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The Mutated Acetolactate Synthase Gene from Rice as a Non-Antibiotic Selection Marker for Transformation of Bamboo Cells

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ABSTRACT

Previously, we developed a particle bombardment-mediated transformation protocol in Phyllostachys nigra bamboo by expressing hygromycin phosphotransferase gene (*HPT*) and neomycin phosphotransferase II gene (*NPT II*). Although these marker genes could introduce to several tissue cultured organs (e.g. leaves, buds, and calli) of Phyllostachs bamboo species, some organs showed a high susceptibility and/or a low selectivity to hygromycin and kanamycin. In this report, therefore, we describe advantages and technical details for generating stable transgenic bamboo cells using the particle bombardment method with the mutated-acetolactate synthase gene (*mALS*) from rice (W548L/S627IOsALS) as a non-antibiotic selection marker. A facile and efficient transformation was achieved with the *mALS* gene and enhanced fluorescent protein gene (*mCherry*). Approximately 490 and 1400 *mCherry*-expressing cells/dish/shot in average were observed in both *P. bambusoides* and *P. nigra* under fluorescent stereo-microscope. Stable transgenic bamboo cell lines were generated in a selection medium supplemented with 0.1 μM of bispyribac-sodium (BS) as ALS inhibitor. The integration of *mALS* gene was identified by *in vivo* ALS enzyme assay and a PCR-restriction fragment length polymerphism (RFLP) based detection procedures.

Keywords: Bamboo; Mutated Acetolactate Synthase Gene; Particle Bombardment; Suspension Culture

1. Introduction

Acetolactate synthase (ALS: EC 2.2.1.6) is the common enzyme in the biosynthetic pathway of the branched-chain amino acids leucine, isoleucine, and valine. A mutated-ALS (*mALS*) gene was isolated from pyrimidinyl carboxy herbicide (Pyriminobac, pyrithiobac-sodium and bispiribac-sodium)-resistant somaclonal variation rice cells and found to have a single amino acid change from serine to isoleucine in a conserved region [1]. Recently, it was developed that the *mALS* gene can be used as a new selectable marker to produce transgenic plants such as *Arabidopsis* [2], rice [3-5], soybean [6], and wheat [7]. These contributions allow us to overcome many difficulties for the selection of transformants of the target plants.

It is very important contribution to improve a new vector system/plural vector choices as powerful tools for understanding physiological/ molecular biological events and/or engineering complex biosynthetic pasway(s) of target plant cells by introducing multiple genes. At present, however, little information is available concerning the transformation of bamboo plants. In order to investigate/improve biosynthetic pathway(s) of bamboo plants, it is essential to develop an efficient transformation protocol for a model cell culture system.

Previously, we developed a cell culture system [8] and a particle bombardment-mediated transformation protocol in *Phyllostachys nigra* bamboo by expressing hygromycin phosphotransferase gene (*HPT*) and neomycin phosphotransferase II gene (*NPT II*) [9]. Although this system has been currently used for a regular transformation technique of bamboo cells in our researches, there is scope for improvement such as a high toxicity and/or a low selectivity of antibiotics in some organs (e.g. leaves and buds) or cell lines as shown in **Figure 1(A)**. In this report, therefore, we described advantages and technical details for generating stable transgenic bamboo cells using the particle bombardment method with *mALS* gene as a non-antibiotic selection marker.

2. Materials and Methods

2.1. Bamboo Cell Cultures

Callus cultures of two bamboo species, both *P. bam-*

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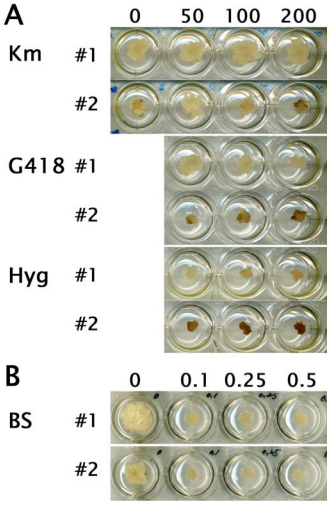

Figure 1. Effects of antibiotics and BS on the proliferation of bamboo cells; (A) Antibiotics condition; (B) BS condition. #1: *P. nigra*, #2: *P. bambusoides*. 50, 100, 200 mg/l of Km, G418, and Hyg; 0.1, 0.25, and 0.5 µM of BS were added to the medium, respectively.

busoides and *P. nigra*, were induced from young bamboo shoots as described by Ogita [8]. Suspension cell cultures were also generated as described by Ogita *et al*. [9]. MSp680 medium supplemented with 680 $mg \cdot l^{-1}$ KH_2PO_4 and 10 µM 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (Picloram) was used for the basal medium.

In order to maintain their stable morphology and synchronous growth under suspension culture condition, sedimented cell volume (SCV) was adjusted every 2 weeks. Briefly, 2.5 ml cells in SCV were initially transferred to 100 ml liquid MSp680 medium, *i.e.* 2.5% (v/v) initial cell density, in a 300-ml flask. The flasks were placed on a rotary shaker with a speed of 100 rpm for 2 weeks in the dark at 25˚C. The SCV was measured by holding a liquid suspension cells for 15 minutes in a 50 ml centrifugation tube graduated in milliliters. In order to investigate susceptibilities to antibiotics, 0, 50, 100, and 200 mg/l of kanamycin (Km), geneticin (G418), and hygromycin (Hyg) were added to the MSp680 medium. Two-ml each of resulted media were poured into a well of 12-well microplate. A portion of callus (ca. 50 mg fresh weight) was transferred to each well and cultured for 3 weeks. All cultures were incubated in the dark at 25˚C.

2.2. Vectors for Transformation

The pBIH1-IG vector [10], carrying the hygromycin phosphotransferase gene (*HPT*) and the neomycin phosphotransferase II gene (*NPT II*) was used as the backbone vector. The original intron-GUS sequence of the pBIH1-IG at the *Xba*I and *Sac*I sites located between the cauliflower mosaic virus 35S promoter (CaMV35Sp) and the nopaline synthase terminator (NOSt) was replaced with a red florescent protein gene (*mCherry*) originated from the *pmCherry* vector (Clontech Laboratories, Inc., USA). The pBIH1-RFP vector [9] was used as a control vector for transient transformation of bamboo cells. The cassette (CaMV35Sp::*mCherry*::NOSt) was amplified from the pBIH1-RFP and introduced to the multi-cloning site of pSTARA (R-4) vector harboring the mutated ALS gene (W548L/S627I*Os*ALS) under the control of ALS promoter and ALS terminator (Kumiai Chemical Industry Co., Ltd., Japan). The resulted pSTARA-RFP vector was used for stable transformation of bamboo cells.

2.3. Transformation

Gold particles, 0.6 µm in diameter, were coated with the plasmid DNA according to Ogita *et al*. [9]. The bombardment was carried out once using the Biolistic Particle Delivery System (PDS-1000/He, Bio-Rad, USA) at 1100 psi of helium pressure. Seven to fourteen days after bombardment, the cells were transferred to a selection medium supplemented with 0.1 µM of bispyribac-sodium (BS) as an ALS inhibitor. They were then subcultured at 2- to 4-week intervals onto fresh selection medium.

2.4. Detection of Transformants

Transient expression of *mCherry* gene in bombarded bamboo cells was observed under a fluorescence stereo microscope (SteREO Lumar.V12, Zeiss, Germany).

The transformed suspension cells were collected and observed using a laser scanning microscope (LSM510 META, Zeiss, Germany) for characterization of the *mCherry*-expressing cells. Genomic DNA was extracted from transformed cells with an ISOPLANT II kit (Nippon gene, Japan) and subjected to PCR analysis using two sets of gene specific primers; for *mALS*, 5'-TGGTCTGGGCGCAATGGGATTTG-3' and 5'-CAAC-AAGTATGGCCCTGGAGTCTC-3'; for *mCherry*, 5'-CCACTACTTGAAGCTGTCC-3' and 5'-GGGGAGTCCCTTGTACAGCTCGTCCATGC-3'. PCR was performed in a 20 µl reaction mixture containing 2 µl of 2mM dNTP, 2 µl of 10 × Blend Taq buffer, 0.5 unit of Blend Taq polymerase (Toyobo, Japan), 1 µl of primers (10 µM) and 100 ng of template genomicDNA (10 ng of template in case of vector control). The amplification reaction was carried out under the conditions of up to 40-cycle of denaturation at 96˚C for 30 s, annealing at 60˚C for 30 s and extension at 72˚C for 1 min. For detection of *mALS* gene, the amplified DNA fragment (388-bp) was treated with *Mfe*I (New England Biolabs, USA) in a 10 µl reaction mixture containing 1 µl of of 10 × FD Green buffer, 1 µl of *Mfe*I (20 unit) and 5 µl of template DNA

at 37˚C for 30 min. The restriction fragments (194 and 198-bp) which were derived from *mALS* were identified. The resulted DNAs were detected with ethidium bromide staining after agarose gel electrophoresis.

In vivo ALS assay was performed using 24-well micro plate according to the procedure of Kawai *et al*. [2] with minor modifications. Bamboo cultured cells (ca. 0.2 g fresh weight) were put into a well and incubated in 1 ml of 25% Murashige and Skoog salt medium containing 10 mM of sodium pyruvate as a substrate, 500 μM 1,1-cyclopropanedicarboxylic acid (CPCA) as a ketol-acid reductoisomerase (KARI) inhibitor, and w/wo 0.1 μM of BS as ALS inhibitor. The micro plate was incubated at 25˚C in the dark for 24 - 48 hr. After the incubation, cells were transferred into a 1.5 ml tube. To determine ALS activity, 500 μl of milliQ water was added to the samples and they were heated to 60˚C for 5 min and then kept for 25 min at room temperature. Aliquots of 100 μl were taken and mixed with 10 μl of 5% (w/v) H_2SO_4. The acidified solutions were incubated at 60˚C for 30 min to facilitate the decarboxylation of acetolactate to acetoin. Then, 50 μl of 0.5% (w/v) creatin and 50 μl of 5% (w/v) 1-naphthol dissolved in 2.5 N NaOH were added to the samples. To allow color development, the samples were incubated at 37˚C for 30 min. The reddish resultant reaction mixtures were judged as acetoin accumulation.

3. Results and Discussion

3.1. Effects of Antibiotics and BS

We preliminary investigated the susceptibility to antibiotics in *P. bambusoides* and *P. nigra* cell lines (**Figure 1(A)**). *P. nigra* cell line which is our regular cell line (#1) showed insusceptibility toward Km and moderate susceptibilities toward G418 and Hyg. On the other hand, *P. bambusoides* cell line (#2) expressed strong susceptibilities toward all antibiotics tested. In Hyg condition, *P. bambusoides* cells hardly browned within 3 weeks. Then we investigated the effect of BS and found that the addition of 0.1 μM BS clearly suppressed proliferation of bamboo cells without browning (**Figure 1(B)**). Lower concentration of BS (<0.1 μM) did not inhibit cell growth (data not shown). Similar results had been reported in rice calli [5] and *Arabidopsis* seedlings [2].

3.2. Transformation of Bamboo Cells

A portion of suspended cells (ca. 0.5 ml in SCV) was placed in a center of Petri dish which contained the MSp680 medium with 10 μM Picloram. The DNA-coated microprojectiles, 498 μg gold particles with 0.83 μg DNA were bombarded. The transient *mCherry* gene expression was detected at 24 hours after a bombardment as shown in **Table 1**. We found that pSTARA-RFP worked efficiently in bamboo cells and showed higher transient gene expression (1402 positive cells/dish/shot in average) than that of control pBIH1-RFP (556 positive cells/dish/shot in average) in *P. nigra* cell line under our regular protocol [9]. Then we checked the adaptability of pSTARA-RFP in *P. bambusoides* cell line and found 491 positive cells/dish/shot in average efficiently expressed *mCherry*.

During subcultures at 2- to 4-week intervals onto the fresh selection medium supplemented with 0.1 μM BS, a large number of BS-resistant cell lines which showed strong expression of *mCherry* gene was easily detected (ca. 40 - 100 transformed cell lines/dish/shot were obtained after 4 month of subcultures). This frequency is about the same as pBIH1-RFP in *P. nigra* [9] and in this experiment. In *P. bambusoides*, however, any resistant cell line could not grow from pBIH1-RFP bombarded cells under the Hyg condition (data not shown). This might be caused by a strong susceptibility to Hyg. In some transformed cell lines strong visible pink fluorescence feature could be seen under the natural light condition as shown in **Figure 2**. Furthermore, detailed clear cytological images could be observed by using these transformed cells as shown in **Figure 3**. These results are the evidences that a target transgene will be highly expressed in bamboo cells by pSTARA vector.

3.3. Stable Expression of *mALS* Gene in Bamboo Cells

Genomic DNA was extracted from 3 randomly selected

Table 1. Transient *mCherry* gene expression in bamboo suspension cells.

Vector	Number of *mCherry*-expressing cells/dish/shot	Mean ± SD
pBIH1-RFP (*P. nigra*)	437	556 ± 127
	438	
	440	
pSTARA-RFP (*P. nigra*)	948	1402 ± 186
	1015	
	1154	
	1704	
	1735	
pSTARA-RFP (*P. bambusoides*)	379	491 ± 85
	403	
	440	
	742	

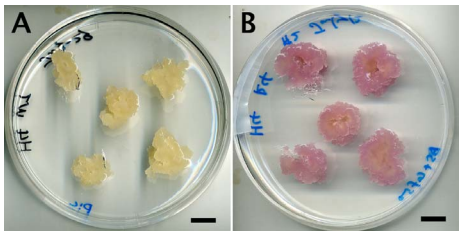

Figure 2. Characteristics of bamboo cultured cells. (A) Non-transformed cell line; (B) Transformed cell line expressing *pSTARA-RFP*. Scale bars = 1 cm.

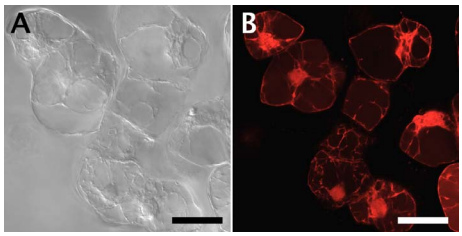

Figure 3. LSM imaging of transformed bamboo cells. The objective lens was a Plan-Apochromat 20 × 0.8. (A) Transmitted light; (B): Plane scan; He-Ne laser 543 nm, 15.0%; Main Beam Splitter: HFT488/543; Beam Splitter 1: mirror; Beam Splitter 2: NFT 545; Filter; LP 560. Scale bars = 50 µm.

independent transformed cells lines as mentioned above and subjected to PCR analysis using gene specific primers for *mCherry* and *mALS*. The expected PCR products of each gene were successfully identified (**Figure 4**).

In order to confirm the expression level of *mALS* enzyme in the BS-resistant bamboo cells, we performed an *in vivo* ALS assay. The assay is based on the colorimetric method which reflects acetoin accumulation depending on the ALS enzyme activity in bamboo cells treated with CPCA with/without BS as follows [2]. Acetolactate synthesized by ALS is subsequently converted to dihydroxyisovalerate by KARI in the branched-chain amino acid biosynthetic pathway. In the presence of CPCA, conversion of acetolactate to dihydroxyiso-valerate is inhibited and acetolactate accumulates in both non-transformed cell line (WT) and transformed cell line (R-4). However, in the presence of both CPCA and BS, the accumulation of acetolactate only occurs in the R-4 cell line harboring *mALS* gene as acetolactate synthesis is not affected by BS. The accumulated acetolactate can be converted to acetoin as shown in **Figure 5**. The endogenous ALS activity of the WT cell line was clearly inhibited by BS. Similar results were obtained in the all PCR-positive cell lines tested.

4. Conclusion

Since hygromycin was used for the selection of transformants in monocotyledonous plant species, such as barley [11], rice [12,13], and sugarcane [14], we preliminary investigated the efficacy of *HPT* gene as a selection marker for transformed bamboo cells [9]. In order to improve transformation technology for engineering/understanding complex biosynthetic pathway(s) of bamboo plants, we described here an illustration of technical details for generating stable transgenic bamboo suspension cells using the particle bombardment method with pSTARA vector harboring the mutated ALS gene derived from rice. In addition, we also informed several advantages of co-expression of *mCherry* gene as visible marker with *mALS* gene. To the best of our knowledge, this is the first detailed information on the transformation of cultured bamboo cells, both *P. bambusoides* and *P. nigra*, by adapting *mALS* gene from rice. We are certain that these transgenic techniques can be used as a powerful tool for further characterization of physiological and molecular biological events of bamboo species.

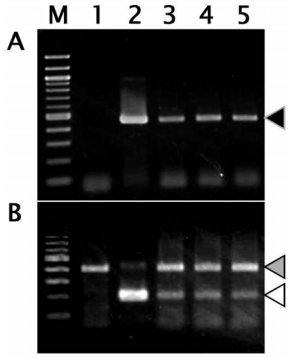

Figure 4. PCR of transformed bamboo cells. M; 100-bp ladder marker, lane 1; non-transformed cell line, lane 2; vector control, lanes 3-5; transformed cell lines. The expected fragments (closed triangle in (A); 468-bp for *mCherry*, gray triangle in (B); 388-bp for ALS genes, open triangle; 194 and 198-bp for the *Mfe*I digested fragments of amplified *mALS* were detected from vector control and genomic DNAs of transformed cells.

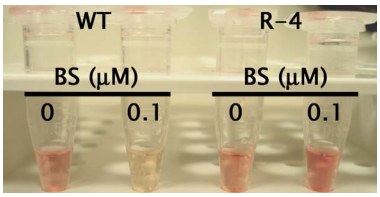

Figure 5. Evaluation of the ALS enzyme activity by *in vivo* ALS assay. The reddish resultant reaction mixtures indicate acetoin accumulation.

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