

AgarTrap: A Simplified *Agrobacterium*-Mediated Transformation Method for Sporelings of the Liverwort *Marchantia polymorpha* L.

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The liverwort *Marchantia polymorpha* L. is being developed as an emerging model plant, and several transformation techniques were recently reported. Examples are biolistic- and *Agrobacterium*-mediated transformation methods. Here, we report a simplified method for *Agrobacterium*-mediated transformation of sporelings, and it is termed Agar-utilized Transformation with Pouring Solutions (AgarTrap). The procedure of the AgarTrap was carried out by simply exchanging appropriate solutions in a Petri dish, and completed within a week, successfully yielding sufficient numbers of independent transformants for molecular analysis (e.g. characterization of gene/protein function) in a single experiment. The AgarTrap method will promote future molecular biological study in *M. polymorpha*.

Keywords: AgarTrap • *Agrobacterium tumefaciens* • Bryophytes • Genetic transformation • *Marchantia polymorpha* • *Rhizobium radiobacter*.

Abbreviations: AgarTrap, agar-utilized transformation with pouring solutions; CaMV, *Cauliflower mosaic virus*; CTAB, cetyltrimethylammonium bromide; LB, Luria–Bertani; NOS, nopaline synthase; Tak-1, Takaragaike-1; Tak-2, Takaragaike-2; T-DNA, transfer DNA.

Introduction

Within land plants, liverworts, mosses, hornworts and vascular plants, the liverwort species is an important group to elucidate how land plants evolved from aquatic ancestors and adapted to life on land; phylogenomic analyses strongly suggest that liverworts seem to be the sister to all other land plants (Qiu et al. 2006). Therefore, the study of liverwort plants is believed to promote the understanding of plant evolution. To date, the dioecious liverwort *Marchantia polymorpha* L. has been well studied at the genomic level; the chloroplast and the mitochondria genomes and the Y chromosome were first sequenced in land plants (Ohyama et al. 1986, Oda et al. 1992, Yamato et al. 2007). Since 2008, a whole-genome sequencing project is being

carried out at the DOE Joint Genome Institute (<http://www.jgi.doe.gov/>) (JGI project ID: 1007435). Further, genetic transformation methods for *M. polymorpha*, e.g. biolistic- and *Agrobacterium*-mediated transformation techniques, have been developed (Nasu et al. 1997, Takenaka et al. 2000, Chiyoda et al. 2008, Ishizaki et al. 2008, Kubota et al. 2013). More recently, development of a gene targeting technique was also reported (Ishizaki et al. 2013a). During the past few years, many studies on *M. polymorpha* using molecular information and molecular techniques have been reported (Era et al. 2009, Tougane et al. 2010, Ishizaki et al. 2012, Kanamoto et al. 2012, Okumura et al. 2012, Takemura et al. 2012, Ueda et al. 2012, Era et al. 2013, Ishizaki et al. 2013b, Kanazawa et al. 2013, Ogasawara et al. 2013, Takemura et al. 2013, Ueda et al. 2013). Thus, the liverwort *M. polymorpha* is an emerging model plant.

Agrobacterium-mediated transformation of *M. polymorpha* was originally reported using a suspension-cultured cell line (Nasu et al. 1997). Recently, a transformation method for in planta, not for suspension-cultured cells, was successfully developed using sporelings (thalli grown from spores) (Ishizaki et al. 2008). The method is highly productive, i.e. hundreds of transformants could be obtained from a sporangium in a single experiment. Because the method could produce a lot of transformants, it may be suitable for the production of a transfer DNA (T-DNA) inserted mutant library. However, the method requires stepwise culture with liquid and solid media, and the resulting transformants become entangled, via their rhizoids, due to the shaking during liquid culture. Thus, after transformation by this method, isolation of independent transgenic lines is difficult. To promote the molecular biological study in *M. polymorpha*, improvement of the transformation technique is necessary.

In this study, we report a simplified method for genetic transformation using sporelings of *M. polymorpha* mediated by *Agrobacterium tumefaciens* (recently also known as *Rhizobium radiobacter*). The method is extremely simple as transformation is carried out in a 60 mm Petri dish simply by exchanging appropriate solutions. We call the procedure 'AgarTrap' (Agar-utilized Transformation with Pouring

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Solutions). The AgarTrap method consists of the following three steps: (I) growth of *M. polymorpha* and *A. tumefaciens*; (II) inoculation of *M. polymorpha* with *A. tumefaciens*; and (III) selection of transgenic *M. polymorpha*. A flow chart summarizing the three steps of the AgarTrap procedure is provided in **Supplementary Fig. S1**.

Results

To develop and evaluate the AgarTrap method, we constructed a binary vector, *pMpGWB103-Citrine*, harboring two marker genes for hygromycin B phosphotransferase (HPT) and Citrine fluorescent protein (Griesbeck et al. 2001) (see **Fig. 4A**). The gene products confer hygromycin B resistance and yellow fluorescence, respectively, to the resulting transformants. The AgarTrap method with the binary vector will be described and discussed in detail in the following paragraphs.

Growth of *M. polymorpha* and *A. tumefaciens* (step I)

For imbibition and germination of spores and for growth of sporelings of *M. polymorpha*, M51C solid medium (1% agar) supplemented with 2% sucrose was utilized (Ishizaki et al. 2008). The solid medium was made in a 60 mm disposable sterile polystyrene Petri dish, and spores of *M. polymorpha* were spread on the solid medium. Spores on the medium were incubated for 3 d at 22°C under continuous white light of 75 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (FL40SW, NEC Corporation). After incubation, sporelings, each consisting of several cells, were grown and observed under a stereomicroscope (**Fig. 1A**). Note that a stereomicroscope equipped with a fluorescent module is recommended for the observation, because Chl fluorescence could be used to identify sporelings growing on solid medium (**Fig. 1B**). In parallel, *A. tumefaciens* was streaked on Luria–Bertani (LB) solid medium (1% agar) in a 90 mm Petri dish and incubated at 28°C for 2–3 d (**Supplementary Fig. S2A**).

Inoculation of *M. polymorpha* with *A. tumefaciens* (step II)

To inoculate sporelings with *A. tumefaciens*, the bacterial solution was directly poured onto sporelings grown in a 60 mm Petri dish (**Supplementary Fig. S2B**). To prepare a bacterial solution, *A. tumefaciens*, cultured for 2–3 d, was detached from the LB agar with a toothpick, and suspended in 1 ml of transformation buffer (10 mM MgCl_2 ; 10 mM MES-NaOH, pH 5.7; 150 μM acetosyringone). After the entire surface was wetted for approximately 1 min, excess bacterial solution was removed with an aspirator or micropipet. Because sporelings remained fixed on the solid medium, possibly by their rhizoids, only excess bacterial solution could be removed easily. For co-cultivation of sporelings with *A. tumefaciens*, the Petri dish was incubated for 3 d at 22°C under continuous white light of 75 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

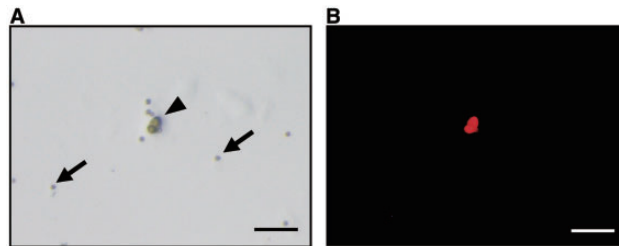


Fig. 1 A 3-day-old sporeling of *M. polymorpha*. (A) Transmitted light image of a sporeling (arrowhead) at 3 d on M51C solid medium (1% agar) supplemented with 2% sucrose. Arrows indicate ungerminated spores. (B) Chl fluorescence image of the same sporeling on M51C solid medium. The bar represents 100 μm .

Selection of transgenic *M. polymorpha* (step III)

Because *A. tumefaciens* had grown slightly on the surface of the medium during co-cultivation, it was removed by washing with sterile water. Subsequently, 1 ml of selection buffer was poured onto the solid medium on which co-cultivated sporelings were grown. The selection buffer includes antibiotics. In the selection buffer of this study, hygromycin (10 $\mu\text{g ml}^{-1}$) and claforan (100 $\mu\text{g ml}^{-1}$) were used as antibiotics for selection of transformants and elimination of *A. tumefaciens*, respectively. Note that the amount of antibiotics is based on the volume of solid medium. For example, when using a 10 ml volume of solid medium, the concentrations of hygromycin and claforan are adjusted to 100 μg and 1 mg, respectively, in the 1 ml selection buffer. The structure and fluorescence of transgenic cells could be clearly observed within 3 d after pouring selection buffer (**Fig. 2A**). Within 2 weeks, transgenic thallus and rhizoids were grown (**Fig. 2B**). After maturation of the thallus, a transgenic G1 gemma (Ogasawara et al. 2013) was successfully obtained (**Fig. 2C**). Because the gemma originated from a single cell (Barnes and Land 1908), the G1 gemma is a non-chimeric transformant.

Optimization of the AgarTrap procedure

To optimize the AgarTrap procedure described above, we considered several factors that influence transformation efficiency. In this subsection, we provide information that will help with optimization for future users. Each experiment was basically performed under 3 d pre-culture, 3 d co-culture, an *Agrobacterium* concentration of $\text{OD}_{600} = 0.5$ and 150 μM acetosyringone, except for the investigated factors.

In step I, several pre-culture periods were compared. When pre-culture periods of 1, 2, 3, 5 and 7 d as were tested, the highest transformation efficiency (approximately 15%) was shown after 3 d (**Fig. 3A**; **Supplementary Table S1**). A small number of transformants were successfully obtained with low efficiency (approximately 4%), even 1 and 2 d pre-culture. However, because rhizoids of 1- or 2-day-old sporelings were probably too undeveloped for anchoring in agar, some of the sporelings were displaced from the solid medium when the transformation buffer was poured. When sporelings

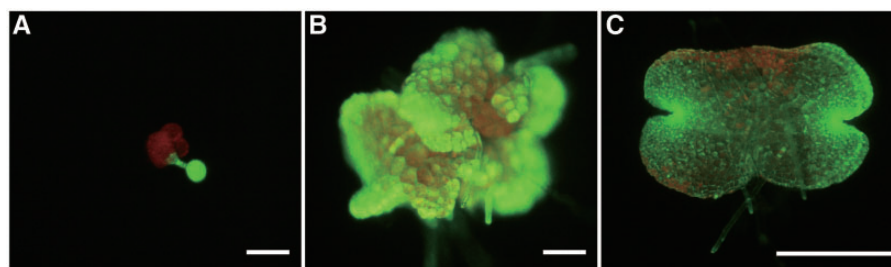


Fig. 2 Fluorescence images of transgenic thalli. (A) Transgenic cells at 3 d post-transformation. The bar represents 100 μm . (B) Transgenic thallus with rhizoids at 2 weeks post-transformation. The bar represents 100 μm . (C) A transgenic G1 gemma. The gemma was obtained from the gemma cup of an AgarTrap-produced transgenic thallus (approximately 1-month-old). The bar represents 500 μm . Chl (red) and Citrine (yellow) fluorescence images were taken by stereofluorescence microscopy.

pre-cultured for 5 and 7 d were used, the transformation efficiency was decreased, compared with 3 d pre-culture. These observations indicate that the appropriate growth stage leads to higher transformation efficiency when using the AgarTrap method.

In step II, the following factors were investigated: (i) the concentrations of *A. tumefaciens* and (ii) acetosyringone in transformation buffer, and (iii) the co-culture period. When the concentration of *A. tumefaciens* in the transformation buffer was compared, no significant difference in transformation efficiency was found at an OD_{600} of 0.5–3.0 (Fig. 3B; Supplementary Table S2). As regards the concentration of acetosyringone, 0, 15, 150 and 1,500 μM were compared. No transformant was obtained using concentrations of 0 and 15 μM , and the highest transformation efficiency was obtained using 150 μM (Fig. 3C; Supplementary Table S3). The results indicate that acetosyringone is necessary for the AgarTrap method with *M. polymorpha*. The transformation efficiency using 1,500 μM was lower than that with 150 μM , indicating that a high concentration of acetosyringone leads to a negative effect on transformation efficiency (Fig. 3C; Supplementary Table S3). When the co-culture period was compared, 3 d proved to result in a higher efficiency than 1 d, although a few transformants could be obtained after only 1 d co-culture (Fig. 3D; Supplementary Table S4).

T-DNA integration into the genome

To check T-DNA integration into the genome, PCR analysis was performed using genomic DNA isolated from 18 independent transgenic thalli. First, we checked whether *Agrobacterium* remains around transgenic thalli. Because an endogenous gene, *virD2*, of *A. tumefaciens* could not be amplified by PCR (Supplementary Fig. S3), it was confirmed that almost all *Agrobacterium* was eliminated by the washing and selection steps. In all lines, the marker gene *Citrine* could be amplified by PCR, suggesting successful T-DNA integration into the genome by the AgarTrap method (Fig. 4B, upper panel).

Both male and female plants exist, because *M. polymorpha* is dioecious. A previous study revealed that *Agrobacterium*-mediated transformation could transform both male and

female individuals of *M. polymorpha* (Ishizaki et al. 2008). To check whether the AgarTrap method can transform both male and female sporelings, PCR-based genotyping of the transformants was performed with primer sets (see the Materials and Methods for details) detecting specific male and female loci (Fujisawa et al. 2001). When 18 independent transgenic thalli were checked, eight males and 10 females were detected (Fig. 4B, lower panel). The results indicate that the AgarTrap method is able to transform both male and female *M. polymorpha* sporelings with similar efficiencies.

To confirm the T-DNA integration, Southern blotting analyses were performed. Six transgenic lines (three male lines and three female lines) were selected, and subjected to Southern blotting analysis by digesting genomic DNA with *HindIII* or *SacI*, for which sites are present in the T-DNA region (Fig. 4A). An alkaline phosphatase-labeled DNA fragment of the *Citrine* sequence was used as a probe (Fig. 4A). The results indicate that the six transformants are independent lines, and have 1–4 copies of T-DNA insertions (Fig. 4C).

Discussion

In this report, we described a simplified transformation method called the AgarTrap for the emerging model plant *M. polymorpha*. The AgarTrap method is rapid and simple; the transformation procedure can be completed in a single dish within a week. Although *M. polymorpha* is dioecious, the AgarTrap method could transform both male and female sporelings without making a distinction in terms of sex.

In the previous method of Ishizaki et al. (2008), transgenic immature thalli produced after co-cultivation of *A. tumefaciens* with sporelings were entangled via their rhizoids, due to the shaking in liquid culture medium, thereby limiting further analysis including cell observation. However, because Ishizaki's method produced hundreds of transformants in a single experiment, it may be suitable for the production of a huge mutant library with T-DNA insertions. In contrast, the AgarTrap method produced individual, non-entangled transgenic immature thalli inserted into the solid medium with their rhizoids during development. Consequently, independent transgenic

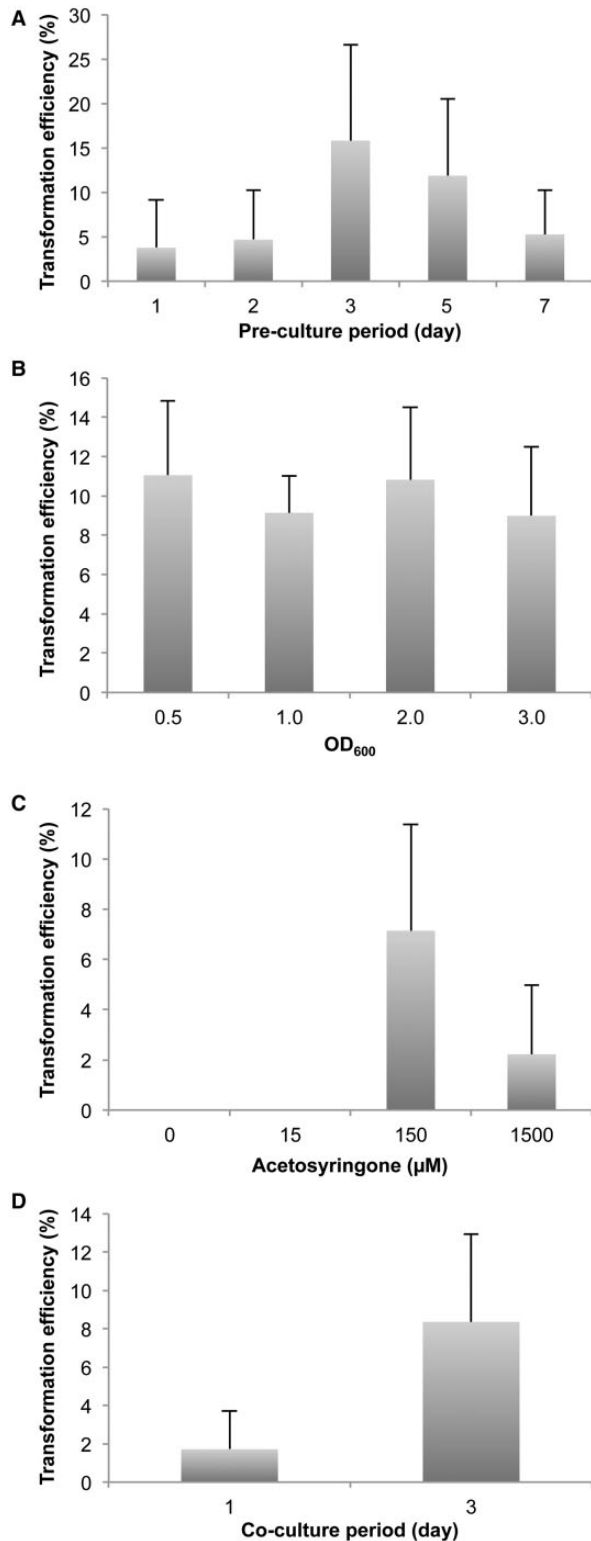


Fig. 3 Optimization of the AgarTrap procedure with reference to transformation efficiency. Each experiment was basically performed under 3 d pre-culture, 3 d co-culture, an *Agrobacterium* concentration of OD₆₀₀ = 0.5 and 150 μM acetosyringone, except for the investigated factors. (A) Effect of a pre-culture period from 1 to 7 d. Bars represent the SDs of triplicate experiments (see also [Supplementary Table S1](#)). (B) Effect of the *Agrobacterium* concentration. *Agrobacterium tumefaciens*

plants can be efficiently obtained and easily subjected to further investigation. The present study showed clear structure and fluorescence of transgenic *Marchantia* cells 3 d after transformation. Thus, the AgarTrap method seems to allow extended cell biology research of transformed plants, including subcellular localization analysis using fluorescent proteins.

Regarding the pre-culture period in the previous method, no transformant was obtained with pre-culture of 0 and 3 d, and the most efficient pre-culture period was 7 d (Ishizaki et al. 2008). On the other hand, in the AgarTrap, transformants could be obtained with 1 and 2 d pre-culture periods, and the most efficient period was 3 d. The pre-culture period required to obtain transformants using the AgarTrap method is shorter than that in the previous method. This might be explained by the growth rate of the sporelings. The sporelings on the solid medium might grow faster than in the liquid medium, because the sporelings grown on the solid medium could obtain air easily, and receive more light, compared with the sporelings in liquid medium. Further examination of the growth rate of sporelings may enhance optimization of the pre-culture period using the AgarTrap method.

In the case of some plant species, the *Agrobacterium* concentration (OD₆₀₀) for co-cultivation with plants is known to influence the transformation efficiency. However, in the AgarTrap method, different concentrations of *Agrobacterium* did not influence the transformation efficiency. Although it remains to be determined what caused the unexpected results, the *Agrobacterium* concentration at an OD₆₀₀ of 0.5 might already be saturated for transformation of sporelings with the AgarTrap method. Nevertheless, an excess amount of *Agrobacterium* during the co-cultivation step might cause a negative effect with the AgarTrap procedure, because the overgrown *Agrobacterium* was difficult to eliminate during subsequent washing and the selection steps.

Because acetosyringone is a phenolic compound that induces transcription of the virulence genes of *Agrobacterium*, the acetosyringone concentration is important for optimization of the transformation procedure (Gelvin 2000). For *Agrobacterium*-mediated transformation of *M. polymorpha* in the previous method, the most efficient acetosyringone concentration was 100 μM (Ishizaki et al. 2008). A similar effect of acetosyringone was found in the present AgarTrap method. In both the previous (Ishizaki et al. 2008) and present methods, acetosyringone was essential for the co-cultivation because no transformants were obtained without addition of

Fig. 3 Continued

was suspended at OD₆₀₀ = 0.5, 1.0, 2.0 and 3.0 in transformation buffer. Bars represent the SDs of triplicate experiments (see also [Supplementary Table S2](#)). (C) Effect of acetosyringone concentrations of 0, 15, 150 and 1,500 μM in the transformation buffer. Bars represent the SDs of experiments repeated four times (see also [Supplementary Table S3](#)). (D) Effect of the co-culture period. Co-culture periods of 1 and 3 d were compared. Bars represent the SDs of experiments repeated five times (see also [Supplementary Table S4](#)).

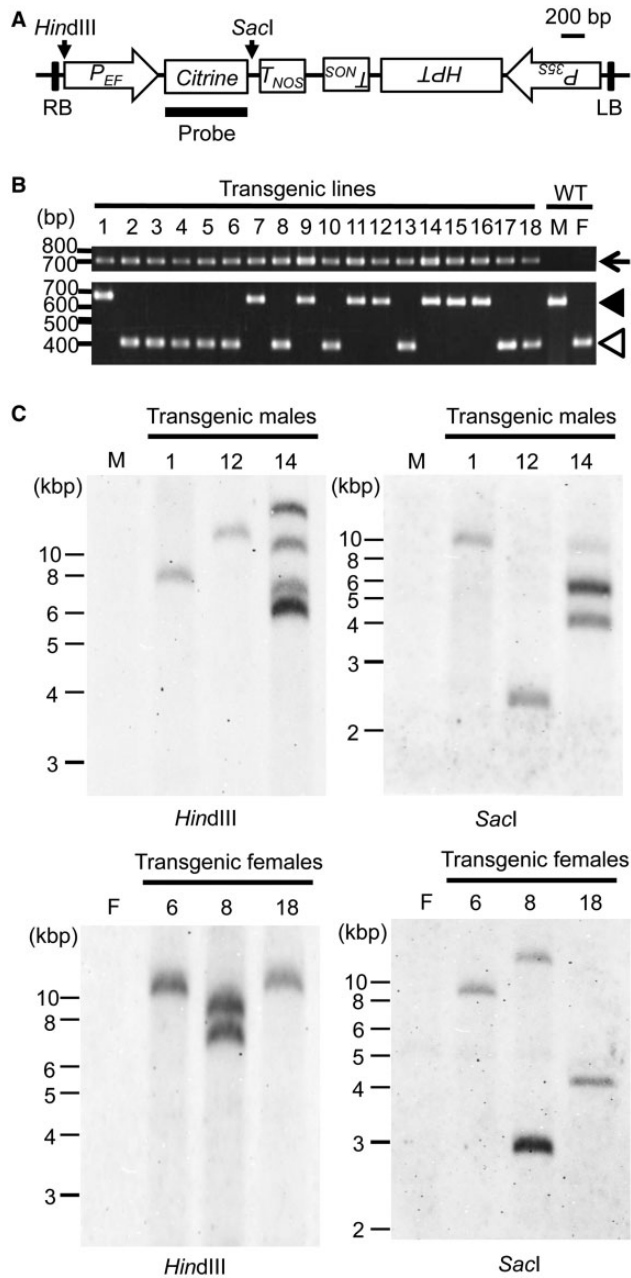


Fig. 4 T-DNA integration into the genome. (A) The T-DNA region of the binary vector, *pMpGWB103-Citrine*, is illustrated. RB, right border; *P_{EF}*, promoter of the gene for *M. polymorpha* elongation factor; *Citrine*, gene encoding an improved yellow fluorescent protein; *T_{NOS}*, terminator of the gene for *A. tumefaciens* nopaline synthase; *HPT*, gene for hygromycin B phosphotransferase; *P_{35S}*, *Cauliflower mosaic virus* 35S promoter; and LB, left border. Arrows indicate restriction sites for *HindIII* and *SacI*. Upper and lower bars show a scale of 200 bp and a probe region for Southern blot analysis, respectively. (B) PCR experiments were performed with genomic DNA, as a template, isolated from transgenic thalli. The arrow indicates the *Citrine* transgene (720 bp). Filled and open arrowheads show PCR-amplified DNA fragments for male- (*rbm27*: 663 bp) and female- (*rhf73*: 406 bp) specific loci, respectively. The DNA size markers are indicated on the left. Numbers (1–18) show independent transgenic lines. Lanes M and F indicate male and female wild-type plants, respectively. (C) Southern

acetosyringone. Although the previous method obtained transformants at 1, 3 and 10 μ M (Ishizaki et al. 2008), the AgarTrap method could not generate any transformant at 15 μ M. When the transformation buffer containing acetosyringone was poured onto the agar medium in the AgarTrap, some acetosyringone might permeate into the agar. In this context, transformation with the solid medium may require a higher concentration of acetosyringone than with the liquid medium.

The co-cultivation step is important in the *Agrobacterium*-mediated transformation procedure, because the T-DNA region is transferred and incorporated into the plant genome during co-cultivation. As in the case in the previous study on the co-culture period of *M. polymorpha* transformation (Ishizaki et al. 2008), in the AgarTrap, 3 d co-culture led to significantly higher transformation efficiency, compared with 1 d co-culture. Because T-DNA is integrated into the *M. polymorpha* genome during the co-cultivation step, a longer period for co-cultivation would increase the opportunity for integration. However, since multiple T-DNA incorporations might be induced when longer co-cultivation is performed, empirical optimization for research purposes is necessary. In the previous study, the copy number of T-DNA insertion was reported as 1–5 copies (Ishizaki et al. 2008). Comparably, when a 3 d co-culture was performed in the AgarTrap, the copy number of T-DNA insertion was found to be 1–4 copies. Taken together, when the AgarTrap method is used, <3 d co-culture is suitable for isolation of transgenic plants with a single T-DNA insertion, whereas >3 d co-culture is suitable for isolation of transgenic plants with multiple T-DNA insertions.

More recently, another transformation method for *M. polymorpha* using regenerated thalli was reported (Kubota et al. 2013). In this method, developing sporangia by crossing was not necessary, and the transformation could be performed in various genetic backgrounds, e.g. male, female and/or mutants (Kubota et al. 2013). Using this method, our group efficiently produced only male transgenic *M. polymorpha* (Ogasawara et al. 2013). However, similar to the previous transformation method for sporelings (Ishizaki et al. 2008), it also requires stepwise culture with liquid and solid medium (Kubota et al. 2013). Like the AgarTrap method in the present study, improved transformation procedures for the regeneration of thalli will be developed in the near future.

Fig. 4 Continued

blot analysis. Among the 18 lines (B), six lines were randomly selected and subjected to DNA gel blotting. Numbers 1, 12 and 14 are males, and numbers 6, 8 and 18 are females. Lanes M and F indicate male and female wild-type plants, respectively. Genomic DNA (5 μ g) was digested with *HindIII* or *SacI*, and loaded in each lane. An alkaline phosphatase-labeled DNA fragment including the *Citrine* sequence (720 bp) was used as a probe (see A). The DNA size marker is indicated on the left. The results of the Southern blot analyses suggest possible copy numbers of T-DNA of 1, 1, 2, 1, 4 and 1 copies in transgenic lines 1, 6, 8, 12, 14 and 18, respectively.

In summary, we developed a simplified transformation method, AgarTrap, for *M. polymorpha*, which consists of an effortless transformation procedure, and provides the method for the *Marchantia* research community. The AgarTrap method will promote the dissection of the molecular mechanisms sustaining the life cycle of the liverworts and help to understand plant evolution. Further, the AgarTrap procedure may be utilized for transformation of other land plants. In particular, since cryptogrammic plants such as liverworts, mosses, hornworts and ferns propagate using spores, procedures similar to the AgarTrap method may contribute to the development of new transformation technology for sporelings of those plants.

Materials and Methods

Plant materials and growth conditions

Marchantia polymorpha were grown in a culture room (temperature, 22°C; humidity, approximately 40%). Males, accession Takaragaike-1 (Tak-1), and females, accession Takaragaike-2 (Tak-2), of *M. polymorpha* were asexually maintained on M51C medium with 1% agar (M51C agar) under 75 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ continuous white light (FL40SW, NEC Corporation) as described previously (Ogasawara et al. 2013). Formation of sexual organs was induced by continuous white fluorescent light and fluorescent light containing the far-red spectrum (FL20S-FR-74, Toshiba Lighting & Technology Corporation), and F₁ spores were obtained by crossing Tak-1 with Tak-2. The intensity of fluorescent light was measured with a light meter (LI-250A, LI-COR Biosciences).

Construction of binary vector

To construct a binary vector containing the *Citrine* gene, Gateway cloning technology (Invitrogen) was used. *Citrine* cDNA was amplified by PCR with primers 5'-GGGACAAGT TGTACAAAAAGCAGGCTCCATGGTGAGCAAGGGCGAG-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAC TGTACAGCTCGTCC-3'. The amplified DNA fragment was mixed with an entry vector, pDONR207, and the BP reaction was performed according to the manufacturer's instructions (Invitrogen). The resulting plasmid, pDONR207-*Citrine*, was mixed with a destination vector, pMpGWB103. After the LR reaction was carried out to clone the *Citrine* gene into the pMpGWB103 vector, the resulting binary vector, pMpGWB103-*Citrine* (Fig. 4A), was transformed into *A. tumefaciens* strain GV2260 (Deblaere et al. 1985). Transformed *A. tumefaciens* were rapidly frozen in 30% glycerol by liquid nitrogen and stored at -80°C until use. The pMpGWB103-*Citrine* vector possesses a spectinomycin resistance gene to select bacterial transformants. In the T-DNA region, a hygromycin resistance gene, *hygromycin B phosphotransferase* (*HPT*), driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter (*P*_{35S}) and the terminator of *A. tumefaciens* nopaline synthase (*T*_{NOS}), was cloned for selection of transgenic plants (Fig. 4A). Expression of the *Citrine* gene was controlled by the promoter region of a

gene for elongation factor (*P*_{EF}) from *M. polymorpha* (Althoff et al. 2013) to avoid yellow fluorescence from the transformed *A. tumefaciens* around the resulting transgenic plants, because the CaMV 35S promoter was confirmed to express Citrine in *A. tumefaciens* strain GV2260 (Supplementary Fig. S4).

Microscopic observation

Sporelings of *M. polymorpha* were observed using a MZ16F stereo fluorescence microscope (Leica Microsystems). Chl and Citrine fluorescence were determined with a fluorescence module (excitation filter 480/40 nm and barrier filter LP 510 nm), and the images were taken with an Olympus DP73 digital camera.

Calculation of transformation efficiency

Spores from a sporangium were divided into several tubes; the spores in one tube were germinated and grown without any treatment as a control, and the spores in other tubes were subjected to the AgarTrap method. To avoid counting sporelings with transient expression that could be observed a few days after pouring selection buffer, the transformants were not counted until approximately 2 weeks after pouring selection buffer. To calculate the transformation efficiency (%), the number of transformed sporelings was divided by the number of non-transformed sporelings, and the resulting number was multiplied by 100. Actual numbers of transformants are presented in Supplementary Tables S1–S4.

PCR genotyping of male and female thalli

To isolate genomic DNA for PCR genotyping, detached thallus (approximately 15 mm × 15 mm) was ground in extraction buffer (100 mM Tris-HCl, pH 9.5, 1 M KCl, 10 mM EDTA) on ice. The crude extract was incubated at 95°C for 10 min and then incubated on ice for 5 min. After centrifugation at 13,000 r.p.m. for 5 min, the supernatant was transferred to a new tube. The supernatant was diluted 10-fold with sterile distilled water to make a template for PCR with Mighty Amp DNA polymerase (TAKARA). For detection of male thalli, primers 5'-CCAAGTCCGGCAGAAT CAAGT-3' and 5'-TTCATCGCCCGCTATCACCTTC-3' were used to amplify the *rbm27* locus (663 bp) (Fujisawa et al. 2001). For detection of female thalli, primers 5'-GACGACGAAGATGTGGAT GAC-3' and 5'-GAAACTTGCCCGTGTGACTGA-3' were used to amplify the *rhf73* locus (406 bp) (Fujisawa et al. 2001). For PCR-based genotyping of male and female thalli, all four primers were mixed at equal concentrations to make a primer master mixture. To amplify the *Citrine* transgene (720 bp), the following primers were used: 5'-ATGGTGAGCAAGGGCGAGGAGCTGTTACC-3' and 5'-TTACTTGTACAGCTCGTCCATGCCGAGAGT-3'. To amplify the *virD2* gene (500 bp) from *A. tumefaciens*, the following primers were used: 5'-ATGCCGATCGCGCTCAAGTAATCATT CGC-3' and 5'-ATCTTTTCCGTAAGCCGTCATAATTCAGC-3'.

Genomic DNA extraction

Genomic DNA for Southern blotting was isolated from approximately 1-month-old thalli by the cetyltrimethylammonium

bromide (CTAB) method. The tissues were frozen in liquid nitrogen and ground using a pestle and mortar. The ground tissues (approximately 3 g) were incubated in 5 ml of 1.5× CTAB buffer (1.5% CTAB, 75 mM Tris–HCl pH 8.0, 15 mM EDTA, 1.05 M NaCl) with gentle rotation at 55°C for 20 min. Subsequently, 6 ml of chloroform was added and mixed at room temperature. After centrifugation at 1,800 × g for 10 min at 20°C, the aqueous phase was transferred to a new plastic tube, and 500 µl of 10% CTAB buffer (10% CTAB, 0.7 M NaCl) was added. A 5 ml aliquot of chloroform was added and centrifuged at 1,800 × g for 10 min at 20°C. The aqueous phase was transferred to a new plastic tube, and 10 ml of CTAB precipitation buffer (1% CTAB, 50 mM Tris–HCl pH8.0, 10 mM EDTA) was added. The sample was incubated for 30–60 min at room temperature. After centrifugation at 10,000 × g for 20 min at 4°C, the precipitate was dissolved in 1 M NaCl. DNA was precipitated with isopropanol and suspended in Tris-EDTA buffer. RNase A (final 10 µg ml⁻¹) was added, and incubated for 30–60 min at 37°C. After extraction with chloroform and isopropanol, precipitated DNA was resuspended in Tris-EDTA buffer.

Southern blot analysis

A 5 µg aliquot of genomic DNA was digested with *Hind*III or *Sac*I at 37°C. The resulting digested DNA was subjected to electrophoresis on a 1% agarose gel, and transferred to a Hybond-N+ nylon membrane (GE Healthcare) by using a vacuum transfer device (BIO CRAFT). AlkPhos Direct Labelling Reagents (GE Healthcare) were used for making a probe (Fig. 4A) labeled with alkaline phosphatase and for hybridization. The DNA fragment including the *Citrine* sequence for the probe was amplified by PCR with the following primers: 5'-ATGGTGAGCAAGGGCGAGGAGCTGTCCACC-3' and 5'-TACTTGTACAGCTCGTCCATGCCGAGAGT-3'. CDP-Star Detection Reagent (GE Healthcare) was used to detect hybridized probes using chemiluminescence.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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