

Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method

Xiuren Zhang, Rossana Henriques, Shih-Shun Lin, Qi-Wen Niu & Nam-Hai Chua

Laboratory of Plant Molecular Biology, Rockefeller University, 1230 York Avenue, New York, New York 10021, USA. Correspondence should be addressed to N.-H.C. (chua@mail.rockefeller.edu).

Published online 29 June 2006; doi:10.1038/nprot.2006.97

Collective efforts of several laboratories in the past two decades have resulted in the development of various methods for *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana*. Among these, the floral dip method is the most facile protocol and widely used for producing transgenic *Arabidopsis* plants. In this method, transformation of female gametes is accomplished by simply dipping developing *Arabidopsis* inflorescences for a few seconds into a 5% sucrose solution containing 0.01–0.05% (vol/vol) Silwet L-77 and resuspended *Agrobacterium* cells carrying the genes to be transferred. Treated plants are allowed to set seed which are then plated on a selective medium to screen for transformants. A transformation frequency of at least 1% can be routinely obtained and a minimum of several hundred independent transgenic lines generated from just two pots of infiltrated plants (20–30 plants per pot) within 2–3 months. Here, we describe the protocol routinely used in our laboratory for the floral dip method for *Arabidopsis* transformation. Transgenic *Arabidopsis* plants can be obtained in approximately 3 months.

INTRODUCTION

Plant transformation is a process of genetic manipulation by which foreign genes are introduced into plant cells and stably integrated in the plant genomes, and the transformed cells are regenerated to obtain transgenic plants. The conventional transformation method typically includes preparation of transformation-competent plant cells or tissues, delivery into plant cells of foreign genes mainly by *Agrobacterium tumefaciens* or by the biolistic method, selection of transformed cells that have stably incorporated foreign genes, and regeneration of transformed cells into transgenic plants¹. This transformation method has been widely used to produce transgenic plants, including many agriculturally important crops, whose tissue culture systems are well established². Because it involves a tissue culture and plant regeneration step, this approach is painstaking and time consuming. It also requires skilled labor and relatively expensive laboratory facilities for its execution. Further, this method can result in undesired DNA modification and somaclonal variation during the processes of plant dedifferentiation and differentiation, which is mostly due to the stress imposed by the *in vitro* cell culture protocol³.

A plant transformation method that excludes the use of tissue culture and plant regeneration would greatly reduce the time required to produce transgenic plants, and such a method was first described as “in planta” transformation almost 20 years ago⁴. In their pioneering work, Feldmann and Marks cocultivated germinating seeds of *Arabidopsis thaliana* with an *Agrobacterium tumefaciens* strain harboring a disarmed Ti plasmid and a binary vector, and amazingly, they achieved stable transgenic lines, although the transformation rates were very low⁴. Several years later, the Pelletier group infiltrated flowering *Arabidopsis* plants with *Agrobacterium* and dramatically improved the transformation efficiency⁵. This version of transformation approach was often referred to as the “*Agrobacterium* vacuum infiltration method”⁵. Briefly, it involves uprooting of flowering *Arabidopsis* plants, vacuum infiltration of the plants using an *Agrobacterium* cell suspension, re-planting, harvesting of seed several weeks later, and screening for primary transformants on medium containing

the appropriate selective agent (usually an antibiotic or a herbicide). These transformation procedures were later simplified and substantially improved⁶. In the improved version, the steps of uprooting and replanting of infiltrated-plants are omitted. The vacuum-aided infiltration of inflorescences⁵ was replaced by the use of a surfactant (Silwet L-77), which had already been shown, in the formulation of some pesticides, to help chemicals enter the plant tissues. All these modifications simplified the initial procedure. The *Arabidopsis* flower buds were simply dipped in an *Agrobacterium* cell suspension containing 5% sucrose (wt/vol) and 0.01–0.05% Silwet L-77 (vol/vol) to allow uptake of the agrobacteria into female gametes^{7,8}. This new and extremely simple transformation scheme was popularly referred to as “the floral dip method”⁶.

Compared with traditional transformation methods that require tissue culture and plant regeneration, the floral dip transformation method⁶ offers several advantages and opens up new opportunities. First, it requires minimal labor, relatively inexpensive equipment and few specialized reagents, and can be successfully executed even by non-specialists. Second, it is easily scalable and therefore allows production of a large number of independent *Arabidopsis* transgenic lines within a short period. Although the overall transformation efficiency of the floral dip method may not be high, the total number of seed produced by an *Arabidopsis* plant ensures that sufficient transgenic events can be recovered even in a single transformation experiment. An *Arabidopsis* plant produces thousands of seeds by self-pollination, and seeds from one treated plant can be screened on one or two 150 × 150 × 25 mm Petri dishes. Third, the floral dipping procedure can be used not only for introducing specific gene constructs into *Arabidopsis* plants but also for larger-scale transformation projects such as generation of a library of enhancer-trapped lines or of mutant lines tagged by T-DNA. Finally, the floral dip method easily maintains genomic stability in *Arabidopsis thaliana* transgenic plants, which can otherwise be harmed by the tissue culture-based transformation³. The success and popularity of this floral transformation procedure

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is reflected by its high citation index since its description^{5,6}. Similar to floral dip, flowering spray also works very well with *Arabidopsis*⁹.

Whereas the floral dip transformation method works well for the majority of *Arabidopsis thaliana* ecotypes, for example, Col-0, Ws-0, Nd-0 and No-0, its efficiency is reduced in the Ler-0 ecotype⁶. Using a similar protocol, Curtis and Nam¹⁰ reported the successful production of transgenic radish (*Raphanus sativus* L. *longipinnatus* Bailey) with optimum transformation efficiency. In addition, the floral transformation methods including vacuum infiltration have been successfully used to produce transgenic Pakchoi *Brassica rapa*¹¹, *Arabidopsis lasiocarpa*¹² and rapeseed *Brassica napus*¹³.

These findings extend the utility of the floral dipping method beyond the *Arabidopsis* species, though presently it is still limited to species belonging to the *Brassicaceae* family.

Floral dip transformation methods have been previously reviewed by its inventors and others either in published form^{14,15} or on the web, for example S. Clough and A. Bent's Simplified *Arabidopsis* Transformation Protocol (<http://www.cropsci.uiuc.edu/~a-bent/protocol.html>). Our extensive use of the floral dip method¹⁴ over the years to generate transgenic *Arabidopsis* plants in the laboratory has allowed us to fine-tune its various steps. Here, we describe our streamlined protocol.

MATERIALS

REAGENTS

- *Agrobacterium* strain(s): ABI (refs. 16, 17), GV3101 (ref. 16), EHA105 (ref. 18), LBA 4404 (ref. 19) or others
- 0.05% agarose (wt/vol). Autoclaved and stored at room temperature.
- MS medium (see REAGENT SETUP)
- Liquid LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter; Bio 101)
- 5% (wt/vol) sucrose, prepared fresh (Step 8)
- Silwet L-77 (Lehle Seeds)
- Selection plates see (REAGENT SETUP)
- 70% (vol/vol) ethanol
- 50% Clorox bleach/50% sterile double distilled water/0.05% Tween, prepared fresh

EQUIPMENT

- Growth chambers or light room or greenhouse, adjustable to long-day condition of 16 h light/8 h dark, 20 °C, and short-day condition of 8 h light/16 h dark, 20 °C.

- Plant pots: e.g., 4 in. × 4 in.
- Laminar-flow hood
- Acrodisc Syringe Filter with 0.2- μ m membranes (Pall Life Sciences)
- Petri dishes: 150 × 150 × 25 mm (Falcon 3025)
- Sample Saks (VWR Scientific)

REAGENT SETUP

MS medium and selection plates Autoclave MS medium (4.3 g Murashige & Skoog salts, 10 g sucrose, 0.5 g MES, 8 g agar per liter; pH 5.7), cool to approximately 50 °C before pouring into Petri dishes (MS solid medium). To prepare selection plates add into MS medium (~50°C) the appropriate antibiotics and herbicide (filter-sterilized) in the following concentrations: kanamycin (final concentration 50 mg L⁻¹), phosphinothricin (herbicide, also known as PPT or Basta; 10 mg L⁻¹ for tissue culture selection), hygromycin (25 mg L⁻¹) and carbenicillin (100 mg L⁻¹).

PROCEDURE

Growing healthy *Arabidopsis* plants until they begin to bolt and produce floral inflorescences

1 | Germinate seeds and grow seedlings. There are two different methods (A and B, below) for this purpose. Generally we recommend the standard method (A). However, in particular cases, such as with mutations that cause reduced viability and low germination frequency, we suggest that users follow the more laborious (B). We also use (B) to quickly prepare plants for transformation or to re-transform a transgenic line with a second construct.

(A) Standard *Arabidopsis* germination procedure

- (i) Suspend seed in 0.05% agarose and keep them in darkness for 3 d at 4 °C to break dormancy. This short period of stratification might not be completely necessary but certainly allows uniform and maximal seed germination.
- (ii) Spread around 20–30 seeds on wet soil in each 4 in. × 4 in. pot.

(B) Alternative *Arabidopsis* germination procedure

- (i) Sterilize and place seeds on MS medium, referring to Steps 15–18 below. Stratify seeds by keeping them in darkness at 4 °C for 3 d.
- (ii) Germinate seeds and grow the seedlings in long-day conditions (16 h light/8 h dark, 20 °C) for 2 weeks, and then transfer them to wet soil (~12 seedlings/pot).

2 | Cover soil with a nylon screen or a piece of cheesecloth and secure with a rubber band.

▲ **CRITICAL STEP** This prevents loss of soil when the plants are inverted for dipping. In procedure 1(A) whereas some may prefer to spread seeds first in wet soil and then cover the soil with netted screens, others may like to carry out the two steps in reverse. In procedure 1(B) it is necessary to make holes in the netted screens to allow growth of the transferred seedlings.

3 | Grow plants in a growth chamber or a greenhouse under short days for 3–4 weeks, then move them to long-day conditions to induce flowering (**Fig. 1a**).

▲ **CRITICAL STEP** Pots and trays must be well watered until the first two true leaves are developed in the case of procedure 1(A). For example, we place the pots in a tray and cover the pots with plastic domes to maintain high humidity for the first 2 weeks. In the case of procedure 1(B) we keep high humidity for the transferred seedlings for 3–4 d before removing the plastic covers. Healthy plants are a prerequisite for a successful transformation. Following these recommendations is important in order to obtain big plants that can produce a large number of seeds.



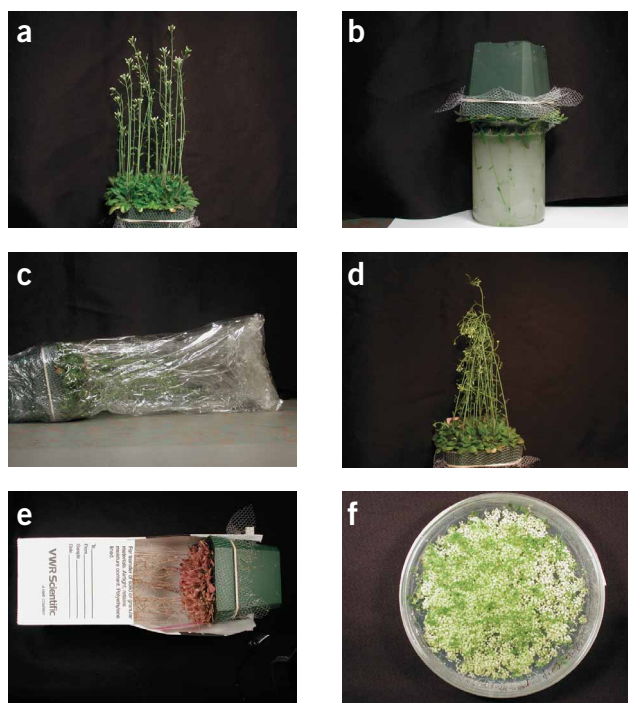


Figure 1 | Stages during the floral dip transformation method. Specific steps are discussed in the text. (a) A good stage for floral dipping is when a pot of healthy plants contain approximately 20–30 inflorescences and some maturing siliques. Siliques are routinely clipped off in our lab (see Step 5). (b) Invert plants and dip their aerial parts in an *Agrobacterium* cell suspension for 10 s. (c) Wrap the dipped plants with plastic films to maintain high humidity for 16–24 h. (d) Remove the plastic covers and grow plants in a growth chamber for 1 month. (e) Dry and harvest seeds with a sample bag. (f) Select primary transformants. Transgenic plantlets are readily distinguished from non-transgenic plants by their green true leaves and roots that penetrate into the selection medium. In this experiment, we selected primary transformants using kanamycin (+ carbenicillin) and obtained more than 100 transgenic lines on a single selection plate. Note that non-transformed seedlings germinated as well but their cotyledons became chlorotic and bleached soon after germination, whereas true transformants were very healthy, with green cotyledons and true leaves, and developed roots that penetrated into the medium.

4| If there is a need to delay the transformation experiment when constructs are not ready, or to produce plants with more immature flower clusters, remove the first bolts to allow proliferation of many secondary inflorescences from axillary buds of the rosette. Plan on treating plants with *Agrobacterium* cell suspension 6–8 d after clipping of first bolts.

5| If you wish to enhance relative nutrient supply to newly

transformed cells, and/or reduce the number of wild-type seeds (increasing transformation efficiency) and reduce contamination of bacteria and fungi during screening of primary transformants, remove the siliques found on plants to be used for transformation. Although this additional step requires extra labor, we prefer to carry out this pruning as it increases transformation efficiency.

Floral dipping transformation

6| Start preparing the *Agrobacterium* strain that harbors the gene of interest in a binary vector by inoculating a single *Agrobacterium* colony into 5-ml liquid LB medium containing the appropriate antibiotics for binary vector selection. Incubate culture at 28 °C for 2 d.

7| Use this feeder culture to inoculate a 500-ml liquid LB with the appropriate antibiotics and grow the culture at 28 °C for 16–24 h. We exclusively use cells that grow to the stationary phase (OD ~ 1.5–2.0).

▲ **CRITICAL STEP** Confirm the presence of the desired construct in the *Agrobacterium* strain used for transformation either by digestion with the appropriate restriction enzymes or by PCR with specific primers. If necessary, sequence the genes to be transferred. We routinely use the *Agrobacterium* strains (ABI, EHA105, LBA 4404), but believe that other *Agrobacterium* strains should work as well. In addition, this feeder *Agrobacterium* culture could be used again in a future transformation experiment by simply keeping aliquots stored at 4 °C up to 1 month.

8| Collect *Agrobacterium* cells by centrifugation at 4,000g for 10 min at room temperature, and gently resuspend cells in 1 volume of freshly made 5% (wt/vol) sucrose solution with a stirring bar.

9| Add Silwet L-77 to a concentration of 0.02% (vol/vol) (100 µl per 500 ml of solution) and mix well immediately before dipping. Transfer the *Agrobacterium* cell suspension to a 500-ml beaker.

▲ **CRITICAL STEP** We normally use Silwet L-77 up to a concentration of 0.02% (vol/vol), as higher concentrations might be toxic. 400–500 ml of *Agrobacterium* cell suspension is sufficient for transformation of at least six pots of plants. We normally dip two pots for a single gene construct. To generate transgenic plants transformed with two independent constructs, re-suspend two types of *Agrobacterium* cells, each carrying one construct, in 300 ml of a 5% sucrose solution. Add an appropriate amount of Silwet L-77 and mix them well in a bigger beaker before dipping. We prefer to use at least four pots of plants for double transformation experiments. To obtain a higher frequency of transformation, especially when one pot of plants is used per construct, or for double transformation, we dip plants twice separated by a 7-d interval.

! **CAUTION** Appropriate masks and gloves should be used for skin and eye protection as Silwet L-77 might be toxic.

10| Invert plants and dip aerial parts of plants in the *Agrobacterium* cell suspension for 10 s with gentle agitation. We dip not only inflorescences but also the rosette to soak shorter axillary inflorescences. Remove dipped plants from the solution and drain the treated plants for 3–5 s. A film of liquid coating the plants should then be visible (**Fig. 1b**).

▲ **CRITICAL STEP** Make sure to submerge all bolts in the solution during dipping. Sometimes it is necessary to tilt the plants to ensure submergence of the shorter axillary floral buds. It is important to remove excess liquid by draining plants for a few

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seconds. Otherwise, the flower buds will be damaged if they are soaked in the solution for too long. Alternatively, apply the *Agrobacterium* suspension to the floral buds using a micropipettor (Pipetman) or spray and make sure that a film of liquid coats the surfaces of all buds on the inflorescences. For transformation of *Arabidopsis* ecotype Ler-0, we prefer to use the vacuum infiltration method. Briefly, we resuspend *Agrobacterium* cells in a solution containing 10 mM MgCl₂, 5% sucrose (wt/vol) and 0.02% Silwet L-77. We submerge inflorescences in this suspension and vacuum infiltrate them for 10 min (instead of the 10 s used for floral dip of other ecotypes).

11| Cover dipped plants with a plastic cover or wrap them with plastic film. Lay down the treated plants on their sides for 16–24 h to maintain high humidity (Fig. 1c).

▲ **CRITICAL STEP** Do not expose treated plants to high temperatures or excessive light, to prevent them from heating up. Treat the remaining *Agrobacterium* cell suspension with 1 volume of Clorox bleach to kill the bacteria before disposal of the suspension.

12| Remove the cover next day (Fig. 1d). Send the treated plants back to the greenhouse or the growth chamber, and allow them to grow normally for 1 month. Withhold watering when siliques turn brown. Wrap drying plants and loose bolts with a piece of wax paper such as a Sample Sak or other means (Fig. 1e).

▲ **CRITICAL STEP** Abnormal embryo development and incomplete seed maturation can occur if watering of transformed plants is terminated too early or if the drying is too rapid. This may result in transformed seedlings containing only a single cotyledon or without any radicle or true leaves.

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13| Collect dry seeds using a sieve mesh.

■ **PAUSE POINT** At this moment, harvested seed can be stored for a few months at room temperature or at least a year at 4 °C before screening.

Screening of primary transformants

14| Pour selection plates (150 × 150 × 25 mm) containing carbenicillin and the appropriate antibiotics or herbicide.

▲ **CRITICAL STEP** Carbenicillin is absolutely necessary for decontamination as harvested seeds are heavily covered with agrobacteria, which might not be completely killed by seed sterilization. We routinely use this chemical to prevent bacterial contamination.

15| Sterilize seeds by first treating them with 50× volume of 70% ethanol for 1 min and mixing the seed suspension thoroughly.

▲ **CRITICAL STEP** Always use only one-half of the amount of seed harvested. Screening this half amount of seed will yield sufficient number of transformants for most experiments. More important, if contamination occurs or if a mistake, for example, use of inappropriate antibiotics, is made in the first experiment the remaining seed can be used for a second trial and this could save you three months.

16| Continue the sterilization process by treating the seeds with a 50× volume of 50% bleach/50% water/0.05% Tween for 10 min and vortexing the suspension vigorously every 2 min.

17| Rinse the seed three times with sterile water. In the last washing step, the wash should appear clear and without any yellow color; if it does not, this indicates that residual bleach is still present in the wash.

18| Re-suspend the sterilized seeds in sterile 0.05% agarose (40 μl seed per ml agarose) and spread the seed-agarose suspension onto selection plates. Plate 3–4 ml seed-agarose mixture on each 150 × 150 × 25 mm plate. Dry plates under a laminar flow hood until agarose dries up and seeds become stable on the plate.

▲ **CRITICAL STEP** Double-check the selection marker in the binary construct before plating seeds to ensure that plates with the appropriate selective agent are used.

19| Vernalize seeds by placing them at 4 °C for 3 d.

20| Move plates to a tissue culture room or a growth chamber under continuous light (50–100 microeinsteins m⁻¹ s⁻¹) or long-day conditions. After 7–10 d, transformants should be readily distinguished as seedlings with healthy green cotyledons and true leaves and roots that extend into the selective medium (Fig. 1f). For kanamycin (+ carbenicillin) selection, non-transformed seedlings can germinate as well, but their cotyledons will become bleached very soon. For Basta (+ carbenicillin) selection, the cotyledons of non-transformants turn yellow or pale a little bit later (approximately 2–3 weeks after germination). By contrast, on hygromycin (+ carbenicillin) selection plates, non-transformants germinate as well and their cotyledons can remain green for more than 1 month after germination; however, the cotyledons are smaller than those of true transformants, and normally roots develop properly only in the true transformants. All these selections share one thing in common: only true transformants can produce true leaves, whereas non-transformants will not. Carbenicillin inhibits growth of

Agrobacterium cells and may have some effect on the growth of primary roots as well. Once plants are moved to MS plates without the antibiotics, or directly to soil, root growth will resume soon after transplantation.

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21| Transfer potential transformants to a fresh selection plate once their true leaves emerge. Allow them to grow for another 1–2 weeks to ensure that they are true transgenic lines.

22| Transplant plantlets to water-saturated soil and cover the tray with a plastic film to maintain high humidity for 2 d. Move the tray to a growth chamber or greenhouse and grow plants under continuous light for seed collection.

▲ **CRITICAL STEP** To avoid breaking plantlets during transplantation, gently remove from the root any chunks of agar that are sticking to it before planting.

23| As an alternative to *in vitro* screening (Steps 14–22), primary transformants carrying the *bar* selection marker can be directly screened on soil in a greenhouse or growth chamber by spraying with the herbicide Basta or its chemically synthesized form, glufosinate ammonium^{14,20}. Briefly, spread non-sterile seed onto moistened soil under normal conditions similar to those described in Step 3. Cover the soil and tray with a clear plastic film until the germinated seedlings develop four to six leaves. Initiate the herbicide spraying when the cotyledons are visible, normally around 8–10 d after sowing. We usually spray the seedlings two times per week with a diluted solution of 250 mg L⁻¹ of herbicide (commercially available Challenge 60 from AgrEvo). True transformants will develop, while non-transformants will become chlorotic and eventually die after 3–4 weeks of herbicide treatment²¹.

▲ **CRITICAL STEP** It is important to spray the plants at some distance from the top. Direct spraying into the plants will cause them to tilt and bend toward the soil surface, and these tilted seedlings easily become rotten. Furthermore, trays containing the plants are normally covered during the selection period, but it is critical to leave a bit of space to allow some air flow to prevent contamination with fungi.

! **CAUTION** Appropriate masks should be used during spraying of herbicides, and spraying should be done in a separate room from where the plants are kept.

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● **TIMING**

- Growth of *Arabidopsis* plants: 2 months
- Growth of agrobacteria and floral dipping transformation: 3 d
- Seed set and maturation of transformed seeds: 1 month
- Screening of primary transformants: 10–14 d

? TROUBLESHOOTING

For troubleshooting guidance, see **Table 1**.

TABLE 1 | Troubleshooting table.

STEP	PROBLEM	SOLUTION
12	Plants producing lots of flowers but few seed	Too much water and low light intensity are likely the causes. Drain excess water so that it barely covers the tray. Increase light intensity and allow more space between pots.
20	No transformants	Incorrect constructs, non-transformed <i>Agrobacterium</i> cells and inappropriate antibiotics are likely reasons. Confirm constructs by sequencing if necessary, and verify that <i>Agrobacterium</i> cells contain the plasmids. Meanwhile, check that the correct antibiotics are used at the appropriate concentration.
23	All the sowed seeds grow after spraying	Spraying was initiated too late and non-transformants develop well enough to survive the herbicide. Start spraying earlier. To prevent flooding the seedlings, we suggest that plants should be watered from underneath their trays rather than directly onto the soil.

ANTICIPATED RESULTS

The methods described here should routinely yield at least one transformant for every 100 seeds harvested from *Agrobacterium*-transformed plants. Normally, it is not difficult to obtain hundreds of independent transgenic plants from two pots of treated plants (about 50–60 plants). For double transformation, it is possible to obtain several dozen of independent double transgenic lines from four pots of dipped plants. For the Ler-0 ecotype, the transformation efficiency is reduced to about 30% of that of other ecotypes.



ACKNOWLEDGMENTS We thank R. Catala-Rodriguez, I.-C. Jang, J. Xu and other members of the Chua laboratory for fruitful discussions. Work in the Chua laboratory is supported in part by US National Institutes of Health grant GM 44640.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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