The Plant Journal (1998) **16**(6), 735–743

TECHNICAL ADVANCE

Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*

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Summary

The *Agrobacterium* vacuum infiltration method has made it possible to transform *Arabidopsis thaliana* without plant tissue culture or regeneration. In the present study, this method was evaluated and a substantially modified transformation method was developed. The labor-intensive vacuum infiltration process was eliminated in favor of simple dipping of developing floral tissues into a solution containing *Agrobacterium tumefaciens*, 5% sucrose and 500 microliters per litre of surfactant Silwet L-77. Sucrose and surfactant were critical to the success of the floral dip method. Plants inoculated when numerous immature floral buds and few siliques were present produced transformed progeny at the highest rate. Plant tissue culture media, the hormone benzylamino purine and pH adjustment were unnecessary, and *Agrobacterium* could be applied to plants at a range of cell densities. Repeated application of *Agrobacterium* improved transformation rates and overall yield of transformants approximately twofold. Covering plants for 1 day to retain humidity after inoculation also raised transformation rates twofold. Multiple ecotypes were transformable by this method. The modified method should facilitate high-throughput transformation of *Arabidopsis* for efforts such as T-DNA gene tagging, positional cloning, or attempts at targeted gene replacement.

Introduction

Plant transformation technology offers an array of opportunities for basic scientific research and for modification of food and fiber crops. Transgenic plants are typically produced by complex methods that require careful preparation of plant cells or tissues, introduction of DNA using *Agrobacterium tumefaciens* or particle bombardment, selection of transformed cell lines, and regeneration of plants (Christou, 1996; Hooykaas and Schilperoort, 1992; Siemens and Schieder, 1996; Weising *et al.*, 1988). These transformation methods require time, skilled labor and relatively expensive laboratory facilities. In contrast, the '*Agrobacterium* vacuum infiltration' method is a relatively new and simple procedure for transformation of *Arabidopsis thaliana* (Bechtold *et al.*, 1993). In its original form, the method involved the growth of *Arabidopsis* to flowering stage, uprooting of plants, application of *Agrobacterium* to whole plants via vacuum infiltration in a sucrose/hormone growth medium, re-planting, collection of seed a few weeks later, and identification of transformed progeny by selection on media containing antibiotic or herbicide (Bechtold *et al.*, 1993). The technique, which can be viewed as an extension of earlier *in planta* transformation methods (Chang *et al.*, 1994; Feldmann and Marks, 1987; Feldmann, 1992; Katavic *et al.*, 1994), offered a substitute for widely utilized *Arabidopsis* transformation methods that involved root tissue culture and plant regeneration (e.g. Valvekens *et al.*, 1988). With vacuum infiltration and other *in planta* transformation methods, most transformed progeny are genetically uniform (non-chimeric) and the somaclonal variation associated with tissue culture and regeneration is minimized. Transformed progeny are typically hemizygous for the transgene at a given locus, suggesting that transformation occurs after the divergence of anther and ovary cell lineages (Bechtold *et al.*, 1993; Feldmann, 1992). Likely targets of heritable transformation are therefore the gametophyte-progenitor tissues, mature gametophytes, or recently fertilized embryos.

The primary reasons for the popularity of the *Agrobacterium* vacuum infiltration method have been its simplicity and reliability. The elimination of tissue culture and regeneration greatly reduces hands-on time, and success can be achieved by non-experts (Bechtold *et al.*, 1993; Bent and Clough, 1998). Transformed plants can be obtained at sufficiently high rates so that the procedure can be used not only to introduce specific gene constructs into plants, but also as a random mutagenesis method for gene-tagging (e.g. Azpiroz-Leehan and Feldmann, 1997; Hirsch *et al.*, 1998; Koncz *et al.*, 1989; Mollier *et al.*, 1995; Richardson *et al.*, 1998). The primary drawback of the method is that successful application has only been reported for one plant species, *Arabidopsis thaliana*.

To improve this very widely used *Arabidopsis* transformation method and to gain insights that may facilitate transformation of other plant species, we sought to test a number of parameters involved in the transformation

Received 26 June 1998; revised 15 October 1998; accepted 26 October 1998.
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method. Some of these parameters had also been suggested and explored in unpublished studies by ourselves and other *Arabidopsis* researchers (e.g. http://genome-www.stanford.edu/cgi-bin/biosci_arabidopsis). The present report offers a substantially simplified protocol that is the product of controlled and replicated studies. Components of the infiltration medium have been eliminated, as has the laborious vacuum infiltration process and the uprooting and re-planting of plants during application of *Agrobacterium*. Plant growth stages have been identified at which a maximal number of transformants can be obtained. Expertise with recombinant DNA methods is still required to generate the desired gene constructs, but stable introduction of a DNA construct into the *Arabidopsis* genome is possible with minimal labor, equipment or specialized reagents. These modifications may be most important for use in larger scale transformation projects such as enhancer-trapping and other forms of gene-tagging mutagenesis, 'focused shotgun complementation' during the late stages of a positional cloning project, or attempts at site-specific gene replacement.

Results

Plant growth stage

Of the many variables present in the germ-line transformation protocol, one of the most important is the developmental stage of the plant at the time of inoculation with *Agrobacterium*. Although it has previously been shown that transformation can occur when *Agrobacterium* is applied to rosettes at the site of initiating bolts or even to seed just prior to planting (Chang *et al.*, 1994; Feldmann and Marks, 1987; Feldmann, 1992; Katavic *et al.*, 1994), reproducible high-frequency transformation is reliably achieved with plants in which the inflorescence has developed and floral buds are evident (Bechtold *et al.*, 1993). To identify the inflorescence developmental stage that is most susceptible to transformation, we used vacuum infiltration to inoculate plants that were at different growth stages: (A) primary bolts clipped, secondary bolts about 1–5 cm (no open flowers); (B) primary bolts clipped, secondary bolts about 2–10 cm (few open flowers); (C) primary bolts not clipped, many open flowers and starting to produce siliques; and (D) mature bolts, many siliques already developed. In six out of seven replications, the highest rate of transformation overall was achieved using plants from group B (Figure 1a). These plants had many immature floral buds visible at the time of inoculation. In all replications, plants in group D were the least susceptible to transformation (Figure 1a). Similar results were obtained in other experiments (see, for example, Figure 3).

When multiple containers (pots) of *Arabidopsis* are planted and grown together, plants flower at slightly different rates. In all subsequent experiments, pots of plants were divided into treatment groups such that all groups had a similar distribution of plant types. Despite this, the developmental variation from pot to pot within treatment groups may have been a main factor contributing to the standard error within experimental treatments in the studies reported in this work. A second source of variation may have been that plants for these studies were grown under greenhouse conditions rather than in growth chambers.

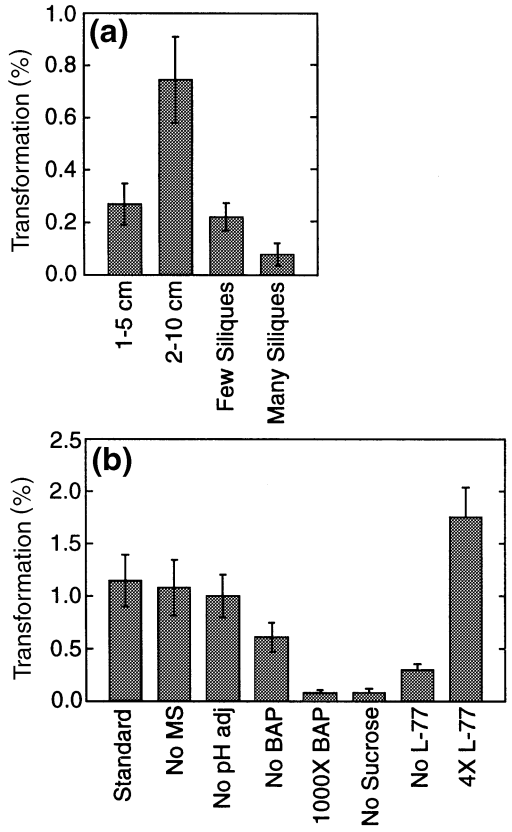

Figure 1. Effects of inflorescence developmental stage and inoculation medium composition on the rate of transformation.
For each treatment, three or more pots of 6–20 plants were inoculated under vacuum with bacterial suspension at OD_{600} = 0.8. Values are mean ± SE.
(a) Transformation of plants of different height/developmental stage. (b) Effect of modified inoculation media on transformation rate (standard inoculation medium contained $\frac{1}{2}\times$ MS medium, pH 5.7, 44 nM BAP, 5% sucrose, 0.005% Silwet L-77).

Confirmation of selection protocol

The validity of the kanamycin plate selection method used to detect transformed progeny seedlings was confirmed by DNA hybridization experiments. A DNA fragment from the *nptII* gene at the border of the T-DNA region of pBINm-gfp5-ER (Haseloff *et al.*, 1997) was used to probe blots of electrophoretically separated genomic DNA from kanamycin-resistant plants or kanamycin-sensitive controls.

Genomic DNA was restricted with *Hin*dIII enzyme to reveal junction fragments that extended beyond the T-DNA and into adjacent host DNA or into a repeat copy of the T-DNA. Bands that differed in size from the hybridizing *Hin*dIII fragment of the native pBINm-gfp5-ER were obtained in all of 42 randomly selected putative transformants examined, and no hybridizing bands were observed in control DNA from non-transformed *Arabidopsis* (data not shown). In addition, no spontaneous kanamycin resistant plants were observed among the approximately 3000 seeds from uninoculated plants that were plated on selective medium as a control in the experiments (total $>$ 30 000 seed). This selection method has also been confirmed in other studies (e.g. Bent *et al.*, 1994).

The pBINm-gfp5-ER construct utilized in these studies delivers a gene for green fluorescent protein to the plant. Expression of the green fluorescent phenotype was clearly observed in adult leaves from 17 of 19 kanamycin resistant, putatively transformed Col-0 plants that were tested, and not in non-transformed controls. Various levels of fluorescence were observed among the different transformants. One line was carried to the T_6 generation by self-fertilization, and those T_6 plants still expressed the green fluorescent phenotype.

Simplification of the inoculation medium

In earlier protocols for transformation of *Arabidopsis* by *Agrobacterium* vacuum infiltration, *Agrobacterium* cells were grown in a rich medium, harvested by centrifugation and then suspended in a pH-adjusted inoculation medium consisting of Murashige and Skoog salts and vitamins, benzylamino purine (BAP, a cytokinin), and sucrose (Bechtold *et al.*, 1993). Use of Silwet L-77 surfactant has also become common. We examined the relative contributions of these media components to transformation success using vacuum infiltration. Figure 1(b) shows that the absence of MS medium had no effect on transformation rates. The standard medium is pH adjusted to 5.7, but we found that pH adjustment was unnecessary (Figure 1b). The pH of our media was typically above 6.0 prior to pH adjustment, which is outside the optimal range for effective induction of *Agrobacterium vir* gene expression (Mantis and Winans, 1992). Removal of BAP from the inoculation medium caused a twofold decrease in transformation rate in one experiment, but had no significant effect in subsequent experiments (Figure 1b and data not shown). Interestingly, a 1000-fold excess of BAP (44 mM, a level common to some other plant transformation protocols), was detrimental, giving rates 5–8 times lower than without BAP. Sucrose was found to be an important component of the inoculation medium. Elimination of sucrose resulted in few or no transformants in the tested samples of about 3000 seeds per treatment (Figure 1b). The only other important component of the inoculation medium was surfactant. In the experiments that used vacuum to aid inoculation, the lack of the surfactant Silwet L-77 caused a three- to fourfold decrease in transformation efficiency, whereas a fourfold increase in Silwet L-77 (to 0.02%) almost doubled the transformation rate (Figure 1b). In a summarizing experiment performed separately from those reported in Figure 1(b), an inoculation medium of just sucrose and surfactant worked as well as the complex medium, verifying that these are the only essential components of the inoculation medium (transformation rate mean $\pm$ standard error of 0.52% $\pm$ 0.19 for simplified medium versus 0.50% $\pm$ 0.16 for complex medium).

Table 1. Floral dip versus vacuum-assisted inoculation

Treatment	% Transformation
Experiment A:	
Dip/ + L77	0.47 $\pm$ 0.16
Vacuum/ + L77	0.56 $\pm$ 0.13
Experiment B:	
Dip/-L77	0.07 $\pm$ 0.06
Dip/ + L77	0.23 $\pm$ 0.07
Vacuum/-L77	0.49 $\pm$ 0.20
Vacuum/ + L77	0.50 $\pm$ 0.16

A. tumefaciens inoculum resuspended to OD_{600} = 0.8 in MS Medium with BAP, 5% sucrose, and (where indicated) 0.005% Silwet L-77. Values are mean $\pm$ SE.

Vacuum versus floral dip

Vacuum infiltration is used to introduce bacteria into intercellular spaces within plant tissue, but an alternative is to simply submerge above-ground parts of the plant into an *Agrobacterium* solution for a few seconds, with no vacuum. We found that the use of vacuum was not necessary for successful transformation. In an initial experiment, the use of low amounts of surfactant (0.005% Silwet L-77) gave transformation rates for dipped plants that were comparable to those obtained with vacuum infiltration (Table 1). In a second experiment, the transformation rate was twofold lower with dipping as opposed to vacuum (Table 1). In the experiments reported in Table 1 and in other experiments, we noted that Silwet L-77 was less critical for success with vacuum infiltration but that it reproducibly enhanced transformation success in dip treatments. The optimal growth stage for transformation by floral dip was when plants contained numerous unopened floral buds, similar to that reported in Figure 1(a). The floral dip method required considerably less time and effort than vacuum infiltration, and resulted in greater seed yields (data not shown), so subsequent experiments focused on optimization of this approach.

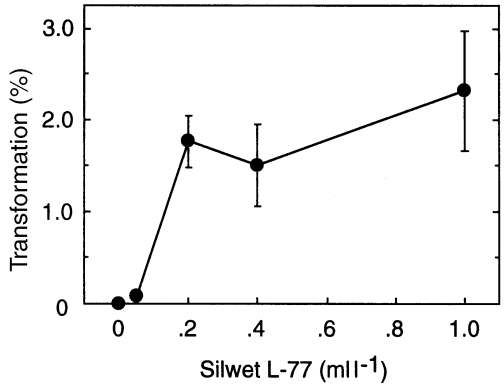

Figure 2. Effect of concentration of Silwet L-77 on transformation rates following dip inoculation.
For each treatment, three or more pots of 6–20 plants were dipped into *Agrobacterium* and 5% sucrose supplemented with the specified amount of Silwet L-77. Bacterial inocula were at OD_{600} = 0.8. Values are mean ± SE.

Table 2. Effect of various sugars on transformation

Sugar	% Transformation
No sugar	0.04 ± 0.01
Sucrose, 0.5%	0.40 ± 0.13
Sucrose, 1.25%	0.34 ± 0.03
Sucrose, 2.5%	0.47 ± 0.13
Sucrose, 5%	0.36 ± 0.08
Sucrose, 10%	1.42 ± 0.25
Glucose, 0.5%	0.14 ± 0.08
Glucose, 1.25%	0.11 ± 0.08
Glucose, 5%	0.76 ± 0.29
Glucose, 10%	0.33 ± 0.12
Mannitol, 5%[a]	death
5% food-grade sucrose	0.48 ± 0.27

Plants dipped in *A. tumefaciens* resuspended to OD_{600} = 0.8 in aqueous Silwet L-77 (0.05%) with sugar as noted. Values are mean ± SE.
[a]Silwet L-77 0.005% for mannitol treatment.

A comparison of different amounts of surfactant in the inoculation medium showed that levels of Silwet L-77 between 0.02% and 0.1% gave about 20-fold greater rates of transformation than did 0.005% when plants were inoculated by dipping (Figure 2). If the percentage of Silwet L-77 surfactant was increased from 0.005% to 0.05% or 0.1%, transformation rates by dipping were as high or higher than those obtained using vacuum (e.g. compare Figure 2 and Figure 1). We used 0.05% Silwet L-77 in subsequent experiments where plants were inoculated by floral dip. However, caution is advised in the use of high surfactant levels as these higher levels can cause necrosis of plant tissue in some environments.

Analysis of the simplified inoculation medium

Experiments that manipulated the inoculation medium (Figure 1b) indicated that the only significantly beneficial components of the standard medium were the sucrose and the Silwet L-77 surfactant. In subsequent experiments using the floral dip method and a modified inoculation medium containing only sugar and 0.05% Silwet L-77, we determined that the rate of transformation did not change significantly at various sucrose concentrations between 0.5 and 5% (Table 2). The three- to fourfold increase in transformation rate when sucrose was used at 10% in this set of experiments was not consistently observed (for example, in a separate experiment an average transformation rate of 1.62% was obtained with 5% sucrose while only a 0.81% rate was obtained with 10% sucrose). Table 2 also shows that less expensive food-grade sucrose (C&H brand) or 5% glucose (approximately the same molar amount as 10% sucrose) could adequately substitute for reagent-grade sucrose. Inoculation medium containing mannitol was deleterious to the plants. When inoculated with *Agrobacterium* suspended in 5% mannitol, above-ground plant tissues became water-soaked and then showed severe leaf necrosis or plant death 2–4 days after inoculation. Similar necrosis occurred when *Agrobacterium* was left out of the mannitol inoculation medium.

Further analysis of the protocol

Analysis of individual siliques from *Agrobacterium*-treated plants has suggested that individual flowers of a certain developmental stage are preferentially transformed (Bechtold *et al.*, 1993; C. Desfeux, S. Clough and A. Bent, unpublished results). Transformed seeds are found in a minority of the siliques on a given plant and these siliques are not evenly distributed throughout the plant. If flower transformation is dependent on developmental stage then flowers at a certain stage are likely to be particularly receptive to transformation, and we reasoned that multiple *Agrobacterium* inoculations over the course of flowering might result in increased numbers of transformants. When *Agrobacterium* was applied to plants by floral dip three times with 6 days between each application, transformation rates and overall yield of transformants increased approximately twofold (Figure 3 and data not shown). However, application of *Agrobacterium* over a 2 week period at intervals of every fourth day or less was very detrimental to plant health. Although some transformants were obtained following these more frequent inoculations (Figure 3), most plant tissue died and fewer seeds were recovered from those plants.

For successful transformation to occur, the *vir* gene operons of *Agrobacterium* must be transcriptionally active (Winans, 1992). In nature, wound sites often offer *vir*-inducing signals such as phenolic compounds, hexoses and low pH. In tissue culture transformation methods, the phenolic compound acetosyringone is sometimes added

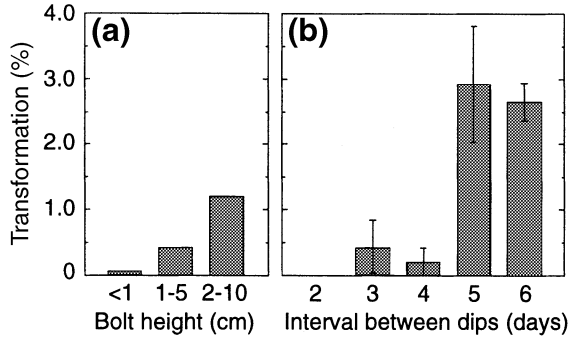

Figure 3. Effect of repetitive dip inoculations on transformation.
Inocula consisted of 5% sucrose, 0.05% Silwet L-77, and bacteria suspended to OD_{600} = 0.8. Values are mean ± SE.
(a) Plants dip-inoculated only once during the same growth period as the plants that were dipped multiple times.
(b) Plants that were dip-inoculated at the indicated day intervals during a 15 day period commencing the day after primary inflorescences were clipped.

to induce expression of the *vir* genes. When we attempted to pre-induce the *vir* genes by growing *Agrobacterium* for 20 h in a standard *vir*-inducing liquid medium (Liu *et al.*, 1992) instead of in rich liquid medium, the rate of transformation did not change significantly (1.03-fold over the value for cells grown in rich liquid medium). Growth on solid *vir*-inducing CIB medium (Fullner *et al.*, 1994) at 19°C led to a fourfold decrease in transformation rate. This decrease may have been attributable to medium composition, to growth on solid medium, or to growth temperature, but was at any rate not favorable.

In earlier work that used vacuum infiltration for inoculation, we also examined growth phase and cell density issues in the preparation and use of *Agrobacterium* inoculum. In our earlier standard protocol, cells were grown to stationary phase (OD_{600} ≈2.0), pelleted and resuspended in inoculation medium to OD_{600} of approximately 0.8 (Bent and Clough, 1998). When *Agrobacterium* was grown to stationary phase and then resuspended at various concentrations of inoculum ranging from OD_{600} = 0.15–1.75, there was little difference in transformation rate (Table 3). We also found that cells grown to very late stationary phase (prepared from cultures grown for 84 h) and diluted to OD_{600} = 0.8 transformed just as efficiently as younger cultures. In a separate experiment, *Agrobacterium* cultures grown to OD_{600} ≈2.0 or OD_{600} = 0.8 were harvested and resuspended to OD_{600} = 0.8; the stationary-phase culture transformed at a threefold higher rate than the late-log phase culture (mean ± standard error of 0.39% ± 0.13 versus 0.12% ± 0.06).

Our earlier standard protocol called for plants to be placed under a plastic dome or other humidity retaining covers for 12–24 h after inoculation. As a test for the contribution of the dome treatment to transformation rate, an experiment was performed in which seven separate but similarly prepared *Agrobacterium* cultures were used to treat paired groups of plants. For each of the seven pairs, one pot was placed under a dome and the other was left uncovered. Covering plants overnight after inoculation gave the same or significantly higher rates in all cases, with overall transformation rates (mean ± standard error) of 0.24% ± 0.08 for domed plants versus 0.10% ± 0.03 for uncovered plants.

Table 3. Effect of Agrobacterium inoculum density on rate of transformation

Inoculum OD_{600}	% Transformation
0.15	0.50 ± 0.02
0.42	0.21 ± 0.05
0.80	0.51 ± 0.14
1.10	0.51 ± 0.09
1.75	0.57 ± 0.15
0.8 (84 h)	0.50 ± 0.05

Plants inoculated by vacuum infiltration with *A. tumefaciens* in MS Medium with BAP, 5% sucrose and 0.005% L-77. All bacteria resuspended from a fresh overnight liquid culture, except '84 h' from culture grown for 84 h. Values are mean ± SE.

Different ecotypes and Agrobacterium *strains*

To determine if the *Arabidopsis* ecotype Col-O was unique in being transformable by simple dipping of plants in *Agrobacterium* suspended in 5% sucrose and 0.05% Silwet L-77, we attempted to transform other *Arabidopsis* ecotypes using this floral dip method. Ecotypes Ws-O, Nd-O, No-O were transformed at rates similar to Col-O. In contrast, Ler-O, Dijon-G and Bla-2 transformed at 10- to 100-fold lower rates (data not shown). In one of the experiments, zero transformants were obtained with Ler-0. In experiments that examined the use of other *Agrobacterium* strains, LBA4404 (Ooms *et al.*, 1982), EHA105 (Hood *et al.*, 1993) and Chry105 (S. Clough, unpublished results) were used successfully to transform ecotype Col-0 by the floral dip method.

Discussion

In 1993, Bechtold and colleagues published their original method for transformation of *Arabidopsis* by *Agrobacterium* vacuum infiltration (Bechtold *et al.*, 1993). Since that time a number of modifications to their protocol have been suggested (e.g. http://genome-www.stanford.edu/cgi-bin/biosci_arabidopsis), but no formal reports of controlled experiments to further assess this transformation method were available until very recently (Richardson *et al.*, 1998). The present study was initiated to provide such an assessment and to identify improvements that facilitate high-throughput transformation. Enhancements and simplifications to *Arabidopsis* transformation were identified that

make a relatively convenient protocol easier and even more convenient.

We found that *Arabidopsis* transformants could be obtained at a high rate (0.5–3% of all progeny seed) simply by dipping flowering plants in *Agrobacterium* that are suspended in a solution containing sucrose (or glucose) and the surfactant Silwet L-77. Plant tissue culture media, pH adjustments, plant growth regulators, and the growth or use of *Agrobacterium* at particular culture densities were not essential to successful, high-rate transformation. Most importantly, vacuum infiltration was found to be unnecessary as long as a suitable surfactant was used. The modified protocol allows large-scale treatment of *Arabidopsis* populations in a greenhouse setting without need for a vacuum apparatus or for uprooting and re-planting of plants. Combination of this method with a high-throughput selection method (such as the sand-bed/Basta approach of Bouchez *et al.*, 1993) allows efficient generation of thousands of independent *Arabidopsis* transformants.

The three main requirements for successful transformation were: (1) correct plant developmental stage (maximum number of unopened floral bud clusters); (2) sugar; and (3) surfactant and/or vacuum to aid infiltration. Investigators can use vacuum, surfactant, or both to assist delivery of bacteria to target plant cells – we obtained similar rates of transformation by either method. We also found that transformation can be achieved without vacuum or surfactant, as well as without sucrose or glucose, but only inconsistently or rarely. While other surfactants may work, Silwet L-77 was originally chosen (Whalen *et al.*, 1991) because it reduces surface tension more than most surfactants and, at doses with low phytotoxicity, greatly enhances entry of bacteria into relatively inaccessible plant tissues. Use of surfactant also allowed transformation upon spraying of *Arabidopsis* floral tissues with *Agrobacterium* (data not shown). However, the spray method may pose containment and cross-contamination problems, and raises worker safety issues (Silwet L-77 aerosols can be harmful to corneal tissues in particular).

There is apparently an ideal stage of floral development either for access of *Agrobacterium* to target tissue or for susceptibility of that tissue to transformation. This ideal stage was targeted more successfully by repeat inoculations on different days. However, the benefit of repeat inoculations (in our hands, a two- to threefold increase in transformation rate) may not always justify the added effort required. We found that plants were harmed by excessive re-application of *Agrobacterium* at less than 4 day intervals.

Covering of inoculated plants with a plastic dome to maintain high humidity during the first 12–24 h after inoculation was beneficial, giving about twice as many transformants. We assume that the dome prolongs the availability of surface water through which the motile *Agrobacterium* cells can swim to reach potential target cells, although humidity may be equally important in maintaining host tissues in an accessible configuration. The effect of dome covers may vary depending on temperature, day length, light intensity, and other environmental factors that vary between laboratories.

T-DNA transfer from *Agrobacterium* to the host requires, among other things, activation of *Agrobacterium vir* gene expression by the products of *virA* and *virG* (Winans, 1992). Expression of *vir* genes is very low when *Agrobacterium* is grown in rich liquid media such as LB. Despite this, the vacuum infiltration method has been widely successful using *Agrobacterium* grown in rich media, and we found that growth of *A. tumefaciens* strain GV3101(pMP90)(pBin-gfp5-ER) in standard *vir*-inducing medium did not substantially improve transformation rates. Most transformation events apparently occur after *Agrobacterium* has adjusted metabolically to the host environment, and may occur days after the plant is inoculated. However, an appropriate match between the genotypes of the host and the *Agrobacterium* strain, Ti plasmid and binary vector may still be important. The quantitative studies presented in this work utilized GV3101(pMP90), a strain based on the C58 and pTiC58 genotypes that have proven to be productive in use with *Arabidopsis* (Bechtold *et al.*, 1993; Katavic *et al.*, 1994; Koncz and Schell, 1986). The floral dip method was also successful when used with *Agrobacterium* strains LBA4404, EHA105 and Chry105.

The floral dip method was very successful for transformation of multiple *Arabidopsis* ecotypes including Col-0, Ws-0, Nd-0 and No-0, but was not equally efficient with all ecotypes. Our poor success with Ler-0 may have been attributable to low compatibility with *Agrobacterium*, or to the growth stage of the plants used. The inflorescence of Ler-0 develops differently from those of most other ecotypes, and the inflorescences on Ler-0 plants used in our experiments may have been developmentally either behind or ahead of the ecotypes such as Col-0. Others have also found that Ler-0 is more difficult or variable than ecotypes such as Col-0 when used for *in planta* transformation, but there are many informal and formal reports of successful transformation of this ecotype by *in planta* methods (e.g. http://genome-www.stanford.edu/cgi-bin/biosci_arabidopsis, Richardson *et al.*, 1998). Ecotype-specific differences in susceptibility of *Arabidopsis* to *Agrobacterium* have also been documented for tissue culture-based transformation systems (Nam *et al.*, 1997).

Reports of the use of *Agrobacterium* vacuum infiltration or similar methods for transformation of plant species other than *Arabidopsis* are not available. This may be due in part to insufficient effort, given that a 10-fold or 100-fold lower transformation rate than is found with *Arabidopsis* would require the screening of 10 000 or more seed. One motivation for the present study was to identify a maximally

effective approach using *Arabidopsis*, a plant that is amenable to transformation by application of *Agrobacterium* to floral tissue, so that similar methods can be applied to other species. A key task beyond the scope of the present study will be to identify the target tissue that is initially transformed when *Arabidopsis* is treated using this protocol. The success of the floral dip method implies that the germ-line tissues that are transformed are, at some point during the procedure, exposed and accessible to *Agrobacterium* that has been passively applied to floral tissues by mere dipping.

Earlier versions of this method and related methods have been known as '*Agrobacterium* vacuum infiltration' or '*In planta* transformation' (Bechtold *et al.*, 1993; Chang *et al.*, 1994; Feldmann, 1992; Katavic *et al.*, 1994). With respect to the present method these names are either inaccurate or ambiguous in that they have been used previously to describe substantially different methods. To provide a distinct name that is descriptive of the process, we propose the term 'floral dip' for methods in which plants are transformed by direct application of *Agrobacterium* to floral tissues without use of vacuum or other pressurizing devices. Our preferred method for transformation of *Arabidopsis* can be found under Experimental procedures, and a concise floral dip protocol is available at http://www.cropsci.uiuc.edu/~a-bent/protocol.html, or upon request from the authors.

Experimental procedures

Plant growth

Arabidopsis plants were grown to flowering stage in a shaded greenhouse, 24°C day/20°C night, with supplemental high-pressure sodium lighting activated for 18 h days when outdoor light levels dropped below 250 μEinsteins m^{-2} s^{-1}. Unless otherwise noted, the plants used were *Arabidopsis* ecotype Col-0 plants genetically marked with the *rps2–201* allele of resistance gene *RPS2* (Kunkel *et al.*, 1993). Plants were typically planted 1–2 per 25 cm^2 pot or 6–20 per 64 cm^2 pot in moistened potting soil (Sunshine Mix #1; Sun Gro Horticulture Inc., Bellevue, WA, USA). To prevent the soil in larger pots from falling into inoculation medium, soil was mounded slightly above the rim of plant containers, seeds were planted, and soil was then covered with nylon window screen or tulle (veil) fabric and secured by a rubber band. To obtain more floral buds per plant, inflorescences were clipped after most plants had formed primary bolts, relieving apical dominance and encouraging synchronized emergence of multiple secondary bolts. Plants were infiltrated or dipped when most secondary inflorescences were about 1–10 cm tall (4–8 days after clipping).

Standard protocols for culture of Agrobacterium tumefaciens *and inoculation of plants*

Agrobacterium tumefaciens strain GV3101(pMP90) (Koncz and Schell, 1986) carrying the binary plasmid pBINm-gfp5-ER (Haseloff *et al.*, 1997) was used in all experiments for which data are shown. Unless noted, bacteria were grown to stationary phase in liquid culture at 25–28°C, 250 r.p.m. in sterilized LB (10 g tryptone, 5 g yeast extract, 5 g NaCl per litre water) carrying added kanamycin (25 μg ml^{-1}). Cultures were typically started from a 1:100 dilution of smaller overnight cultures and grown for roughly 18–24 h. Cells were harvested by centrifugation for 20 min at room temperature at 5500 ***g*** and then resuspended in infiltration medium to a final OD_{600} of approximately 0.80 prior to use. The revised floral dip inoculation medium contained 5.0% sucrose and 0.05% (i.e. 500 μl l^{-1}) Silwet L-77 (OSi Specialties, Inc., Danbury, CT, USA). The earlier 'standard' inoculation medium (Bent and Clough, 1998) consisted of 1/2 strength Murashige & Skoog Basal Medium (Sigma Chemicals #M-5519, St. Louis, MO, USA), 5.0% sucrose, 44 nM benzylamino purine (10 μl l^{-1} of a 1-mg ml^{-1} stock in DMSO; Sigma Chemicals #B-3274), 0.005% Silwet L-77, pH adjusted to 5.7. Other media were as noted.

For floral dip, the inoculum was added to a beaker, plants were inverted into this suspension such that all above-ground tissues were submerged, and plants were then removed after 3–5 sec of gentle agitation. For vacuum infiltration, beakers carrying plants submerged in inoculum were placed into a chamber (bell jar) and a vacuum was applied until air bubbles were drawn from plant tissues. Using a vacuum pump, this typically required 30–60 sec. The vacuum was then held for 5–15 more seconds, during which time the solution boiled vigorously, and the vacuum was then released rapidly. Dipped or vacuum infiltrated plants were removed from the beaker, placed in a plastic tray and covered with a tall clear-plastic dome to maintain humidity. Plants were left in a low light or dark location overnight and returned to the greenhouse the next day; care was taken to keep domed plants out of direct sunlight. Domes were removed approximately 12–24 h after treatment. Plants were grown for a further 3–5 weeks until siliques were brown and dry, keeping the bolts from each pot together and separated from neighboring pots using tape and/or wax paper. Seeds were harvested by gentle pulling of grouped inflorescences through fingers over a piece of clean paper. The majority of the stem and pod material was removed from the paper by gentle blowing and seeds were stored in microfuge tubes and kept at 4°C under dessication.

Selection of putative transformants using an antibiotic marker

Seeds were surface sterilized by liquid or vapor-phase methods. For liquid sterilization, seeds were first treated with 95% ethanol for 30–60 sec, then with 50% bleach (2.625% sodium hypochlorite, final volume) containing 0.05% Tween 20 for 5 min, followed by three rinses with sterile water. For vapor-phase sterilization, seeds in open containers were placed into a 10 l desiccator jar, placed in a fume hood. Just prior to sealing the desiccator, a 250 ml beaker containing 100 ml bleach was added and 3 ml concentrated HCl was carefully added into the bleach. The desiccator with chlorine fumes remained sealed 4 or 15 h. To select for transformed plants, sterilized seeds were suspended in 0.1% sterile agarose and plated on kanamycin selection plates at a density of approximately 3000 seeds per 150 × 15 mm^2 plate, cold-treated for 2 days, and then grown for 7–10 days in a controlled environment at 24°C under 23 h light 50–100 μEinsteins m^{-2} s^{-1}. Selection plates contained 1/2X MS medium (Sigma Chemicals #M-5519), 0.8% agar (Sigma Chemicals #A-1296), 50 μg ml^{-1} kanamycin monosulfate. In some

experiments, Petri plates and lids were sealed with surgical tape for the first week of growth. Excess moisture during growth was removed by briefly opening the plates and shaking moisture off the lid. Transformants were identified as kanamycin-resistant seedlings that produced green leaves and well-established roots within the selective medium. Some transformants were grown to maturity by transplanting, preferably after the development of 3–5 adult leaves, into heavily moistened potting soil. Where tested, genomic DNA from kanamycin-resistant plants was digested with *Hin*dIII restriction enzyme, electrophoretically separated, blotted and then probed with a 1.9 kb *Pst*I fragment of the *nptII* gene from pBINm-gfp5-ER essentially as in Ausubel *et al*. (1997). Transformation rates, expressed as 'percentage transformation', were calculated as [(# kanamycin-resistant seedlings)/(total # seedlings tested)] $\times$ 100.

Fluorescence microscopy

Expression of green fluorescent protein was assayed by viewing excised leaves in water mounts on an Olympus Vanox-T microscope fitted with a 20-BP405 nm excitation filter and a 455 nm dichroic mirror.

Acknowledgements

This work was supported by a grant from the North Central Soybean Research Board.

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