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# Highly efficient *Agrobacterium*-mediated transformation of suspension-cultured cell clusters of rice (*Oryza sativa* L.)

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ABSTRACT

Development of a large-scale transformation procedure is essential for generating T-DNA insertion lines and FOX libraries and for gene targeting studies. Suspension culture may be one of the best sources for producing a large number of transgenic cells; however, suspension-cultured cell clusters have been reported to have low *Agrobacterium*-mediated transformation frequencies in rice. In this investigation, we found that rice calli produce some unidentified substance critical for *Agrobacterium*-mediated transformation of rice that is released in the liquid culture media. By co-cultivating 3-day old suspension-cultured cells with *Agrobacterium* on filter paper moistened with enriched N6 media containing suspension-cultured cell media, more than $10^4$ stable transformants were routinely obtained from 1 g of suspension-cultured cell clusters. Transformation efficiency was about 60-times higher than that obtained from calli co-cultured in N6 media alone. Also, judging from the average weight of the cell clusters, using suspension-cultured cell clusters resulted in about 10-fold enhancement of the transformation efficiency compared with that of calli subcultured on solid media.



## 1. Introduction

Electroporation, particle bombardment and *Agrobacterium*-mediated transformation have all been used successfully to transfer DNA to rice (rice transformation) [1–3]. *Agrobacterium*-mediated transformation of rice has recently been improved by several approaches in order to generate a large number of transformants needed to generate T-DNA insertion and FOX libraries, as well as for gene targeting studies [4–6]. Gene targeting has become a routine technique in mammals for knocking out gene functions. Numerous experiments showed that gene targeting (homologous recombination) occurred at frequencies of $10^{-2}$ or higher in ES cells [7]. Unfortunately, similar frequencies have not been obtained in plant gene targeting experiments, where the observed gene targeting frequencies occur at a much lower frequency of $10^{-4}$ to $10^{-5}$ [5,8–9]. Zinc-finger nucleases (ZFNs) have been used to make site-specific genome modifications in animal cells, and a recent report indicates that high-frequency ZFNs-stimulated gene targeting is also possible in plants [10]. Terada et al. developed a reproducible gene targeting procedure using a positive–negative selection system in which fertile, transgenic rice plants with the Waxy or Adh2 genes disrupted by insertion of the hpt gene were obtained [8,9]. The success of this team was due to their use of a very large number of calli initiated from 2000 mature seeds in each experiment. Because of such enormous efforts to obtain gene disrupted mutants, gene targeting has not become a routine technique in plants.

Rapid cell division is expected to be advantageous for T-DNA integration, and rapid cell proliferation is a characteristic of suspension cultures [11]. Therefore, suspension culture may be one of the best sources for producing a large number of transgenic cells. In addition, suspension culture is suitable for producing a large number of small clusters of cells that are easier to handle in large numbers. Therefore, establishment of suspension-culture system in which high transformation efficiencies can be maintained for small clusters of cells would be advantageous for developing an efficient gene targeting system for rice. To date, suspension-cultured cell clusters have been reported to have low transformation frequencies as transformation events occurred only in large clusters of cells [11,12]. The watery condition of the suspension cultures might be responsible for the low transformation frequency [11].

In a previous study, we succeeded in establishing a highly efficient transformation system in rice by co-cultivating large rice calli (2–4 mm in diameter) with *Agrobacterium* on three layers of filter paper moistened with liquid media instead of using solid media

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[6]. In this report, we demonstrate that calli produce a substance critical for *Agrobacterium*-mediated transformation of rice that is easily released into liquid medium. Using this nurse media, we have established a high efficiency *Agrobacterium*-mediated rice transformation system using cell suspension cultures of well dispersed embryogenic cells that are easier to handle in large numbers.

## 2. Materials and methods

### 2.1. Preparation of rice cultures and media

Rice seeds were obtained from the Genetic Resources Center, National Institute of Agrobiological Sciences, Japan. Establishment of cell suspension cultures was based upon the method of Ozawa and Komamine [13]. Mature seeds (*Oryza sativa L. cv*. Nipponbare) were sterilized in 10% sodium hypochlorite for 30 min. After three rinses with sterilized water, the seeds were placed on solid N6 medium [14] supplemented with 2,4-D (2 mg/L), proline (10 mM), casein hydrolysate (300 mg/L), sucrose (30 g/L) (N6D), and Gelrite (3 g/L). The cultures were incubated at 30 °C using a 16 h light (50 $\mu$mol $m^{-2}$ $s^{-1}$) and 8 h dark cycle. Plastic Petri dishes (9 cm diameter) sealed with surgical tape (Surgical tape-21N, Nichiban Co., Ltd. Japan) were used for the induction step. Calli (3 weeks old) derived from seed were transferred to 100-mL Erlenmeyer flasks that contained 15 mL of liquid N6D media. Cell clusters were subcultured by transferring 0.2 mL (packed cell volume) into 15 mL fresh media in 100 mL Erlenmeyer flasks every 3 days. The cultures were incubated at 30 °C using a 16 h light (50 $\mu$mol $m^{-2}$ $s^{-1}$) and 8 h dark cycle on a gyratory shaker (90 rpm). After 3 days, the cell clusters were used for transformation.

Enriched N6 and AAM media containing suspension-cultured cell media (nurse media) was prepared as follows: calli (3 weeks old) derived from rice seeds were subcultured on the same fresh medium for 3 days, then 10 g (fresh weight) of rice calli (100 seeds usually produced an average of 10 g of calli) were transferred to 20 mL of N6D medium supplemented with glucose (5 g/L) and L-cysteine (100 mg/L) (N6AS) or AAM medium [3] containing acetosyringone (0 or 15 mg/L) and incubated at 20 °C on a gyratory shaker (80 rpm) for 2 h. Suspension-cultured cell media was centrifuged, and the resulting supernatant was filter-sterilized and used as 1N6 or 1AAM nurse media. Nurse medium diluted with an equal volume of N6AS or AAM medium is referred to as 1/2 N6 nurse or 1/2 AAM nurse medium. Similarly, two-fold dilution of 1/2 nurse medium yielded 1/4 nurse medium. All media except N6AS and AAM media were adjusted to pH 5.7 and autoclaved at 15 psi for 12 min at 120 °C. N6AS and AAM media were adjusted to pH 5.4 and autoclaved at 15 psi for 12 min at 120 °C. L-Cysteine, kanamycin sulfate, hygromycin B, acetosyringone, and Meropen were filter-sterilized.

### 2.2. Rice transformation procedure

The rice transformation procedure was based upon the method of Ozawa [6]. *Agrobacterium* strain EHA101 harboring pCAMBIA1301 (http://www.cambia.org/daisy/cambia/home.html) was cultured on AB medium [15] containing kanamycin sulfate (50 mg/L), hygromycin B (50 mg/L) and agar (15 g/L) (Wako Ltd., Japan) for 3 days at 28 °C in the dark. They were collected and suspended in AAM or AAM nurse medium (1, 1/2 and 1/4 AAM nurse medium) containing acetosyringone (0 or 15 mg/L). For *Agrobacterium* infection, the density of the bacterial suspension was adjusted ($OD_{600}$ = 0.05). Calli were subcultured every 3 days in N6D liquid medium or N6D solid medium for 1–5 times. After 3 days of culture, the clusters of suspension-cultured cells were washed with N6AS or N6 nurse medium (1, 1/2 and 1/4 N6 nurse medium) containing acetosyringone (0 or 15 mg/L) and then immersed in a bacterial suspension for 2 min. Calli subcultured on solid medium were directly immersed in a bacterial suspension for 2 min. Excess bacteria were removed by blotting the cell clusters on filter paper. The cell clusters were transferred onto a single piece of sterilized filter paper (9 cm in diameter, No. 2, ADVANTEC, Japan, comparable to Whatman Grade No. 2 filter paper) placed in a 9 cm diameter Petri dish containing 1.8 mL of liquid N6AS or N6 nurse media (1, 1/2 and 1/4 N6nurse medium) containing acetosyringone (0 or 15 mg/L).

The plates were sealed with Parafilm (Pechiney Plastic Packaging Company, USA) to prevent evaporation of the medium and co-cultivated for 3 days at 25 °C in the dark. Cell clusters were then washed once in sterile water containing Meropen (Dainippon Sumitomo Pharma, Japan) (50 mg/L) to remove *Agrobacterium*. The co-cultured cell clusters were blotted dry on a filter paper and plated on N6D supplemented with Meropen (25 mg/L), hygromycin B (50 mg/L) and Gelrite (3 g/L) (N6Hy). The plates were sealed with surgical tape and incubated at 30 °C using a 16 h light (50 $\mu$mol $m^{-2}$ $s^{-1}$) and 8 h dark cycle.

After about 2 weeks, proliferating hygromycin-resistant (Hm-resistant) calli were transferred to the same fresh medium and cultured for 7–10 days. Then Hm-resistant calli were transferred to MS regeneration medium [16] containing naphthyl-acetic acid (0.1 mg/L), kinetin (2.5 mg/L), casein hydrolysate (2 g/L), sucrose (30 g/L), sorbitol (20 g/L), Meropen (25 mg/L), hygromycin B (50 mg/L) and Gelrite (4 g/L) and sealed with Parafilm to prevent evaporation of the medium. The cultures were incubated at 30 °C using a 16 h light (50 $\mu$mol $m^{-2}$ $s^{-1}$) and 8 h dark cycle. Regenerated shoots were transferred to hormone-free MS medium containing sucrose (30 g/L), Meropen (25 mg/L) and Gelrite (3 g/L) and cultured at 30 °C before potting.

### 2.3. Measurement of transformation frequency and regeneration ability

Co-cultured clusters were subjected to hygromycin selection for 7 days and then stained with X-Gluc solution [17]. The transformation efficiency was calculated as the average number of cell foci showing GUS staining (GUS+) within each callus. Also, after 2 weeks of cultivation on N6Hy medium, the transformation efficiency was expressed as the number of Hm-resistant and GUS-positive calli/initial number of cell clusters inoculated × 100%. Leaves of plants regenerated from Hm-resistant calli were transferred to X-Gluc solution. Regeneration efficiency was calculated as the number of regenerated Hm-resistant and GUS-positive green plants/number of Hm-resistant calli inoculated × 100%. Each experiment was repeated at least three times.

## 3. Results and discussion

Calli derived from seeds were transferred to 100-mL Erlenmeyer flasks containing 15 mL N6D liquid medium. After 3 days of culture, the clusters of cells were used for *Agrobacterium*-mediated transformation. Table 1 shows the effect of nurse media on the transformation efficiency. When suspension-cultured cell clusters were co-cultured on a piece of filter paper moistened with 1.8 mL of N6 co-cultivation medium, about 15 GUS-positive foci per cluster were observed. The number of GUS-positive foci per cluster was significantly increased (>2-fold) at $\alpha$ = 0.05 (*t*-test) when calli were co-cultured on a piece of filter paper moistened with 1.8 mL of liquid 1/2 N6 nurse medium. The transformation efficiency was not increased when 1/4 and 1N6 nurse media were used.

Next, we examined the transformation efficiency of suspension-cultured cell clusters subcultured for 1–5 times. Calli were subcultured every 3 days in N6D liquid medium or N6D solid