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Note

Highly efficient G-AgarTrap-mediated transformation of the *Marchantia polymorpha* model strains Tak-1 and Tak-2

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Abstract The liverwort *Marchantia polymorpha* L. is an important model species for investigating land plant evolution. Effective genetic transformation techniques are crucial for plant molecular biology and simplified or improved techniques for specific cultivars or strains can accelerate research. Over the past several years, we developed a simple *Agrobacterium*-mediated transformation technique for *M. polymorpha* named AgarTrap (Agar-utilized transformation with pouring solutions). AgarTrap is an easy technique that involves pouring the appropriate solutions onto plant materials on a single solid plate of medium. We recently improved AgarTrap using gemmalings (G-AgarTrap) of the *M. polymorpha* female model strain BC3-38 and achieved a transformation efficiency of nearly 100%. Based on this improved technique, in the current study, we adopted two factors (sealing the Petri dish with Parafilm and dark treatment during co-cultivation) and optimized two factors (*Agrobacterium* strain and pre-culture period) of the improved G-AgarTrap for other model strains of *M. polymorpha*, the male strain Takaragaike-1 (Tak-1) and the female strain Takaragaike-2 (Tak-2). After optimization, the transformation efficiency of Tak-1 using G-AgarTrap was as high as 55% compared to approximately 30% using the previous protocol. Furthermore, using Tak-2, we achieved a transformation efficiency of nearly 100%. Our improved G-AgarTrap technique for Tak-1 and Tak-2 represents a promising tool for promoting the study of *Marchantia*.

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

The dioecious liverwort *Marchantia polymorpha* is a model species used to study land plant evolution, as it belongs to the bryophytes, the basal group of all land plants (Bowman et al. 2016; Puttick et al. 2018; Qiu et al. 2006; Wickett et al. 2014). Much is known about the taxonomy, development, physiology, and genetics of *M. polymorpha* (Bowman 2016; Bowman et al. 2016). Genomic analysis of this species has recently advanced substantially, as its whole genome sequence was completed in 2017 (Bowman et al. 2017). To date, various molecular techniques including transformation and genome editing methods have been established to study *M. polymorpha* (Ishizaki et al. 2016). Especially, because genetic transformation techniques are necessary for molecular analysis, several transformation methods such as *Agrobacterium*- and particle bombardment-mediated methods for *M. polymorpha* were developed (Chiyoda et al. 2008; Ishizaki et al. 2008; Kubota et al. 2013; Nasu et al. 1997; Takenaka et al. 2000).

Over the past several years, to promote the study of *M. polymorpha*, we developed AgarTrap (Agar-utilized transformation with pouring solutions), a simplified *Agrobacterium*-mediated transformation method for *M. polymorpha* (Tsuboyama and Kodama 2014). The basic operation of AgarTrap simply involves pouring the appropriate solutions onto plant materials on solid medium. First, *M. polymorpha* tissues are plated onto solid medium. Second, transformation buffer, containing *Agrobacterium*, is poured onto the tissues on the solid medium. Finally, selection buffer containing antibiotics is poured onto the solid medium (Tsuboyama and Kodama 2014). To date, we have developed three types of AgarTrap methods using *M. polymorpha* sporelings (S-AgarTrap), intact gemmae/gemmalings (G-AgarTrap), and mature thallus pieces (T-AgarTrap) (Tsuboyama and Kodama 2014; Tsuboyama-Tanaka and Kodama 2015; Tsuboyama-Tanaka et al. 2015). Among these, G-AgarTrap appears to be the most useful. G-AgarTrap can be used to produce many transformants with a uniform genetic background, since each gemma originates from a single cell in a gemma cup on a mature thallus (Barnes and Land 1908; Kato et al. 2017; Shimamura 2016).

In our previous study using G-AgarTrap, however, the transformation efficiency was low; the transformation efficiency of male strain Tak-1 was approximately 30%

Abbreviations: AgarTrap, Agar-utilized transformation with pouring solutions; G-AgarTrap, AgarTrap using gemmalings; LB, Luria-Bertani; S-AgarTrap, AgarTrap using sporelings; T-AgarTrap, AgarTrap using pieces of mature thallus; Tak-1, Takaragaike-1; Tak-2, Takaragaike-2.

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and that of female strain BC3-38, which was produced by three-time backcross with Tak-1, was approximately 60% (Ishizaki et al. 2008; Tsuboyama-Tanaka and Kodama 2015). Therefore, we recently improved G-AgarTrap using the BC3-38 strain and achieved a transformation efficiency of almost 100% (Tsuboyama et al. 2018). In the current study, we adopted these improvements (sealing the Petri dish with Parafilm and dark treatment during co-cultivation) and optimized two factors, the *Agrobacterium* strain used and the pre-culture period of gemmalings, for the *M. polymorpha* model strains Tak-1 (male strain) and Tak-2 (female strain). Note that Tak-2 strain had not previously been tested with the AgarTrap method (Tsuboyama-Tanaka and Kodama 2015).

To optimize the G-AgarTrap method for Tak-1 and Tak-2, we used gemmae obtained from 1-month-old *M. polymorpha* strain Tak-1 and Tak-2 thalli that had been maintained in a culture room at 22°C in 75 μmol photons m^{-2} s^{-1} continuous white light from florescent tube bulbs on half-strength Gamborg's B5 (1/2 B5) medium with 1% agar, pH 5.5 (Gamborg et al. 1968; Tsuboyama and Kodama 2018). To identify the transformants, we used *Agrobacterium* harboring the *pMpGWB103-Citrine* vector encoding Citrine yellow fluorescent protein (Citrine) and hygromycin B phosphotransferase (HPT) (Tsuboyama and Kodama 2014). The *pMpGWB103* is a Gateway binary vector for *M. polymorpha*, in which a promoter of *ELONGATION FACTOR1α* of *M. polymorpha* is located at the upstream of the Gateway cassette and a *HPT* gene is driven by double enhancer version of cauliflower mosaic virus 35S promoter (Ishizaki et al. 2015). Transformed cells appeared greenish-yellow (Citrine fluorescence), and non-transformed cells appeared red (chlorophyll fluorescence) under a MZ16F fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany). Percentages of transformed gemmalings in the gemmalings examined are shown as transformation efficiency in Figures 1–3. To calculate the transformation efficiency, transformants were counted at least 2 weeks after selection to avoid counting transiently transformed gemmalings as positive transformants (Tsuboyama-Tanaka and Kodama 2015).

To prepare the materials for AgarTrap, *Agrobacterium* cells that had been stored in 30% glycerol at −80°C were streaked onto LB solid medium and incubated at 28°C for 2–3 days. The G-AgarTrap procedure was performed using the three steps described below. We recently presented more detailed protocols for AgarTrap describing the specific operations and post-AgarTrap procedure (Tsuboyama and Kodama 2018). Briefly, the steps are as follows: (1) Pre-culture step: gemmae are plated onto 10 ml solid medium containing 1/2 B5 supplemented with 1% sucrose and 1% agar and cultured for a few days under continuous light. (2) Co-culture step: transformation buffer (10 mM $MgCl_2$; 10 mM MES-NaOH, pH 5.7; 150 μM acetosyringone; *Agrobacterium* OD_{600}=0.5) is poured onto the gemmalings, and excess buffer is removed with an aspirator after 1 min. Based on our previous study, the gemmalings are cultured for 2 days in a Parafilm-sealed Petri dish in the dark (Tsuboyama et al. 2018). (3) Selection step: the gemmalings and surface of the solid medium are washed twice with sterile water, and 1 ml selection buffer (100 μg ml^{-1} hygromycin B and 1 mg ml^{-1} Claforan) is poured onto the solid medium. After a few weeks of culture, transformants can be obtained.

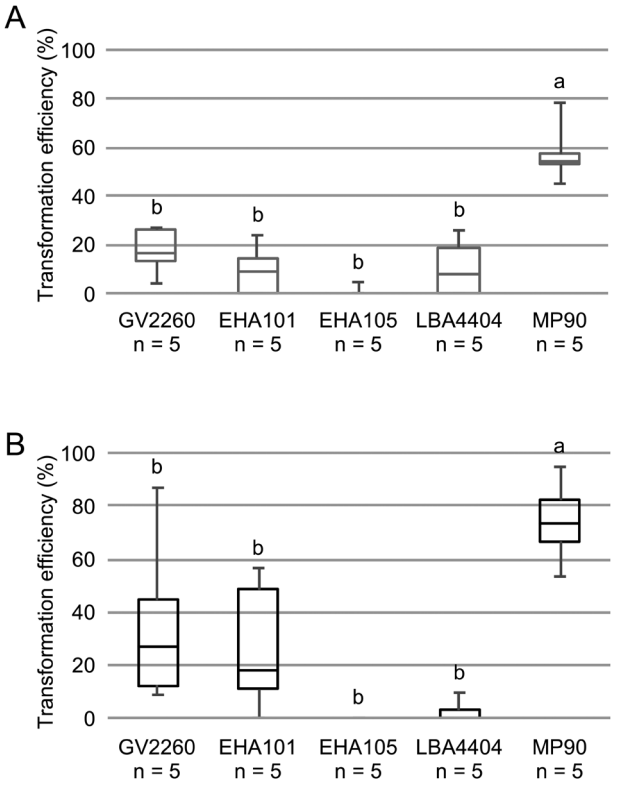

Figure 1. Transformation efficiency of G-AgarTrap using five *Agrobacterium* strains: GV2260, EHA101, EHA105, LBA4404, and MP90. (A) Transformation efficiency of AgarTrap using gemmalings from the male strain Tak-1. (B) Transformation efficiency of AgarTrap using gemmalings from the female strain Tak-2. (A, B) The experiments were performed using gemmalings subjected to 2 days of pre-culture and 2 days of co-culture in the dark in Parafilm-sealed Petri dishes. Different letters indicate a significant difference (Tukey's Test; $p<0.05$).

To improve G-AgarTrap for use with Tak-1 and Tak-2, we tested various *Agrobacterium* strains and pre-culture periods. First, we tested five *Agrobacterium* strains, GV2260, EHA101, EHA105, LBA4404, and MP90 (Deblaere et al. 1985; Hood et al. 1986, 1993; Koncz and Schell 1986; Ooms et al. 1982; Tsuboyama et al. 2018), using gemmalings after 2 days of pre-culture. When Tak-1 gemmalings were used, the median transformation efficiencies of GV2260, EHA101, EHA105, LBA4404, and MP90 were 16.1% (mean: 17.2%), 8.7% (mean: 9.5%), 0.0% (mean: 0.9%), 8.3% (mean: 10.6%), and 54.2%

(mean: 57.5%), respectively (Figure 1A). When Tak-2 gemmalings were used, the median transformation efficiencies of GV2260, EHA101, EHA105, LBA4404 and MP90 were 26.9% (mean: 35.9%), 17.9% (mean: 26.7%), 0.0% (mean: 0.0%), 0.0% (mean: 2.5%), and 73.3% (mean: 74.0%), respectively (Figure 1B). Although EHA101 was the most efficient *Agrobacterium* strain for use with BC3-38 gemmalings (Tsuboyama et al. 2018), MP90 was the most suitable strain for both Tak-1 and Tak-2 gemmalings (Figure 1A, B). Indeed, for *Arabidopsis thaliana*, the most suitable *Agrobacterium* strains differ among ecotypes and/or tissues (Akama et al. 1992; Chateau et al. 2000). Similarly, the most suitable *Agrobacterium* strains might differ among *M. polymorpha* strains and/or tissues.

In our previous study with Tak-1 gemmalings cultured under continuous light, the experiments were performed using a 1-day pre-culture period, followed by 3 days of co-culture with *Agrobacterium* strain GV2260 (Tsuboyama-Tanaka and Kodama 2015). The median transformation efficiency of Tak-1 gemmalings was 27.9% (mean: 31.3%) (Tsuboyama-Tanaka and Kodama 2015). However, when co-cultivation is performed under dark conditions, a longer pre-culture period is likely needed because *M. polymorpha* tissues grow slowly during co-cultivation in the dark (Tsuboyama et al. 2018). To optimize the pre-culture period of Tak-1 gemmalings in the present study, we tested the effects of pre-culture for 0–5 days, followed by co-cultivation with *Agrobacterium* strain MP90. When using Tak-1 gemmalings, the median transformation efficiencies of gemmalings pre-cultured for 0, 1, 2, 3, 4, and 5 days were 0.0% (mean: 2.9%), 13.6% (mean: 14.1%), 36.4% (mean: 46.1%), 33.5% (mean: 35.7%), 55.0% (mean: 52.9%), and 11.5% (mean: 11.8%), respectively (Figure

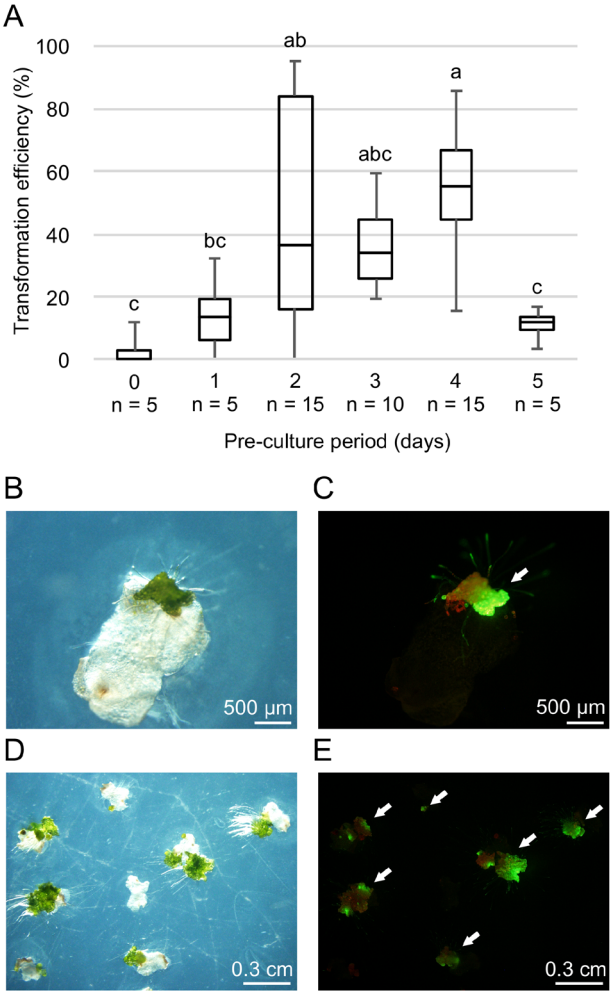

Figure 2. Effects of different pre-culture periods of Tak-1 gemmalings on transformation efficiency. (A) Transformation efficiency of Tak-1 gemmalings pre-cultured for 0–5 days. The experiment was performed by co-culturing the gemmalings for 2 days with *Agrobacterium* strain MP90 in Parafilm-sealed Petri dishes in the dark. Different letters indicate a significant difference (Tukey–Kramer's test; $p<0.05$). (B–E) Bright field (B, D) and fluorescence (C, E) microscopy images of Tak-1 gemmalings at 18 days after pouring selection buffer. G-AgarTrap was performed by pre-culturing the gemmalings for 2 days, followed by 2 days of co-culture with *Agrobacterium* strain MP90 in Parafilm-sealed Petri dishes in the dark. (C, E) Arrows indicate transformants.

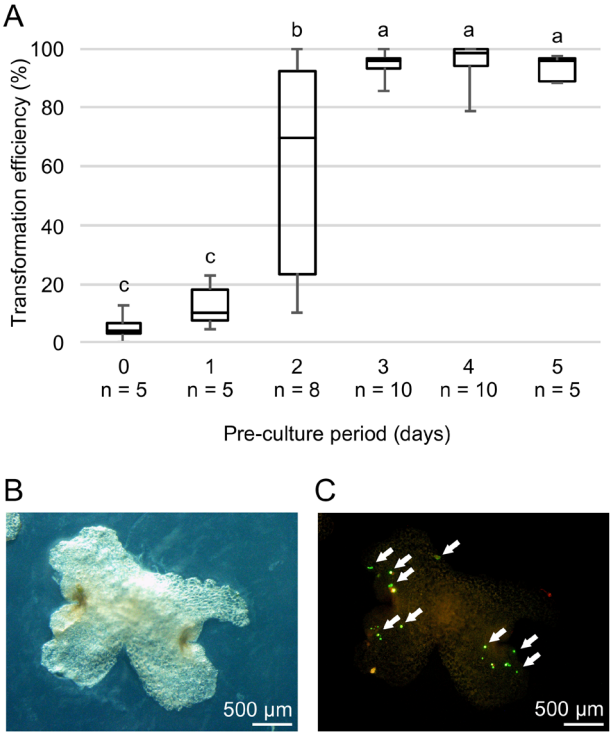

Figure 3. Effects of different pre-culture periods of Tak-2 gemmalings on transformation efficiency. (A) Transformation efficiency of Tak-2 gemmalings pre-cultured for 0–5 days. The experiment was performed by co-culturing the gemmalings for 2 days with *Agrobacterium* strain MP90 in Parafilm-sealed Petri dishes in the dark. Different letters indicate a significant difference (Tukey–Kramer's test; $p<0.05$). (B, C) Bright field (B) and fluorescence (C) microscopy images of Tak-2 gemmalings at 15 days after pouring selection buffer. G-AgarTrap was performed by pre-culturing the gemmalings for 3 days, followed by 2 days of co-culture with *Agrobacterium* strain MP90 in Parafilm sealed Petri dish in the dark. (C) Arrows indicate representative transformed cells.

2A). Therefore, the most suitable pre-culture period was 2–4 days (Figure 2A). Compared to the previous method (Tsuboyama-Tanaka and Kodama 2015), a longer pre-culture period was needed because we performed co-cultivation in the dark, as expected. At approximately 2 weeks after pouring the selection buffer, Tak-1 transformants were obtained (Figure 2B–E).

Similarly, when we tested the use of 0–5-day pre-culture periods for Tak-2 gemmalings, the median transformation efficiencies of gemmalings pre-cultured for 0, 1, 2, 3, 4, and 5 days were 4.0% (mean: 5.1%), 10.0% (mean: 12.5%), 70.0% (mean: 60.5%), 95.9% (mean: 95.3%), 98.7% (mean: 95.6%), and 95.8% (mean: 93.4%), respectively (Figure 3A). The highest transformation efficiency was nearly 100% at 3–5 days of pre-culture (Figure 3A). When using Tak-2 gemmalings, several transformed cells were often contained in a single gemmaling (Figure 3B, C). The highest median transformation efficiency using Tak-1 and Tak-2 was 55.0% and 98.7%, respectively (Figures 2A, 3A). These results suggest that Tak-2 gemmalings are much more easily infected by *Agrobacterium* than Tak-1 gemmalings.

In this study, we succeeded in improving the transformation efficiency of G-AgarTrap using *M. polymorpha* model strain Tak-1 (55.0% in the present study and 27.9% in the previous study) (Tsuboyama-Tanaka and Kodama 2015). Additionally, we developed the G-AgarTrap method for use with the Tak-2 strain and achieved a transformation efficiency of almost 100%. The highly efficient G-AgarTrap methods for *M. polymorpha* model strains Tak-1 and Tak-2 developed in this study should be useful for the research community of *M. polymorpha*.

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