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BRIEF REPORT

High transformation efficiency in *Arabidopsis* using extremely low *Agrobacterium* inoculum [version 1; peer review: 1 approved, 1 approved with reservations]

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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 ($OD_{600} \approx 2.0$) in large cultures and resuspended in inoculation medium to $OD_{600} \geq 0.8$. 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

Approval Status

	1	2
version 2 (revision) 16 Sep 2020		
version 1 13 May 2020	view	view
<p>1. Ying Wang, Carleton University, Ottawa, Canada</p> <p>Shelley Hepworth , Carleton University, Ottawa, Canada</p> <p>2. Zhanyuan J Zhang, University of Missouri, Columbia, USA</p> <p>Any reports and responses or comments on the article can be found at the end of the article.</p>		

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Author roles: Wang Y: Data Curation, Formal Analysis, Investigation, Writing – Original Draft Preparation; Yaghmaiean H: Conceptualization, Investigation, Methodology; Zhang Y: Conceptualization, Funding Acquisition, Methodology, Project Administration, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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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 improvements. *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 $OD_{600}\geq 0.8$ (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 cm² (8 cm × 8 cm) pot in moistened potting soil initially and transplanted to 64 cm² 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

Figure 1. The effect of *Agrobacterium* 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 ($p < 0.05$).

(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 from $OD_{600} = 1$ 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.

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)

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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Current Peer Review Status:

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Reviewer Report 27 May 2020

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Zhanyuan J Zhang

Plant Biotechnology Innovation Laboratory, Division of Plant Sciences, University of Missouri, Columbia, MO, USA

The work showed an interesting result that is contrary to the common practice in *Arabidopsis* floral dip transformation. The authors demonstrated that the use of an unusually low concentration of *Agrobacterium* inoculum (OD600=0.002) could achieve the same transformation rate as a much higher concentration (OD600=1) does. They also discover a practical implication by using a small amount of *Agrobacterium* culture. The manuscript is well-written and results are convincing with a sound conclusion. The statistical method was appropriate as well.

Minor revisions will be needed, though:

Please indicate the *Agrobacterium* cell density (OD600 value (values)) at the time of harvest before the *Agrobacterium* cells were resuspended to OD600=0.001-1.0. This is an important growth parameter for *Agrobacterium*. If other users would harvest *Agrobacterium* cells at a too early stage, say, well before the log growth phase, with the same cell density of OD600=0.002, they may not obtain the same transformation rate. In other words, the description of the *Agrobacterium* growth phase (lag, log, stationary, etc.) to be harvested will be important.

"*Agrobacteria*" should be "*Agrobacterium*" when it is used as an adjective such as *Agrobacterium* concentration. Please make corrections throughout the paper.

The other reviewer has pointed out all the written and grammatical issues that I agree with, so no need for me to raise them again.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant tissue culture and transformation and plant molecular biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 04 Sep 2020

Yuelin Zhang, University of British Columbia, Vancouver, Canada

We sincerely appreciate the support and constructive reviews from the reviewers. We have revised our manuscript according to the comments. Our point-to-point responses to comments are listed below.

Minor revisions will be needed, though:

Please indicate the Agrobacterium cell density (OD600 value (values)) at the time of harvest before the Agrobacterium cells were resuspended to OD600=0.001-1.0. This is an important growth parameter for Agrobacterium. If other users would harvest Agrobacterium cells at a too early stage, say, well before the log growth phase, with the same cell density of OD600=0.002, they may not obtain the same transformation rate. In other words, the description of the Agrobacterium growth phase (lag, log, stationary, etc.) to be harvested will be important.

This is an excellent point. The OD600 value of the Agrobacterium cell density at the time of harvest is now added to the Method section. They were between 1.5-1.8, which was before reaching the stationary phase.

"Agrobacteria" should be "Agrobacterium" when it is used as an adjective such as Agrobacterium concentration. Please make corrections throughout the paper.

Corrected as suggested.

Competing Interests: No competing interests.

Reviewer Report 26 May 2020

<https://doi.org/10.5256/f1000research.25880.r63373>

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Ying Wang

Department of Biology, Institute of Biochemistry, Carleton University, Ottawa, ON, Canada

Shelley Hepworth

Department of Biology, Institute of Biochemistry, Carleton University, Ottawa, ON, Canada

The floral dip method for the transformation of Arabidopsis plants has been a revolutionary tool in plant biology. Several refinements to the popular protocol of Clough and Bent (1998) have been published.

This report shows that Agrobacterium cultures diluted to as low as OD600=0.002 yielded a transformation rate similar to infection with regular-density suspensions (OD600=1). Down-scaling in this way can save time, space, and expense -- especially important for high-throughput transformation experiments.

The article is well-written and the data convincing. Other steps of the transformation process such as plant preparation and downstream selection remain labor intensive, so follow-up experiments are important. The Arabidopsis Col-0 ecotype used in this study is relatively easy to transform. It will be interesting to see if low Agro concentrations are equally suitable for other ecotypes / mutant genetic backgrounds or if the method can be combined with other efficiencies like making the Agrobacterium solution directly from plates to further avoid culturing and sub-culturing steps.

Minor comments:

1. Please see [attached PDF](#) for small corrections in grammar.
2. Floral dip transformation is feasible in quite a few other species besides the ones listed (e.g. Bastaki and Cullis, 2014, references therein).
3. Clarify the figure legend of Figure 1. Was this one representative experiment of six pots of plants? Or an average of the three independent trials mentioned in the materials and methods.
4. Unable to access the underlying raw dataset - check the file is attached to the link.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

No

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant development, Arabidopsis thaliana, molecular genetics, flowering, meristems, transcription factors, gene expression, transgenic plants

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 04 Sep 2020

Yuelin Zhang, University of British Columbia, Vancouver, Canada

We sincerely appreciate the support and constructive reviews from the reviewers. We have revised our manuscript according to the comments. Our point-to-point responses to comments are listed below.

Minor comments:

1. Please see attached PDF for small corrections in grammar.

Thanks a lot for your suggestion. We have made the suggested corrections.

2. Floral dip transformation is feasible in quite a few other species besides the ones listed (e.g. Bastaki and Cullis, 2014, references therein).

References for floral dip transformation in species other than Arabidopsis have been added to the revised manuscript as suggested.

3. Clarify the figure legend of Figure 1. Was this one representative experiment of six pots of plants? Or an average of the three independent trials mentioned in the materials and methods.

Figure 1 is one representative experiment of six pots of plants. This is clarified in the revised legend for figure 1.

4. Unable to access the underlying raw dataset - check the file is attached to the link.

We checked the access to the raw dataset, there is no problem with the link. The data is under "Archive of OSF Storage" inside the "File" sign shown on the left side of the page.

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Competing Interests: No competing interests.

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