

## ARTICLE



# An Efficient Agrobacterium-Mediated Genetic Transformation Method for a Monocot Model Plant, *Brachypodium distachyon*

Sharanya Tripathi and Jeong-Il Kim\*

Department of Integrative Food, Bioscience and Biotechnology, Chonnam National University, Gwangju, Korea

Received: April 5, 2022

Accepted: May 3, 2022

\*Corresponding author :

Jeong-Il Kim

Department of Integrative Food,  
Bioscience and Biotechnology, Chonnam  
National University, Gwangju, Korea  
Tel : +82-62-530-2189  
E-mail : kimji@jnu.ac.kr

Copyright © 2022 Institute of Agricultural Science  
& Technology, Chonnam National University.

This is an Open Access article distributed  
under the terms of the Creative Commons  
Attribution Non-Commercial License  
(<http://creativecommons.org/licenses/by-nc/4.0>)  
which permits unrestricted non-commercial  
use, distribution, and reproduction in any  
medium, provided the original work is  
properly cited.

### ORCID

Sharanya Tripathi

<https://orcid.org/0009-0003-1120-9512>

Jeong-Il Kim

<https://orcid.org/0000-0003-4177-4048>

## Abstract

Model plants are necessary and important for advances in various research areas of plant biology. Compared with *Arabidopsis thaliana*, a dicot model plant that is widely used thus far, monocot model plants are not popular in using plant biology research. In this regard, *Brachypodium distachyon* is suggested as a new and alternative monocot model plant for economically valuable temperate grasses and cereals such as wheat and barley, and also for bioenergy crops such as switchgrass and miscanthus. One of the major constraints in performing plant research would be the difficulty of genetic transformation and its low frequency. Here, we provide a detailed and improved method for *Agrobacterium*-mediated genetic transformation of *B. distachyon*, based on our experimental experience. With hygromycin resistance as a selectable marker, we could obtain approximately 40%–60% of the transformation efficiency using this method, in which the process takes approximately 26 weeks to obtain T<sub>1</sub> seeds starting from co-cultivation of immature embryos with *Agrobacterium* cells. Here, we also describe about important factors possibly contributing to the genetic transformation of *B. distachyon*.

## Keywords

*Brachypodium distachyon*, monocot, model plant, tissue culture, genetic transformation

## Introduction

Model plants are essential for the advances in plant biology research [1]. So far, *Arabidopsis thaliana* has been widely used as a model plant. However, it is a dicotyledonous plant, so monocot model plants are necessary because monocots include cereals and grasses that provide the sources of foods and biofuels [2]. *Oryza sativa* (rice) has been used as a monocot model plant. However, it is a semi-aquatic tropical grass, i.e., not a suitable monocotyledonous plant for small grain cereals such as *Triticum aestivum* (wheat), *Hordeum vulgare* (barley), and *Avena sativa* (oats), and also for bioenergy crops such as miscanthus and switchgrass [3]. These considerations have led to the development of *Brachypodium distachyon* (hereafter, Brachypodium) as a new and alternative monocot model plant for temperate cereals and bioenergy crops [4–6].

Brachypodium has typical characteristics for being a model plant such as small stature (approximately 20–30 cm), diploidy (2n=10), short life cycle (~4 months), small genome (~270 Mb), self-pollination, and easy cultivation [3,5,7,8]. In addition, a series of genetic transformation methods for Brachypodium have been reported [9–14]. More recently, genome editing methods of Brachypodium are also developed using *Agrobac-*

*terium tumefaciens* (hereafter, *Agrobacterium*) for transformation [15]. Furthermore, numerous resources have been developed for working with Brachypodium, including a large germplasm collection and sequenced mutant collections, in addition to the completion of genome sequencing [16,17]. Therefore, Brachypodium research is moving forward rapidly as being a widely used monocot model plant.

For Brachypodium research, one of the major constraints would be the establishment of an efficient genetic transformation system. Genetic transformation makes possible to transfer gene(s) from one organism to another, which would not be possible using traditional plant breeding methods. Among the genetic transformation methods, *Agrobacterium*-mediated transformation is currently the predominant technology used to generate transgenic plants [18]. In addition, immature embryos are frequently used as explant materials for the efficient plant transformation, due to their high regeneration capacity [19]. Thus, we intended to describe about tissue culture and *Agrobacterium*-mediated transformation methods for Brachypodium using immature embryos, based on our experimental experience with two Brachypodium inbred lines, Bd21 and Bd21-3. Here, starting with the growth of Brachypodium plants to obtain immature embryos, we summarize the steps for the transformation procedures to obtain transgenic T<sub>1</sub> plants efficiently. In addition, we explain about possible factors contributing to the genetic transformation of Brachypodium with the timeline.

## Brachypodium Plant Growth

### 1. Growth conditions

Seeds of *B. ditachyon* inbred lines (Bd21 or Bd21-3) were taken in a 50 mL conical tube and soaked with tap water for 20 min. Then, lemmas were removed completely to ensure seed germination using fingertips, and surface-sterilized in 70% (v/v) ethanol for 2 min, followed by 2% (w/v) sodium hypochlorite (NaOCl) for 7 min with gentle shaking. After rinsing in sterile distilled water (DW) 4-5 times, the de-husked seeds were kept submerged in DW inside the conical tube and placed at 4°C for 7 days in the dark to ensure synchronous seed germination. The de-husked and surface-sterilized seeds were then placed on half-strength of Murashige and Skoog media (½MS) supplemented with 1% sucrose and 0.6% phytoagar, and grown under 16/8 or 18/6 hr light/dark cycle with 24.5 W/m<sup>2</sup> of light intensity. Two-week-grown plantlets on the plates were transferred to autoclaved soil. During the transfer, the phytoagar should be removed completely with DW. As a note, seeds can be placed directly on the soil, but plant phenotypes such as tiller numbers and stem thicknesses appear to be better when the seeds are cultured on the ½MS plate first. In addition, due to dormancy of Bd21 seeds, it is recommended to use seeds at least 3 weeks after harvesting.

### 2. Collection of immature embryos

Around 10 plants were grown in a pot with 15 cm diameter under 18/6 hr light/dark cycle at 22°C with the light intensity of 24.5 W/m<sup>2</sup>. The plants were watered twice a

week and HYPONeX was given once a month as fertilizer. It is notable that the soil should not be dried out during the growth. After 7–9 weeks from the date of soil transfer, immature embryos with suitable sizes (~0.3 mm) could be collected for the induction of embryogenic calli by checking the state of embryos everyday. For seed amplification, seeds were harvested when *Brachypodium* seeds became completely dry by collecting them in paper envelopes. After incubating the envelopes in 20°C desiccator for at least 3 weeks, seeds were stored at 4°C in airtight containers with silica gels.

## Materials for Tissue Culture and Transformation

For the genetic transformation of *Brachypodium*, *A. tumefaciens* strain AGL1 is recommended due to its high virulence. In addition, vectors harboring hygromycin resistance as a selectable marker, such as pCAMBIA1301, are recommended over other selectable markers because of the strong and rapid selection. Stock solutions of phytohormones, antibiotics, and other reagents are listed in Table 1. In addition, culture media for *A. tumefaciens* and *B. distachyon* are listed in Table 2.

## The Efficient Method for *Agrobacterium*–Mediated Transformation of *Brachypodium*

We summarize a practical method for *Brachypodium* transformation with 18 steps, including the culture for the induction of embryogenic calli (STEP 1 to 8), *Agrobacterium*–mediated transformation (STEP 9 to 13), selection of transformed calluses (STEP 14 and 15), and regeneration of transgenic plantlets (STEP 16 to 18).

**STEP 1.** After plant growth in soil for 7 to 9 weeks, spikelets carrying seeds with

**Table 1.** Stock solutions for tissue culture and transformation of *Brachypodium*

Phytohormones	
2,4-D (2.5 mg/mL)	Dissolve 50 mg 2,4-D (Sigma, D7299) in 20 mL of 95% ethanol, and aliquot and store at –20°C (up to 6 months)
Kinetin (0.2 mg/mL)	Dissolve 4 mg Kinetin (Duchefa Biochemie, K0905) in few drops of 1 N NaOH and adjust volume to 20 mL with DW, aliquot after 0.2 μm filter-sterilization, and store at –20°C (up to 6 months)
Antibiotics	
Carbenicillin (100 mg/mL)	Dissolve 5 g Carbenicillin disodium (Duchefa Biochemie, C0109) in 50 mL of DW, aliquot after filter-sterilization, and store at –20°C (up to 6 months)
Kanamycin (50 mg/mL)	Dissolve 2.5 g Kanamycin sulfate (AG Scientific, K-1022) in 50 mL of DW, aliquot after filter-sterilization, and store at –20°C (up to 6 months)
Cefotaxime (300 mg/mL)	Dissolve 15 g Cefotaxime sodium (Duchefa Biochemie, C0111) in 50 mL of DW, aliquot after filter-sterilization, and store at –20°C (up to 6 months)
Hygromycin (40 mg/mL)	Dissolve 2 g Hygromycin-B (AG Scientific, H1012) in 50 mL of DW, aliquot after filter-sterilization, and store at –20°C (up to 6 months)
Other reagents	
CuSO <sub>4</sub> (1 mg/mL)	Dissolve 50 mg of CuSO <sub>4</sub> ·5H <sub>2</sub> O (Sigma, C8027) in 50 mL of DW, wrap the tube with aluminum foil, and store at 4°C in the dark (up to 6 months)
Acetosyringone (200 mM)	Dissolve 0.392 g Acetosyringone (Bioplus Chemicals, 40100297-2) in 10 mL of DMSO, aliquot after filter-sterilization, and store at –20°C (up to 6 months)

**Table 2.** Preparation of culture media for *Agrobacterium* and *Brachypodium*

For <i>Agrobacterium</i>	
MG/L medium (per liter)	5 g tryptone (Gibco, 211705); 2.5 g yeast extract (BD Biosciences, 212750); 5 g NaCl (Duchefa Biochemie, S0520); 5 g Mannitol (Duchefa Biochemie, M0803); 0.1 g MgSO <sub>4</sub> (Sigma, M7506); 0.25 g K <sub>2</sub> HPO <sub>4</sub> (Sigma, P3786); 1.2 g Glutamic acid (Sigma, G6904)
<ul style="list-style-type: none"> <li>· Adjust pH to 7.2 with 0.1 N NaOH, add 15 g Bacto agar (BD Biosciences, 214010), and autoclave.</li> <li>· After cool-down, add 1 mL of 200 mM Acetosyringone, 1 mL of Carbenicillin (100 mg/mL) and a second antibiotics depending on the vector used for transformation (ex., Kanamycin).</li> <li>· Pour into petri dishes, and store at 4°C (up to 2 weeks).</li> </ul>	
For <i>Brachypodium</i>	
Callus induction medium (CIM, per liter)	4.43 g Linsmaier and Skoog medium (Duchefa Biochemie, L0230); 30 g Sucrose (Duchefa Biochemie, S0809); 0.6 mL CuSO <sub>4</sub> stock solution (1 mg/mL); 1 mL 2,4-D stock solution (2.5 mg/mL)
<ul style="list-style-type: none"> <li>· Adjust pH to 5.8 with 0.1 N KOH, and autoclave.</li> <li>· For liquid CIM, store in a sterile glass bottle at 4°C (up to 2 weeks).</li> <li>· For solid CIM, add 6 g Phytoagar (Duchefa Biochemie, P1003) before autoclave. After cool-down, pour into petri dishes, and store at 4°C (up to 2 weeks).</li> </ul>	
Selection medium (SM, per liter)	CIM+Cefotaxime+Hygromycin (ex., depending on the selectable marker)
<ul style="list-style-type: none"> <li>· Add 6 g Phytoagar to CIM before autoclave.</li> <li>· After cool-down, add 1 mL of Cefotaxime stock solution (300 mg/mL) and 1 mL of Hygromycin stock solution (40 mg/mL), stir well, pour into petri dishes, and store at 4°C (up to 2 weeks).</li> </ul>	
Regeneration medium (RGM, per liter)	4.43 g Linsmaier and Skoog medium (Duchefa Biochemie, L0230); 30 g Maltose (Duchefa Biochemie, M0811)
<ul style="list-style-type: none"> <li>· Adjust pH 5.8 with 0.1 N KOH, add 6 g Phytoagar, and autoclave.</li> <li>· After cool-down, add 1 mL of Kinetin stock solution (0.2 mg/mL), 1 mL of Cefotaxime stock solution (300 mg/mL), and 1 mL of Hygromycin stock solution (40 mg/mL), stir well, pour into petri dishes, and store at 4°C (up to 2 weeks).</li> </ul>	
Rooting medium (RM, per liter)	4.43 g Murashige and Skoog medium (Duchefa Biochemie, M0222); 30 g Sucrose (Duchefa Biochemie, S0809)
<ul style="list-style-type: none"> <li>· Adjust pH 5.7 with 0.1 N KOH, add 2.5 g Phytigel (Sigma, P8169), and autoclave.</li> <li>· After cool-down, add 1 mL of Cefotaxime stock solution (300 mg/mL), and 1 mL of Hygromycin stock solution (40 mg/mL), stir well, pour into petri dishes, and store at 4°C (up to 2 weeks).</li> </ul>	

immature embryos were taken (Fig. 1a and 1b). Then, the immature embryos with approximately 0.3 mm in length (Fig. 1c) were collected in a 50 mL conical tube containing a few mL of DW to avoid desiccation. The time for the collection of immature embryos is when caryopses have grown completely to the end of the palea and seeds are still soft and pliable, which is a critical step to obtain good embryogenic calli.

**STEP 2.** Lemmas were removed from the seeds very carefully using fingers, and the de-husked seeds were placed back into the tube containing a few mL of DW.

**STEP 3.** After removal of water in the tube, seeds were surface-sterilized in 70% (v/v) ethanol for 2 min, followed by 2% (w/v) sodium hypochlorite (NaOCl) for 5 min with gentle agitation. After rinsing in sterile DW 4–5 times, kept the seeds in the corning tube with 5 mL of sterile DW. This step and all the following steps should be carried

out in a laminar-flow hood.

**STEP 4.** Approximately 10 seeds were placed on a sterile petri dish and immature embryos were isolated using a blunt forcep with a blade. The forcep was used to hold the seed and the blunt side of the blade was used to make gentle pressure on the seed near the immature embryo (Fig. 1d and 1e). The immature embryo locates at the lower end of the seed (i.e., very close to the surface), so gentle pressure is enough to take out the whole immature embryo from the seed without damage. A microscope can be used for this step if it is difficult to identify the immature embryo with naked eyes. Immature embryos with small sizes (~0.3 mm) are translucent and capable of producing good embryogenic calluses (Fig. 1f), whereas bigger size embryos (more than 0.5 mm) are white or yellow in color and usually produce soft, watery, or disorganized calli.

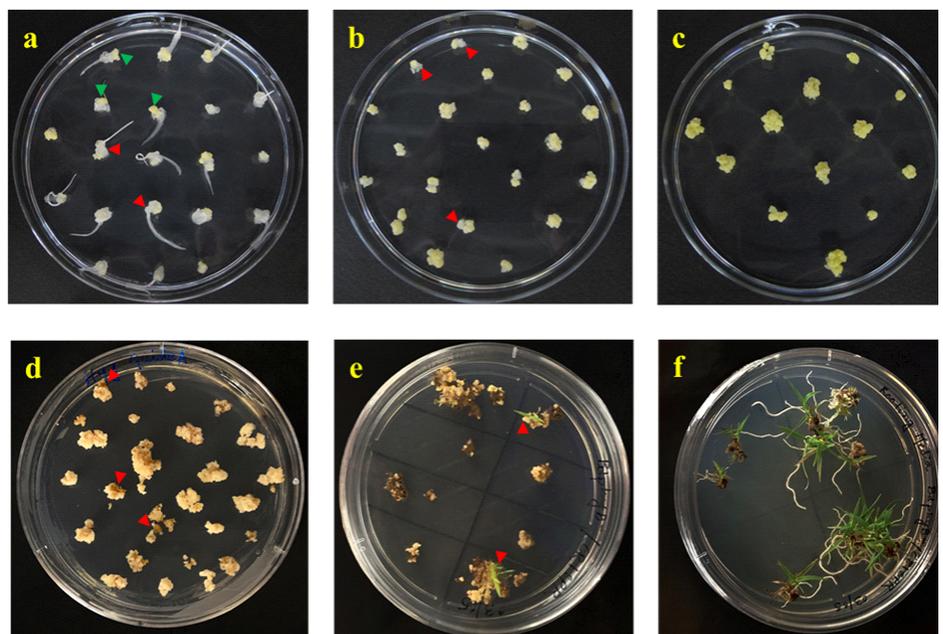


**Fig. 1.** Isolation of immature embryos for the induction of embryogenic calli. (a) Seeds obtained from different developmental stages. The seeds were arranged from the youngest on the left to the oldest on the right. Yellow arrows indicate caryopses developed during the growth. Immature embryos could be collected at the time when the caryopsis has grown close to the end of palea. Counting from left to right, the 8th and 9th seeds are ideal for the isolation of immature embryos. (b) Seeds after the removal of lemmas. The seeds are arranged from the youngest on the left to the oldest on the right, and color changes are observed from light green to yellow with maturity. Yellow arrows indicates caryopses, and the 4th and 5th seeds are ideal to dissect for immature embryos. (c) A picture depicting immature (~0.3 mm) and mature (~1 mm) embryos. Counting from the left, the 1st and 2nd embryos are immature ones that can be used for the induction of embryogenic calli, and the 3rd is a mature embryo. Scale bar, 1 mm. (d) The pressure point to push out the immature embryo from the seed. Red box indicates the position containing the immature embryo. (e) The immature embryo marked by the red circle. The blade should be held away from the immature embryo in all the steps to avoid damage. (f) Intact immature embryo isolated from seed. Marked by the red arrow.

**STEP 5.** Isolated immature embryos were transferred onto CIM plates using forcep tips. Around 20 immature embryos were placed on the plate (90×15 mm). It does not matter whether the scutellum side is facing upward or downward on the medium. However, the immature embryos should not be pushed inside the medium: i.e., surface tension is enough to hold the immature embryo on the surface of the CIM plate.

**STEP 6.** Petri dishes were sealed with clean tape and placed at 25°C for 3 to 4 weeks in the dark. In general, embryogenic calli began to form approximately a week after the incubation. Quality of the embryogenic calli could be determined by its color and structure, i.e., yellow and organized structure. It is notable that embryogenic calli of Bd21-3 are yellower than those of Bd21. In addition, it takes 3 weeks for Bd21 and 4 weeks for Bd21-3 to obtain embryogenic calli for sub-culturing to fresh CIM plates.

**STEP 7.** After 3–4 weeks, only yellow embryogenic parts from each callus were cut and transferred to fresh CIM plates (Fig. 2a), whereas amorphous parts were discarded. The plates were further incubated for 2 more weeks. In this step, it is important to select only embryogenic (i.e., high quality) calli by avoiding damaging or mashing calli. Very sharp blades should be used to make clean cuts of a callus into 4 to 5 pieces (~1 mm), and around 20 pieces of embryogenic calli were kept in one plate.



**Fig. 2.** Brachypodium transformation stages from embryogenic callus induction to root formation. (a) Three-week-old calli of Bd21 on CIM. The green arrows indicate yellow and compact embryogenic calli, and red arrows indicate white and watery calli. (b) Five-week-old calli in the first sub-culture. Red arrows indicate some watery calli that should be removed as many as possible during the next sub-culture. (c) Six-week-old calli. At this step, most calli are yellow and compact, due to the removal of watery calli. (d) Calli after 7 days of incubation on selection media containing hygromycin. Non-transformed calli became brown or black (red arrows). (e) Shoot induction after 3 weeks of incubation on regeneration media. Red arrows show shoots generated from the calli. (f) Root formation after 2 weeks of incubation on rooting media. Plantlets with 2–3 roots were transferred on soil to harvest  $T_1$  seeds.

**STEP 8.** After 2 weeks, each callus was cut into 3–4 pieces (~2 mm), transferred to fresh CIM plates, and incubated for 1 week. At this step, the calli should be appeared more organized than those in the previous sub-culture (Fig. 2b). At the end of this sub-culture, each immature embryo usually generates around 16–20 embryogenic calli.

**STEP 9.** Three days before transformation, *A. tumefaciens* (usually strain AGL1) harboring a vector with a selectable marker such as hygromycin resistance gene was cultured overnight at 28°C in 1 mL of LB containing 1 µL of carbenicillin (100 mg/mL), 1 µL of acetosyringone (200 mM), and the antibiotic for selection depending on the vector used. Next morning, 200 µL of the culture was transferred and evenly distributed on MG/L medium plates by spreading (Fig. 3a), and incubated at 28°C for 2 days.

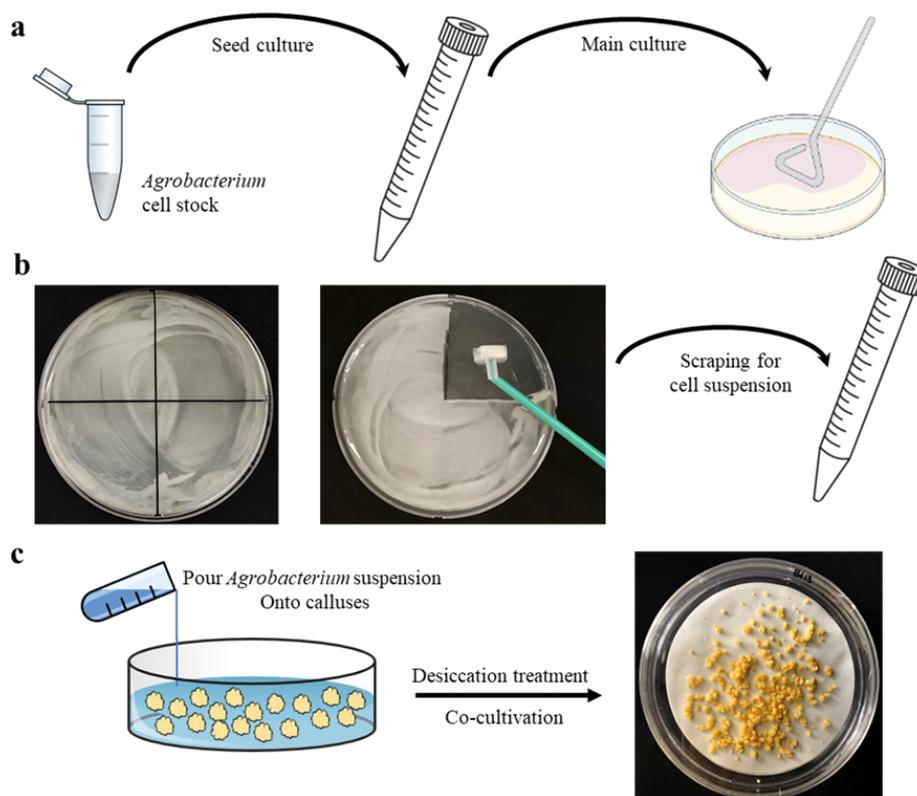
**STEP 10.** After the growth, *Agrobacterium* cells were scraped from the plates using a cell scraper (SPL Life Sciences, 90020) and transferred to a sterile 50 mL conical tube containing 10 mL of liquid CIM (Fig. 3b). Then, the cells were completely suspended using a vortex and adjusted O.D<sub>600</sub> to 0.6 using a spectrophotometer by adding more suspension or liquid CIM. Generally, 30 mL of *Agrobacterium* cell suspension is enough to transform around 200 calluses. 30 µL of 200 mM acetosyringone stock solution was added to the 30 mL of the suspension before the inoculation with embryogenic calluses.

**STEP 11.** Around 200 calluses, as shown in Fig. 2c, were taken from the CIM plates, and transferred in a sterile petri dish (100×40 mm) containing a few mL of liquid CIM to avoid desiccation. The *Agrobacterium* suspension were poured onto the calluses, the dishes were swirled for 30 s, and kept still for 5 min (Fig. 3c). At this step, breaking the calluses should be avoided as much as possible. Then, the *Agrobacterium* suspension were removed carefully using a pipette with sterile tips.

**STEP 12.** Two sterile and dry filter papers (125 mm in diameter) were placed in a petri dish (150×20 mm), and the *Agrobacterium*-inoculated calluses were transferred carefully onto the filter papers using a forcep (Fig. 3c). The calluses were left uncovered under the laminar-flow hood for 7–10 min for drying. As a note, since there is no washing step, this desiccation treatment is very important for avoiding the overgrowth of *Agrobacterium* cells during the *Brachypodium* transformation.

**STEP 13.** After the desiccation treatment, calluses were divided into two parts and transferred to two petri dishes (150×20 mm), each containing approximately 100 calluses on dry filter papers (Fig. 3c). The plates were then incubated at 25°C for 3 days in the dark. As a note, the calluses should not be over-crowded on the filter papers to provide enough spaces between the calluses for proper drying. After the co-cultivation, calluses appeared dry and shrunken, which is important to avoid *Agrobacterium* overgrowth in next steps. If the calluses appear yellow, moist, and healthy, the desiccation treatment has not been done properly, resulting in a high risk of *Agrobacterium* overgrowth problem. It is also notable that, once *Agrobacterium* overgrowth happens, it is almost impossible to kill the cells, so all the calluses would be eventually discarded. Thus, the control of *Agrobacterium* overgrowth is critical for the success of *Brachypodium* transformation.

**STEP 14.** After 3 days of co-cultivation, calluses were transferred directly onto the SM plates with hygromycin, and incubated at 25°C for a week in the dark. For



**Fig. 3.** A schematic diagram to show the steps for infection and co-cultivation of embryogenic calli with *Agrobacterium* cells. (a) Growing *Agrobacterium* cells. Starting from cell stock of *Agrobacterium* carrying a vector, cells were grown first for seed culture in LB broth, and then for main culture on a MG/L plate. (b) Preparation of *Agrobacterium* cell suspension. After incubation of 2 days at 28°C, *Agrobacterium* cells were scraped from the plate, in which one-fourth was suspended in liquid CIM with acetosyringone. (c) Co-cultivation step. *Agrobacterium* cell suspension was poured onto the calluses in a petri dish. Then, the *Agrobacterium*-inoculated calluses were transferred carefully onto the filter papers in a dish and left uncovered under the laminar-flow hood for 7–10 min for drying.

convenience, the petri dish (90×20 mm) is divided in 16 boxes by drawing 3 horizontal and 3 vertical lines at the bottom of the plate using a marker, and each callus including broken pieces is kept in one box to make enough spaces between the calluses and also for maintaining independent transgenic lines.

**STEP 15.** After a week, calluses were sub-cultured on fresh SM plates and incubated at 25°C for 2 weeks in the dark. As a note, the appearance of calluses varies depending on the selection agent, in which hygromycin is the strongest selection agent so most of the non-transformed calluses turn brown or black within a week on the SM plates (Fig. 2d). If a callus appears mostly brown/black but a small part remains healthy, only the healthy part could be transferred to fresh SM plates. As another note, if a callus is broken into pieces during the transfer, all the broken pieces should be kept in the same box on the plate, which will be considered as one independent line.

**STEP 16.** After 2 weeks, healthy calluses were transferred to RGM plates and incu-

bated at 28°C for 3 weeks under 16/8 hr light/dark cycle. During the incubation, usually green dots and shoots are emerged from the calluses (Fig. 2e). As a note, incubation at 28°C is helpful for a faster shoot regeneration than that at 25°C.

**STEP 17.** After 3 or 4 weeks, the shoots with 1–2 cm lengths were transferred to RM plates. At the same time, calluses with green dots were sub-cultured on fresh RGM plates one more time at 28°C for 3 weeks. Once shoots with 1–2 cm length were formed, they were also transferred to the RM plates.

**STEP 18.** The rooting status on the RM plates were checked periodically, and the plantlets with 2–3 healthy roots around 2–5 cm in length (Fig. 2f) were transferred to the soil. During the transfer, the phytigel should be removed carefully from the roots with DW. After the soil transfer, pots were kept covered with a clear plastic bag for 7 days to avoid desiccation. After around 12–16 weeks, the T<sub>1</sub> seeds of putative transgenic plants could be harvested.

**TIMELINE.** The process for the induction of embryogenic calli (STEP 1 to 8), starting from 7 to 9 week-old plants, takes approximately 6 weeks for Bd21 (i.e., 3+2+1 weeks) and 7 weeks for Bd21-3 (i.e., 4+2+1 weeks). The process from co-cultivation to rooting (STEP 13 to 18) takes around 9 or 10 weeks [Co-cultivation, 3 days; Selection, 1+2=3 weeks; Regeneration, 3–4 weeks; Rooting, 3 weeks]. In addition, the time to harvest T<sub>1</sub> seeds from plantlets with roots requires about 12–16 weeks, if 16/8 hr light/dark cycle was applied. Considering that embryogenic calli are supplied constantly, the overall process from co-cultivation to obtain T<sub>1</sub> seeds takes approximately 22–26 weeks, respectively. For the efficient control of *Brachypodium* transformation, it is recommended to grow new batch of plants in every 2 weeks to ensure a steady supply of immature embryos when needed.

## Conclusion

Using this method described above, we could have successfully obtained transgenic *Brachypodium* plants of two inbred lines, Bd21 and Bd21-3. When transformation efficiencies were estimated from the number of hygromycin-resistant plantlets per the number of embryogenic calluses used for co-cultivation, it was ~40% for Bd21 (~20 plantlets out of 50 embryogenic calluses) and ~60% for Bd21-3 (~30 plantlets out of 50 embryogenic calluses). Therefore, the transformation efficiencies obtained with this method are comparable with previous reports [9,10,13]. There are several factors contributing to the genetic transformation of *Brachypodium*. First, the quality of embryogenic calli is important (STEP 4 to 8). It is necessary to use immature embryos of around 0.3 mm in size (should be less than 0.5 mm) for the callus induction. In addition, only yellow and compact calli or yellowish parts with organized structure should be used for transformation by removing white and watery calli or amorphous parts. Second, co-cultivation step is critical for the successful transformation (STEP 11 to 13). Considering no washing step in this method, *Agrobacterium* overgrowth should be controlled by the desiccation treatment before co-cultivation. Once *Agrobacterium* overgrowth occurs, the calluses cannot be used for further steps. It is notable that over-desiccation

should be also avoided. Third, growing *Brachypodium* plants in a healthy condition and seed storage are important for consistent transformation. The seeds should be harvested after proper desiccation period, but watering must not be stopped while seeds are still green. If any moisture remains in the seeds, germination might not be good. Thus, seed storage at 4°C is recommended under very low humidity condition using an airtight container. Other factors such as *Agrobacterium* strain and selectable marker need to be also considered for the transformation efficiency. In our experience, *Agrobacterium* strain AGL1 grown on MG/L medium plates showed the best performance in *Brachypodium* transformation, compared with other strains such as GV3101, LBA4404, and EHA105. For the selectable marker, hygromycin resistance was the best in selecting transgenic plantlets, compared with herbicide resistance. When we used the hygromycin resistance for the selection, almost all the hygromycin-resistant plantlets are real transgenic plants. Since *Brachypodium* is now generally used as a monocot model plant, in addition to the dicot model plant *A. thaliana*, the method described here will provide potential benefits to the researchers who are interested in using *B. distachyon* as an alternative model system.

## Conflict of Interest

The authors declare no potential conflict of interest.

## Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (Ministry of Science and ICT, MSIT) (grant no. 2021R1A2C1012562).

## References

1. Stepanova AN. Plant biology research: what is next? *Front Plant Sci.* 2021;12:749104.
2. Scholthof KBG, Irigoyen S, Catalan P, Mandadi KK. *Brachypodium*: a monocot grass model genus for plant biology. *Plant Cell.* 2018;30:1673-1694.
3. Brkljacic J, Grotewold E, Scholl R, Mockler T, Garvin DF, Vain P, et al. *Brachypodium* as a model for the grasses: today and the future. *Plant Physiol.* 2011;157:3-13.
4. Draper J, Mur LAJ, Jenkins G, Ghosh-Biswas GC, Bablak P, Hasterok R, et al. *Brachypodium distachyon*. A new model system for functional genomics in grasses. *Plant Physiol.* 2001;127:1539-1555.
5. Opanowicz M, Vain P, Draper J, Parker D, Doonan JH. *Brachypodium distachyon*: making hay with a wild grass. *Trends Plant Sci.* 2008;13:172-177.
6. Girin T, David LC, Chardin C, Sibout R, Krapp A, Ferrario-Méry S, et al. *Brachypodium*: a promising hub between model species and cereals. *J Exp Bot.* 2014;65:5683-5696.

7. Kellogg EA. *Brachypodium distachyon* as a genetic model system. *Annu Rev Genet.* 2015;49:1-20.
8. Betekhtin A, Hus K, Rojek-Jelonek M, Kurczynska E, Nibau C, Doonan JH, et al. In vitro tissue culture in *Brachypodium*: applications and challenges. *Int J Mol Sci.* 2020;21:1037.
9. Alves SC, Worland B, Thole V, Snape JW, Bevan MW, Vain P. A protocol for *Agrobacterium*-mediated transformation of *Brachypodium distachyon* community standard line Bd21. *Nat Protoc.* 2009;4:638-649.
10. Vogel J, Hill T. High-efficiency *Agrobacterium*-mediated transformation of *Brachypodium distachyon* inbred line Bd21-3. *Plant Cell Rep.* 2008;27:471-478.
11. Thole V, Vain P. *Agrobacterium*-mediated transformation of *Brachypodium distachyon*. In: Dunwell JM, Wetten AC, editors. Vol. 847. *Transgenic plants: methods and protocols*. Totowa, NJ: Humana; 2012. pp. 137-149.
12. Collier R, Bragg J, Hernandez BT, Vogel JP, Thilmony R. Use of *Agrobacterium rhizogenes* strain 18r12v and paromomycin selection for transformation of *Brachypodium distachyon* and *Brachypodium sylvaticum*. *Front Plant Sci.* 2016;7:716.
13. Chen F, Liu Q, Vogel JP, Wu J. *Agrobacterium*-mediated transformation of *Brachypodium distachyon*. *Curr Protoc Plant Biol.* 2019;4:e20088.
14. Yu G, Wang J, Miao L, Xi M, Wang Q, Wang K. Optimization of mature embryo-based tissue culture and *Agrobacterium*-mediated transformation in model grass *Brachypodium distachyon*. *Int J Mol Sci.* 2019;20:5448.
15. Hus K, Betekhtin A, Pinski A, Rojek-Jelonek M, Grzebelus E, Nibau C, et al. A CRISPR/Cas9-based mutagenesis protocol for *Brachypodium distachyon* and its allopolyploid relative, *Brachypodium hybridum*. *Front Plant Sci.* 2020;11:614.
16. The International *Brachypodium* Initiative. Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature.* 2010;463:763-768.
17. Hasterok R, Catalan P, Hazen SP, Roulin AC, Vogel JP, Wang K, et al. *Brachypodium*: 20 years as a grass biology model system; the way forward? *Trends Plant Sci.* 2022;27:1002-1016.
18. Hwang HH, Yu M, Lai EM. *Agrobacterium*-mediated plant transformation: biology and applications. *Arabidopsis Book.* 2017;15:e0186.
19. Vogel JP, Garvin DF, Leong OM, Hayden DM. *Agrobacterium*-mediated transformation and inbred line development in the model grass *Brachypodium distachyon*. *Plant Cell Tissue Organ Cult.* 2006;84:199-211.