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Review —Invited Paper—

AgarTrap Protocols on your Benchtop: Simple Methods for *Agrobacterium*-mediated Genetic Transformation of the Liverwort *Marchantia polymorpha*

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Abstract *Agrobacterium*-mediated genetic transformation is a powerful technique in plant biology. We recently developed a simplified *Agrobacterium*-mediated genetic transformation method for the liverwort *Marchantia polymorpha*, named AgarTrap (agar-utilized transformation with pouring solutions). AgarTrap is easy to perform; all procedures can be completed within a week using a single plate of solid medium, and basic operations involve simply pouring the appropriate solutions onto the solid medium. Thus far, we have developed three types of AgarTrap methods (S-AgarTrap, G-AgarTrap, and T-AgarTrap) using three different *M. polymorpha* tissues: sporelings, intact gemmalings, and mature thallus pieces, respectively. Each AgarTrap method can be used to transform tissues at high efficiency, thereby producing sufficient numbers of transformants for study. The ease and efficiency of these AgarTrap methods will likely prompt widespread molecular biological analyses of *M. polymorpha*. In this review, we describe the basic characteristics of the three AgarTrap methods and present the detailed protocols used in our laboratory.

Key words: AgarTrap, *Agrobacterium tumefaciens*, bryophyte, genetic transformation, *Marchantia polymorpha*.

Genetic transformation techniques are important for promoting and facilitating molecular analysis of plants. To date, several transformation techniques, such as *Agrobacterium*-, particle bombardment-, and polyethylene glycol (PEG)-mediated transformation, have been developed for use in many plant species (Newell 2000). Among these techniques, *Agrobacterium*-mediated transformation is widely used because it does not require expensive equipment. However, almost all *Agrobacterium*-mediated transformation methods reported to date are labor-intensive, as they often require plant materials to be transferred from one medium to another and/or the use of large volumes of liquid medium for co-cultivation with *A. tumefaciens* (Wang 2015).

The liverwort *Marchantia polymorpha* is a model species for examining the evolution of land plants, since liverwort is a sister species of all land plants (Bowman et al. 2017; Qiu et al. 2006). Similar to the conventional methods described above (Wang 2015), molecular analysis of *M. polymorpha* has previously been performed using *Agrobacterium*-mediated transformation of cultured cell lines, sporelings, and regenerating thalli (Ishizaki et al. 2008; Kubota et al. 2013; Nasu et al. 1997). We recently developed a labor- and time-saving *Agrobacterium*-mediated transformation method for *M. polymorpha* named AgarTrap (agar-utilized transformation with pouring solutions) (Tsuboyama and Kodama 2014). Unlike conventional methods, the AgarTrap procedure does not involve transferring tissues or the use of liquid medium. Instead, the appropriate buffer solutions are simply poured onto *M. polymorpha* tissue in a single 60 mm Petri dish of solid medium (Figure 1) (Tsuboyama and Kodama 2014). To date, we have developed three distinct AgarTrap methods for transformation of sporelings (S-AgarTrap), intact gemmalings (G-AgarTrap), and mature thallus pieces (T-AgarTrap) (Tsuboyama and Kodama 2014; Tsuboyama-Tanaka and Kodama 2015; Tsuboyama-Tanaka et al. 2015).

S-AgarTrap can be used to produce numerous transformants from sporelings, since it involves the use of abundant spores produced via sexual reproduction (Tsuboyama and Kodama 2014). Thus, S-AgarTrap is suitable for the large-scale production of transformants, such as T-DNA insertion mutants. However, because

Abbreviations: AgarTrap, Agar-utilized Transformation with Pouring Solutions; G-AgarTrap, AgarTrap using gemmalings; S-AgarTrap, AgarTrap using sporelings; T-AgarTrap, AgarTrap using mature thallus pieces; LB, Luria-Bertani; Tak-1, Takaragaike-1; Tak-2, Takaragaike-2; T-DNA, transfer DNA.

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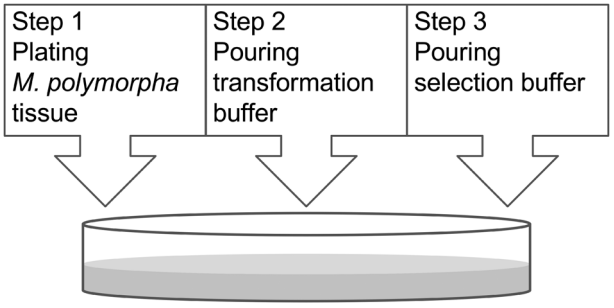

Figure 1. The concept of AgarTrap. AgarTrap involves three steps: (1) plating *M. polymorpha* tissue, (2) pouring transformation buffer, and (3) pouring selection buffer.

spores are produced by sexual reproduction, S-AgarTrap cannot be used to produce transformants with a uniform genetic background. G-AgarTrap, which involves intact gemmalings, can be used to produce transformants with a uniform genetic background, because each gemma develops from a single cell generated within the gemma cup on a mature thallus by asexual reproduction (Barnes and Land 1908; Tsuboyama-Tanaka and Kodama 2015). Like G-AgarTrap, T-AgarTrap, which uses mature thallus pieces, can be used to produce transformants with a uniform genetic background, as each thallus piece is prepared by cutting a single mature thallus (Tsuboyama-Tanaka et al. 2015). However, because fewer thallus pieces (with dimensions of approximately 5×5 mm) than gemmae (0.5×0.5 mm) can be cultured on a single Petri dish, fewer transformants can be obtained by T-AgarTrap than by G-AgarTrap using a single 60 mm Petri dish; several dozen transformants can be obtained on a single Petri dish via G-AgarTrap, but only a few transformants can be obtained via T-AgarTrap. T-AgarTrap may be suitable for use with previously produced transformants and/or mutants that cannot produce any gemmae.

We have optimized several experimental conditions for AgarTrap, including the pre-culture period for *M. polymorpha* tissue, the co-culture period with *A. tumefaciens*, the density of *A. tumefaciens* (OD_{600} in the transformation buffer), the acetosyringone concentration in the transformation buffer, the medium composition, and the *A. tumefaciens* culture conditions (Tsuboyama and Kodama 2014; Tsuboyama-Tanaka and Kodama 2015; Tsuboyama-Tanaka et al. 2015). The transformation efficiency of the S-, G-, and T-AgarTrap method is approximately 20%, 30–60%, and 50–70%, respectively (Tsuboyama and Kodama 2014; Tsuboyama-Tanaka and Kodama 2015; Tsuboyama-Tanaka et al. 2015). In addition, we recently improved several factors for G-AgarTrap during the co-culture period, as well as the transformation efficiency (Tsuboyama et al. 2018; Tsuboyama et al. unpublished data). Currently, the transformation efficiency of G-AgarTrap is approximately 50–60% for *M. polymorpha* strain Tak-1 (a male strain), and almost 100% for Tak-2 and BC3-38 (female strains) (Tsuboyama et al. 2018; Tsuboyama et al. unpublished data).

In this review, we present the AgarTrap protocols currently used in our laboratory. The equipment and reagents needed to perform these AgarTrap methods are listed in Tables 1 and 2.

Preparation of *M. polymorpha* and *A. tumefaciens* cultures

M. polymorpha strains: Strains Takaragaike-1 (Tak-1), Takaragaike-2 (Tak-2), and BC3-38 are used in these protocols. Tak-1 is a male strain, and Tak-2 and BC3-38 are female strains (Ishizaki et al. 2008; Tsuboyama-Tanaka and Kodama 2015). BC3-38 is the third backcross line between Tak-1 and Tak-2. The thalli are maintained on half-strength Gamborg's B5 (1/2 B5) medium solidified with 1% agar at pH 5.5 under 75 μmol photons $m^{-2}\,s^{-1}$ of continuous white light in a culture room at 22°C (Gamborg et al. 1968). Spores are produced by crossing Tak-1 and Tak-2 and are stored at −80°C until use. Gemmae and mature thalli pieces are obtained from 3- to 6-week-old thalli.

A. tumefaciens strains: The GV2260 strain can be used in all AgarTrap methods (Deblaere et al. 1985; Tsuboyama and Kodama 2014; Tsuboyama-Tanaka and Kodama 2015; Tsuboyama-Tanaka et al. 2015). However, for G-AgarTrap, the most efficient *A. tumefaciens* strain differs depending on the *M. polymorpha* strain used; *A. tumefaciens* strain GV3101::pMP90 (Koncz and Schell 1986) is the most efficient strain for use with Tak-1 and Tak-2, whereas EHA101 (Hood et al. 1986) is the most suitable strain for BC3-38 (Tsuboyama et al. 2018; Tsuboyama et al. unpublished data). *A. tumefaciens* cells harboring a binary vector are stored in 30% glycerol at −80°C until use. Before use, the *A. tumefaciens* cells are streaked onto LB solid medium supplemented with antibiotics and incubated at 28°C for 2–3 days.

AgarTrap protocols

AgarTrap involves three major steps: (1) plating of *M. polymorpha* tissue, (2) pouring transformation buffer, and (3) pouring selection buffer (Figure 1). A flowchart of the AgarTrap procedure and post-operations is shown in Figure 2. All AgarTrap procedures should be performed under sterile conditions (i.e., in a laminar flow hood), except for the streaking of *A. tumefaciens* onto LB solid medium.

Step 1: Plating of M. polymorpha tissue

Plate *M. polymorpha* tissue onto 10 ml of half-strength Gamborg's B5 solid medium supplemented with 1% agar and 1% sucrose at pH 5.5 in a disposable 60 mm sterile

Table 1. Equipment list.

Equipment	Use purpose
Laminar flow hood	General operations of AgarTrap
Micropipette and Micropipette tips (in a laminar flow hood)	General operations of AgarTrap
Gas burner (in a laminar flow hood)	Sterilization of forceps
Curved forceps (in a laminar flow hood)	Streaking gemmae
Forceps (in a laminar flow hood)	Planting thallus pieces
Aspirator (for use in a laminar flow hood)	Removing liquid
Incubator (28°C)	Culture of *A. tumefaciens*
Concentration measuring instrument (OD_{600})	Adjust of *A. tumefaciens* density
Tabletop centrifuge	Precipitation of spores
Vortex	Suspension of spores Suspension of *A. tumefaciens*
pH meter	Making solid medium for AgarTrap

Table 2. Products and reagents list.

Product name	Manufacturer	Product ID number
1.5 ml tube	WATSON	131-7155C
15 ml tube	AS ONE	1-3500-01
50 ml tube	Corning	430921
60×15 mm Petri dish	INA·OPTIKA	196001
90×15 mm Petri dish	AS ONE	3-1491-01
90×20 mm Petri dish	AS ONE	1-8549-04
Acetosyringone	Sigma-Aldrich	D134406
Agar	SSK sales	BOP
Cefotaxime	SANOFI	199121197
Fluorescent lamp	NEC	FL40SW
Fluorescent lamp	HITACHI	FL40SSW/37-B
Gamborg's B5 Medium Salt Mixture	Wako	399-00621
Glass beads	AS ONE	6-567-03
Hygromycin B	Wako	085-06153
KOH	Wako	168-03855
LB Agar	nacalai tesque	20067-85
LB broth	invitrogen	12780-052
MES	nacalai tesque	21623-26
$MgCl_2$	Wako	135-00165
Microporus tape (1.25 cm)	3M	1530-0
Microporus tape (2.5 cm)	3M	1530-1
NaClO	Wako	194-02216
NaOH	nacalai tesque	31511-05
Parafilm	Bemis	PM-996
Spectinomycin	Wako	195-11531
Sucrose	Wako	196-00015
TritonX-100	SIGMA	T-9284

Petri dish. This medium is referred to hereafter as solid medium.

(i) Spores (S-AgarTrap)

1. Resuspend sporangia that had been stored at −80°C in 400 μl sterile ultrapure water (e.g., autoclaved Milli-Q water) in a 1.5 ml tube by pipetting and/or vortexing.
   Tip: Because the spore germination rate is highly variable due to the different conditions used to produce sporangia, adjust the number of sporangia as needed.
2. After pipetting, transfer the spore suspension to a new 1.5 ml tube and discard the remaining husks. If the sporangia are contaminated by fungi and/or bacteria, add 400 μl sterilizing solution (0.4% HClO; 0.2% Triton X-100) to the spore suspension and incubate it for 1 min.
3. Precipitate the spores using a tabletop centrifuge and discard the supernatant. Add 400 μl sterile ultrapure water to the tube and wash the spores by pipetting and/or vortexing. Precipitate the spores again using a tabletop centrifuge and discard the supernatant. This washing procedure is performed twice.
4. After washing, resuspend the spore sediments in

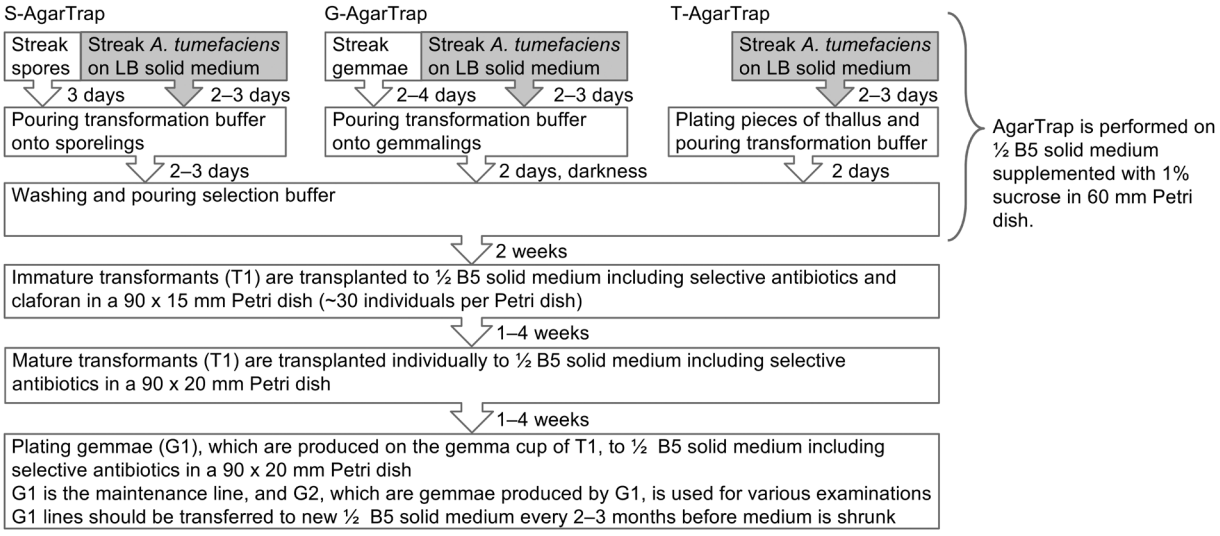

Figure 2. Flowchart of the AgarTrap protocols. *M. polymorpha* tissues are cultured in the light at 22°C in a culture room. *A. tumefaciens* is cultured at 28°C in an incubator.

500 μl sterile ultrapure water (Figure 3A).
Tip: Adjust the final volume depending on the number of Petri dishes used for transformation.

5. Place five sterile glass beads (diameter: 2.5–3.5 mm) onto the solid medium (Figure 3B).
6. Pour approximately 50 μl of spore suspension onto the glass beads (Figure 3C).
7. Shake the Petri dish to allow the spores to spread uniformly across the plate.
8. To retrieve the used glass beads, invert the Petri dish and tap it at the bottom.
9. Allow the used glass beads to fall into the lid of the Petri dish (Figure 3D), and retrieve them by tapping the lid.
10. Seal the Petri dish with microporous tape and culture the spores in the light for 3 days in a culture room (pre-culture step).
11. On the same day, streak *A. tumefaciens* cells onto LB solid medium and culture them at 28°C for 3 days in an incubator.

(ii) Gemmae (G-AgarTrap)

1. Drop sterile ultrapure water (300 μl) onto the solid medium (Figure 3E).
2. Place approximately 50 gemmae into the water droplet (Figure 3F).
3. Spread the gemmae onto the medium using curved-tipped forceps sterilized with a gas burner (Figure 3G).
   Tip: Be careful not to scratch the surface of the solid medium.
4. Seal the Petri dish with microporous tape, and culture the gemmae in the light for 2–4 days (for pre-culture) in a culture room.
5. On the same day or day after plating gemmae, streak *A. tumefaciens* cells onto LB solid medium and culture them at 28°C for 2–3 days in an incubator.

(iii) Mature thallus pieces (T-AgarTrap)

1. Streak the *A. tumefaciens* cells onto LB solid medium and culture them for 2–3 days at 28°C in an incubator. This step should be performed before plating the thallus pieces, which do not require pre-culture.
2. Cut mature thalli into approximately 5×5 mm pieces with forceps and place 10–15 thallus pieces onto solid medium (Figure 3H). Note that the thallus pieces do not require pre-culture.
   Tip: Thallus pieces can be obtained from whole thalli, as the transformation efficiency does not differ among pieces obtained from different positions of the thallus. When plating thallus pieces on solid medium, they must be flat to avoid the overgrowth of A. tumefaciens.

Step 2: Pouring transformation buffer containing A. tumefaciens

A. tumefaciens cells cultured on LB solid medium for 2–3 days at 28°C are used for co-culture (Figure 4A).

1. Skim cultured *A. tumefaciens* cells from the surface of the LB solid medium with a 1,000 μl micropipette tip (Figure 4B, C) and resuspend them in transformation buffer (150 μM acetosyringone, 10 mM $MgCl_2$, 10 mM MES-NaOH, pH 5.7).
2. Adjust the density of the *A. tumefaciens* cells in the transformation buffer to OD_{600}=0.3–0.5 (Figure 4D).
   Tip: The A. tumefaciens *density may need to be*

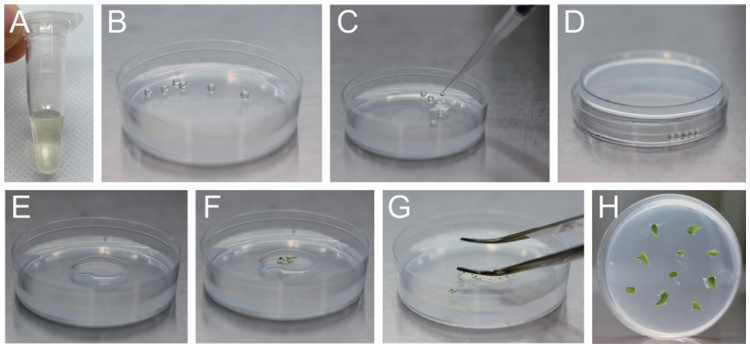

Figure 3. Photographs of steps in the method used to plate *M. polymorpha* tissue. (A) Spore suspension. (B) Sterile glass beads on solid medium. (C) Pouring spore suspension onto the glass beads. (D) Glass beads on the lid of a Petri dish. (E) Droplet (300 μl) of sterile water on solid medium. (F) Gemmae in a water droplet. (G) Spreading gemmae using curved forceps. (H) Plating approximately 5×5 mm mature thallus pieces on solid medium.

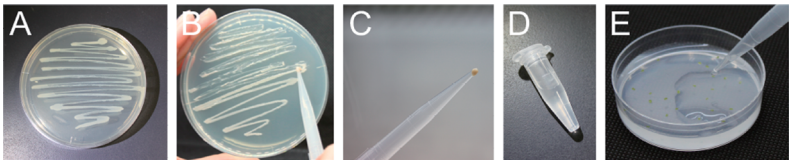

Figure 4. Preparation of *A. tumefaciens* in transformation buffer. (A) *A. tumefaciens* after 2 days of culture on LB solid medium. (B) Skimming *A. tumefaciens* with a 1,000 μl micropipette tip. (C) *A. tumefaciens* on the micropipette tip. (D) Tube of 1 ml transformation buffer containing *A. tumefaciens* at OD_{600}=0.5. (E) Pouring transformation buffer onto gemmalings on solid medium.

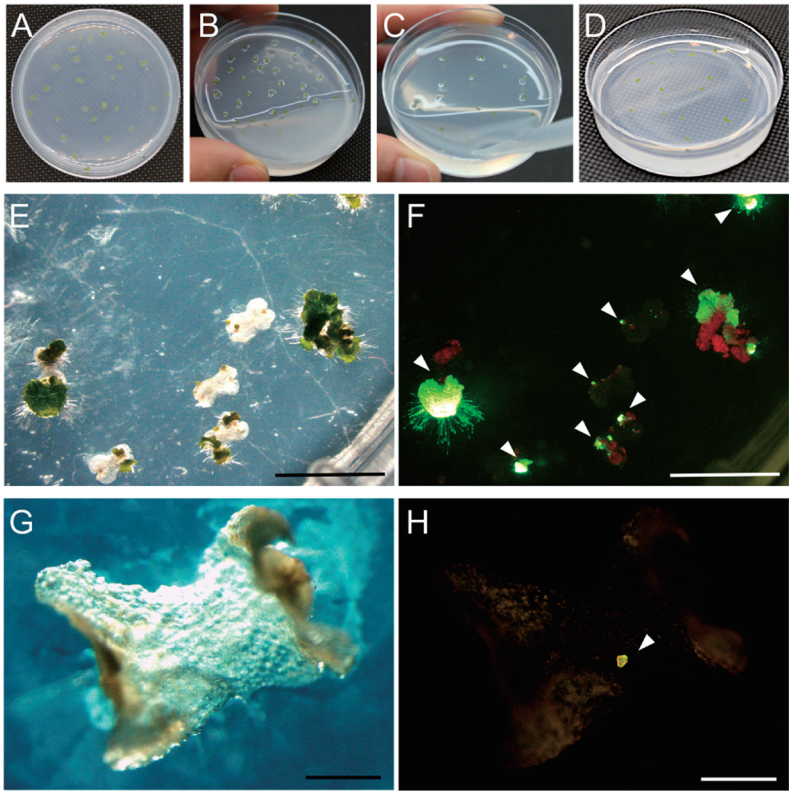

Figure 5. Washing *M. polymorpha* and selecting transformants. (A) *A. tumefaciens* growing on the surface of gemmalings after 2 days of co-culture. (B) Washing *M. polymorpha*. (C) Removing sterile water containing *A. tumefaciens* after washing. (D) One milliliter of selection buffer on the solid medium. (E–H) Cells transformed with *A. tumefaciens* harboring *pMpGWB103-Citrine* (Addgene Plasmid #79137) and selected on solid medium containing 10 mg l^{-1} hygromycin. Transformants are observed under a microscope (MZ16F stereo fluorescence microscope, Leica Microsystems, Wetzlar, Germany). (E, F) Transmitted light image (E) and fluorescence image (F) of transformants produced from BC3-38 gemmalings transformed with *Agrobacterium* strain EHA101 and selected for 14 days. Bar indicates 50 mm. Arrowheads indicate transformed cells (F). (G, H) Transmitted light image (G) and fluorescence image (H) of transformants produced from Tak-1 gemmalings transformed with *Agrobacterium* strain GV3101::pMP90 and selected for 15 days. Bar indicates 500 μm. Arrowhead indicates transformed cells (H).

optimized for each laboratory, because the growth rate of A. tumefaciens *is highly dependent on various factors.*

3. Pour 1 ml transformation buffer onto the *M. polymorpha* tissue on solid medium after pre-culture (Figure 4E).
   Tip: For S-AgarTrap, the transformation buffer should be spread over the entire surface of the solid medium because the sporelings cannot be detected with the naked eye.
4. After 1 min, remove excess transformation buffer from the medium with an aspirator or micropipette while tilting the Petri dish.
5. Seal the Petri dish with microporous tape or Parafilm and incubate at 22°C for 2–3 days. In the case of G-AgarTrap, incubate the Petri dish in darkness condition during co-cultivation.

Step 3: Pouring selection buffer containing antibiotics

After co-cultivation, a thin layer of *A. tumefaciens* cells covers the *M. polymorpha* tissues and the surface of the solid medium (Figure 5A). To remove the *A. tumefaciens* cells, wash the specimens using sterile ultrapure water. When sterile water is poured onto the solid medium, the *A. tumefaciens* cells float, whereas *M. polymorpha* is attached to the solid medium by its rhizoids (Figure 5B). Water (containing only *A. tumefaciens)* can easily be removed with an aspirator or micropipette while tilting the Petri dish (Figure 5C).

1. Pour 4 ml sterile water onto the *M. polymorpha* tissue and the surface of the solid medium, which are covered with a thin layer of *A. tumefaciens* cells.
   Tip: We normally use a 5 ml micropipette to pour 4 ml of sterile water into the Petri dish. If you do not have this type of micropipette, use a 1,000 µl micropipette and pour 1 ml of sterile water into the Petri dish.
2. To efficiently remove the *A. tumefaciens*, gently tilt the Petri dish from left to right several times.
3. Remove the water containing *A. tumefaciens* with an aspirator or micropipette while tilting the Petri dish (Figure 5B, C). This washing procedure is performed twice.
4. After washing, pour 1 ml selection buffer containing a selective antibiotic (e.g., 100 $\mu g\,ml^{-1}$ hygromycin) and 1 $mg\,ml^{-1}$ cefotaxime onto the solid medium (Figure 5D). After pouring, do not discard the selection buffer on the surface of the solid medium; the solid medium will absorb the 1 ml selection buffer.
   Tip: The amount of antibiotic used should be adjusted according to the volume of solid medium. For example, when using 10 ml of solid medium treated with 100 µg hygromycin and 1 mg cefotaxime, the M. polymorpha *and* A. tumefaciens *cells receive 10 $\mu g\,ml^{-1}$ hygromycin and 100 $\mu g\,ml^{-1}$ cefotaxime.*
5. Seal the Petri dish with microporous tape and incubate it in the light for 2–3 weeks.
6. After culture, the transgenic cells survive, whereas almost all non-transgenic cells perish (Figure 5E, F).
   Tip: In both G-AgarTrap and T-AgarTrap, all cells in the explant tissues might appear to be dead during selection because these tissues contain only a few transformed cells (Figure 5G, H).
7. After 2–3 weeks of selection, the solid medium might have shrunk due to drying. To efficiently grow the thalli, transfer the transformants to a new 90 mm Petri dish containing ½ B5 solid medium including a selective antibiotic (e.g., 10 $\mu g\,ml^{-1}$ hygromycin) and 100 $\mu g\,ml^{-1}$ cefotaxime (~30 individuals per 90 mm Petri dish) *(see also Figure 2)*.
   Tip: After AgarTrap, it is not necessary to add sucrose to the medium. Cefotaxime must be added to the ½ B5 solid medium to avoid the regrowth of A. tumefaciens in the first transfer. In the second transfer, cefotaxime is not needed.

Concluding remarks

To simplify the *Agrobacterium*-mediated transformation procedure for *M. polymorpha*, we omitted labor-intensive steps such as the use of liquid medium and the transfer of plant tissues to multiple media. Our simplified procedure, named AgarTrap, involves trapping *M. polymorpha* tissues on solid agar medium and allowing the appropriate solutions to be absorbed into the medium. Because these AgarTrap methods are not only simple but also highly efficient, we believe that the AgarTrap technique will promote the molecular analyses of *M. polymorpha*. Furthermore, we are currently evaluating the use of the AgarTrap technique for genetic transformation of other plant species. We hope that the AgarTrap concept will be widely adopted by the plant science community.

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References

Barnes CR, Land WJG (1908) Bryological papers. II. The origin of the cupule of *Marchantia*. *Bot Gaz* 46: 401–409

Bowman JL, Kohchi T, Yamato KT, Jenkins J, Shu S, Ishizaki K, Yamaoka S, Nishihama R, Nakamura Y, Berger F, et al. (2017) Insights into land plant evolution garnered from the *Marchantia polymorpha* genome. *Cell* 171: 287–304.e15

Deblaere R, Bytebier B, De Greve H, Deboeck F, Schell J, Van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid derived vectors for *Agrobacterium*-mediated gene transfer to plants. *Nucleic Acids Res* 13: 4777–4788

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 50: 151–158

Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J Bacteriol* 168: 1291–1301

Ishizaki K, Chiyoda S, Yamato KT, Kohchi T (2008) *Agrobacterium*-mediated transformation of the haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. *Plant Cell Physiol* 49: 1084–1091

Koncz C, Schell J (1986) The promoter of the TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol Gen Genet* 204: 383–396

Kubota A, Ishizaki K, Hosaka M, Kohchi T (2013) Efficient *Agrobacterium*-mediated transformation of the liverwort *Marchantia polymorpha* using regenerating thalli. *Biosci Biotechnol Biochem* 77: 167–172

Nasu M, Tani K, Hattori C, Honda M, Shimaoka T, Yamaguchi N, Katoh K (1997) Efficient transformation of *Marchantia polymorpha* that is haploid and has a very small genome DNA. *J Ferment Bioeng* 84: 519–523

Newell CA (2000) Plant transformation technology: Developments and applications. *Mol Biotechnol* 16: 53–66

Qiu YL, Li L, Wang B, Chen Z, Knoop V, Groth-Malonek M, Dombrovska O, Lee J, Kent L, Rest J, et al. (2006) The deepest divergences in land plants inferred from phylogenomic evidence. *Proc Natl Acad Sci USA* 103: 15511–15516

Tsuboyama S, Kodama Y (2014) AgarTrap: A simplified *Agrobacterium*-mediated transformation method for sporelings of the liverwort *Marchantia polymorpha* L. *Plant Cell Physiol* 55: 229–236

Tsuboyama S, Nonaka S, Ezura H, Kodama Y (2018) Improved G-AgarTrap: A highly efficient transformation method for intact gemmalings of the liverwort *Marchantia polymorpha*. *bioRxiv* 329839

Tsuboyama-Tanaka S, Kodama Y (2015) AgarTrap-mediated genetic transformation using intact gemmae/gemmalings of the liverwort *Marchantia polymorpha* L. *J Plant Res* 128: 337–344

Tsuboyama-Tanaka S, Nonaka S, Kodama Y (2015) A highly efficient AgarTrap method for genetic transformation of mature thalli of the liverwort *Marchantia polymorpha* L. *Plant Biotechnol* 32: 333–336

Wang K (2015) *Agrobacterium* Protocols, Third Edition. Springer, New York