

1 **Title:**

2 Improved G-AgarTrap: A highly efficient transformation method for intact gemmalings
3 of the liverwort *Marchantia polymorpha*

4

5 **Authors:**

6 Shoko Tsuboyama¹, Satoko Nonaka², Hiroshi Ezura², and Yutaka Kodama^{1*}

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8 **Author Addresses:**

9 ¹Center for Bioscience Research and Education, Utsunomiya University, Tochigi
10 321-8505, Japan. ²Tsukuba Plant Innovation Research Center, University of Tsukuba,
11 Ibaraki 305-8572, Japan

12

13 ***Corresponding Author:**

14 Yutaka Kodama

15 350 Mine-machi, Utsunomiya city, Tochigi 321-8505, Japan.

16 Tel: +81-28-649-8154

17 Fax: +81-28-649-8651

18 E-mail: kodama@cc.utsunomiya-u.ac.jp

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1 **Abstract**

2 Liverworts are key species for studies of plant evolution, occupying a basal position
3 among the land plants. *Marchantia polymorpha* has emerged as a highly studied model
4 liverwort, and many relevant techniques, including genetic transformation, have been
5 established for this species. *Agrobacterium*-mediated transformation is widely used in
6 many plant species because of its low cost. Recently, we developed a simplified
7 *Agrobacterium*-mediated method for transforming *M. polymorpha*, known as AgarTrap
8 (agar-utilized transformation with pouring solutions). The AgarTrap procedure, which
9 involves culturing the liverwort tissue in various solutions on a single solid medium,
10 yields up to a hundred independent transformants. AgarTrap is a simple procedure,
11 requiring minimal expertise, cost, and time.

12 Here, we investigated four factors that influence AgarTrap transformation
13 efficiency: (1) humidity, (2) surfactant in the transformation buffer, (3) *Agrobacterium*
14 strain, and (4) light/dark condition. We adapted the AgarTrap protocol for transforming
15 intact gemmalings, achieving an exceptionally high transformation efficiency of 97%.
16 The improved AgarTrap method will enhance the molecular biological study of *M.*
17 *polymorpha*. The present study also provides new possibilities for improving
18 transformation techniques for a variety of plant species.

19

1 **Key words**

2 AgarTrap, *Agrobacterium*, bryophyte, liverwort, *Marchantia polymorpha*,

3 transformation, transformation efficiency, transgenic

4

1 **Introduction**

2 *Marchantia polymorpha* is a dioecious liverwort, the sister group to all other land
3 plants¹. This species has therefore been extensively studied to enhance our
4 understanding of land plant evolution, with research focusing on its taxonomy,
5 development, and physiology; furthermore, its nuclear, chloroplast, and mitochondrial
6 genomes have all been sequenced^{2–6}. The rapidly expanding *M. polymorpha* research
7 community has recently developed various molecular biology techniques to study this
8 key species, including particle bombardment- and *Agrobacterium*-mediated
9 transformation, plastid transformation, homologous recombination-mediated gene
10 targeting, and TALEN- and CRISPR/Cas9-mediated genome editing^{7–16}.

11

12 *Agrobacterium*-mediated transformation is widely used for many plant species because
13 it does not require any expensive equipment¹⁷. This technique involves three steps: (1)
14 preparation of plant material, (2) co-culture of the material with *Agrobacterium*
15 *tumefaciens* containing a recombinant transfer DNA (T-DNA), and (3) antibiotic
16 selection of transgenic cells. During the co-culture step, T-DNA is transferred from the
17 *Agrobacterium* into the plant cell, where it is integrated into the genome to facilitate the
18 expression of its constituent genes. Previous studies have determined that the co-culture
19 conditions are the most important aspect of transformation efficiency, with the
20 *Agrobacterium* strain used, duration of co-culture, *Agrobacterium* density, temperature,
21 co-culture medium, and surfactants used having the greatest impact^{18–22}.

22

1 Recently, we developed a simplified *Agrobacterium*-mediated transformation method
2 for *M. polymorpha*, which we named AgarTrap (agar-utilized transformation with
3 pouring solutions)^{23–25}. Like the general *Agrobacterium*-mediated transformation
4 procedure, AgarTrap consists of three steps: (1) pre-culture of *M. polymorpha* tissue, (2)
5 co-culture of the tissue with *Agrobacterium* containing recombinant T-DNA, and (3)
6 selection of transgenic cells. A unique feature of AgarTrap is that none of these steps
7 require a liquid medium culture; rather, the appropriate solutions are simply poured onto
8 the solid medium in a single Petri dish (Fig. 1)^{23–25}. We previously developed and
9 optimized AgarTrap for use with *M. polymorpha* sporelings (S-AgarTrap), intact
10 gemmae/gemmalings (G-AgarTrap), and pieces of mature thallus (T-AgarTrap),
11 achieving transformation efficiencies of approximately 20%, 60%, and 70%,
12 respectively^{23–25}. Despite its low transformation efficiency, S-AgarTrap results in
13 numerous transformants, because spores are produced abundantly, rendering it suitable
14 for the large-scale production of transformants (e.g., T-DNA insertion mutants)²³.
15 However, because spores are produced by sexual reproduction, S-AgarTrap
16 transformants do not have a uniform genetic background. G-AgarTrap can be used to
17 produce transformants in a genetically uniform background, because the gemmae
18 develop from single cells asexually generated within the gemma cup on a mature
19 thallus^{24,26}. Similarly, T-AgarTrap results in transformants with uniform genetic
20 backgrounds, because the cells are obtained from mature thalli²⁵; however, fewer
21 individual transformants were obtained using T-AgarTrap than G-AgarTrap despite their
22 respective transformation efficiencies, because the pieces of mature thallus were larger

1 than the gemmae and fewer could be included in a single Petri dish. Thus, of these three
2 methods, G-AgarTrap appears to be the best choice for producing transgenic *M.*
3 *polymorpha*; however, because the transformation efficiency of G-AgarTrap was
4 relatively low (approximately 60%), this approach needed improvement. As the
5 co-culture step is the most critical for efficient transformation^{18,22}, the transformation
6 efficiency of G-AgarTrap would likely be improved by optimizing this step.

7

8 In our previous study, we optimized several factors of AgarTrap transformation,
9 including the pre-culture period of *M. polymorpha* tissue, the duration of co-culture,
10 *Agrobacterium* density (OD₆₀₀ in transformation buffer), acetosyringone concentration
11 in the transformation buffer, medium composition, and *Agrobacterium* culture
12 conditions^{23–25}. In the present study, we investigated four additional co-culture factors:
13 (1) humidity, (2) surfactant in the transformation buffer, (3) *Agrobacterium* strain, and
14 (4) light/dark condition. We also fine-tuned the pre-culture period, ultimately achieving
15 an exceptionally high transformation efficiency for the G-AgarTrap procedure, of nearly
16 100%.

17

18 **Results**

19 **Humidity conditions during co-culture**

20 In our previous study, gemmalings (BC3-38) were pre-cultured for one day and
21 co-cultured with *Agrobacterium* for three days on ½ B5 medium supplemented with 1%
22 sucrose, which resulted in a median transformation efficiency of 57.0% (mean: 59.2%)

1 (Fig. 2a)²⁴. Permeable microporous tape was used to seal the Petri dishes containing the
2 solid medium; therefore, the humidity to which the plants were exposed depended on
3 the humidity of the culture room. The humidity of the culture room was kept at
4 approximately 40% with a humidifier, as in our previous study²⁴. In the present study,
5 we tested whether humidity differences in the co-culture step influence transformation
6 efficiency. Without the humidifier, the humidity in the culture room decreased to
7 approximately 20%. When gemmalings were co-cultured with *Agrobacterium* at 20%
8 humidity, the median transformation efficiency was decreased by 8.1%, and the mean
9 efficiency decreased by 10.5% (Fig. 2a, see also Supplementary Table S1). These results
10 suggested that higher humidities during the co-culture step increase transformation
11 efficiency; however, it can be challenging to control the humidity in culture rooms
12 precisely, because humidity fluctuates depending on the location and/or season.

13

14 To maintain a high humidity in the Petri dishes during co-culture, we sealed the dishes
15 with Parafilm, which is more airtight than microporous tape. When using Parafilm,
16 almost all gemmalings co-cultured for three days suffered from an overgrowth of
17 *Agrobacterium* such as Supplementary Fig. S1, suggesting that the growth of this
18 bacterium is enhanced by high humidity. Since it was difficult to completely eliminate
19 the bacteria in the subsequent selection step when they were overgrown, the co-culture
20 period was shortened to two days when using Parafilm, which increased the median
21 transformation efficiency to 62.3% (mean: 59.6%) in the 20% humidity condition (Fig.
22 2a, see also Supplementary Table S1). These results indicate that the high humidity in

1 Parafilm-sealed Petri dishes during the co-culture step increases the transformation
2 efficiency, while shortening the required duration of the co-culture period from three
3 days (at 40% humidity when sealed with microporous tape) to two days.

4

5 Next, we investigated the pre-culture period of gemmae/gemmalings required when
6 sealing the dishes with Parafilm during the co-culture step. The gemmalings were
7 pre-cultured for 0, 1, 2, 3, and 4 days in a Petri dish sealed with microporous tape, then
8 co-cultured for two days in a Petri dish sealed with Parafilm, which led to median
9 transformation efficiencies of 0% (mean: 0.6%), 74.1% (mean: 62.6%), 74.1% (mean:
10 70.3%), 47.4% (mean: 45.8%), and 9.1% (mean: 12.2%), respectively (Fig. 2b, see also
11 Supplementary Table S2). These results indicate that pre-culture periods of one and two
12 days are optimal.

13

14 The use of Parafilm-sealed Petri dishes shortened the period required for the AgarTrap
15 co-culture step. For the following investigations, we used fixed conditions of a two-day
16 pre-culture, a two-day co-culture with *Agrobacterium* strain GV2260 in the light in Petri
17 dishes sealed with Parafilm, and no surfactant in the transformation buffer. These
18 conditions were varied as described below, to investigate their impact on transformation
19 efficiency.

20

21 **Surfactants in transformation buffer**

22 In previous studies of *Agrobacterium*-mediated transformation, it was reported that the

1 use of surfactants in the co-cultivation medium during co-culture increased the
2 transformation efficiency^{27,28}. We therefore examined whether surfactants in the
3 transformation buffer influenced the efficiency of G-AgarTrap.

4

5 To determine a suitable surfactant for *M. polymorpha* transformation, a survival test was
6 performed using three surfactants, Silwet L-77, Triton X-100, and Tween 20. We added
7 various concentrations of these surfactants to the transformation buffer and treated the
8 pre-cultured gemmalings with this buffer. After two days of co-culture, the survival
9 rates of the gemmalings were estimated. Four concentrations of Silwet L-77 (0.01%,
10 0.02%, 0.05%, and 0.1%) were analyzed, resulting in mean survival rates of 100%,
11 100%, 98.8%, and 11.7%, respectively (Fig. 3a). When 0.01%, 0.02%, 0.05%, or 0.1%
12 Triton X-100 was used, the mean survival rates of the gemmalings were 100%, 100%,
13 99.2%, and 63.5%, respectively (Fig. 3b). Because gemmalings could not survive in the
14 higher concentrations of Silwet L-77 and Triton X-100, these surfactants may be toxic
15 to *M. polymorpha*. By contrast, when Tween 20 concentrations of 0.01%, 0.02%, 0.05%,
16 and 0.1% were tested, the mean survival rate was 100% for all concentrations (Fig. 3c).
17 Tween 20 seemed to have no effect on gemmaling growth, and was therefore selected
18 for use as a surfactant.

19

20 We assessed whether the use of Tween 20 in the transformation buffer increased the
21 efficiency of G-AgarTrap. Tween 20 concentrations of 0%, 0.01%, 0.02%, 0.05%, and
22 0.1% resulted in median transformation efficiencies of 57.6% (mean: 59.3%), 80.0%

1 (mean: 74.1%), 77.4% (mean: 73.8%), 70.6% (mean: 65.8%), and 54.8% (mean:
2 61.1%), respectively (Fig. 3d, see also Supplementary Table S3). These results showed
3 that the use of 0.01–0.02% Tween 20 in the transformation buffer slightly increased the
4 efficiency of G-AgarTrap transformation; however, the differences were not statistically
5 significant. Nevertheless, when the gemmalings were co-cultured in transformation
6 buffer, the solutions lacking surfactant were often repelled by the plants, requiring
7 careful manipulation to ensure proper coverage. When surfactants such as Tween 20
8 were added to the transformation buffer, this hydrophobicity was counteracted;
9 therefore, the addition of surfactants improves the ease of performing G-AgarTrap
10 transformations.

11

12 *Agrobacterium* strain

13 *Agrobacterium* strains influence the efficiency of *Agrobacterium*-mediated
14 transformations in other plant species, with the most effective strain being dependent on
15 the plant species or transformation method used^{29–33}. For the transformation of *M.*
16 *polymorpha* above, and in the previous G-AgarTrap study, the GV2260 strain was
17 used²⁴. To assess the best strain for G-AgarTrap transformation, we compared the
18 efficiencies of the technique using five *Agrobacterium* strains, GV2260, EHA101,
19 EHA105, LBA4404, and MP90^{34–38}. The median transformation efficiencies using these
20 strains were 61.0% (mean: 57.6%), 96.7% (mean: 93.8%), 47.6% (mean: 47.2%),
21 28.3% (mean: 26.2%), and 9.2% (mean: 18.1%), respectively (Fig. 4a, see also
22 Supplementary Table S4). The use of *Agrobacterium* strain EHA101 resulted in over a

1 90% efficiency in eight out of 10 transformations, and 100% efficiency on four
2 occasions (Fig. 4a, see also Supplementary Table S4). EHA101 was therefore the
3 superior strain for AgarTrap, contributing consistently high levels of transformation
4 efficiency (Fig. 4a, see also Supplementary Table S4), which also resulted in the
5 presence of many transformed cells within each gemmaling (Fig. 4b). Conversely,
6 MP90 was not suitable for AgarTrap, as its use resulted in a 0% efficiency for two of 10
7 transformations, and only ever resulted in one or a few transformed cells within a single
8 gemmaling (Fig. 4a, c, see also Supplementary Table S4).

9

10 We assessed the combined use of the most efficient *Agrobacterium* strain, EHA101, and
11 0.01–0.02% Tween 20 as a surfactant. When gemmalings were transformed with
12 EHA101 in the presence of 0.01% Tween 20, the median transformation efficiency was
13 95.5% (mean: 93.1%), which was similar to the efficiency of EHA101-mediated
14 transformations without a surfactant (Supplementary Fig. S2, see also Supplementary
15 Table S5). The median transformation efficiency of EHA101 using 0.02% Tween 20 as
16 a surfactant decreased to 17.6% (mean: 40.1%) (Supplementary Fig. S2, see also
17 Supplementary Table S5). Thus, when using EHA101, 0.01% Tween 20 yields better
18 results than 0.02% Tween 20.

19

20 **Light/dark condition during co-culture**

21 In previous studies of *Agrobacterium*-mediated transformation, light and dark
22 conditions were reported to influence the transformation efficiency^{39–41}. All previous

1 studies of AgarTrap were performed under continuous white light conditions (75 μmol
2 photons $\text{m}^{-2} \text{s}^{-1}$)^{23–25}. When *M. polymorpha* was co-cultured with *Agrobacterium* strain
3 GV2260, the median transformation efficiencies under light and dark conditions were
4 61.5% (mean: 61.3%) and 97.1% (mean: 95.3%), respectively (Fig. 5a, see also
5 Supplementary Table S6). Additionally, the combined use of the most efficient
6 *Agrobacterium* strain, EHA101, and dark conditions during the co-culture period
7 resulted in a median transformation efficiency of 100% (mean: 97.0%), which was the
8 highest efficiency observed in this study. Of the seven transformations performed in
9 darkness using EHA101, a transformation efficiency of 100% was achieved five times
10 (Fig. 5a, b, see also Supplementary Table S6). Numerous cells in each gemmaling were
11 transformed under the dark condition when using either GV2260 or EHA101 (Fig. 5c,
12 d). Thus, for the G-AgarTrap transformation of *M. polymorpha*, the transformation
13 efficiency when gemmalings were co-cultured with *Agrobacterium* under dark
14 conditions was higher than that under light conditions.

15

16 Discussion

17 To improve the efficiency of the G-AgarTrap transformation of *M. polymorpha*, we
18 focused on optimizing the co-culture step for the following four factors: (1) humidity,
19 (2) surfactant in the transformation buffer, (3) *Agrobacterium* strain, and (4) light/dark
20 condition. Among these factors, humidity, *Agrobacterium* strain, and light/dark
21 condition contributed to increases in transformation efficiency.

22

1 Because AgarTrap is performed on a solid medium, we predicted that humidity might
2 influence the transformation efficiency. We found that high humidities during co-culture
3 promoted transformation efficiency, and that sealing the Petri dishes with Parafilm
4 instead of microporous tape could overcome the problem of low culture room humidity.
5 The high humidity also enhanced *Agrobacterium* growth, suggesting that this bacterium
6 is sensitive to drying out. Sealing the Petri dishes with Parafilm might better maintain a
7 high internal humidity than sealing the Petri dishes with microporous tape. The
8 enhancement of *Agrobacterium* observed in Petri dishes sealed with Parafilm might
9 promote transformation efficiency; however, the overgrown bacteria were difficult to
10 completely eliminate in the subsequent selection step of G-AgarTrap. When Parafilm
11 was used to seal the Petri dishes during two days of co-culture, efficient pre-culture
12 periods were one and two days. This result was consistent with our previous study using
13 microporous tape-sealed Petri dishes, in which the humidity was approximately 40%²⁴.
14 This suggests that the gemmaling cell states arising after 1–2 days of pre-culture might
15 be the most suitable for transformation.

16
17 In the *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, the use of a
18 surfactant, Silwet L-77, increases the transformation efficiency by reducing the surface
19 tension of the aqueous solution^{27,42}. In the present study, we did not find any significant
20 improvement in transformation efficiency when using a range of surfactants; however,
21 the addition of surfactants simplified the procedure by reducing the hydrophobicity of
22 the gemmalings, which otherwise repelled the transformation solution. When 0.05% and

1 0.1% Tween 20 were used, the transformation efficiency using *Agrobacterium* GV2260
2 was decreased relative to the efficiency when using 0.01% and 0.02% Tween 20
3 solutions, even though ~1% Tween 20 did not cause damage to *M. polymorpha*
4 gemmalings. The solutions did not appear to affect the survival rate of *M. polymorpha*;
5 therefore, the higher concentrations (0.05% and 0.1%) of Tween 20 might affect the
6 bacterium itself. The inclusion of Tween 20 when using the more effective EHA101
7 strain also requires caution, because the transformation efficiency was greatly decreased
8 with a 0.02% concentration of Tween 20 in the transformation solution. EHA101 might
9 therefore be more sensitive to Tween 20 than GV2260.

10

11 The transformation efficiency of G-AgarTrap varied significantly with the use of
12 different *Agrobacterium* strains; the strains yielding the highest and lowest efficiencies
13 were EHA101 and MP90, respectively. In a previous study using tomato (*Solanum*
14 *lycopersicum*), it was suggested that differences in transformation efficiency using
15 different *Agrobacterium* strains was caused by variations in the plant tissue mortality³²,
16 which might also be the case in the present study. Additionally, for many methods using
17 *Agrobacterium*-mediated plant transformation, the co-culture medium was optimized
18 for transformation, but was also used for the culture of both plant material and
19 *Agrobacterium*. By contrast, in AgarTrap, the co-culture was performed on a solid
20 medium (½ B5 supplemented with 1% sucrose in agar) optimized for the growth of *M.*
21 *polymorpha*, but not optimized for *Agrobacterium*. Thus, the solid medium might
22 negatively affect *Agrobacterium*, leading to differences in transformation efficiency as a

1 result of differences in the adaptability of the *Agrobacterium* strains to the medium.

2

3 It was previously reported that the EHA101 and EHA105 strains are genetically almost
4 identical, as EHA105 was developed by the removal of a kanamycin resistance gene
5 from EHA101³⁷; however, in G-AgarTrap, we found a remarkable difference in
6 transformation efficiency when using EHA101 in comparison with EHA105. This might
7 suggest that they are less genetically similar than previously thought. This possibility
8 remains to be investigated.

9

10 Previous reports using intact tobacco (*Nicotiana tabacum*) seedlings, *A. thaliana* root
11 segments, and tepary bean (*Phaseolus acutifolius*) calli suggested that light enhanced
12 transformation efficiency^{39,41}, but another report using carnation (*Dianthus*
13 *caryophyllus*) stem explants reported that dark conditions resulted in a higher proportion
14 of transformants⁴⁰. No significant differences in transformation efficiency were
15 observed between light and dark conditions in garlic (*Allium sativum*)⁴³. These
16 differences suggest that the effects of light on transformation efficiency might depend
17 on the plant species or tissue used. In the present study, we found that performing the
18 co-culture in darkness significantly enhanced the transformation efficiency. The
19 dark-mediated improvement in transformation efficiency for carnation stem explants
20 was previously suggested to be caused by an increased susceptibility to infection in the
21 etiolated tissue, and/or by an enhanced *Agrobacterium* activation⁴⁰. A subsequent report
22 confirmed that *Agrobacterium* activation is greater in darkness⁴⁴. Plants are more

1 susceptible to infection by pathogens at night, because the reactive oxygen species
2 produced by photosynthesis enhance their resistance to attack⁴⁵. Taken together, we
3 hypothesize that the dark-mediated activation of *Agrobacterium* and the increased
4 susceptibility to infection in the gemmaling cells in darkness result in the observed
5 improvement in transformation efficiency when performing the AgarTrap co-culture in
6 the dark compared with the light condition.

7

8 In this study, we successfully developed a highly efficient G-AgarTrap procedure by
9 making several modifications (high humidity, darkness, *Agrobacterium* strain EHA101)
10 to the co-culture step. The improved G-AgarTrap technique will benefit future
11 molecular biology studies of *M. polymorpha*. These improved conditions may also be
12 applicable to other AgarTrap methods (S- and T-AgarTrap). Furthermore, understanding
13 the biological mechanisms underpinning the benefits of these improvements may
14 contribute to the enhancement of the many other transformation technologies using
15 *Agrobacterium* applied to various plant species.

16

17 **Methods**

18 **Plant materials and growth conditions**

19 *Marchantia polymorpha* (L.) gemmae/gemmalings of BC3-38, the female line of the
20 third backcross generation created in the crossing of Takaragaike-1 (male line) and
21 Takaragaike-2 (female line), were used in this study. BC3-38 was provided by Dr.
22 Takayuki Kohchi (Kyoto University, Kyoto, Japan). The plants were maintained on

1 half-strength Gamborg's B5 ($\frac{1}{2}$ B5) medium^{46,47} containing 1% agar (BOP; SSK Sales
2 Co., Ltd., Shizuoka, Japan), pH 5.5, in a 90-mm disposable sterile Petri dish. *M.*
3 *polymorpha* tissues were illuminated with 75 μmol photons $\text{m}^{-2} \text{ s}^{-1}$ continuous white
4 light (FL40SW; NEC Corporation, Tokyo, Japan) in a culture room maintained at
5 around 22°C with air conditioning. The gemmae/gemmalings subjected to G-AgarTrap
6 transformation were obtained from one- to two-month-old thalli.

7

8 **G-AgarTrap**

9 The basic procedure of G-AgarTrap was previously reported²⁴. Gemmae were sown on
10 approximately 10 mL $\frac{1}{2}$ B5 solid medium (1% agar) supplemented with 1% sucrose, pH
11 5.5, in a 60-mm disposable sterile Petri dish, and pre-cultured into gemmalings. For the
12 co-culture, 1–3 mL transformation buffer (10 mM MgCl₂; 10 mM MES-NaOH, pH 5.7;
13 150 μM acetosyringone; *Agrobacterium* OD₆₀₀ = 0.5) was poured over the gemmalings,
14 with the excess being removed after 1 min using an aspirator or micropipette. Four
15 factors were considered, including sealing of the Petri dish with Parafilm, the
16 *Agrobacterium* strain used, the addition of a surfactant (0.01–0.1% Tween 20) in the
17 transformation buffer, and dark treatment during the co-culture period. After
18 co-cultivation, the *Agrobacterium* was twice washed from the gemmalings and solid
19 medium with 1–4 mL sterile water, and then 1 mL selection buffer containing
20 antibiotics (100 μg hygromycin B and 1 mg Claforan) was poured over the gemmalings
21 and the solid medium. After culturing for a few weeks, the transformed cells had grown
22 and the non-transgenic cells had died²⁴.

1

2 ***Agrobacterium* preparation for G-AgarTrap**

3 *Agrobacterium tumefaciens* harboring the *pMpGWB103-Citrine* vector, which encodes
4 bacterial aminoglycoside resistance (*aadA*), was stored in 30% glycerol at -80°C. On
5 the same day that the gemmae were sown on the ½ B5 medium (the first step in the
6 G-AgarTrap procedure), *Agrobacterium* was streaked onto Luria-Bertani (LB) solid
7 medium (1% agar) supplemented with 100 mg L⁻¹ spectinomycin and incubated at 28°C
8 for 2–3 days (Supplementary Fig. S1a). The *Agrobacterium* was then suspended in
9 transformation buffer at OD₆₀₀ = 0.5 (Supplementary Fig. S1b). Surfactant (0.01–0.1%
10 Silwet L-77, Triton X-100, or Tween 20) was included in the transformation buffer. A
11 1-mL aliquot of transformation buffer was poured onto each Petri dish during the
12 co-culture step.

13

14 **Microscopy observation**

15 *M. polymorpha* gemmalings were observed using a MZ16F stereo fluorescence
16 microscope (Leica Microsystems, Wetzlar, Germany). Chlorophyll fluorescence and
17 Citrine fluorescence (in transgenic cells) were determined using a fluorescence module
18 (excitation filter: 480/40 nm; barrier filter: LP 510 nm). Images were taken using a
19 DP73 digital camera (Olympus, Tokyo, Japan).

20

21 **Transformation efficiency**

22 The transformation efficiency was evaluated using the binary vector

1 *pMpGWB103-Citrine*, which was transformed into *Agrobacterium* as described
2 previously^{23–25}. The T-DNA of *pMpGWB103-Citrine* possessed two marker genes
3 encoding hygromycin B phosphotransferase and Citrine fluorescent protein^{23–25}. To
4 identify stable transformants, *M. polymorpha* gemmalings were selected for their ability
5 to grow on the antibiotic hygromycin B (10 µg mL⁻¹), and their yellow fluorescence was
6 observed using fluorescence microscopy more than two weeks after the selection buffer
7 was poured (transient expression of Citrine has not been observed after this time)^{23–25}. A
8 gemmaling containing one or more transformed cells was considered transformed²⁴. The
9 transformation efficiency (%) was calculated as the number of transformed gemmalings
10 divided by the total number of gemmalings, multiplied by 100. Approximately 10–50
11 gemmalings per Petri dish were served for transformation. The median transformation
12 efficiency was considered to be representative, and the mean was also reported to
13 facilitate comparisons with previous studies. Statistics were analyzed by t-test, Tukey's
14 test, or Tukey-Kramer's test.

15

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- 18

19 **Acknowledgments**

20 This work was supported by Grant-in-Aid for JSPS Fellows Grant Number 15J09907
21 (S.T.-T.), the Plant Transgenic Design Initiative of the University of Tsukuba (S.N., H.E.,
22 and Y.K.), and the JST-ERATO Numata Organelle Reaction Cluster Grant Number

1 JPMJER1602 (Y.K.).

2

3 **Author contributions**

4 S.T., S.N., H.E. and Y.K. designed the research. S.T. and Y.K. wrote the paper. S.T.
5 performed the examinations.

6

7 **Additional information**

8 Competing financial interests: The authors declare no competing financial interests.

9

10 **Figure legends**

11 **Fig. 1** Flowchart of G-AgarTrap. Step I: Pre-culture of *M. polymorpha*
12 gemmae/gemmalings on ½ B5 supplemented with 1% sucrose, and *Agrobacterium* on
13 LB agar medium. Step II: Co-culture of *M. polymorpha* gemmalings with
14 *Agrobacterium* on ½ B5 supplemented with 1% sucrose. Step III: Washing of *M.*
15 *polymorpha* gemmalings and selection of transgenic cells on ½ B5 supplemented with
16 1% sucrose.

17

18 **Fig. 2** Effect of sealing culture dishes with Parafilm on transformation efficiency. (a)
19 Comparison among the use of microporous tape to seal Petri dishes during co-culture in
20 a culture room at approximately 40% and 20% humidity, and the use of Parafilm to seal
21 Petri dish during co-culture in a culture room at approximately 20% humidity. For both
22 examinations using microporous tape, gemmalings were subjected to a one-day

1 pre-culture before a three-day co-culture. For examinations using Parafilm, gemmalings
2 were subjected a one-day pre-culture before a two-day co-culture. All examinations
3 were performed with *Agrobacterium* strain GV2260 under light. Different letters
4 indicate a significant difference (Tukey's test; P<0.05). *These raw data were reported
5 in Tsuboyama-Tanaka & Kodama 2015. (b) Effect of the duration of gemmaling
6 pre-culture prior to the use of Petri dishes sealed with Parafilm during the co-culture.
7 All examinations were performed after a two-day co-culture with *Agrobacterium* strain
8 GV2260 under light. Different letters indicate a significant difference (Tukey-Kramer's
9 test; P<0.05).

10

11 **Fig. 3** Effect of adding surfactant to the transformation buffer on gemmaling survival
12 rates and transformation efficiency. (a, b, c) The survival rates were estimated for
13 gemmalings treated with various concentrations of Silwet L-77 (a), Triton X-100 (b),
14 and Tween 20 (c). (d) The effect of adding Tween 20 to the transformation buffer on
15 transformation efficiency. All examinations were performed following a two-day
16 co-culture with *Agrobacterium* strain GV2260 under light, in Petri dishes sealed with
17 Parafilm. The same letters indicate no significant difference (Tukey-Kramer's test;
18 P<0.05).

19

20 **Fig. 4** Effect of *Agrobacterium* strain on transformation efficiency. (a) The
21 transformation efficiency of G-AgarTrap using five *Agrobacterium* strains, GV2260,
22 EHA101, EHA105, LBA4404, and MP90. All examinations were performed following

1 a two-day co-culture under light, in Petri dishes sealed with Parafilm. Different letters
2 indicate a significant difference (Tukey's test; P<0.05). (b, c) Fluorescence images of
3 transient marker expression in a gemmaling transformed using EHA101 (b) and MP90
4 (c), cultured for three days after treatment with the selection buffer. Red and
5 yellow-green indicate chlorophyll and Citrine fluorescence, respectively. Scale bar, 500
6 μm . Arrows indicate representative transformed cells.

7

8 **Fig. 5** Effect of dark treatment on transformation efficiency. (a) Transformation
9 efficiency following co-culture under light and dark conditions using *Agrobacterium*
10 strain GV2260, or following dark culture using strain EHA101. The effects of the light
11 and dark conditions were examined following a two-day co-culture on Petri dishes
12 sealed with Parafilm. Different letters indicate statistically significant differences
13 (Tukey's test; P<0.05). (b) Transmitted light image (left) and fluorescence image (right)
14 of stable marker expression in transformants generated using EHA101 in the dark,
15 which were cultured under light for two weeks after treatment with the selection buffer.
16 Scale bar, 0.5 cm. Arrows indicate representative transformants. (c) Fluorescence image
17 of transient marker expression in a gemmaling transformed in darkness using GV2260,
18 and cultured under light for three days after treatment with the selection buffer. (d)
19 Fluorescence image of transient marker expression in a gemmaling transformed in
20 darkness using EHA101, and cultured under light condition for five days after treatment
21 with the selection buffer. For (c) and (d), the scale bar represents 500 μm , red and
22 yellow-green indicate chlorophyll and Citrine fluorescence, respectively, and arrows

1 indicate representative transformed cells.

2

Sowing *M. polymorpha* gemmae on
½ B5 supplemented with 1% sucrose

Streaking
Agrobacterium on LB
solid medium

pre-culture: 1–3 days

Pouring transformation buffer

2–3 days
at 28°C

co-culture: 2 days

Washing and pouring selection buffer

selection: 2 weeks

Fig.1

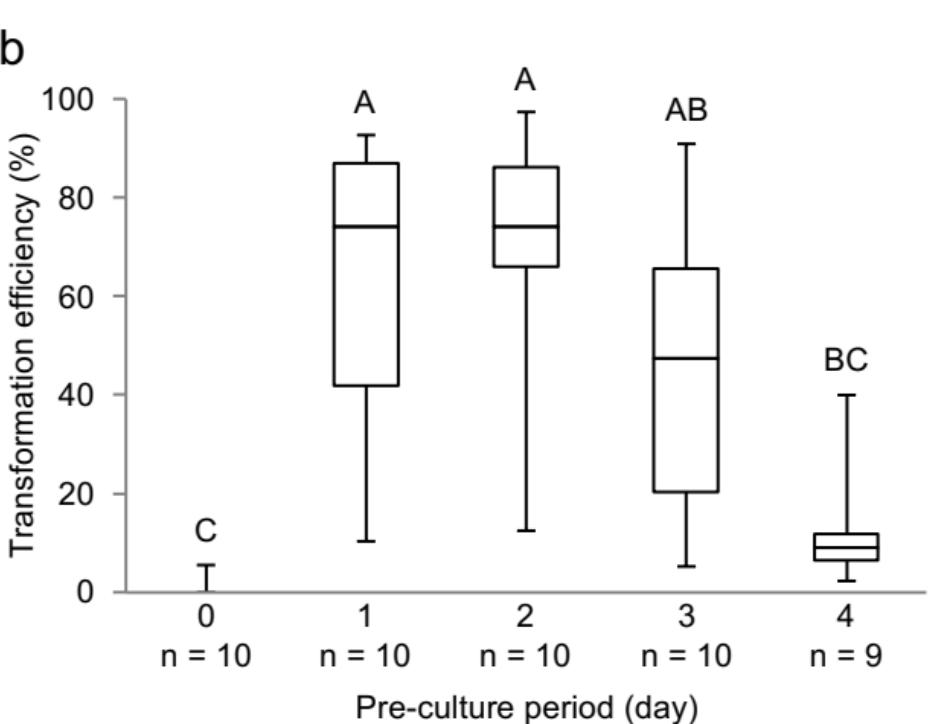
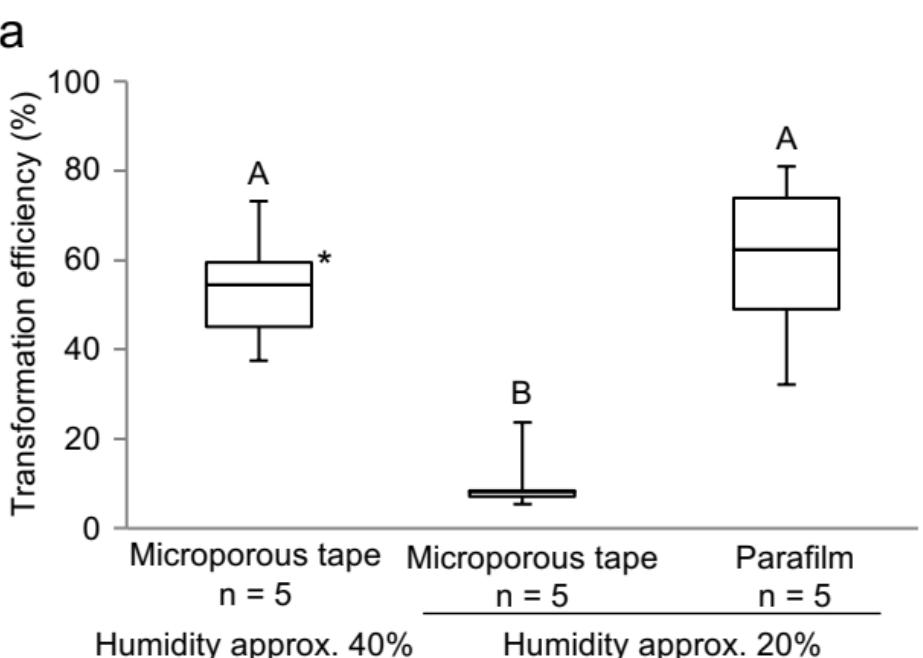


Fig. 2

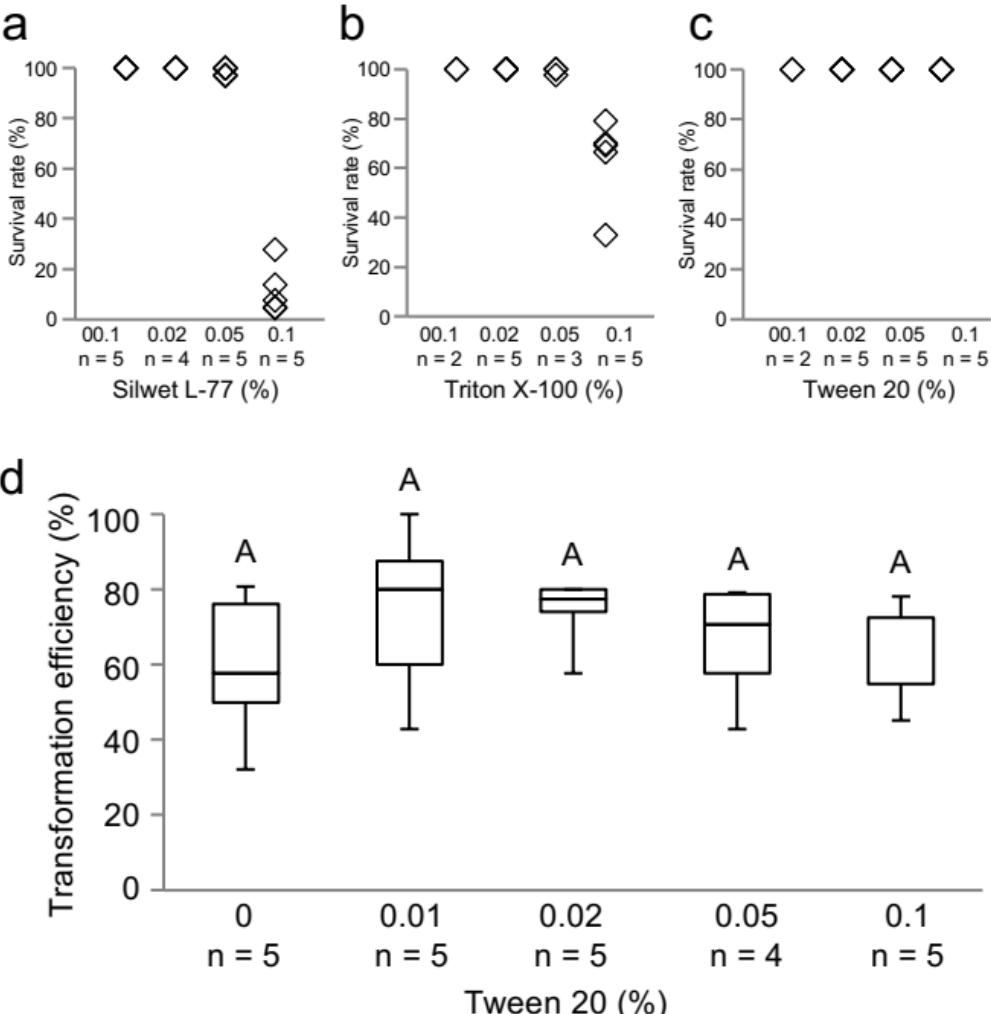


Fig. 3

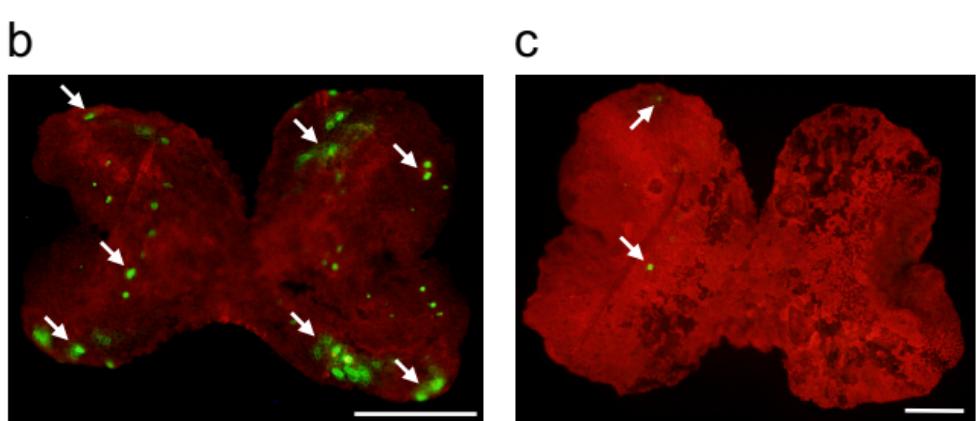
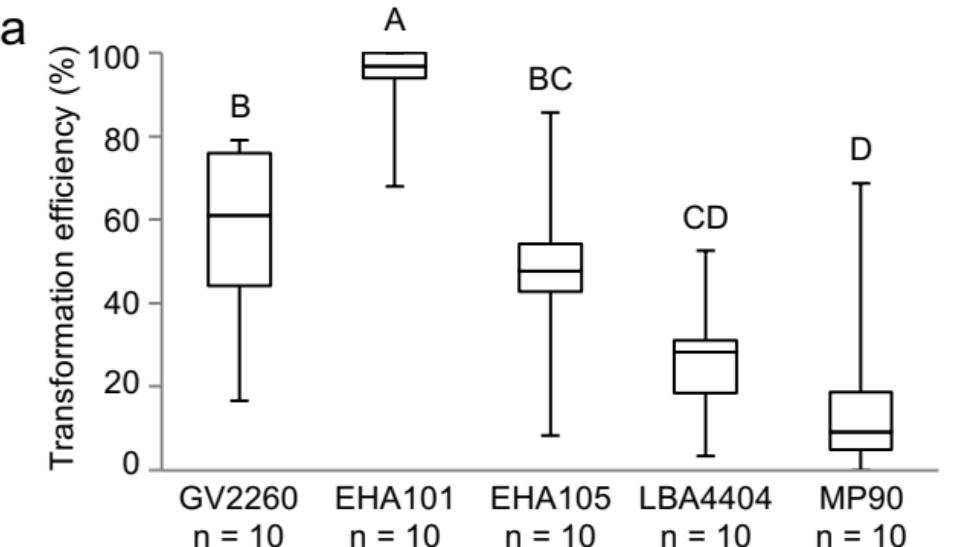


Fig. 4

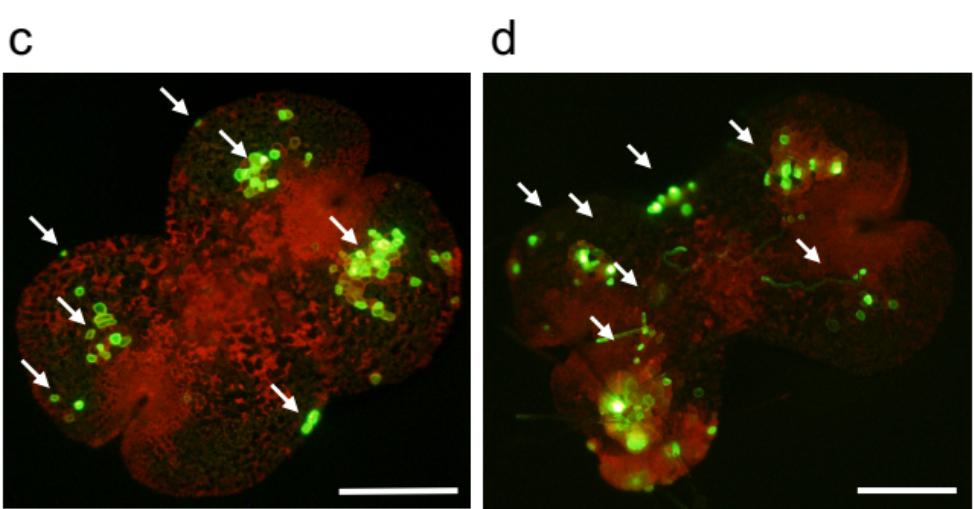
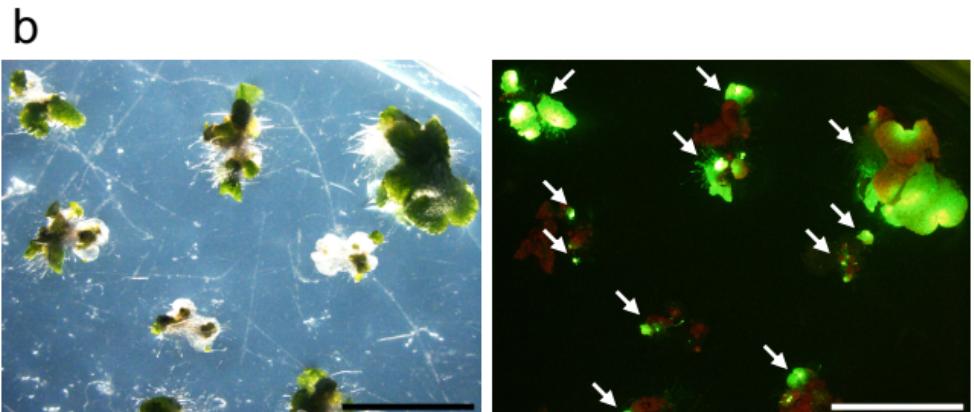
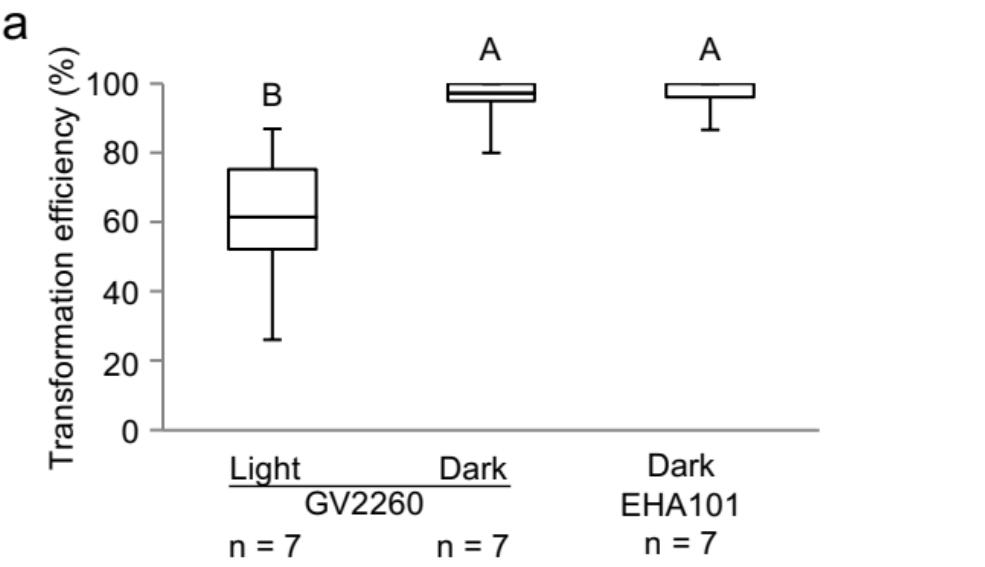


Fig. 5