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Development of Transgenic Orchardgrass (*Dactylis glomerata* L.) Using *Agrobacterium tumefaciens*

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Abstract

To produce of transgenic orchardgrass, the seed-derived calli of orchardgrass (*Dactylis glomerata* L.) co-cultivated with *Agrobacterium tumefaciens* EHA101 harboring binary vector pIG121-Hm were selected with hygromycin and then transferred onto N6 regeneration medium containing 1 mg/ℓ of NAA, 5 mg/ℓ of kinetin, 250 mg/ℓ of carbenicillin and 50 mg/ℓ of hygromycin. The efficiency of transformation was differed on cultivars, that is, 'Potomac' appeared 12% of transformation efficiency while 'Amba' did 5.5%. The addition of acetosyringone during co-cultivation was a key to successful transformation of orchardgrass. Transgene fragments were identified by PCR analysis and the constitutive expression of GUS gene was confirmed by Northern blot analysis.

(**Key words** : Acetosyringone, *Agrobacterium tumefaciens*, Orchardgrass (*Dactylis glomerata* L.), Transformation)

I. Introduction

Production of transgenic plants through the introduction of foreign gene is rapidly becoming a general technology for a variety of plant species including many economically important crops (Gasser and Fraley, 1989). Gene transfer methods developed for plants include *Agrobacterium*-mediated DNA transfer, electroporation and PEG-mediated gene transfer into protoplasts, and microprojectile bombardment into intact cells (Horsch et al., 1984; Potrykus et al., 1985; Krol et al., 1990). Transformation methods based on the use of soil bacterium *Agrobacterium tumefaciens* are still preferred in many instances because it does not require protoplasts, its higher transformation efficiency and a more predictable pattern of foreign DNA integration than any other transformation techniques (Czernilofsky et al., 1986). Unfortunately, monocots and particularly the graminaceous crop species have been considered to be outside of the *Agrobacterium* host range (Decleene, 1985). However, in recent years, there are many reports on the transformation of monocots using *Agrobacterium*. Hiei et al (1994) reported the transformation frequency of *japonica* type rice as high as dicots and demonstrated the Mendelian transmission of the introduced DNA to the progeny. In spite of the establishment of *Agrobacterium*-mediated transformation of monocots, the transformation of orchardgrass is limited due to the low efficiency of tissue culture. Thus, for the stable supply of tissue cultured material and the establishment of efficient regeneration system, we previously reported the regeneration of orchardgrass from seed-derived calli and suspension cells (Lee et al., 1998; 2000). In the present report, we describe the advanced methodology for genetic transformation of orchardgrass via co-cultivation of seed-derived calli with *Agrobacterium tumefaciens*.

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Ⅱ. Materials and Methods

1. Plant materials

Orchardgrass varieties, Amba and Potomac which were kindly distributed from National Livestock Research Institute were used in *Agrobacterium*-mediated transformation. Seed sterilization and callus induction were carried out according to the procedure described by Lee et al (1998; Table 1). Both of the transformed and nontransformed plants were grown in a greenhouse at approximately 28℃ with a 16/8-hour light/ dark cycle.

2. Transformation of *Agrobacterium tumefaciens*

A binary vector, pIG121-Hm (Hiei et al., 1994) was used for the transformation of orchardgrass. pIG121-Hm was transformed into *Agrobacterium tumefaciens* EHA101 by two cycles of freeze-thaw method (Horsch et al., 1984). The transformed *Agrobacterium* was spreaded onto AB agar medium (Table 1; Chilton et al., 1974) containing 50 mg/ℓ kanamycin and hygromycin, respectively and incubated at 28℃ for 3 days under dark condition. The *Agrobacterium* suspended in 30 mℓ of AA medium (Table 1; Muller and Grafe, 1978) containing 30 mg/ℓ acetosyringone was used for callus infection.

3. Transformation of orchardgrass

The calli induced from dehusked mature seeds of orchardgrass were soaked in Agrobacterium culture for 3 min and co-cultured on the N6 co-culture medium (Table 1) containing 30 mg/ℓ acetosyringone at 28℃ for 3 days under dark condition. After co-culture, the calli were washed with N6 medium containing 500 mg/ℓ carbenicillin and transferred onto N6 selection medium (Table 1) containing 500 mg/ℓ carbenicillin and 50 mg/ℓ hygromycin and incubated at 25℃ for 3 weeks under light condition. The selected calli were induced plant regeneration onto N6 regeneration medium (Table 1) containing 1 mg/ℓ NAA, 5 mg/ℓ kinetin, 250 mg/ℓ carbenicillin, and 50 mg/ℓ hygromycin.

4. Identification of transgenic orchardgrass

In order to identify the transformation of the regenerated plants, a PCR analysis was conducted.

Table 1. Media used for tissue culture and transformation of orchardgrass

Medium	Composition
N6 callus induction	N6 salts and vitamins, 30 g/ℓ sucrose, 3 mg/ℓ dicamba, 2 g/ℓ gelrite, pH 5.8
AA	AA salts and amino acids, B5 vitamins, 20 g/ℓ sucrose, 2 mg/ℓ 2,4-D, 0.2 mg/ℓ kinetin, 30 mg/ℓ acetosyringone, pH 5.8
AB	3 g/ℓ K_2HPO_4, 1 g/ℓ Na_2HPO_4, 1 g/ℓ NH_4Cl, 0.3 g/ℓ $MgSO_4 \cdot 7H_2O$, 0.15 g/ℓ KCl, 0.01 g/ℓ $CaCl_2$, 2.5 mg/ℓ $FeSO_4 \cdot 7H_2O$, 5 g/ℓ glucose, 15 g/ℓ agar, pH 7.2
YEP	10 g/ℓ Bacto peptone, 10 g/ℓ Bacto yeast extract, 5 g/ℓ NaCl, 15 g/ℓ agar, pH 7.2
N6 co-culture	N6 salts and vitamins, 30 g/ℓ sucrose, 10 g/ℓ glucose, 2 mg/ℓ 2,4-D, 30 mg/ℓ acetosyringone, pH 5.8
N6 selection	N6 salts and vitamins, 30 g/ℓ sucrose, 2 mg/ℓ 2,4-D, 2 g/ℓ gelrite, 500 mg/ℓ carbenicillin, 50 mg/ℓ hygromycin, pH 5.8
N6 regeneration	N6 salts and vitamins, 30 g/ℓ sucrose, 1 mg/ℓ NAA, 5 mg/ℓ kinetin, 2 g/ℓ gelrite, 250 mg/ℓ carbenicillin, 50 mg/ℓ hygromycin, pH 5.8
MS hormone free	MS salts and vitamins, 30 g/ℓ sucrose, 50 mg/ℓ hygromycin, pH 5.8

Genomic DNA was isolated from leaves of orchardgrass as described by Murray and Tompson (1980) and 100 ng of each sample was subjected to PCR analysis. PCR was performed with specific primers either for 35S promoter and GUS gene in the pIG121-Hm. The two primers used for PCR analysis were 35S sense (35Ss1; 5'-TTCAACAAA-GGGTAATATCCGG-3') and GUS antisense (GusAs; 5'-CAATTCCACAGTTTTCGCGATC-3'). The following settings were used with 1 U of Taq DNA polymerase (Takara): denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min and extension at 72℃ for 1 min. Thirty cycles of amplification were performed. The PCR products were analyzed by 1.2% agarose gel electrophoresis.

5. Northern blot analysis

Total RNA was isolated using the phenol-SDS extraction and LiCl precipitation method (Ausubel et al., 1987). RNA was quantified by measuring at the A_{260}, and samples containing 15 μg of total RNA were fractionated on 1.2% formaldehyde agarose gel. Gels were blotted onto Hybond-N nylon membranes (Amersham, UK) with 10×SSC. The membrane was hybridized with a ^{32}P-labeled GUS gene, washed with 0.2×SSC and 0.1% SDS at 55℃ for 1 h and then autoradiographed.

Ⅲ. Results and Discussion

1. *Agrobacterium*-mediated transformation of orchardgrass

The binary vector, pIG121-Hm which was used for the transformation of orchardgrass contained hygromycin and kanamycin resistance genes as selectable markers and GUS (β-glucuronidase) gene as a reporter gene under the control of CaMV (cauliflower mosaic virus) 35S promoter (Fig. 1). Thus, CaMV 35S promoter and GUS gene were used in PCR analysis for identification of transformation and GUS gene was used in the Northern blot analysis for identification of normal expression of introduced gene. The plasmid, pIG121-Hm was transferred into *Agrobacterium tumefaciens* EHA101 by direct *Agrobacterium* transformation method for the transformation of orchardgrass.

The mature seeds of orchardgrass cultivars, Amba and Potomac were dehusked, surface-sterilized, and incubated on N6 medium containing 3 mg/ℓ dicamba for callus induction. The induced calli were inoculated with the suspension of *Agrobacterium tumefaciens* EHA101 harboring pIG121-Hm, co-cultured for 3 days, and transferred onto N6 selection medium. After 3 weeks on the N6 selection medium, the calli were transferred onto N6 regeneration medium. Two weeks after transfer to the N6 regeneration medium, the formation of green spots were observed and the initiation of shoot buds occurred after 3~5 weeks. The induced shoots were transferred to hormone-free MS medium for root induction. (Fig. 2). After root development, these plantlets were transferred to potting soil and grown in a greenhouse condition for sampling for the confirmation of transformation. No morphological

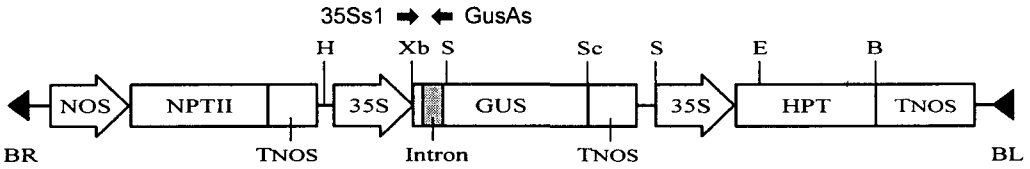

Fig. 1. Binary vector, pIG121-Hm used for the transformation of orchardgrass. BR, right border; BL, left border; NPTⅡ, neomycin phosphotransferase; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase; NOS, nopaline synthase promoter; 35S, CaMV 35S promoter; T_{NOS}, nopaline synthase terminator; B, *Bam*HI; E, *EcoR*I; H, *Hind*III; S, *Sal*I; Sc, *Sac*I; Xb, *Xba*I. Arrows represent the positions and orientations of primers (35Ss1 and GusAs) used for PCR analysis.

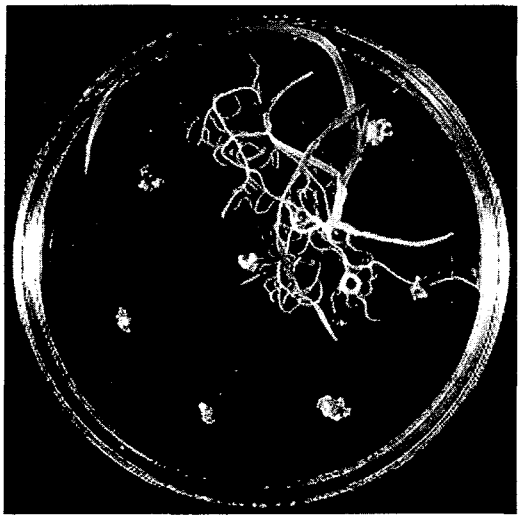

Fig. 2. Plant regeneration from seed-derived callus of orchardgrass transformed with *Agrobacterium tumefaciens*. The plantlet was selected on N6 medium containing 50 mg/ ℓ of hygromycin.

differences were observed between transformed and nontransformed plants.

2. Analysis of transgenic orchardgrass

To determine the correct integration of the foreign DNA in the orchardgrass genome, the orchardgrass genomic DNAs by PCR analysis were identified using specific primers for CaMV 35S promoter (35Ss1) and GUS gene (GusAs) in the pIG121-Hm. The region of 0.4 kb in the CaMV 35S promoter and GUS gene was amplified and seperated on the 1.2% agarose gel. As shown in Fig. 3, the DNA fragments of 0.4 kb size were detected in most regenerted plants except #7 and #10.

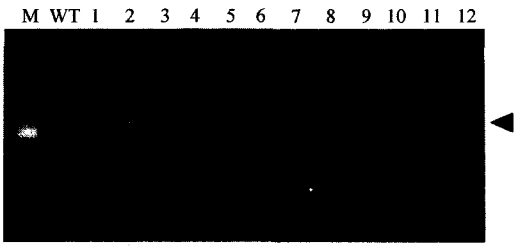

Fig. 3. Confirmation of transgenic plants by PCR amplification using genomic DNAs from wild-type (WT) and transgenic plants. The primers 35Ss1 and GusAs were used for identification of transformation. Arrowheads represent amplified PCR products. Numbers indicate independent transgenic lines.

The factors affecting the transformation efficiency of monocotyledonary plants using *Agrobacterium tumefaciens* included the genotype of plant, the type and stage of tissues infected, the conditions for tissue culture, the kind of vector and the strain of *Agrobacterium* (Hiei et al., 1997). In this experiment, genotypic difference of transformation efficiency was also observed. That is, the efficiency of transformation was differed from cultivar to cultivar, that is, 'Potomac' appeared to be 12% of transformation efficiency while 'Amba' did 5.5% (Table 2).

In order to identify the normal expression of introduced gene in transgenic orchardgrass, a Northern blot analysis was conducted. Fifty micrograms of total RNA was subjected to formaldehyde agarose gel electrophoresis and transferred to a Hybond-N nylon membrane. The amounts of total RNA loaded were normalized with those of ribosomal RNA (Fig. 4B). The membrane was

Table 2. Transformation efficiency (T.F.) by *Agrobacterium tumefaciens* EHA101 in orchardgrass cultivars Amba and Potomac

Cultivar	No. of calli co-cultivated (A)	No. of calli with HygR (%)	Transformed plants (B)	T.F. (%) (B/A)
Amba	200	97 (48.5)	11	5.5
Potomac	200	129 (64.5)	24	12

hybridized with a ^{32}P-labeled GUS gene. As shown in Fig. 4A, transcripts of 2.0 kb length were detected in all transgenic plants. And different levels of the GUS transcripts were detected in the transgenic plants suggesting that copy numbers integrated and insertion position in the genome might be responsible for the different levels of the GUS transcripts (Kuhlemeier et al., 1987; Larkin and Scowcroft, 1981). These results indicated that mRNAs of the correct size were transcribed from the foreign construct and accumulated in the transgenic orchardgrass.

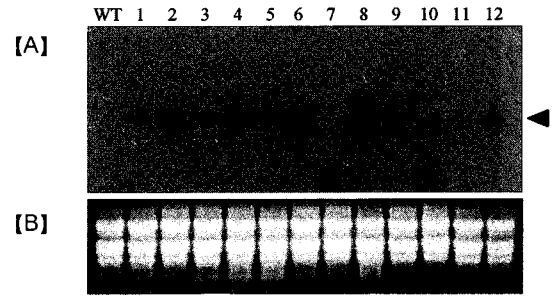

Fig. 4. Northern blot analysis of transgenic orchardgrass. [A] Total RNA was isolated from the leaves of wild-type (WT) and transgenic plants. Numbers indicate independent transgenic lines. Each lane was loaded with 15 μg of total RNA. Transcripts were hybridized with ^{32}P-labeled *Sal* I fragment of GUS gene. Arrowheads represent hybridization bands. [B] Ethidium bromidestained RNA served as a loading control.

It is known that wounded plant cells exude phenolic compounds such as acetosyringone and α-hydroxyacetosyringone, which activate *vir* gene of Ti plasmid (Stachel et al., 1985). These signal molecules appear to be very important in the recognition by *Agrobacterium* of suitable host. However, monocotyledons, particularly grasses, appeared not to produce these compounds, or if they do, the levels would not be sufficient to serve as a signal (Smith and Hood, 1995). Hiei et al. (1994) reported that the addition of 100 μM acetosyringone was a key to successful transformation of rice. Similar results were obtained in this experiment. No plants were obtained at all without acetosyringone while 35 transgenic plants were obtained with acetosyringone in the course of co-cultivation (data not shown). Thus, the addition of acetosylingone both into *Agrobacterium* culture and co-culture medium was very important in the transformation of orchardgrass.

These results indicated that *Agrobacterium*-mediated transformation of orchardgrass, a monocotyledonary plant was applicable like in dicotyledonous plants. As the process, however, was more complicated compared to the transformation of dicotyledonous plants and the regeneration efficiency was still low, continuous efforts for rapid regeneration and for elevated transformation efficiency would be required.

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