Plant Biotechnology 27, 349–351 (2010)

Technical Note

The floral inoculating protocol: a simplified *Arabidopsis thaliana* transformation method modified from floral dipping

Mari Narusaka[1], Tomonori Shiraishi[2], Masaki Iwabuchi[1], Yoshihiro Narusaka[1,*]

[1] Research Institute for Biological Sciences (RIBS) Okayama, Okayama 716-1241, Japan; [2] Laboratory of Plant Pathology and Genetic Engineering, Faculty of Agriculture, Okayama University, Okayama 700-8530, Japan
* E-mail: yo_narusaka@bio-ribs.com Tel: +81-866-56-9450 Fax: +81-866-56-9453

Received December 3, 2009; accepted April 16, 2010 (Edited by J. Yamaguchi)

Abstract The floral dip protocol mediated by *Agrobacterium tumefaciens* is the most widely used transformation method for *Arabidopsis thaliana*. The "floral dip" process in which *A. thaliana* flower buds are dipped in an *Agrobacterium* cell suspension requires large volumes of bacterial cultures grown in liquid media, large shakers and centrifuges, and experimental space for them. These factors limit the number of transformations that can occur at once. We established that *A. thaliana* can be transformed by inoculating 5 μl of *Agrobacterium* cell suspension in flower buds, thus avoiding the use of large volumes of *Agrobacterium* culture. Using this modified protocol, we obtained 15–50 transgenic plants per transformation from each pot containing 3 *A. thaliana* plants. The protocol is satisfactory to be used for subsequent analyses. This simplified method, without floral dipping, which requires large volumes of *Agrobacterim* culture, offers as efficient a transformation as previously reported protocols. This method reduces the required workload, cost, time, and space. Furthermore, an important aspect of this modified protocol is that it allows many independent transformations to be performed at once.

Key words: *Agrobacterium*, *Arabidopsis*, floral dip, floral inoculation, transformation.

The development of DNA sequence technologies and genome sequencing projects has resulted in the rapid progress of gene function studies. In plant functional genomic studies, transformation of plants is a powerful and useful research tool for gene discovery, new insights into gene function, and investigation of genetically controlled characteristics. In addition, plant transformation technology offers opportunities for basic scientific studies. Furthermore, it enables the introduction of novel, useful genes into crops and the creation of new genetically modified organisms within a short period of time.

The first method to describe an *in planta* transformation included the use of tissue culture and plant regeneration (Feldmann and Marks 1987). In *A. thaliana*, the *Agrobacterium* vacuum (Bechtold et al. 1993) and floral dip (Clough and Bent 1998) are efficient methods to generate transgenic plants. These methods allow plant transformation without the need for tissue culture. Therefore, the floral dip protocol markedly advanced the ease of creating transformants in *A. thaliana*, and is the most widely used transformation method. These procedures were later simplified and substantially improved (Davis et al. 2009; Zhang et al. 2006), which significantly reduced the required workload, cost, and time as compared with earlier procedures.

However, these transformation methods have problems. The floral dip process in which *A. thaliana* flower buds are dipped in an *Agrobacterium* cell suspension, requires large volumes of bacterial cultures grown in liquid media, large shakers and centrifuges, and experimental space for them. These factors limit the number of transformations. Here we describe an improved method for *Agrobacterium*-mediated transformation that does not require large volumes of bacterial liquid cultures necessary for the process of floral dipping.

Until now, only a limited number of constructs could be transformed into plants at a time because of difficulties in growing large volumes of *Agrobacterium* due to limits in both shaker capacity and experimental space. Therefore, we focused on improvements in the floral dipping procedure. The problem of space and volume can be solved by employing small culture volume. Each plant is transformed with 30–50 μl of bacteria grown in 2 ml of liquid culture. Our present method is a simple modification of the protocol reported by Clough and Bent (1998). We describe our protocol below.

Recent papers (Liu et al. 2008; Zhang et al. 2006) illustrate the floral dipping process. Clough and Bent

This article can be found at http://www.jspcmb.jp/

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

(1998) reported that neither Murashige and Skoog (MS) salts and hormones nor optical density (OD) of *Agrobacterium* makes a difference for transformation efficiency. An *Agrobacterium* cell suspension containing 0.01–0.05% Silwet L-77 (vol/vol) was employed to allow uptake of *Agrobacteria* into female gametes, instead of vacuum-aided infiltration of inflorescences.

1. Plant health is an important factor and healthy *A. thaliana* plants should be grown until they are flowering.
   There are 2 different methods: standard procedure (A) and quick procedure (B) (Zhang et al. 2006). We generally use the quick method, which is useful for rare seeds and seeds with low germination frequency. It is also used to retransform a transgenic line with a second construct.
   Standard procedure (A): Suspend seeds in 0.1% (wt/vol) agar solution and keep them in darkness for 2–4 days at 4°C to break dormancy. Sow seeds on wet soil (potting soil No. 2 for cutting, Dio Chemicals, Ltd., Tokyo, Japan) in a 3-inch pot and grow under long days (16-h light/8-h dark) at 22°C.
   Quick procedure (B): Sterilize seeds by treating with 70% (vol/vol) ethanol for 1 min and immerse it in sodium hypochlorite solution containing 1% available chlorine and 0.02% (vol/vol) Tween 20 for 7 min. Wash seeds 5 times with sterile distilled water. Place seeds on MS medium (1×MS plant salt mixture [Wako Pure Chemical Industries, Osaka, Japan], 1×Gamborg's vitamin solution [Sigma-Aldrich], 1% (wt/vol) sucrose, 0.05% (wt/vol) MES, and pH 5.7 adjusted with 1 N KOH) containing 0.8% (wt/vol) Bacto agar (Difco). Keep them in darkness for 2–4 days at 4°C to break dormancy. Grow under long days (16-h light/8-h dark) for 3 weeks at 22°C, and then transfer them to wet soil (Step 1(A)) in a 3-inch pot (3 seedlings/pot) without covering with a bridal veil, window screen, or cheesecloth.
2. Clip the first bolts to encourage proliferation of secondary bolts (Figure 1A). Plants will be ready approximately 4–6 days after clipping (Figure 1B).
3. Prepare *Agrobacterium tumefaciens* strain carrying the gene of interest on a binary vector. Spread a single *Agrobacterium* colony on an LB agar plate with suitable antibiotics. Incubate the culture at 28°C for 2 days.
4. Use feeder culture to inoculate a 2-ml liquid culture in LB with suitable antibiotics to select for the binary plasmid in a 10–15 ml Falcon tube at 28°C for 16–24 h. You can use mid-log cells or a freshly saturated culture (Clough and Bent 1998).
   Optional: If needed, keep 500 μl of *Agrobacteria* culture in 25% (vol/vol) glycerol stock at −80°C.
5. Spin down 1.5 ml of *Agrobacterium* cell suspension using 2 ml Eppendorf tubes and resuspend in 1–1.2 ml transformation buffer (1/2×MS plant salt mixture, 1×Gamborg's vitamin solution, 5% (wt/vol) sucrose, and pH 5.7, adjusted with 1 N KOH). Adjust of the OD_{600} value is not required. Each small pot containing 3 plants will require approximately 150 μl of culture.
   Optional: You may use 5% (wt/vol) sucrose solution instead of the transformation buffer.
6. Just before inoculation, add Silwet L-77 to a concentration of 0.02% (vol/vol), and mix well immediately.
   Optional: If using the transformation buffer, add 0.01 $\mu g\ ml^{-1}$ 6-benzylaminopurine (BAP) just before transformation.
7. Put 5 μl of *Agrobacterium* inoculum in the flower buds (Figure 1B, C, D). Each plant is inoculated

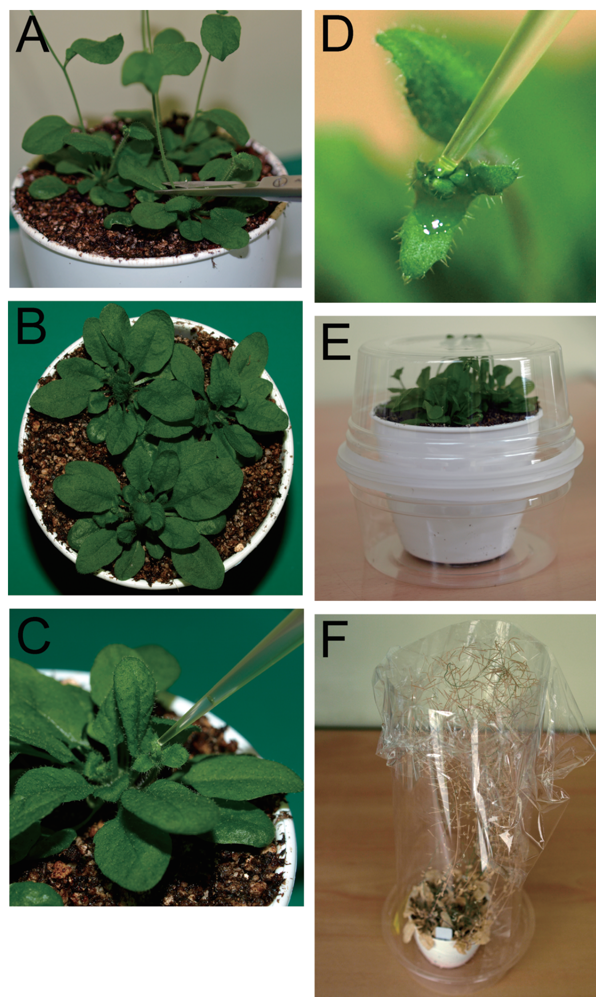

Figure 1. Floral inoculating transformation of *Arabidopsis thaliana*. (A) Clipping the first bolts. (B) The growth stage for transformation begins when the plants have just started to flower after clipping the first bolts. (C, D) Using a micropipettor, inoculate 5 μl of *Agrobacterium* in flower buds. (E) Place inoculated plants under a dome or cover for 16 to 24 h to maintain high humidity. (F) Remove the cover and grow the plants in a greenhouse or growth chamber until maturity.

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

Table 1. Transformation efficiency by the floral inoculation method.

Vector	Antibiotic marker(final concentration)	Ecotype	% Transformation[a]
pBI101	kanamycin (30 μg ml^{-1})	Columbia (Col-0)	0.32 ± 0.02
		Wassilewskija (Ws-0)	0.86 ± 0.12
pGWB1[b]	kanamycin (30 μg ml^{-1}) hygromycin (20 μg ml^{-1})	Wassilewskija (Ws-0)	0.31 ± 0.05

[a] Values are mean± SE.
[b] Refer to Nakagawa et al. (2007).

with 30–50 μl of *Agrobacterium* inoculum.

8. Place inoculated plants under a dome or cover for 16–24 h to maintain high humidity (Figure 1E). Avoid excessive exposure to light.
   Optional : For higher rates of transformation, newly forming flower buds may be inoculated with *Agrobacterium* 2–3 times at 7-day intervals.
9. Water and grow plants normally, tying up loose bolts with wax paper, tape, stakes, twist-ties, or other means. Stop watering as seeds become mature (Figure 1F).
10. Harvest dry seed. Transformants are usually all independent, but are guaranteed to be independent if they come from separate plants.
11. Surface-sterilize seeds by immersion in 70% (vol/vol) ethanol for 1 min, followed by immersion in sodium hypochlorite solution containing 1% available chlorine and 0.02% (vol/vol) Tween 20 for 10 min. Then, wash seeds 5 times with sterile distilled water.
12. To select for transformed plants, liquid-sterilized seeds were resuspended in approximately 8 ml of 0.1% (wt/vol) agar solution containing 2 mg ml^{-1} claforan (Aventis Pharma AG). They were then sowed in MS medium (Step 1 (B)), 0.8% Bacto agar and appropriate antibiotics or herbicide selective markers in the following concentrations: kanamycin (final concentration 30 μg ml^{-1}), hygromycin (20 μg ml^{-1}) and bialaphos (7.5 μg ml^{-1}). Claforan is necessary for decontamination of *Agrobacteria*.
13. Transplant putative transformants to soil (Step 1(A)). Grow, test, and use.

By this modified protocol we obtained 15–50 transgenic plants per transformation from each pot containing 3 *A. thaliana* plants (Table 1). The protocol is satisfactory to be used for subsequent analyses. This simplified method without inverting plants or floral dipping, which requires large volumes of *Agrobacterium* culture, offers as efficient a transformation as previously reported protocols. The method allows us to reduce the required workload, cost, time, and space. Furthermore, an important aspect of this modified protocol is that it allows many independent transformations to be performed at once.

Acknowledgements

We would like to thank Nami Hosaka (RIBS, Okayama) for their technical assistance and Tsuyoshi Nakagawa (Shimane Univ.) for providing pGWB1. This work was supported in part by an Industrial Technology Research Grant Program in 2004 and 2009 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan, by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) to YN, and by a Grant-in-Aid for Scientific Research (KAKENHI) (21580060 to YN, 21780038 to MN).

References

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C R Acad Sci Paris, Life Sci* 316: 1194–1199

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium* mediated transformation of *Arabidopsis thaliana*. *Plant J* 16: 735–743

Davis AM, Hall A, Millar AJ, Darrah C, Davis SJ (2009) Protocol: Streamlined sub-protocols for floral-dip transformation and selection of transformants in *Arabidopsis thaliana*. *Plant Meth* 5: 1–7

Feldmann KA, Marks MD (1987) *Agrobacterium* mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non-tissue culture approach. *Mol Gen Genet* 208: 1–9

Liu NY, Zhang ZF, Yang WC (2008) Isolation of embryo-specific mutants in *Arabidopsis*: plant transformation. *Meth Mol Biol* 427: 91–100

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J Biosci Bioeng* 104: 34–41

Zhang X, Henriques R, Lin SS, Niu QW, Chua NH (2006) *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat Protocol* 1: 1–6

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology