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Note

A highly efficient AgarTrap method for genetic transformation of mature thalli of the liverwort *Marchantia polymorpha* L.

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Abstract *Marchantia polymorpha* L. has been established as a model liverwort species for understanding plant evolution, and several genetic transformation technologies have been developed. Recently, we described a simplified *Agrobacterium*-mediated genetic transformation method termed AgarTrap (Agar-utilized Transformation with Pouring Solutions) that uses sporelings (S-AgarTrap) or gemmae/gemmalings (G-AgarTrap), both of which easily produce a sufficient number of independent transformants. In this study, we report the development of a novel AgarTrap protocol using mature pieces of liverwort thalli (T-AgarTrap). Optimal transformation efficiency was approximately 70%, and 100% transformation was achieved in a few experimental cases. The efficiency of T-AgarTrap was much greater than those of S- and G-AgarTrap methods. This highly efficient T-AgarTrap protocol seems to promote molecular research with *M. polymorpha*.

Key words: AgarTrap, *Agrobacterium tumefaciens*, genetic transformation, liverworts, thallus.

Liverworts are important for understanding how plants have adapted from aquatic to terrestrial environments because liverworts are sister to all other land plants (Qiu et al. 2006). The dioecious liverwort *Marchantia polymorpha* L. has recently been recognized as a model species. *Agrobacterium*-mediated genetic transformation techniques have been employed to study *M. polymorpha* (Ishizaki et al. 2008; Kubota et al. 2013; Nasu et al. 1997; Tsuboyama and Kodama 2014; Tsuboyama-Tanaka and Kodama 2015). We recently developed a simplified transformation method, termed AgarTrap (Agar-utilized Transformation with Pouring Solutions), using sporelings (S-AgarTrap) or intact gemmae/gemmalings (G-AgarTrap) (Tsuboyama and Kodama 2014, Tsuboyama-Tanaka and Kodama 2015). The AgarTrap procedure was performed by pouring appropriate solutions into a single Petri dish, thereby producing a sufficient number of independent transformants. Because spores produced by sexual reproduction can be obtained in quantity, S-AgarTrap produced a large number of transformants (Tsuboyama and Kodama 2014). Gemmae produced by asexual reproduction were developed in gemma cups on matured thalli (Barnes and Land 1908). Because gemmae and thalli are genetically identical, G-AgarTrap produced transformants with uniform genetic backgrounds (Tsuboyama-Tanaka and Kodama 2015). Average transformation efficiencies optimized using M51 medium were approximately 10% and 50% for S- and G-AgarTrap methods, respectively; maximum transformation efficiencies for S- and G-AgarTrap methods were 27.7% and 80.4%, respectively (Tsuboyama and Kodama 2014, Tsuboyama-Tanaka and Kodama 2015). In this study, we developed a highly efficient AgarTrap method using samples from mature thalli, termed T-AgarTrap.

To develop the T-AgarTrap method, Takaragaike-1 (Tak-1) and BC3-38 were used as male and female plants, respectively (Tsuboyama-Tanaka and Kodama 2015). In addition, we used *Agrobacterium tumefaciens* (GV2260) (Deblaere et al. 1985), possessing a binary vector (*pMpGWB103-Citrine*) with two marker genes for hygromycin B phosphotransferase (HPT) and Citrine fluorescent protein (Tsuboyama and Kodama 2014). *M. polymorpha* tissues were grown in a culture room (temperature: 22°C, humidity: approximately 40%), and asexually maintained on half-strength Gamborg's B5 (1/2 B5) medium (Gamborg et al. 1968) with 1% agar (BOP, SSK Sales Co., Ltd., Shizuoka, Japan) under 75 μmol photons $m^{-2}s^{-1}$ of continuous white light (FL40SW, NEC Corporation, Tokyo, Japan).

Abbreviations: AgarTrap, Agar-utilized Transformation with Pouring Solutions; G-AgarTrap, AgarTrap using gemmae/gemmalings; LB, Luria–Bertani; S-AgarTrap, AgarTrap using sporelings; T-AgarTrap, AgarTrap using matured thalli pieces; Tak-1, Takaragaike-1; T-DNA, transfer DNA.

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The T-AgarTrap method using matured thalli pieces was developed by modification of S- and G-AgarTrap methods. Similar to the S- and the G-AgarTrap procedures, the T-AgarTrap method consisted of three steps. First, 1-month-old mature thalli were obtained and cut into approximately 5×5 mm pieces using tweezers. Both apical and basal pieces were used. Pieces were placed in a 60 mm disposable sterile polystyrene Petri dish containing 10 ml of M51C solid medium. Second, pieces were co-cultured with *A. tumefaciens*. One ml transformation buffer (10 mM $MgCl_2$; 10 mM MES-NaOH, pH 5.7; 150 μM acetosyringone) containing *A. tumefaciens* was directly poured over thalli pieces, and excess buffer was removed using a micropipette or aspirator. Finally, thalli pieces and the surface layer of the medium were washed using sterile water, and 1 ml selection buffer (appropriate antibiotic reagent and 1 mg ml^{-1} claforan) was poured over the pieces and solid medium.

When *pMpGWB103-Citrine* was used, Citrine fluorescent-transformed cells were observed 3 days after pouring selection buffer (100 μg ml^{-1} hygromycin and 1 mg ml^{-1} claforan) (Figure 1A and B). Note that transformed cells were observed in wounded and unwounded regions, and that two or more transformed cells were occasionally found within a piece of thalli. Cells were observed using a MZ16F stereo fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Citrine and chlorophyll fluorescence were determined with a fluorescence module (excitation filter: 480/40 nm, barrier filter: LP 510 nm). Images were obtained using a DP73 digital camera (Olympus, Tokyo, Japan). After approximately 1 month, almost all non-transformed cells declined, and regeneration occurred only in transformed cells (Figure 1C and D). It has been reported that regeneration of wild-type thallus occurred from the basal piece, not the apical piece, when the thallus was cut (Nishihama et al. 2015; Vöchting 1885). However, in our T-AgarTrap experiments, regeneration of transformants could be induced in both basal and apical pieces under selection pressure with hygromycin. In this context, regeneration events because of *Agrobacterium*-mediated transformation differ from regeneration (or cell division) events in the lifecycle of *M. polymorpha*.

To evaluate transformation efficiency, we used two marker genes, *HPT* and *Citrine* (of *pMpGWB103-Citrine*); hygromycin B was used as an antibiotic reagent for selection of transformed cells, and Citrine fluorescence was observed under a fluorescence microscope. To avoid transient expression of marker genes, transformants were determined 2 weeks after pouring selection buffer. To calculate transformation efficiency (%), the number of transformed thalli pieces was divided by 10 (total number of thalli pieces tested in each experiment) and multiplied by 100. Average transformation efficiency and standard error were calculated from 10 independent experiments.

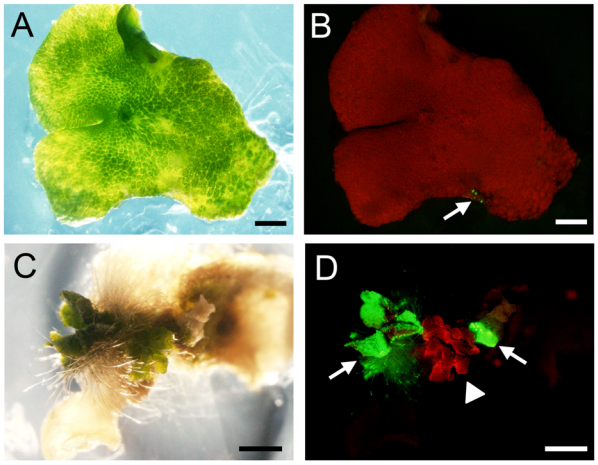

Figure 1. Transmitted light and fluorescence microscope images of thalli pieces transformed by T-AgarTrap. (A) Transmitted light image of a thallus piece 3 days after pouring selection buffer. (B) Fluorescence image of a thallus piece 3 days after pouring selection buffer. (C) Transmitted light image of a thallus piece 1 month after pouring selection buffer. (D) Fluorescence image of a thallus piece 1 month after pouring selection buffer. Transformed cells were observed as yellow-green in colour (Citrine fluorescence; arrows), and non-transformed cells were observed as red (chlorophyll fluorescence; arrowhead). Note that the non-transformed cells declined thereafter by continuous selection with hygromycin. Bar represents 1 mm.

S- and G-AgarTrap methods were optimized by investigating several factors that influence transformation efficiency (Tsuboyama and Kodama 2014, Tsuboyama-Tanaka and Kodama 2015). Based on previous studies, the T-AgarTrap was optimized by observing thalli pieces during pre-culture periods and comparing the following *A. tumefaciens* culture conditions: solid and liquid Luria–Bertani (LB) media. Except for the 2 factors (pre-culture periods and *A. tumefaciens* culture conditions), the following treatments were employed: 0-day pre-culture of *M. polymorpha*, 3 days co-culture with *A. tumefaciens* (OD_{600}=0.5) in M51C medium supplemented with 2% sucrose and *A. tumefaciens* cultured on solid LB medium at 28°C for 2 days (Tsuboyama and Kodama 2014, Tsuboyama-Tanaka and Kodama 2015).

To determine the effect of pre-culture periods, 0- and 3-d pre-cultures were compared. In male thalli, average transformation efficiencies of 0- and 3 days pre-cultures were 47±5% and 41±4%, respectively (Figure 2A). For female thalli, average transformation efficiencies of 0- and 3-day pre-cultures were 59±8% and 53±5%, respectively (Figure 2B). Similar to G-AgarTrap, the transformation efficiency of females was higher than that of males (Figure 2A and B). This difference may be because of genomic variation between males and females, as previously described (Tsuboyama-Tanaka and Kodama 2015).

Average transformation efficiency of S-AgarTrap was

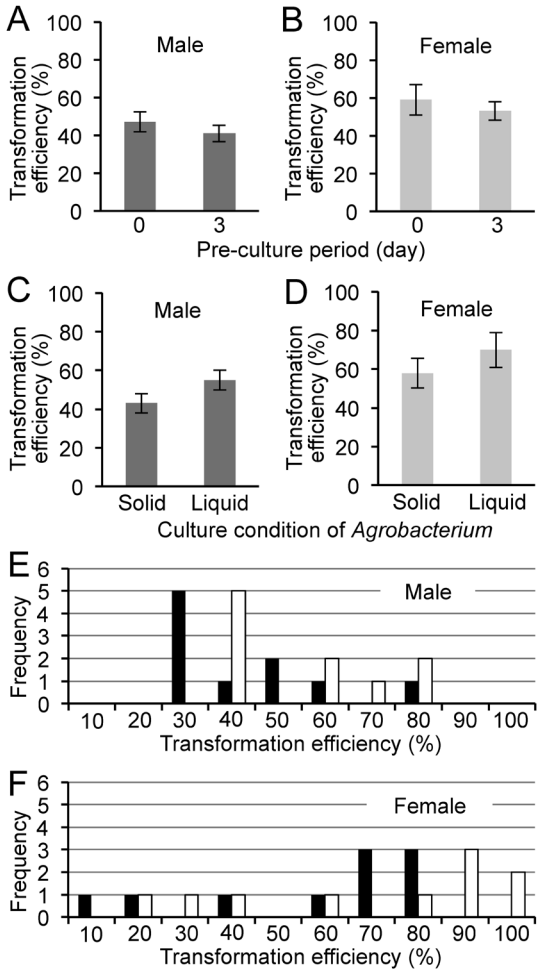

Figure 2. Optimization of T-AgarTrap. (A and B) Thalli pieces during pre-culture period. (A) Transformation efficiencies of male thalli pieces pre-cultured for 0 and 3 days. (B) Transformation efficiencies of female thalli pieces pre-cultured for 0 and 3 days. (C and D) Culture conditions for *Agrobacterium tumefaciens*. (C) Transformation efficiencies of male thalli pieces transformed using *A. tumefaciens* cultured on solid and liquid LB media. (D) Transformation efficiencies of female thalli pieces transformed using *A. tumefaciens* cultured on solid and liquid LB media. Bars represent standard error of 10 experiments. (E and D) The frequency distribution analysis of transformation efficiencies using *A. tumefaciens* cultured on solid (black bar) and liquid (white bar) LB media. Frequency was determined from 10 independent experiments. (E) Frequency distribution of transformation efficiencies of male thalli. (F) Frequency distribution of transformation efficiencies of female thalli.

approximately 10%, and the efficiency of G-AgarTrap was approximately 30% and 50% in male and female plants, respectively (Tsuboyama and Kodama 2014, Tsuboyama-Tanaka and Kodama 2015). The efficiency of T-AgarTrap was much higher than that of other AgarTraps. This difference may be explained by differences in plant material. Intact tissues (sporelings and gemmae/gemmalings) were used in S- and G-AgarTraps (Tsuboyama and Kodama 2014, Tsuboyama-Tanaka and Kodama 2015), whereas thalli pieces were cut (wounded) in the T-AgarTrap method. *A. tumefaciens* may infect thalli pieces more easily than intact tissues.

For the T-AgarTrap method, the efficiency of 0-day pre-cultured thalli was slightly higher than that of the 3-day group for both male and female plants (Figure 2A and B). In S- and G-AgarTraps, a pre-culture period improved efficiency; the highest efficiencies were observed after 3 days of pre-culture for S-AgarTrap and after 1 to 2-s pre-culture for G-AgarTrap (Tsuboyama and Kodama 2014, Tsuboyama-Tanaka and Kodama 2015). Unlike S- and G-AgarTraps, T-AgarTrap did not require pre-culture of *M. polymorpha* (Figure 2A and B). Phenolic compounds released from wounded cells can activate virulence genes in *A. tumefaciens* (Stachel et al. 1985). We speculated that phenolic compounds enhanced transformation efficiency at 0-day pre-culture, followed by a decline because of restoration of wounded cells in the 3-day pre-culture.

Next, culture conditions for *A. tumefaciens* were compared. In previous experiments, *A. tumefaciens* used for S- and G-AgarTraps were cultured using solid LB medium (Tsuboyama and Kodama 2014, Tsuboyama-Tanaka and Kodama 2015). To further improve transformation efficiency, we tested the following culture conditions for *A. tumefaciens*: solid and liquid LB media. *A. tumefaciens* stock in 30% glycerol was preserved at −80°C, streaked on solid LB medium, cultured at 28°C for 2 days and subjected to T-AgarTrap. To test liquid medium, a single colony of *A. tumefaciens* was picked from solid medium and cultured in 2 ml liquid medium at 28°C for 36 h. Further 15 μl of cultured liquid medium was added to 15 ml of new liquid medium. After culture at 28°C for 20–22 h, *A. tumefaciens* were collected by centrifugation and suspended in transformation buffer at OD_{600}=0.5.

For male thalli, average transformation efficiency was 43±5% when solid medium was used (Figure 2C). When *A. tumefaciens* was cultured in liquid medium, efficiency increased to 55±5% (Figure 2C). For female thalli, transformation efficiency was 58±8% on solid medium (Figure 2D), compared with 70±9% in liquid medium (Figure 2D). For both sexes, transformation efficiency was the highest in the liquid medium (Figure 2C and D), suggesting increased activity of *A. tumefaciens* when cultured in liquid LB. After frequency distribution analysis was performed, we confirmed that the liquid medium was effective for both male and female plants (Figure 2E and F). In a few cases with female plants, 100% transformation efficiency was achieved (Figure 2F).

Real-time PCR was performed to estimate T-DNA copy numbers in transformants produced via T-AgarTrap. Use of real-time PCR for estimating the number in transgenic *M. polymorpha* has been previously reported (Tsuboyama-Tanaka and Kodama 2015). Briefly, genomic DNA was isolated using DNeasy Plant Mini Kits (QIAGEN, Hilden, Germany), and an Applied Biosystems 7500 real-

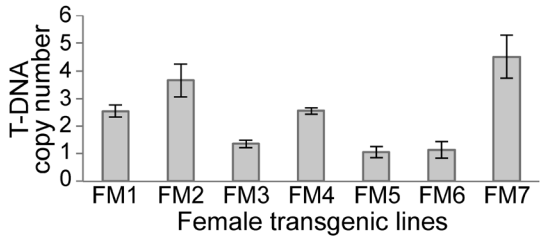

Figure 3. Estimation of T-DNA copy numbers using real-time PCR. Seven female transformants were selected randomly for investigation. Bars represent standard deviations of experiments performed in triplicate.

time PCR system (Life Technologies, Carlsbad, CA, USA) with FastStart Universal SYBR Green Master (ROX; Roche Diagnostics GmbH, Mannheim, Germany) was used. To amplify an internal control gene (286 bp) obtained from an expressed sequence tag (Accession No.: BJ867210) (Ogasawara et al. 2013), the primers 5′-CAA GAA GAG GTT GGG GTC AG-3′ and 5′-GCC GAT TTG GTA GAT TGG TG-3′ were used. To determine T-DNA copy numbers, the primers 5′-AGA ACG GCA TCA AGG TGA AC-3′ and 5′-GTT GGG GTC TTT GCT CAG G-3′ were used to amplify a segment of *Citrine* (164 bp) on the *pMpGWB103-Citrine*. Seven female transformants produced via T-AgarTrap were selected randomly (FM1–7), and subjected to real-time PCR. Three transformants had a single copy (FM3, 5 and 6), 2 transformants had two copies (FM1 and 4) and FM2 and FM7 had 3 and 4 copies, respectively (Figure 3). Based on these results, we concluded that T-AgarTrap produced transformants with both single and multiple T-DNA insertion(s).

Similar to S- and G-AgarTraps, T-AgarTrap is a simple procedure performed by pouring appropriate solutions into a single Petri dish, and it can be completed within a few days. Because pieces of matured thalli are used in T-AgarTrap, the preparation of plant materials is easy compared with other AgarTraps. Because T-AgarTrap requires neither spores nor gemmae, it may be suitable for transformants/mutant(s) that cannot produce both spores and gemmae. The three types of AgarTrap methods (S, G and T) that we developed can be applied to a variety of research scenarios involving *M. polymorpha*. Among the three AgarTrap methods, T-AgarTrap had the highest transformation efficiency; the average and greatest transformation efficiencies were approximately 70% and 100%, respectively. Moreover, similar to G-AgarTrap, T-AgarTrap produced a sufficient number of independent transformants with uniform genetic background. Therefore, the T-AgarTrap method is believed to be useful for molecular studies involving *M. polymorpha*.

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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

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

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

Kubota A, Ishizaki K, 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

Nishihama R, Ishizaki K, Hosaka M, Matsuda Y, Kubota A, Kohchi T (2015) Phytochrome-mediated regulation of cell division and growth during regeneration and sporeling development in the liverwort *Marchantia polymorpha. J Plant Res* 128: 407–421

Ogasawara Y, Ishizaki K, Kohchi T, Kodama Y (2013) Cold-induced organelle relocation in the liverwort *Marchantia polymorpha* L. *Plant Cell Environ* 36: 1520–1528

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

Stachel SE, Messens E, Van Montagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens. Nature* 318: 624–629

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-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

Vöchting (1885) Über die regeneration der Marchantieen. *Jahrbücher für wissenschaftliche Botanik* 16: 367–414