the first study on the herbicide BASTA in this species.

Materials and Methods

Plant materials

Seeds of seashore papalum from derived from seeded variety were used. Matured seeds were surface-sterilized in 70% (v/v) EtOH for 10 min, then in 5% (w/v) commercial bleach (Yuhanrox, Korea) for 10 min with shaking. Callus and plant regeneration protocol was modified by Li & Qu's method (2003) reported in bermudagrass. After rinsing in the sterile distilled water three times, seed were placed on a callus induction medium containing MS basal salts and vitamins (Murashige & Skoog, 1962) supplemented with 2mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma, USA, 30g/l sucrose, 3g/l phytagel (Sigma, USA). The pH of the medium was adjusted to pH5.8 before autoclaving at 121°C for 20 min. Cultured plated were placed in the dark at 25~28°C for 4 weeks and induced calli were subcultured to fresh medium every 2 weeks until they were used for transformation.

Callus were selected in MS salts with B5 vitamins (Gamborg *et al.*, 1968) (Duchefa, the Netherlands), 2g/l Casein hydrolysate (CH) (Sigma, USA), 0.6g/l 3-(N-Morpholino) propanesulfonic acid sodium salt (MES), 30g/l sucrose (pH 5.8), and 3g/l Phytagel.

Agrobacterium-mediated transformation

Agrobacterium tumefaciens strain EHA105 containing the disarmed hyper-virulent plasmid pTiBo542 in the C58 chromo-somal background (Hood *et al.*, 1993) was used for this study. The plasmid pCAMBIA3301 (CAMBIA, Australia) vector carries the *gusA* as a reporter and *bar* gene as a selectable marker as well as the gene of interest, and both gene are controlled under Cauliflower mosaic virus (CaMV35S) promoter (Fig. 1). The *bar* gene encodes the enzyme phosphinothricin acetyltransferase (PAT), which detoxifies phosphinothricin by acetylation into an inactive compound, and the *gusA* gene encodes the enzyme β-glucuronidase (GUS). The plasmid vector was subsequently transferred to *A. tumefaciens* strain EHA105 by the freeze–thaw method (Holsters *et al.*, 1978). Bacterial cultures were grown overnight in YEP liquid medium containing 100 mg/l kanamycin, and 100 mg/ l rifampicin, and collected by low-speed centrifugation at 3,000 rpm for 15 min. For transformation of embryogenic calli explants, bacterial cells were resuspended in liquid co-culture medium (CCM) containing 1/10 MS with B5 vitamins, 2 g/l CH, 0.6 g/l MES, 30 g/l sucrose (pH 5.4) and 200 μM acetosyringone (AS) (Sigma, USA) to a final O.D of 0.5 to 1.0 at 660 nm.
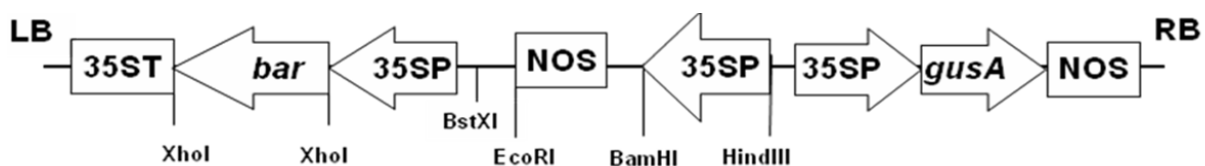

Fig. 1. The map of vector pCAMBIA3301. 35S P, Cauliflower mosaic virus (CaMV) 35S promoter; 35S T, CaMV35S terminator; NOS T, Nopaline synthase terminator; *bar*, phosphinothricin acetyltransferase; *gusA*, β-glucuronidase; LB and RB, left and right of T-DNA borders, respectively.

Embryogenic calli (ca. 100 mg) were immersed for a 1-h in the bacterial suspension containing liquid co-cultivation medium then blotted dry with sterile filter-papers to remove the excess bacteria and placed on CCM solidified with 0.5% (w/v) agarose, followed by incubating at 24°C in the dark for 3 to 5 days. After this period, the infected calli were washed for 30 min with sterile distilled water containing 500 mg l^{-1} cefotaxime and then the calli were transferred to solid CCM containing 3% (w/v) sucrose, 0.3% (w/v) phytagel, 3 mg/l phosphinothricin (PPT) and 300 mg/l cefotaxime. The PPT-resistant calli were subcultured to fresh medium every 2 weeks. Proliferating calli showing green sectors were transferred to the plant regeneration medium which contained MS salts & vitamins supplemented with 1 mg/l 6-benzylaminepurine (BAP) (Sigma, USA), 0.2mg/l α-naphthylacetic acid (NAA) (Sigma, USA), 0.6g/l MES (Duchefa, the Netherlands), 3% (w/v) Sucrose, 0.3% (w/v) Phytagel, 0.5 mg/l gibberellic acid (GA3) (Sigma, USA), 3 mg/l PPT and 300 mg/l cefotaxime. PPT-resistant and well-developed plantlets were transferred to rooting medium containing MS basal salts and vitamins, 0.2 mg/l NAA, 0.6g/l MES, 3% (w/v) sucrose (pH 5.8), and 3g/l Phytagel, 3 mg/l PPT and 300 mg/l cefotaxime. All regenerating cultures were maintained at 25−28°C with a 16 h photoperiod (27-33 μmol/m^2/s). The plants that produced roots were transferred to soil-filled pots and tested for herbicide resistance.

DNA isolation and PCR analysis

Genomic DNA was isolated from the leaves of selected 6 PPT-resistant plants and a wild type plant, as described previously (Kim & Hamada, 2005). The PCR was carried out on the putative transgenic plants by amplifying the coding regions of both *gusA* and *bar* transgenes using the following sets of oligonucleotide primers: for *gusA* gene, forward 5'-tgg tgacgcatgtcgcgcaagac-3' and reverse 5'-ggtgatgataatcgcctgatgcag-3'; for *bar* gene, forward 5'-tacatcgagacaagcacggtcaactt-3' and reverse: 5'-tgccagaaacccacgtcatgccagtt-3'. The expected PCR products were a 413-bp for *bar* and a 648-bp for *gusA*. For the *bar* gene, cycling began with initial denaturation at 95°C for 5 min, then 30 cycles of denaturation at 95°C for 2 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. For the *gusA* gene, cycling began with an initial denaturation at 95°C for 5 min,

then 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 45 s, followed by a final extension of 72°C for 7 min. PCR products were resolved by electrophoresis in 0.8% (w/v) agarose gels.

Chlorophenol red assay and herbicide tolerance assay

The 3',3"-Dichlorophenolsulfonphthalein (Chlorophenol red, CPR) (Sigma, USA), an pH indicator dye that changes color from yellow to violet in the pH range 4.8 to 6.7, assay was used to further test the putative transformed plantlets before transplanting to the greenhouse (Kramer *et al.*, 1993). Plantlets were transferred to rooting medium supplemented with 5 mg/l PPT and 50 mg/l CPR (pH 6.0). They were observed for continued growth, and the medium color shift from red to orange was examined.

The transgenic lines expressing the *bar* gene and wild type plants were analyzed for resistance on a commercial herbicide. Plants were sprayed with a 0.5% (v/v) solution of the non-selective and broad spectrum herbicide Basta® (Bayer Crop Science Ltd., Korea), a commercial formulation of glufosinate ammonium salt. The plants were observed over 1 week to determine resistance or susceptibility to the herbicide.

Results and Discussion

Transformation and regeneration of seashore paspalum plants

Prior to transformation experiment with the *bar* gene, sensitivity of explants to PPT was determined. PPT at 3 and 10 mg/l completely inhibited callus growth (data not shown) and consequently, PPT at 3 mg/l was used for the selection of resistant transformed calli.

The calli infected with *Agrobacterium* strain EHA105 (Hood *et al.* 1993) carrying the binary vector pCAMBIA3301 (CAMBIA, Australia) (Fig. 1) were kept on CCM for 5 days and then the explants were transferred to callus maintaining medium containing PPT for the selection of PPT-resistant calli. After 4 to 6 subcultures, the PPT-resistant calli could be distinguished from the susceptible ones. The susceptible calli had a brown and necrotic or bleached tissue whereas the resistant calli proliferated continuously and developed green sectors. The calli with green sectors which assumed

PPT-resistant were transferred to plant regeneration medium with PPT. In the regeneration stage, most of green sector did not survive in PPT-containing medium and only few green sectors were developed to shoots. After 6 to 10 weeks (3 to 5 times subcultures) cultured in regeneration medium, shoots developed plantlets. These PPT-resistant shoots were transferred to the rooting medium containing PPT, and they were subcultured until they fully developed plants with roots. Plants that formed roots within 3- to 5-week on rooting medium grew vigorously.

PCR analysis

PCR analysis of DNA from the six PPT-resistant individual selected seashore papalum plants gave bands of expected size for the *gusA* gene (Fig. 2A) and for the *bar* gene (Fig. 2B). PCR amplification products were not obtained with DNA from NT (non-transformed wild type) pants. PCR analysis, both the *bar* and *gusA* genes were integrated in the genome of seashore paspalum.

CPR assay and Application of Herbicide

The CPR assay was performed (Kramer *et al.* 1993). The color change of the medium in the CPR assay is pH dependent. As CPR is an indicator of pH, culture vessel(10 cm×5 cm) containing 50 ml of rooting medium containing 3 mg/l PPT adjusted to pH 6 containing and supplemented 50 mg/l CPR which is deep red color at the pH. In medium containing

CPR and PPT, the wild-type plantlet did not change the color of the medium keeping initial deep red color (Fig. 3A); whereas the plant expressing *bar* gene caused medium acidification leading to the orange color of the medium within 2 weeks (Fig. 3B) The PPT-resistant seashore paspalum plantlets formed roots, whereas the wild-type plantlet did not form roots (Fig. 3B), indicating that the introduced *bar* gene is functionally expressed in the transgenic plants. After roots were well established (4-week), the plants were transferred to soil-filled pots boxes under high humidity.

In order to determine whether the herbicide-resistant seashore paspalum expressing *bar* gene, Basta at 0.5% (w/v), which is equal to 900 mg/l of glufosinate ammonium salt, was applied to them by spray. Spraying the herbicide onto the leaves of seashore paspalum plants, wild type plants started necrosis in tissue necrosis within 1 week, followed by extensive browning within 2 weeks; herbicide BASTA-resistant plants remained green (Fig. 4). Thus, seashore paspalum plants expressing *bar* transgene had tolerance to the herbicide without showing
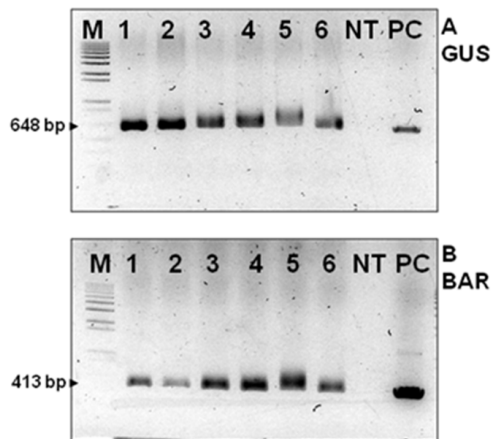

Fig. 3. CPR assay for *bar* gene expression in transformed seashore paspalum plants. The color was obtained after 4 weeks of plant incubation at room temperature. (A) Non-transformed plant (wild type), (B) Transgenic plant sample expressing *bar* gene.

Fig. 2. PCR analysis of *gusA* (A) and *bar* (B) gene in seashore papspalum. M, molecular marker; 1-6, transgenic plants; NT, non-transgenic plant (wild type); PC, positive control (pCAMBIA3301).
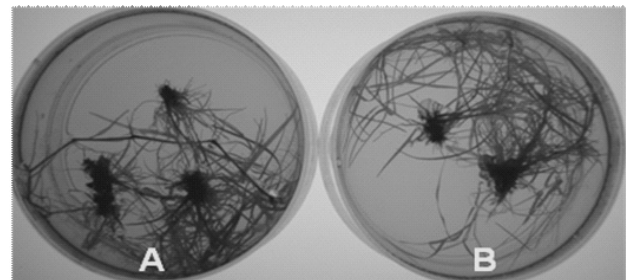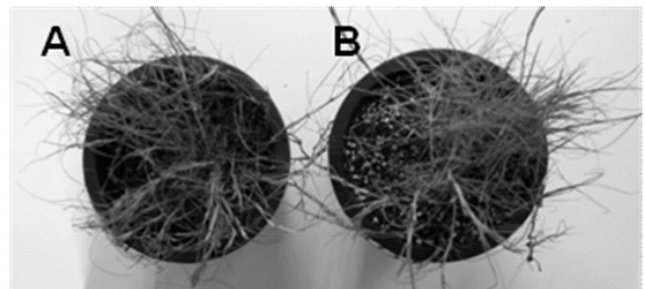

Fig. 4. Application of herbicide BASTA to seashore paspalum plants. (A) Non-transformed plant, (B) Transgenic plant expressing bar gene.

any damage, whereas non-transformed plants were highly susceptible to the herbicide and died within 2 weeks. Therefore, our results showed the herbicide-resistant *bar* gene is successfully transferred and functionally expressed in seashore paspalum plant.

In summary, the generation of *bar* transgenic seashore paspalum line from its seeded variety and it showed high levels of resistance to non-selective herbicide BASTA is described in this study. Following *Agrobacterium*-mediated transformation, a total of 6 plants expressing the *bar* gene as detected by PCR, CPR assay and herbicide were produced.

Herbicide-resistance traits have been reported in many turfgrass species including zoysiagrass (Toyama *et al.*, 2003), creeping bentgrass (Kim *et al.*, 2007), burmudagrass (Li *et al.*, 2005), Kenturky bluegrass (Gao *et al.*, 2006), bahiagrass (*Paspalum notatum* Fkugge) (Sandhu *et al.*, 2007), etc. However, seashore paspalum is used as forage food for herviborous animals and for golf courses, landscape, and roadsides. The most importance of this species is tolerant to high saline areas, in special, coastal region of subtropical and tropical area worldwide (Lee *et al.*, 2004). Breeding efforts initiated in the late 1990s and development of improved cultivar for use on golf course, athletic field, and other landscape areas (Brosnan & Deputy, 2008; Raymer, 2007). Weed control is the one of the most problem in seashore paspalum like other turf species. To control weed problems in seashore paspalum, research was conducted to evaluate the use of slat as an alternative weed control methods. For many weed species which are less tolerance to salts than seashore paspalum, application of salts may fatal injury to certain weed species without damage to seashore paspalum (Lee *et al.*, 2004). However, repetitive application of certain salts may result in severe damage to soil quality and seashore paspalum (Brosnan & Deputy, 2008; Raymer, 2007). Thus, though the salts to weed control in seashore paspalum turfgrass may be used tentatively until herbicide resistant its lines are developed, it is not recommended. Therefore, in this study, we developed seashore paspalum with herbicide resistance and our study showed the possible used of the biotechnology for the development of new traits in seashore paspalum.

Acknowledgement

References

Brosnan, J. T. and J. Deputy. 2008. Seashore paspalum. Turf management TM-1. Cooperative Extension service, University of Hawaii at Manoa.

Carrow, R. N. 2005. Seashore paspalum ecotype responses to drought and root limiting stresses. USGA Turfgrass Envrion. Res. Online 4(13): 1-9.

Gamborg, O. L., R. A. Miller, and K Ojima. 1968. Nutrient requirement suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.

Gao, C. X., L Jiang, M. Folling L. B. Han, and K. K. Nielson. 2006. Generation of large number of transgenic kenturky bluegrass (*Poa pratensis* L.) plants flowing biolstic gene transfer. Plant Cell Rep. 25: 19-25.

http://www.plantaxa.com/paspalum_vaginatum_seashore_paspa lum.xhtml

Holsters, M., D. De Waele, A. Depicker, E. Messens, M. Van Montagu, and J. Schell. 1978. Transfection and transformation of *Agrobacterium tumefaciens*. Mol. Gen. Genom. 163:181-187.

Hood, E. E., S. B. Gelvin, L. S. Melchers, and A. Hoekema. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res. 2: 208-218.

Kim, S. H. and T. Hamada. 2005. Rapid and reliable method of extracting DNA and RNA from sweet potato, *Ipomoea batatas* (L.) Lam. Biotechnol. Lett. 27:841-1845

Kim, S. J., J. Y. Lee, Y. M. Kim, S. S. Yang, O. J. Hwang., N-J. Hong, K-M. Kim, H-Y. Lee, P-S. Song, and J-I. Kim. 2007. *Agrobacterium*-mediated high-efficiency transformation of creeping bentgrass with herbicide resistance. J. Plant Biol. 50: 577-585.

Kramer, C., J. DiMaio, G. K. Carswell, and R. D. Shillito. 1993. Selection of transformed protoplast-derived *Zea mays* colonies with phosphinothricin and a novel assay using the pH indicator chlorophenol-red. Planta 190:454-458.

Jiang, Y., R. Duncan, and R. N. Carrow. 2004. Assessment of low light tolerance of seashore paspalum and bermudagrass. Crop Sci. 44: 587-594.

Lee, G, R. R. Duncan, and R. N. Carrow. 2004. Salinity tolerance of seashore paspalum ecotypes: Shoot growth responses and criteria. HortScience 39: 916-1156.

Li, L., R. Li, S. Fei, and R. Qu. 2005. *Agrobacterium*-mediated transformation of common bermudagrass (*Cynodon dactylon*). Plant Cell Tissue Organ Cult. 83: 223-229.

Li, L. and R. Qu. 2003. Development of highly regenerable callus lines and biolistic transformation of turf-type common bermudagrass [*Cynodon dactylon* (L.) Pers.] Plant Cell Rep. 22:403-407.

Murashege, T, and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures.

Physiol. Plant. 15: 473-497.

Raymer, P. 2007. Breeding seashore paspalum for recreation turf use. In, J. L. Nus (ed.) 2006 USGA Turfgrass and Environmental Research summary. USGA Green Section, Far Hills, NJ. p.36.

Sandhu, S., F. Altpeter, and A. R. Blount. 2007. Apomictic bahiagrass expressing the *bar* gene is highly resistant to glufosinate under field conditions. Crop Sci.47: 1691-1697.

Toyama K., C. H. Bae., J. G. Kang, Y. P. Lim, T. Adachi, K. Z. Riu, P-S. Song, and H-Y. Lee. 2003. Production of herbicide-tolerant zoysiagrass by *Agrobacterium*-mediated transformation. Mol. Cells 16:19−27