Pretreatment and posttreatment in the biolistic transformation of tea plant (*Camellia sinensis*) somatic embryos

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Received November 13, 2019; accepted April 4, 2020 (Edited by N. Ohtsubo)

Abstract  The tea plant (*Camellia sinensis*) contains various metabolic substances, including catechins and caffeine, for which genetic transformation techniques are essential for investigating the associated metabolic pathways. In this study, we sought to optimize the conditions and culture process for particle bombardment-mediated transformation of tea plant somatic embryos. We describe somatic embryo pretreatment for effective transient transformation in biolistic bombardment and the posttreatment conditions of somatic embryos for accelerating differentiation after bombardment. For the purpose of transformation, we used the somatic embryos of *C. sinensis* var. *assamica* ‘Tingamira normal,’ which were cultured in Murashige and Skoog (MS) medium containing 2 mg l−1 indole-3-butyric acid (IBA) and 4 mg l−1 6-benzyladenine (BA) at 25°C ± 2°C under a 16-h photoperiod. With respect to the optimization of particle bombardment conditions for tea somatic embryos, we examined the effects of different Au colloid particle diameters and bombardment pressures, and accordingly established bombardment with 1.0-µm-diameter DNA-coated Au colloid at 1,100 psi as optimal conditions for introducing DNA for the transient expression of GUS. Additionally, we found that transplantation of tea somatic embryos from IBA/BA medium to a hormone-free medium prior to bombardment and incubation in the dark post-bombardment increased the frequency of secondary embryo production. Furthermore, osmotic treatment by culturing the somatic embryos in medium supplemented with 0.4 M mannitol improved transient transformation efficiency. After transformation, the culture of somatic embryos on filter papers or Kimwipes soaked in MS medium facilitated rapid and effective development of the somatic embryos.

Key words: *Camellia sinensis*, microprojectile bombardment, osmotic treatment, somatic embryo.

Introduction

Tea is one of the most popular beverages worldwide, with green, oolong, and black teas all being produced from the same economically important tea plant species, *Camellia sinensis*. Additionally, tea has garnered considerable attention with regard to its beneficial health-related properties, attributable to leaf components such as catechins, caffeine, and theanine (Nagata and Sakai 1985). The transformation techniques for tea plants are important for elucidating the metabolic pathways associated with these components, as well as for breeding new cultivars. For example, transformation is necessary to elucidate the biosynthetic pathway of nicotine in tea plant (Ikka et al. 2018). Although several previous studies have reported tea plant transformation using *Agrobacterium*-mediated methods (Mariya John et al. 2009; Matsumoto and Fukui 1998; Mondal et al. 2001; Qianru et al. 2017; Rana et al. 2016; Sandal et al. 2007; Song et al. 2014), there tends to be limited compatibility between tea genotypes and *Agrobacterium* strains. Furthermore, for tissues infected with *Agrobacterium*, it is necessary to apply an effective regeneration protocol. Microprojectile bombardment is an alternative transformation method for delivering genes into plant cells. In addition, somatic embryos would be a useful material for microprojectile bombardment, as in response to transfer from a medium containing auxin to a hormone-free medium; these embryos can produce transformed secondary embryos from a single bombarded surface cell. We report efficient transformation conditions for...
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the biolistic transformation of somatic embryos and culture conditions for the development of tea plants from somatic embryos. With regard to basic biolistic conditions, we examined Au colloid diameter and burst pressure, and also pretreatment of somatic embryos with mannitol as an osmotic agent. Additionally, we examined whether the transplantation of somatic embryos to a hormone-free medium before and after bombardment treatment would be effective in promoting secondary embryogenesis. Given that the biolistic conditions for increasing transient foreign gene expression are relative to the frequency of stable transformation, we also evaluated conditions via transient expression of the β-glucuronidase (GUS) gene. Furthermore, two paper-based culture methods were developed for accelerating the development of transformed secondary embryos.

Materials and methods

Plant materials

Somatic embryos induced from the seedling buds of Tingamira normal (C. sinensis var. assamica) by Kato (1996) were used as the study material. Tingamira normal is a genetic resource of an Indian black tea strain, and not a commercial cultivar. The somatic embryos were maintained by subculturing for a 50-day period on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 0.2% gellan gum, 3% sucrose, 2 mg l⁻¹ 3-indolebutyric acid (IBA), and 4 mg l⁻¹ 6-benzyladenine (BA). Prior to autoclaving at 120°C and 0.1 MPa for 20 min, the pH of the medium was adjusted to 5.8. The culture conditions were as follows: 25°C ±2°C and a 16-h photoperiod maintained with fluorescent lamps providing approximately 59 µmol m⁻² s⁻¹ photon flux density. To obtain a larger number of somatic embryos, the embryos were transplanted onto hormone-free MS medium to promote secondary embryogenesis (Figure 1).

Bombardment conditions and mannitol pretreatment

For transformation of the somatic embryos, they were subjected to microprojectile bombardment. The bombardment was carried out using the biolistic PDS-1000/He System (Bio-Rad Laboratories, Inc., Hercules, CA, US). The common bombardment conditions were as follows: the target distance was 6 cm and the burst pressure was generated using a rupture disk (Bio-Rad Laboratories, Inc.). Au colloid comprising 0.4 mg of particles coated with 2 µg of plasmid DNA was used for one-shoot bombardment. For the attachment of DNA to the Au surface, the colloid was pretreated with 2.5 M CaCl₂ and 0.1 M spermidine. As delivery vectors, we used pRI201-ANGUS plasmids (Takara Bio Inc., Shiga, Japan), containing an NPT II kanamycin-resistance gene and GUS as the reporter gene, driven by the nopaline synthase and cauliflower mosaic virus 35S promoters, respectively. Twenty-five yellow somatic embryos per Petri dish were shot twice with the Au particles, and thereafter the somatic embryos were cultured in the dark for 30 days and subsequently transferred onto hormone-free MS medium. To investigate the suitable bombardment conditions for the somatic embryos of C. sinensis, we examined combinations of different diameters of Au particles (0.6 and 1.0 µm) and burst pressures (rupture disks of 650, 1,100, and 1,500 psi, all from Bio-Rad Laboratories, Inc.). For the osmotic pretreatment, 25 yellow somatic embryos were cultured overnight on 20 ml MS medium containing 0.1–1.0 M mannitol in a 90-mm-diameter plastic Petri dish at 25°C±2°C in the dark prior to transformation. These experiments were repeated four to six times. The different mannitol conditions were evaluated in terms of the number of blue spots (indicating GUS expression) detected in transformed embryos. Given the differences in sample number and the non-parametric nature of the data, statistical comparisons were performed using the Steel–Dwass test (p<0.05).
Detection of blue spots as an indicator of GUS gene expression

To investigate the transient expression of GUS, the somatic embryos at 3 days post-bombardment were soaked in 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution [0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6] \cdot 3H_2O$, 100 mM NaH$_2$PO$_4 \cdot 2H_2O$ (pH 7.0), 100 mM Na$_2$HPO$_4 \cdot 12H_2O$ (pH 7.0), 5% methanol, 0.3% TritonX-100, and 1.9 mM X-Gluc dissolved in $N,N$-dimethylformamide] overnight at 37°C, after which they were transferred to 70% ethanol for decolorization. The blue spots (indicating gene expression) on the surface of somatic embryos were counted using a stereomicroscope (SZ61; OLYMPUS Co., Tokyo, Japan).

Culture conditions for embryo development

We compared four types of media that promote the differentiation of somatic embryos, with a particular focus on medium solidification. MS medium (pH 5.8) containing 3% sucrose and 2mg l$^{-1}$ IBA and 4 mg l$^{-1}$ BA was used as a basic medium unless specified otherwise. As the standard condition, we used 10ml of MS medium solidified using 0.2% gellan gum in a 40-mm-diameter flat bottom test tube. For the Kimwipe condition, two sheets of Kimwipe (NIPPON PAPER CRECIA Co., LTD., Tokyo, Japan) were folded to form a cylinder, which was placed in a 40-mm diameter flat bottom test tube, followed by the addition of 10ml of MS liquid medium. For the filter paper condition, two filter papers (90 mm diameter No. 131; Toyo Roshi Kaisha,Ltd., Tokyo, Japan) were placed in a 90-mm-diameter plastic Petri dish and soaked with 3ml of MS liquid medium. For the Petri dish condition, a 90-mm-diameter plastic Petri dish was filled with 20ml of MS medium solidified with 0.2% gellan gum. For each of the assessed conditions, 50 Tingamira normal yellow somatic embryos were inoculated and cultured at 23°C under a 16-h photoperiod, with four replicates per treatment.

Results and discussion

Biolistic conditions for tea plant somatic embryos

In order to determine the optimal conditions for biolistic bombardment, we examined the efficacies of six combinations of different Au particle diameters and burst pressures and evaluated transient GUS expression, as indicated by blue spots on the surface of the somatic embryos (Figure 2A). Enumeration of the blue spots on the 25 embryos in each Petri dish revealed that the highest number of blue spots developed on those embryos bombarded at 1,100 psi with both 0.6 and 1.0µm Au colloid particles. Bombardment at the highest pressure, 1,550 psi, was found to result in
a reduction in the number of blue spots (Figure 2B). We accordingly suspect that excessively high pressures might cause irreparable damage to the somatic embryos, and that the cells on the embryo surface may die prior to GUS staining. Furthermore, Au particles with diameter of 1.0 \( \mu \text{m} \) were found to facilitate better DNA delivery than those with a diameter of 0.6 \( \mu \text{m} \). These conditions of biolistic bombardment, which increase transient expression frequency, would result in stable transformation as reported for tobacco (Russell et al. 1992).

**Osmotic stress treatment with mannitol prior to bombardment**

In order to increase the frequency of transformation, the somatic embryos were transferred onto MS medium containing mannitol at concentrations ranging from 0.1 to 1.0 M for 24 h prior to bombardment. We found that mannitol treatment tended to enhance cell transformation efficiency, although concentrations higher than 0.6 M caused a decrease in the number of blue spots (Figure 3). Mannitol concentrations in the range 0.2–0.4 M were considered to be suitable for the transient expression of GUS in the somatic embryos. We assume that higher concentrations of mannitol could affect somatic embryo regeneration. Consistently, Aoshima (2005) has reported that survival of somatic embryos of the tea cultivar Surugawase decreased with an increase in the mannitol concentration of medium, whereas Russell et al. (1992) reported that preconditioning with the addition of 0.2 M raffinose and mannitol plus sorbitol as osmotic reagents were...
effective in promoting the transient expression of GUS following bombardment in tobacco cell suspensions. Similarly, in maize, transient and stable bombardment-mediated transformation was enhanced by osmotic pretreatment and posttreatment on MS medium containing 0.4 M mannitol and/or sorbitol (Vain et al. 1993). We also examined the timing of transfer of somatic embryos from auxin-containing MS medium to hormone-free MS medium in order to induce efficient secondary embryogenesis after bombardment (Figure 4A). The somatic embryo was transferred to hormone-free medium after or before bombardment. In addition, each treatment was carried out with or without 0.2 M mannitol (Figure 4A; Table 1). Transferring somatic

Table 1. Effects of medium conditions before and after bombardment treatment on secondary embryogenesis.

| Treatment* | Culture media conditions | Secondary embryo formation rate (d) = ((a) + (b))/(a) | Transformation rate (e) = (c)/(a)×100 (%) |
|------------|--------------------------|-----------------------------------------------------|------------------------------------------|
| Before bombardment | After bombardment | After 2 weeks | Bombed embryos (a) | Secondary embryos (b) | GUS activity (c) | |
| HF | HF | HF | 50 | 51.5 a | 5.5 ab | 2.0 | 11.0 |
| IBA/BA | IBA/BA | IBA/BA | HF | 50 | 20.5 b | 2.0 a | 1.4 | 4.0 |
| HF-Man | HF-Man | HF | 50 | 60.5 a | 19.5 b | 2.2 | 39.0 |
| IBA/BA-Man | IBA/BA-Man | IBA/BA-Man | HF | 50 | 16.5 b | 3.5 a | 1.3 | 7.0 |

* HF denotes MS medium without plant growth regulator. IBA/BA denotes MS medium containing 2 mg l⁻¹ IBA and 4 mg l⁻¹ BA. Man denotes MS medium supplemented with 0.2 mol l⁻¹ mannitol. Different letters after the mean values indicate statistically significant difference at p = 0.05 (Tukey’s test).
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Embryos from MS medium containing IBA/BA to hormone-free (HF) MS medium prior to bombardment increased the number of secondary embryos obtained (Figure 4B; Table 1). From this experiment, we found that the timing of somatic embryo transfer to a HF medium rather than the mannitol treatment had a more pronounced effect on secondary embryogenesis (Table 1; Figure 4B), with the transfer prior to bombardment being conducive to the induction of secondary embryogenesis (Figure 4B). We also observed that after bombardment, secondary embryogenesis was unaffected by 0.2 M mannitol treatment (Table 1). Accordingly, we established that osmotic pretreatment of tea plant somatic embryos with 0.2–0.4 M mannitol was an optimal treatment for bombardment-mediated transient foreign gene expression.

In contrast to Agrobacterium-mediated gene delivery, which depends on the plant species and bacterial strain, biolistic bombardment is an efficient gene-delivery system that can be applied to a wide range of plant species. Although Agrobacterium-mediated transformation has been applied for tea plants (Mariya John et al. 2009; Matsumoto and Fukui 1998; Mondal et al. 2001; Qianru et al. 2017; Rana et al. 2016; Sandal et al. 2007; Song et al. 2014), the viable combinations of cultivar and bacterial strain tend to be limited, and such limitations have previously been reported for other plants, including rice (Hiei et al. 1994). Agrobacterium-mediated transformation tends to be sub-optimal for tea plants because the catechins in tea tend to inhibit the growth of Agrobacterium cells (Song et al. 2014). Bombardment is an alternative method for delivering genes, and Mohanpuria et al. (2011) applied this method to transform the somatic embryos of tea plants to induce post-transcriptional silencing of caffeine synthase mRNA. Accordingly, the transformation conditions and somatic embryo response found in this study are useful tools to elucidate the metabolic pathway of tea. Furthermore, these transformation techniques are effective for breeding tea plants by genetic modification including genome editing.

**Comparison of medium conditions for tea plant somatic embryo differentiation**

In the present study, the somatic embryos of tea plants were characterized by the development of yellow secondary embryos. When the yellow embryos turned green, they differentiated and did not form secondary embryos. We sought to identify the medium conditions that would be optimal for rapidly obtaining transformed individual plants from somatic embryos. To the best of our knowledge, this is the first report of somatic embryos turning green on medium-soaked papers. The somatic embryos on Kimwipes soaked in MS medium turned green sequentially and underwent differentiation (Figure 5).
differentiated and developed rapidly (Figures 5 and 6), and the green embryos subsequently developed into plantlets (Figure 5). The most effective condition in this regard was Kimwipes soaked in 10 ml of MS medium. In addition, filter paper was easier to handle. Figure 5 shows the embryos at 90 days post-inoculation into each medium, and after turning green, which occurred most rapidly on Kimwipes, the embryos started to differentiate. Figure 6 shows the percentages of the different types of embryos obtained with the different experimental conditions. We also found that when the somatic embryos of Makura Ck-2, which is a Chinese green tea genetic resource, were cultured under the same four conditions, the change tendency tended to be similar to that observed for Tingamira normal (data not shown), indicating that the method is not dependent on genotype and could be generally applicable to tea plants. However, the mechanisms underlying the effect of paper medium on somatic embryo differentiation have yet to be elucidated. We suspect that several factors might contribute to modifying internal hormone localization in somatic embryos under soaked paper conditions. In order to verify this assumption, it will be necessary to quantify the localization of endogenous hormones. Nevertheless, the Kimwipe method is simple to apply, enables the ready preparation and subculturing of cultures, and is conducive to the development and rapid differentiation of somatic embryos. Furthermore, for embryo subculture, liquid medium can be poured into the same test tube.

**Conclusions**

Given that biolistic bombardment is not limited by the suitability of target plant materials, this method can be applied to a wide range of tea cultivars. Several factors contribute to determining transformation efficiency of tea plant somatic embryos; in the present study, we established that a combination of 1.0-µm-diameter Au particles and a burst pressure of 1,100 psi were effective for promoting transient gene expression. Additionally, we found that osmotic pretreatment with 0.2 or 0.4 M mannitol enhanced particle bombardment-mediated transient transformation of tea somatic embryos. Furthermore, we were able to induce secondary embryos characterized by transgene expression by transferring somatic embryos from a medium containing auxin to a hormone-free medium prior to biolistic bombardment. Figure 7 shows the flowchart of the transformation conditions and the procedure for establishing tea plant somatic embryos using microprojectile bombardment. Following bombardment, the culture conditions provided by Kimwipes soaked in MS medium accelerated somatic embryo development compared with that obtained when using 0.2% gellan gum, and we thus established that transformed tea plants can be obtained.
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Despite these significant findings, however, we have yet to solve the problem of inducing clonal cultures. Particularly in Japanese green tea cultivars, a stable culture method has yet to be established. Currently, somatic embryos are induced from the cotyledons in immature seeds (Akula et al. 2000; Furukawa and Tanaka 2004; Kato 1986, 1996; Mondal et al. 2002; Nakamura 1988ab; Wachira and Ogada 1995), which, although a relatively stable method, is not ideal because the embryo is not a clone of the parental plant. Furthermore, owing to the heterozygosity of tea plants, same-genotype progenies are never obtained. Moreover, tea plant seeds take a year to mature, and thus, it is difficult to obtain tea plant seeds of target genotypes in all seasons. With respect to clones of a particular cultivar, as alternatives to naturally crossed seedlings, callus induction and regeneration have been reported (Kato 1985; Nakamura 1989; Timirbaran and Sen 1992); however, these techniques can have associated technical difficulties, including microbial contamination of plant materials and genotype-dependent differential response to hormones. To date, only two studies have reported direct somatic embryo induction in tea plants (Akula and Dodd 1998; Aoshima 2005), with the latter author describing the induction of somatic embryos from the shoot apex-derived callus of the Japanese green tea Surugawase. Somatic embryos from Japanese green tea cultivars should be induced for future breeding, and on the basis of the findings of Aoshima (2005) and Kato (1996), it is our intention to improve the method of somatic embryo induction in several green tea cultivars.

In the future, Japanese green tea transformation by microprojectile bombardment will be a useful technique for both elucidating endogenous metabolic pathways of the components of tea plant cells and breeding of new cultivars through genetic modification and genome editing.

Acknowledgements

We are grateful to Dr. Michiyo Kato and Ms. Ayano Fukizawa-Nemoto for providing the somatic embryos of Tingamira normal and for their valuable advice regarding culture techniques for tea plants. We are also grateful to Dr. Yutaka Tabei (NARO) and Dr. Masao Ohshima (University of Tsukuba) for their helpful suggestions and support with respect to biolistic bombardment. We thank Dr. Junichi Tanaka (NARO) for his useful advice as a tea genome research expert, and also thank all Numazu KOSEN student members of our laboratory for their long-term dedication in continuous somatic embryo culture.
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