Preculture in an enriched nutrient medium greatly enhances the Agrobacterium-mediated transformation efficiency in Arabidopsis T87 cultured cells

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Abstract The Arabidopsis T87 cell line has been widely used in both basic and biotechnological plant sciences. Agrobacterium-mediated transformation of this cell line was reported to be highly efficient when precultured in Gamborg’s B5 medium for a few days. However, because we could not obtain the expected efficiency in our laboratory, we further examined the preculture conditions of Arabidopsis T87 cells in the Agrobacterium-mediated transformation. As a result, we found that preculture in an excess amount of Murashige and Skoog (MS) macronutrients before cultivation in the B5 medium enhanced the transformation efficiency up to 100-fold, based on the transformed callus number on selective gellan gum plates. In this study, transformants were labeled with green fluorescent protein (GFP), and we found multiple fluorescent spots on individual transgenic calli. Therefore, the actual number of transgenic clones seems much more than that of transgenic calli. In our MS macronutrient-rich culture condition, T87 cells tended to aggregate and formed bigger cell clumps, a change that might be related to the enhancement of transformation efficiency. Based on these results, we report an improved protocol of Agrobacterium-mediated transformation of Arabidopsis T87 cells with high efficiency.

Key words: Agrobacterium, Arabidopsis thaliana T87 cell, GFP, stable transformation, transformation protocol.

Plant cell culture is a useful system not only for basic plant sciences but also for genetic engineering to produce useful substances (Eibl et al. 2018; Nagata et al. 1992; Ochoa-Villarreal et al. 2016). Because the cell culture systems are suitable to prepare cell populations of homogeneous physiological properties, they could provide highly reproducible experimental systems, compared with plant bodies or tissue samples.

The Arabidopsis T87 cell line (Axelos et al. 1992) is one of the widely used cultured cells for the following reasons: having photosynthetic ability under light illumination, transformation protocol is established and availability of highly reliable genomic data (Kwiatkowska et al. 2014; Li et al. 2012, 2018). Ogawa et al. reported a highly efficient Agrobacterium-mediated transformation protocol of T87 cells (Ogawa et al. 2008), in which preculture in Gamborg’s B5 medium for a few days before cocultivation with Agrobacterium was crucial to obtain high transformation efficiency. In this study, we further examined the preculture conditions of this cell line in the Agrobacterium-mediated transformation and found that preculture with an excess amount of MS macronutrient (Murashige and Skoog 1962) before cultivation in B5 medium enhanced the transformation efficiency at least 100-fold. Incorporating this new finding, we now report an improved transformation protocol of Arabidopsis T87 cells.

In this study, culture and transformation of Arabidopsis T87 cultured cells (Axelos et al. 1992) were carried out essentially according to Ogawa et al. (Ogawa et al. 2008) with slight modifications. The cells were cultured in mJPL3 medium (Ogawa et al. 2008) at 22°C with shaking (120 rpm) under continuous light (50–70 μE m⁻² s⁻¹). Two-week-old cultured cells were sieved through 1 mm stainless mesh and diluted to 60-fold by the following media (Table 1); mJPL3 medium, mJPL3 + 1/3MSmacro [JPL A (stock A of Axelos et al. 1992), 1/3 strength of Murashige and Skoog Plant Salt Mixture (392-00591, Nihon Pharmaceutical), Murashige and Skoog Vitamin Solution (M3900, Sigma), 15 g l⁻¹ sucrose (30404-45, Nacalai Tesque), 0.1 g l⁻¹ casamino acids (392-00655, Nihon Pharmaceutical), 1 μM NAA (161-04021, Wako), 1% (v/v) 250 mM MES (pH 5.9)
An improved transformation protocol of *A. thaliana* T87 cells

Table 1. The concentration of MS nutrients in preculture media tested in this study.

|                     | mJPL3 | mJPL3+1/3MSmacro | mJPL3+MS |
|---------------------|-------|-----------------|----------|
| **MS macronutrients** |       |                 |          |
| NO$_3^-$             | 0.49  | 0.83            | 1.5      |
| NH$_4^+$             | 0     | 0.33            | 1.0      |
| PO$_4^{3-}$          | 0.30  | 0.63            | 1.3      |
| K$^+$                | 1.0   | 1.3             | 2.0      |
| Ca$^{2+}$            | 0.30  | 0.63            | 1.3      |
| Mg$^{2+}$            | 0.30  | 0.63            | 1.3      |
| **MS micronutrients** | 0.33  | 0.33            | 1.0      |

Each concentration was normalized by the original composition of MS medium (Murashige and Skoog 1962) as 1× strength.

Figure 1. The number of Kanamycin-resistant green calli obtained per 10 ml of cell culture. (A) Cells were pretreated by incubation for a week in the indicated media. (B) Cells were pretreated by incubation in the mJPL3+MS for a week. After that, the media were replaced by the indicated media and cultured for 2 days, and then cocultivated with *Agrobacterium*. Mean±SD of six independent plates are indicated.

Figure 2. Photographs of the T87 cells. (A–C) Green calli observed after two weeks of culture on the Kanamycin-containing plates. White and yellowish calli were not transformants. The preculture media were (A) mJPL3, (B) mJPL3+1/3MSmacro and (C) mJPL3+MS. (D) The green calli in (C) was observed using an OLYMPUS SZX7 system. Green and red spots indicate GFP fluorescence and fluorescence from chloroplasts, respectively. (E) Bright-field image of (D). (F–G) Cell clumps when cultured in mJPL3 (F), and in mJPL3+MS (G) for one week. Scale bar=1 mm.

(345-01625, Dojindo) or mJPL3+MS [JPL A, Murashige and Skoog Plant Salt Mixture, Murashige and Skoog Vitamin Solution, 15 g l$^{-1}$ sucrose, 0.1 g l$^{-1}$ casamino acids, 1 µM NAA, 1% (v/v) 250 mM MES (pH 5.9)]. The detailed composition of the media used in this study was shown in Supplementary Table S1. Cells in the respective media were cultured at 22°C with shaking under continuous light for 1 week, then harvested and 0.5 g wet weight aliquots were resuspended in 100 ml of B5 medium [Gamborg’s B5 medium salt mixture (399-00621, Nihon Pharmaceutical), Gamborg’s B5 vitamin mix (G-1019, Sigma), 1 µM NAA, 30 g l$^{-1}$ sucrose, pH 5.9] or mJPL3+MS medium and cultured for 2 days. Subsequently, 5 ml aliquots of the cell cultures were cocultivated with 5 µl of *Agrobacterium* (GV3101) culture harboring pGreenII MH2 vector (Hellens et al. 2000; Hirashima et al. 2006) in a six-well plate. After 40 to 48 h of cocultivation, cells were washed three times with mJPL3 medium supplemented with 25 mg l$^{-1}$ of meropenem and 30 mg l$^{-1}$ of Kanamycin (113-00343, Wako). After two weeks of culture, green resistant calli were counted. White and yellowish calli were not counted because they were dead or escaped cells against the Kanamycin-based selection.

Figure 1A represents the number of Kanamycin-resistant green calli on the plates from the cells precultured in the respective media, showing that increasing nutrient salt concentration in the preculture media resulted in a higher transformation efficiency. Transformants were hardly obtained when precultured in mJPL3, while those increased ca 30-fold and 100-fold when precultured in mJPL3+1/3MSmacro and mJPL3+MS, respectively.

Figure 1A–C shows the cells on the plates, corresponding to the three treatment samples in Figure 1. The cells precultured in mJPL3 hardly grew on the Kanamycin-containing plate and turned white (Figure 2A), those in mJPL3+1/3MSmacro grew to form calli
(Figure 2B) and those in mJPL3+MS formed bigger green calli (Figure 2C).

Figure 2D, E represents the fluorescence and bright-field images of the cells as in Figure 2C, respectively. We found many green fluorescent protein (GFP) fluorescent spots on the callus, indicating that the number of transformed cells was much higher than that of the green calli. Therefore, we expect that the preculture in the mJPL3+MS medium enhanced the transformation efficiency far more than 100-fold compared with that in mJPL3.

These results indicate that mJPL3+MS medium greatly enhances the Agrobacterium-mediated transformation efficiency when used for preculture medium. Next, we examined if mJPL3+MS also has an enhancing effect when used as a coculture medium. For this purpose, Arabidopsis T87 cells precultured in the mJPL3+MS medium for 1 week were transferred to fresh mJPL3+MS medium instead of B5 medium, and after 2 days, cocultivated with Agrobacterium. However, as shown in Figure 1B, we could not obtain a successful transformation. We observed that Agrobacterium could proliferate in mJPL4+MS medium, suggesting this condition may not suitable for the infection of Agrobacterium.

Based on these results, we propose an improved protocol for the highly efficient transformation of T87 cells (Figure 3). In this protocol, cells are precultured for 1 week in mJPL3+MS medium instead of mJPL3 medium. Subsequent steps of the transformation protocol are essentially the same as Ogawa’s method (Ogawa et al. 2008). If isogenic clones are required rather than a massive number of transformants, we suggest a much shorter time of Agrobacterium cocultivation because Agrobacterium can sufficiently introduce T-DNA to plant genome as early as 6 h postinfection (Shilo et al. 2017).

This protocol is very useful for plant biotechnology but raises the question of how preculture conditions affected the transformation efficiency. In this respect, we should first compare the composition of the tested media as shown in Table 1. These media share very similar components, and the difference mainly lies in their concentration (Supplementary Table S1). Though mJPL3 and mJPL3+1/3MSmacro have the same concentration of MS micronutrients (Table 1), their transformation efficiencies were quite different (Figure 1A). Therefore, the MS micronutrients’ concentrations are less effective for the transformation efficiency, but the MS macronutrients should be the critical factor.

As another angle of the explanation of the transformation efficiency, we are interested in the cell clump size. When the clump size of the T87 cells was bigger, the introduction of the Cre enzyme by electroporation was reported to be highly efficient (Furuhatara et al. 2019). Analogous to this, Agrobacterium-mediated transformation efficiency may also be affected by the cell clump size. In this study, cell clump size tended to be bigger when cultured in mJPL3+MS (about 0.5 mm) (Figure 2F) than in mJPL3 medium (less than 0.1 mm) (Figure 2G). A possible explanation from this angle remains to be examined.

The protocol we propose in this study was really useful when we prepared massive transformants for a large-scale experiment utilizing next-generation sequencing and bioinformatics. Transformation efficiency of the cells is one of the critical factors for preparing transformant libraries for large-scale analysis (Akhtar et al. 2013; Inoue and Ahituv 2015). In this respect, this improved protocol could contribute to the advancement of future plant sciences.

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![Figure 3. An improved protocol of efficient transformation of Arabidopsis T87 cells. One week preculture in the mJPL+MS medium (step 1) is critical to obtain high efficiency.](image-url)
An improved transformation protocol of *A. thaliana* T87 cells

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