BRIEF REPORT

High transformation efficiency in Arabidopsis using extremely low *Agrobacterium* inoculum [version 1; peer review: awaiting peer review]

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Abstract

Agrobacterium-mediated transformation methods have allowed the stable introduction of target genes into the nuclear genomes of recipient plants. Among them, the floral dip approach represents the simplest due to its straightforwardness and high transformation efficiency. In a standard floral dip protocol that most researchers follow, *Agrobacterium* cells are grown to stationary phase (OD_{600}≈2.0) in large cultures and resuspended in inoculation medium to OD_{600}≥0.8. Here, we tested the effects of low *Agrobacterium* inoculum on transformation rate. Our data revealed that the floral dip method still guarantees relatively high transformation rate in *Arabidopsis thaliana* Col-0 ecotype even with very low *Agrobacterium* inoculum (OD_{600}=0.002). Our finding thus simplifies the floral dipping protocol further, which allows transformation with small bacterial culture and enables high-throughput transformation of large numbers of constructs in parallel.

Keywords

Arabidopsis, floral dip, transformation

Open Peer Review

Reviewer Status *AWAITING PEER REVIEW*

Any reports and responses or comments on the article can be found at the end of the article.

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Introduction

Plant transformation integrates foreign genes into the plant nuclear genome. The development of different transformation protocols in various plants has enabled advances in plant molecular biology and crop improvement. *Agrobacterium* is routinely used as a plant gene transformation vehicle as it naturally possesses the ability to transfer a segment of its plasmid DNA (T-DNA) into its host nucleus, which ultimately leads to integration of the T-DNA into the nuclear genome (Tzfira *et al.*, 2004). During 1980s and early 1990s, generating transgenic plants by leaf disc-based *Agrobacterium*-mediated transformation requires laborious plant tissue culture and regeneration steps. In 1993, a simple floral vacuum infiltration method was developed in Arabidopsis for stable transformation, overcoming the tedious tissue culture requirements (Bechtold *et al.*, 1993). Later, the vacuum infiltration step was replaced by floral dipping where the developing floral tissues are dipped into a solution containing *Agrobacterium*, sucrose and the surfactant Silwet L-77 (Clough & Bent, 1998). Because of the simplicity and reliability of this floral dip method, it is now the commonly used transformation method in Arabidopsis. This protocol has also been shown to work in certain *Brassicaceae* plants (Bent, 2006). Floral dip transformation may be feasible in plants such as wheat and *Setaria viridis* (Agarwal *et al.*, 2009).

In *Agrobacterium*-mediated transformation protocols, the concentration of bacterial inoculum has been considered crucial to the success of plant transformation. In the commonly used floral dip protocol, bacterial cells are grown to stationary phase (OD_{600}=2.0), pelleted and resuspended in inoculation medium to OD_{600}≥0.8 (Clough & Bent, 1998; Zhang *et al.*, 2006). Here, we tested whether low concentration of *Agrobacterium* inoculum affects the plant transformation rate. Our data showed that, in contrary to our expectation, using extremely low density of *Agrobacterium* inoculum (OD_{600}=0.002) in floral dip method still warrants relatively high transformation rate in Arabidopsis.

Method

Plant materials and growth conditions

Arabidopsis Col-0 wild type plants were grown in a growth room under long day (16 h light/8 h dark cycle) at 23°C. Seedlings were grown at a density of 30–40 per 64 cm^2 (8 cm × 8 cm) pot in moistened potting soil initially and transplanted to 64 cm^2 pots with eight plants per pot when they were two weeks old. After plants bolted and floral buds are formed (~30-day-old), they were used in floral dip transformation.

Culture of *Agrobacterium tumefaciens*

The plasmid pCambia1305-3flag-NOS was transformed into *Agrobacterium tumefaciens* strain GV3101 (Van Larebeke *et al.*, 1974) by mixing the plasmid DNA with the bacterial cells in a 1mm gap cuvette (BTX, #45-0124) followed by electroporation for 5 millisecond at 1,500 volts using the ECM 399 Electroporation System (BTX, #45-0000) (Gao *et al.*, 2009). The resulting strain was used in the plant transformation experiments. Bacteria were grown overnight in sterilized 4 ml LB media (Bio Basic Inc., #SD7002) with kanamycin, gentamicin and rifampicin antibiotics (50 µg/ml each, Bio Basic Inc. #KB0286, #GB0217, #RB0808) in a 28°C shaker (New Brunswick Scientific Co G25 Controlled Environment Incubator Shaker). Then the overnight culture was diluted into 100 ml LB media with kanamycin (50 µg/ml) and allowed to grow further for 8 h in the same shaker. The bacteria were collected by centrifugation (Thermo Scientific, Sorvall Legend X1R) at 6000 g for 10 min at room temperature and then resuspended in 100 ml floral dip medium to final OD_{600} of 1, 0.1, 0.01, and 0.002 (measured by BioSpec-1601 UV-visible spectrophotometer from SHIMADZU) prior to use. The floral dip medium contained 5.0% (w/v) sucrose (Bio Basic Inc. #SB0498) and 0.01% (v/v) Silwet L-77 (PhytoTechnology Laboratories #S7777) in distilled water.

Floral dip transformation

For floral dip, pots were tilted and floral buds were submerged in bacterial suspension with 30 sec of gentle agitation. The dipped plants were then covered with a tall clear-plastic dome to maintain humidity. Plants were placed in a dark room overnight before being moved back to the growth room. The domes were removed approximately 48 h after the floral dip treatment. Plants were grown for another 30–32 days until siliques became brown and dry. Each pot with 8 plants were transformed separately. For each concentration of *Agrobacterium* inocula, 4–6 pots of plants were transformed depending on the number of plants available for transformation in each experiment, this varied mainly due to the uneven germination of the seeds in each experiment. About 6000 seeds were bulk harvested from the plants grown in a pot. Seeds were harvested by gentle stripping of dried inflorescences by fingers above a piece of clean paper. The debris from the stem and pods was removed from the seeds by gentle blowing. Seeds were kept in a 37°C incubator for two days for desiccation.

Selection of transformants

Prior to selection, seeds were surface sterilized with 20% (v/v) bleach (Clorox Regular Bleach) containing 0.1% (v/v) Tween20 (Sigma-Aldrich #P1379) for 1min, followed by three times rinse with sterile water. The sterilized seeds were suspended in 0.1% (w/v) sterile agar (Bio Basic Inc. #FB0010) and plated on hygromycin selection plates (1/2 MS medium, Murashige & Skoog Basal Medium with Vitamins from PhytoTechnology Laboratories #M519 and 50 µg/ml hygromycin, Bio Basic Inc. #HD0230) at a density of approximately 3000 seeds (0.06 gram by weight) per 92×12mm (diameter×height) petri plate (Sarstedt #82.1473.001). Seeds collected from each pot (4–6 pots for each concentration of *Agrobacterium* inocula) were plated on a separate selection plate. Plates were placed in 4°C refrigerator for two days before moved to a plant growth chamber (16 h light/8 h dark cycle, Conviron Model A1000). The plants were grown at 23°C for 10 days before transformants were identified as hygromycin-resistant seedlings that produced green leaves and well-established roots grown on the selective medium. The experiment was repeated three times by transforming independently grown plants with different concentrations of *Agrobacterium* inocula.

Statistical analysis

Analysis of statistical differences between transformation rates from different concentrations of *Agrobacterium* inocula was performed by one-way ANOVA using Microsoft® Office Excel version 16.35 (20030802).

Results and discussion

Four *Agrobacterium* inocula from high to low concentrations (OD_{600}=1, 0.1, 0.01, 0.002) were used in floral dip transformation to test the effect of bacterial concentration on the transformation rate. As shown in Figure 1 (Underlying data (Wang, 2020)), similar transformation rate (approximately 0.60%) was observed under all tested bacterial concentration. Notably, the transformation efficiency remains unchanged even though the Agrobacterium inoculum was diluted 500 times form OD_{600} = 1 to OD_{600} = 0.002. Therefore, it is feasible to dramatically reduce the Agrobacterium inoculum concentration in the floral dip method. Regardless of the inoculum concentration, transforming eight Arabidopsis plants grown in a single pot produced about 36 T1 transgenic lines on average, which is sufficient for most studies.

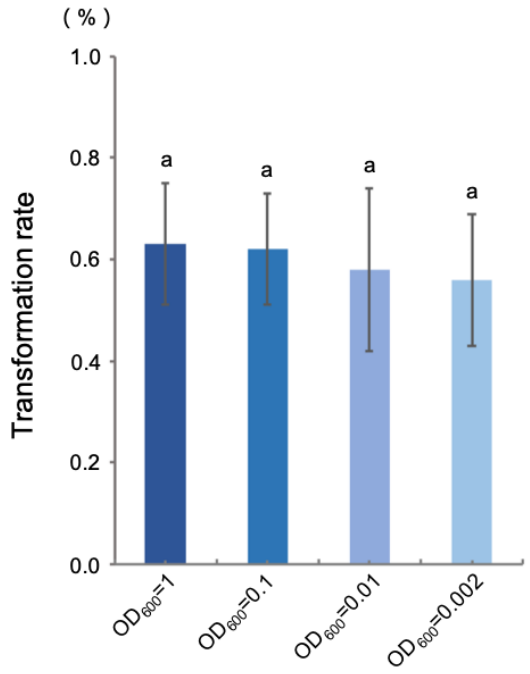

Figure 1. The effect of *Agrobacteria* concentration on transformation rate in floral dip method. Transformation rates were calculated as [(# of hygromycin-resistant seedlings) / (total # seedlings tested)] × 100%. The data are shown as mean ± SE from six independent repeats. The same letters denote no statistically significant difference according to one-way ANOVA ($p<0.05$).

Standard floral dip protocols use high concentrations of *Agrobacterium* inoculum, which requires growing large bacterial cultures (Clough & Bent, 1998; Zhang *et al.*, 2006). Our study showed that *Agrobacterium* inoculum can be diluted to as low as OD_{600} = 0.002 without sacrificing the transformation efficiency. Thus, the volume of bacterial culture used in each transformation experiment could be greatly reduced. For example, diluting 0.1 ml of overnight culture (OD_{600} = 2) to OD_{600} = 0.002 gives ~100 ml bacterial inoculum, which is sufficient in most transformation experiments. Such improvement allows researchers to culture small volume of a large number of *Agrobacterium* strains in parallel and use the diluted cultures to carry out high-throughput transformation of a large number different constructs into Arabidopsis plants.

Data availability

Open Science Framework: High transformation efficiency in Arabidopsis using extremely low Agrobacterium inoculum project.

https://doi.org/10.17605/OSF.IO/YF6AE (Wang, 2020)

This project contains the following underlying data:

- Transformation efficiency.xlsx (raw data of results from transformation using different *Agrobacterium* concentrations)

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