Journal of Plant Research, volume 128, pages 337–344 (2015)
https://doi.org/10.1007/s10265-014-0695-2

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(5) Physiology/Biochemistry/Molecular and Cellular Biology

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Title:
AgarTrap-mediated genetic transformation using intact gemmae/gemmalings of the liverwort *Marchantia polymorpha* L.

Running Title:
AgarTrap method for *Marchantia* gemmae/gemmalings

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Abstract:
The dioecious liverwort, *Marchantia polymorpha* L., is an emerging model plant. Various molecular biological techniques have been optimized for *M. polymorpha* for the past several years, and recently we reported a simplified *Agrobacterium*-mediated transformation method using sporelings (immature thalli from spores) of *M. polymorpha*. This method, termed AgarTrap (Agar-utilized Transformation with Pouring Solutions), completed by exchanging appropriate solutions on a single Petri dish to produce a sufficient number of independent transgenic sporelings. However, because spores are produced by crosses between males and females, the genetic backgrounds of resulting transgenic sporelings are not uniform. To easily produce transgenic liverworts with a uniform genetic background using AgarTrap, we developed an AgarTrap-mediated transformation method using intact gemmae/gemmalings produced by asexual reproduction. Using AgarTrap with male and female gemmae/gemmalings produced a sufficient number of independent transgenic gemmalings with uniform genetic backgrounds. The optimized transformation efficiencies were approximately 30% and 50% in males and females, respectively. As with AgarTrap using sporelings, AgarTrap using intact gemmae/gemmalings will be useful in promoting studies of the molecular biology of *M. polymorpha*.

Keywords:
Agrobacterium, AgarTrap, bryophytes, gemma, Marchantia, transformation.

Introduction

Because phylogenomic analyses have suggested that liverworts are sister to all other land plants, this group of plants is important for understanding how plant species adapted to terrestrial from aquatic environments (Qiu et al. 2006). The dioecious liverwort, *Marchantia polymorpha* L., is an emerging model plant, making studies of *M. polymorpha* helpful for understanding plant evolution. The nuclear genome of *M. polymorpha* has been well studied, along with the mitochondrial and chloroplast genomes, with the Y chromosome being the first sequenced among all land plants (Oda et al. 1992; Ohyama et al. 1986; Yamato et al. 2007). In addition, a whole-genome sequencing project is in progress at the DOE Joint Genome Institute (http://www.jgi.doe.gov/) (JGI project ID: 1007435).

Over the past several years, techniques have been developed to study the molecular biology of *M. polymorpha*, including genetic transformation techniques mediated by biolistic particle delivery and *Agrobacterium tumefaciens* (also known as *Rhizobium radiobactor*) (Nasu et al. 1997; Takenaka et al. 2000; Chiyoda et al. 2008; Ishizaki et al. 2008; Kubota et al. 2013). The first *Agrobacterium*-mediated transformation method for *M. polymorpha* involved cultured cells (Nasu et al. 1997), followed by a transformation method using sporelings (immature thalli from spores) (Ishizaki et al. 2008) and regenerating thalli (Kubota et al. 2013). The latter method does not require spores made by crossing male and female plants, resulting in transformants with a uniform genetic background (Kubota et al. 2013). However, because these *Agrobacterium*-mediated transformation methods require stepwise culture in liquid and solid media (Nasu et al. 1997; Ishizaki et al. 2008; Kubota et al. 2013), these procedures are complicated. In addition, because resulting transformants are aggregated by their rhizoids while being shaken in liquid medium, it was difficult to define independent transformants. Thus, further improvements are required for the *Agrobacterium*-mediated transformation of *M. polymorpha*.

We recently developed an AgarTrap (Agar-utilized Transformation with Pouring Solutions) procedure for simplified *Agrobacterium*-mediated transformation using sporelings of *M. polymorpha*. The AgarTrap method is very simple and only requires pouring appropriate solutions into a Petri dish. This allows a sufficient number of independent transformants, to be obtained in a single experiment (Tsuboyama and Kodama 2014). Similar to a previous method (Ishizaki et al. 2008), however, this method requires spores generated by crossings of male and female plants (i.e. sexual reproduction) (Tsuboyama and Kodama 2014). Because the genetic background of the spores is not uniform, spores are not suitable as for generating transformants with a uniform genetic background. In addition, AgarTrap using sporelings cannot be used to transform infertile mutant/transgenic plants. To overcome these possible limitations, it was necessary to develop an AgarTrap-mediated genetic transformation method that does not require sexual reproduction.

Because gemmae develop from single cells in the gemma cup on a mature thallus (asexual reproduction) (Barnes and Land 1908), the gemma and thallus are genetically identical. This study describes our development of an AgarTrap method using intact gemmae obtained by asexual reproduction and intact gemmalings (immature thalli grown from gemmae).

Materials and methods

Plant materials and growth conditions

M. polymorpha tissues were grown in a culture room (temperature: 22 °C, humidity: approximately 40 %). Takaragaike-1 (Tak-1) and BC3-38 were used as male and female plants, respectively. BC3-38 is a female line of the third backcross generation, which was kindly provided by Dr. Takayuki Kohchi (Kyoto University, Kyoto, Japan). Note that the first backcross generation (BC1) was created via crossing of Takaragaike-2 (Ishizaki et al. 2008) with Tak-1, and the second and the third backcross generations (BC2 and BC3) were generated via crossing of BC1 and BC2 females with Tak-1, respectively. Tak-1 and BC3-38 were asexually maintained on half strength Gamborg's B5 (1/2 B5) medium (Gamborg et al. 1968; Ishizaki et al. 2012) with 1 % agar (BOP, SSK Sales Co., Ltd., Shizuoka, Japan) while illuminated with 75 μmol photons m^{-2} s^{-1} continuous white light (FL40SW, NEC Corporation, Tokyo, Japan). The gemmae subjected to the AgarTrap method were obtained from approximately 1-month-old thalli.

Microscopic observation

The gemmae/gemmalings of *M. polymorpha* were observed using a MZ16F stereo fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Chlorophyll and Citrine fluorescence were determined with a fluorescence module (excitation filter 480/40 nm and barrier filter LP 510 nm). The images were taken with a DP73 digital camera (Olympus, Tokyo, Japan).

Transformation efficiency

The transformation efficiency was evaluated by transformation with the binary vector, *pMpGWB103-Citrine*, which was transformed into *Agrobacterium tumefaciens* strain GV2260 (Deblaere et al. 1985) and used to evaluate transformation efficiency (Tsuboyama and Kodama 2014). The vector possesses two marker genes, encoding hygromycin B phosphotransferase (HPT) and Citrine fluorescent protein (Tsuboyama and Kodama 2014). To identify transformants, cells were selected by growth on the antibiotic hygromycin B, and their yellow fluorescence from Citrine observed by fluorescence microscopy two weeks after pouring the selection buffer. Transformation efficiency (%) was calculated as the number of transformed gemmalings divided by the number of all gemmalings, and multiplied by 100.

Real-time PCR with genomic DNA

Genomic DNA was isolated from approximately 1-month-old-thalli using DNeasy Plant Mini Kits (QIAGEN, Hilden, Germany). Real-time PCR was performed using an Applied Biosystems 7500 real-time PCR system (Life Technologies, Carlsbad, CA, USA) with FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH, Mannheim, Germany). As an internal control, the primers 5'-CAAGAAGAGGTTGGGGTCAG-3' and 5'-GCCGATTTGGTAGATTGGTG-3' were used to amplify part of the *PHOT* gene (286 bp) of *M. polymorpha*, with the sequence of this gene obtained from Expressed Sequence Tag (Accession No.: BJ867210) (Ogasawara et al. 2013). To determine T-DNA copy numbers, the primers 5'-AGAACGGCATCAAGGTGAAC-3' and 5'-GTTGGGGTCTTTGCTCAGG-3' were used to amplify part of the *Citrine* gene (164 bp) of T-DNA in *pMpGWB103-Citrine* (Tsuboyama and Kodama 2014).

Results

Adaptation of the AgarTrap procedure to intact gemmae/gemmalings

The AgarTrap procedure using intact gemmae/gemmalings (termed the G-AgarTrap method) was developed by modifying our previously described AgarTrap method using sporelings (termed the S-AgarTrap method) (Tsuboyama and Kodama 2014). Like the S-AgarTrap method, the G-AgarTrap method consists of 3 steps. The first step is the individual pre-culture of *M. polymorpha* gemmae/gemmalings and *A. tumefaciens*; the second step is their co-culture; and the third step is the selection of transformants using an antibiotic.

Male and female gemmae were obtained from approximately 1-month-old thalli. In the first step, male or female gemmae were sown on M51C or half strength Gamborg's B5 (1/2 B5) solid medium (1 % agar) supplemented with 1–2 % sucrose in a 60 mm disposable sterile polystyrene Petri dish. To separate individual gemmae on the solid medium, the gemmae were suspended in a drop (approximately 500 µL) of sterile water prepared on the solid medium (Fig. S1a), and spread individually (Fig. S1b). The water drops were absorbed by the solid medium within one day, and the 1-day-old gemmalings (Fig. S1c) were fixed on the medium via their rhizoids. Other procedures in the first, second and third steps were identical to the S-AgarTrap method (Tsuboyama and Kodama 2014) (Fig. S2).

A binary vector, *pMpGWB103-Citrine*, was used to develop the G-AgarTrap method. Because the vector contains two marker genes, encoding hygromycin B phosphotransferase (HPT) and Citrine fluorescent protein (Tsuboyama and Kodama 2014), the transformants were determined by both selection on hygromycin and monitoring of fluorescence. After culture for three days in selection buffer (100 µg mL^{-1} hygromycin and 1 mg mL^{-1} claforan), a single transformed fluorescent cell could be observed at the non-peripheral region of each gemmaling (Fig. 1a, b). After pouring selection buffer for 2–4 weeks, almost all non-transgenic cells of the gemmalings decayed in the presence of antibiotic. For example, after pouring selection buffer for 2 weeks, non-transgenic cells had died and a few green living cells were present (Fig. 1c); transgenic fluorescent cells were also observed (Fig. 1d). After 4 weeks, a mature transgenic thallus (T1) was obtained (Fig. 1e, f); however, chimeric transformants may be present in the T1 generation. To isolate non-chimeric transformants only, it was important to obtain each G1 gemma from a T1 thallus because gemmae originated from single cells (Barnes and Land 1908; Ogasawara et al. 2013).

Optimization of G-AgarTrap

During development of the G-AgarTrap method, we optimized five factors that influence transformation efficiency: (I) the pre-culture period for gemmae/gemmalings, (II) the co-culture period with *A. tumefaciens*, (III) *Agrobacterium* concentration in transformation buffer, (IV) use of 1/2 B5 medium and (V) sucrose concentration in the solid medium. Each experiment included pre-culture for one day, co-culture for three days, an *Agrobacterium* concentration in transformation buffer of OD_{600} = 0.5, and use of M51C solid medium (1 % agar) supplemented with 2 % sucrose, except for the factor being investigated. In all experiments, 150 µM acetosyringone was added to the transformation buffer (10 mM $MgCl_2$; 10 mM MES-NaOH, pH 5.7) (Tsuboyama and Kodama 2014). To evaluate transformation efficiency, the *pMpGWB103-Citrine* vector

was also used (Tsuboyama and Kodama 2014).

(I) Pre-culture period for gemmae/gemmalings – Male and female gemmae/gemmalings were pre-cultured for 0, 1, 2 and 3 days, and their transformation efficiencies were evaluated. When testing gemmae pre-cultured for 0 days, the process of discarding the excess transformation buffer in the second step was handled carefully because these gemmae had no rhizoids and were not fixed in agar. Male gemmae pre-cultured for 0, 1, 2 and 3 days had transformation efficiencies of approximately 15 %, 30 %, 20 % and 10 %, respectively (Fig. 2a; Table S1), whereas female gemmae pre-cultured for these times had transformation efficiencies of approximately 20 %, 40 %, 50 % and 40 %, respectively (Fig. 2b; Table S2). The pre-culture period with the highest efficiency was shorter in males (1 day) than in females (2 days).

(II) Co-culture period with *A. tumefaciens* – The transformation efficiencies of male and female gemmalings co-cultured for 1–3 days with *A. tumefaciens* were compared. Males co-cultured for 1, 2, and 3 days showed transformation efficiencies of approximately 0 %, 10 % and 25 %, respectively (Fig. 2c; Table S3), whereas females co-cultured for 1, 2, and 3 days had transformation efficiencies of approximately 1.5 %, 40 % and 45 %, respectively (Fig. 2d; Table S4). Although transformation efficiency of both males and females was increased by extending the co-culture periods, females had a higher transformation efficiency than males (Fig. 2c, d). Thus, subsequent optimizations of *Agrobacterium* concentration in transformation buffer, use of 1/2 B5 solid medium, and sucrose concentration in solid medium were performed using only female gemmae/gemmalings.

(III) *Agrobacterium* concentration in transformation buffer – In optimizing the S-AgarTrap method, we found that different concentrations of *Agrobacterium* (OD_{600} = 0.5, 1.0, 2.0 and 3.0) had no effect on transformation efficiency, suggesting that the *Agrobacterium* concentration was saturated at OD_{600} = 0.5 (Tsuboyama and Kodama 2014). In optimizing the G-AgarTrap method, we compared transformation efficiencies at *Agrobacterium* concentrations at OD_{600} = 0.1, 0.3 and 0.5. The transformation efficiencies at OD_{600} = 0.3 and 0.5 were almost the same, approximately 50 %, but was lower, approximately 30 %, at an *Agrobacterium* concentration of OD_{600} = 0.1 (Fig. 3a; Table S5). This finding indicates that an *Agrobacterium* $OD_{600} > 0.3$ was suitable for the AgarTrap procedure.

(IV) Use of 1/2 B5 medium – Since *M. polymorpha* grows on both M51C and 1/2 Gamborg's B5 media (Ono et al. 1979, Ishizaki et al. 2012), we examined whether M51C and 1/2 B5 media could be used in the G-AgarTrap method. The transformation efficiencies following growth in M51C and 1/2 B5 media were approximately 50 % and 60 %, respectively (Fig. 3b; Table S6). A similar effect was observed with the S-AgarTrap method; with M51C and 1/2 B5 media having transformation efficiencies of 10 % and 20 %, respectively (Fig. S3; Table S7).

(V) Sucrose concentration in solid medium – Because sucrose influences the efficiency of *Agrobacterium*-mediated transformation (Clough and Bent 1998), we tested the effect of sucrose concentration on the G-AgarTrap method. M51C solid media containing 0 %, 1 % and 2 % sucrose had transformation efficiencies of < 1 %, 55 % and 50 %, respectively (Fig. 3c; Table S8), with similar results observed in 1/2 B5 medium (Fig. S4; Table S9). These results indicate that sucrose supplementation of solid medium significantly increased the transformation efficiency of the AgarTrap method.

Quantitative T-DNA integration

The number of copies of T-DNA integrating into transgenic liverworts was estimated by real-time PCR using *M. polymorpha* genomic DNA. Previously, Southern blot analysis was utilized to estimate the number of copies of T-DNA in the transgenic liverworts (Tsuboyama and Kodama 2014), with plants numbers 1, 6, 8, 12, 14 and 18 containing 1, 1, 2, 1, 4 and 1 copies, respectively, of T-DNA by Southern blotting (Tsuboyama and Kodama 2014). Real-time PCR results for five of these plants (Nos. 1, 6, 8, 12 and 18) containing one or two copies of T-DNA, were similar to the Southern blotting results (Fig. S5). In contrast, plant No. 14, which contained four copies of T-DNA on Southern blotting, contained five copies by real-time PCR (Fig. S5). Thus, real-time PCR would be useful for estimating T-DNA copy number in transgenic plants.

Five transgenic male gemmalings (M1–M5) produced by the G-AgarTrap method were randomly selected, and their T-DNA copy numbers estimated by real-time PCR. Two plants (M1 and M3) each had a single copy, two (M2 and M5) had two copies each and one (M4) had seven copies (Fig. 4a). Similar assays in five randomly selected transgenic female gemmalings (FM1–FM5) showed that two (FM1 and FM2) each had a single copy of T-DNA, FM5 had two copies, FM4 had three copies, and FM3 had 16 copies (Fig. 4b). Similar to the S-AgarTrap method, the G-AgarTrap method can introduce both single and multiple transgene(s) into intact gemmae/gemmalings.

Discussion

Our previous study found that the S-AgarTrap method using sporelings of *M. polymorpha* was useful for producing transgenic liverworts for molecular biological studies, because a sufficient number of independent transformants could be obtained using a simple procedure (Tsuboyama and Kodama 2014). However, this method utilized spores obtained by crossing males and females, with the resulting transformants having diverse genetic backgrounds. Furthermore, this method was limited in adding additional genetic transformation to existing transformants because it was necessary to generate transgenic spores. To solve these limitations we developed the G-AgarTrap method using gemmae/gemmalings, which are produced by asexual reproduction.

The transformation efficiency with the G-AgarTrap method (approximately 30–50 %) was higher than that with S-AgarTrap (approximately 10 %) (Tsuboyama and Kodama 2014). This difference may be due to differences in the composition of cells in sporelings and gemmae/gemmalings. The latter may contain specific cell(s) suitable for *Agrobacterium*-mediated transformation, because the initial transformants were usually located at the non-peripheral regions of gemmalings (Fig. 1b). The number of these cells may be higher in females than in males, since the transformation efficiency of females (approximately 50 %) was higher than that of males (approximately 30 %) in the G-AgarTrap system (Fig. 2a, b; Table S1, 2). The difference in transformation efficiency between males and females may have been due to genomic variations (within autosomes and/or sex chromosomes) between Tak-1 (male) and BC3-38 (female) plants.

Agrobacterium-mediated transformation using regenerating thalli has also been used to transform gemmalings, producing different independent transformants with the same genetic background (Kubota et al. 2013). Previously our group successfully produced only male transformants using this method (Ogasawara et al. 2013). However, this method required stepwise culture in liquid and solid media, as well as a long period of pre-culture, 17 days (Kubota et al. 2013). In addition, during pre-culture it was

necessary to prepare plantlets using 2-week-old gemmalings with a special cutting pattern for co-culture (Kubota et al. 2013). In contrast, the G-AgarTrap method required only 1–2 days of pre-culture, but did not require preparation of plantlets with this cutting pattern. Optimal pre-culture periods for male and female gemmalings were 1 and 2 days, respectively (Fig. 2a, b; Table S1, 2), shorter than for sporelings (3 days) (Tsuboyama and Kodama 2014). The shorter pre-culture period may be due to the higher rate of cell division of gemmalings, which may result in more efficient transformation; however, further analysis is necessary.

Although extending the co-culture period may increase transformation efficiency, it may also result in *Agrobacterium* overgrowth. Because overgrown *Agrobacterium* is difficult to eliminate during the washing step, the co-culture period should be as short as possible. In co-culturing male gemmalings with *Agrobacterium*, we found that the transformation efficiency was 2-fold higher at 3 days than at 2 days (Fig. 2c; Table S3). In contrast, the transformation efficiency of female gemmalings was similar after co-culture for 2 and 3 days (Fig. 2d; Table S4). Thus, the optimal co-culture periods for male and female gemmalings were 3 and 2 days, respectively. Co-culture for 1 day was insufficient for both males and females, because few or no transformants were obtained (Fig. 2c, d; Table S3, 4), results similar to those using the S-AgarTrap method (Tsuboyama and Kodama 2014).

Maintaining a low enough concentration of *Agrobacterium* in transformation buffer is important in preventing its overgrowth. In the present study, we found that the transformation efficiencies at OD_{600} of 0.3 and 0.5 were almost the same, whereas the transformation efficiency at OD_{600} of 0.1 was lower (Fig. 3a; Table S5). Thus, *Agrobacterium* was saturated at an OD_{600} of 0.3. Because the transformation efficiencies at OD_{600} = 0.5–3.0 were almost identical using the S-AgarTrap method (Tsuboyama and Kodama 2014), a similar effect would be expected using G-AgarTrap. However, the growth rate of *Agrobacterium* seemed to be dependent on bacterial strains and/or culture conditions. Thus, *Agrobacterium* concentration should be optimized when preparing to use AgarTrap techniques.

In both the G- and S-AgarTrap methods, the transformation efficiency was much higher with 1/2 B5 than with M51C medium (Fig. 3b, S3; Table S6, 7). In contrast, when liquid medium was used for co-cultivation, the transformation efficiency was higher with M51C than with 1/2 B5 medium (Kubota et al. 2013). This discrepancy may have been due to differences in culture conditions, although further analysis is required to determine the cause of this discrepancy.

Assessment of the previous method showed that the addition of sucrose to both the liquid and solid media was essential for efficient transformation (Kubota et al. 2013). Similarly, sucrose promoted efficient *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method (Clough and Bent 1998). In the present study, the supplement of sucrose to the medium increased transformation efficiency approximately 50 fold (from 1% to 50 %), providing further indication that sucrose enhances *Agrobacterium*-mediated transformation using AgarTrap procedures (Fig. 3c; Table S8).

Southern blotting is usually used to estimate the number of copies of T-DNA inserted into recipient genomes. However, Southern blotting analysis is labor-intensive and requires large amounts of genomic DNA. A real-time PCR method has therefore been utilized for the rapid and easy estimation of T-DNA copy number(s) in various

plants (Ingham et al. 2001; Mason et al. 2002; Bubner et al. 2004; Yang et al. 2005). For example, in transgenic tomatoes, copy numbers estimated by real-time PCR were equal to or higher than numbers estimated by Southern blotting analysis, suggesting that the latter may underestimate actual copy numbers (Mason et al. 2002). In using real-time PCR to determine T-DNA copy number in transgenic *M. polymorpha*, we found 1–16 T-DNA insertions in transformants produced by the G-AgarTrap method (Fig. 4a, b), with our real-time PCR estimates equal to or higher than results from Southern blotting analysis (Fig. S5). These findings suggest that real-time PCR determinations may be useful for the rapid analysis of T-DNA copy numbers in large numbers of transgenic *M. polymorpha*.

The optimized S-AgarTrap method required 6 days, 3 days for pre-culture and 3 days for co-culture (Tsuboyama and Kodama 2014). In contrast, the optimized G-AgarTrap method required only 3 days, 1 day for pre-culture and 2 days for co-culture. Thus, the G-AgarTrap is a much more rapid technology that further promotes molecular biological studies using *M. polymorpha*.

Acknowledgments

The authors thank Dr. Christopher Suarez (University of Notre Dame) for critical reading of the manuscript. The authors also thank Dr. Takayuki Kohchi (Kyoto University) for providing Tak-1 and BC3-38 lines. This work was supported by the Plant Transgenic Design Initiative of University of Tsukuba (Y.K.), the Japan Society for the Promotion of Science Research (JSPS) KAKENHI (No. 26840088) (Y.K.) and research projects [CORE Adopted, CDI-F, UU-COE and UU-COE-next] of Utsunomiya University (Y.K).

References

Barnes, C.R. and Land, W.J.G. (1908) Bryological papers. II.The origin of the cupule of *Marchantia*. Bot. Gaz. 46: 401–409.

Bubner, B., Gase, K. and Baldwin, I.T. (2004) Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR. BMC Biotechnol. 4: 14.

Chiyoda, S., Ishizaki, K., Kataoka, K., Yamato, K.T. and Kohchi, T. (2008) Direct transformation of the liverwort *Marchantia polymorpha* L. by particle bombardment using immature thalli developing from spores. Plant Cell. Rep. 27: 1467–1473.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16: 735–743

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

Gamborg, O.L., Miller, R.A. and Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151–158.

Ingham, D.J., Beer, S., Money, S. and Hansen, G. (2001) Quantitative real-time PCR assay for determining transgene copy number in transformed plants. Biotechniques. 31: 132–140.

Ishizaki, K., Chiyoda, S., Yamato, K.T. and 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.

Ishizaki, K., Nonomura, M., Kato, H., Yamato, K.T. and Kohchi, T. (2012) Visualization of auxin-mediated transcriptional activation using a common auxin-response reporter system in the liverwort *Marchantia polymorpha*. J Plant Res. 125: 643–651.

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

Mason, G., Provero, P., Vaira, A.M. and Accotto, G.P. (2002) Estimating the number of integrations in transformed plants by quantitative real-time PCR. BMC Biotechnol. 2: 20.

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

Oda, K., Yamato, K., Ohta, E., Nakamura, Y., Takemura, M., Nozato, N. et al. (1992) Gene organization deduced from the complete sequence of liverwort *Marchantia polymorpha* mitochondrial DNA. A primitive form of plant mitochondrial genome. J. Mol. Biol. 223: 1–7.

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

Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S. et al. (1986) Chloroplast gene organization deduced from the complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. Nature 322: 572–574.

Ono, K., Ohyama, K. and Gamborg, O.L. (1979) Regeneration of the liverwort *Marchantia polymorpha* L. from protoplasts isolated from cell suspension culture. Plant Sci Lett 14: 225–229.

Takenaka, M., Yamaoka, S., Hanajiri, T., Shimizu-Ueda, Y., Yamato, K.T., Fukuzawa, H. et al. (2000) Direct transformation and plant regeneration of the haploid liverwort *Marchantia polymorpha* L. Transgenic Res. 9: 179–185.

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

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

Yamato, K.T., Ishizaki, K., Fujisawa, M., Okada, S., Nakayama, S., Fujishita, M. et al. (2007) Gene organization of the liverwort Y chromosome reveals distinct sex chromosome evolution in a haploid system. Proc. Natl Acad. Sci. USA 104: 6472–6477.

Yang, L., Ding, J., Zhang, C., Jia, J., Weng, H., Liu, W. et al. (2005) Estimating the copy number of transgene in transformed rice by real-time quantitative PCR. Plant Cell Rep. 23: 759–763

Figure Legends

Fig. 1 Transformed cells within gemmalings. (a, c, e) Transmitted light images. (b, d, f) Fluorescence images. Red: chlorophyll fluorescence. Yellow-green: Citrine fluorescence. (a, b) Gemmalings 3 days after pouring selection buffer. Bar represents 500 μm. Arrow indicates a transformed cell (b). (c, d) Transformed cells 2 weeks after pouring selection buffer. Bar represents 500 μm. Arrow indicates transformed cells (d). (e, f) Transgenic thalli one month after pouring selection buffer. Bar represents 1 mm.

Fig. 2 Effect of culture periods on transformation efficiency. (a, b) Effect of pre-culture for 0–3 days on (a) male and (b) female gemmae/gemmalings. Bars represent standard deviations (see also Tables S1 and S2). (c, d) Effect of co-culture for 1–3 days on. (c) male and (d) of female gemmalings. Bars represent standard deviations (see also Tables S3 and S4).

Fig. 3 Effects of *Agrobacterium* concentration and medium conditions on transformation efficiencies of female gemmalings obtained from BC3-38. (a) Effect of *Agrobacterium* concentrations (OD_{600} = 0.1, 0.3 and 0.5 in transformation buffer). Bars represent standard deviations (see also Table S5). (b) Comparison between M51C and half-strength Gamborg's B5 (1/2 B5) solid media. Bars represent standard deviations (see also Table S6). (c) Effect of sucrose in M51C solid medium. Bars represent standard deviations (see also Table S8).

Fig. 4 Estimation of T-DNA copy numbers using real-time PCR. (a) Transgenic lines produced by G-AgarTrap using male gemmalings obtained from Tak-1. Bars represent standard deviations of triplicate experiments. (b) Transgenic lines produced by G-AgarTrap using female gemmalings obtained from BC3-38. Bars represent standard deviations of triplicate experiments.

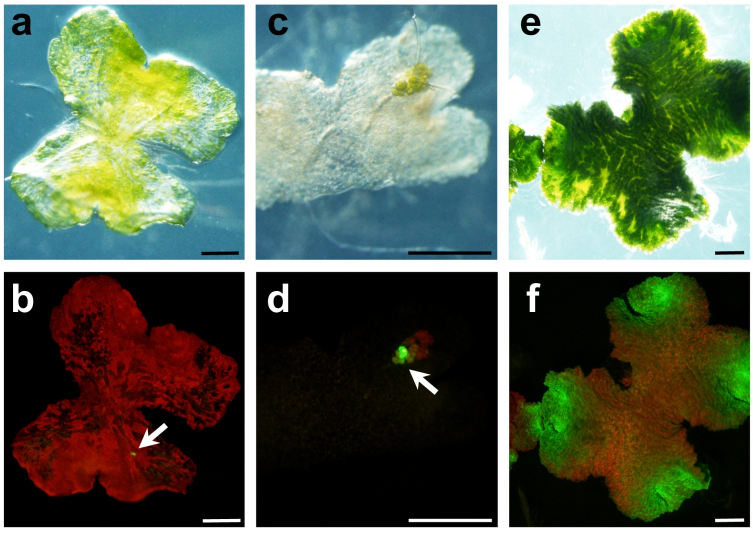
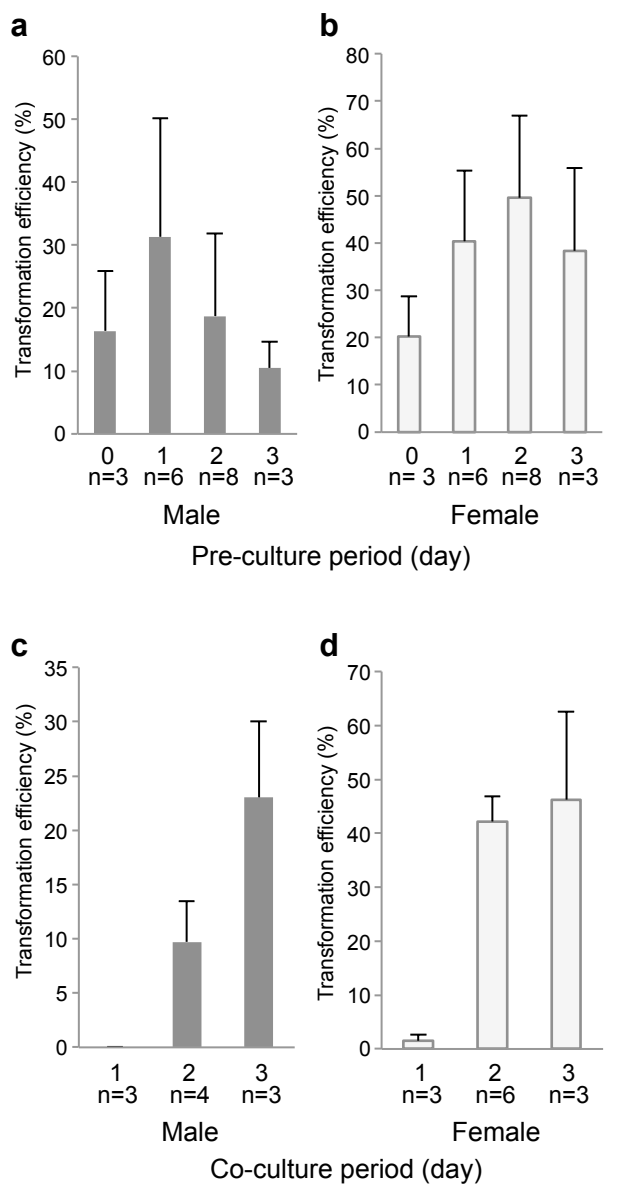
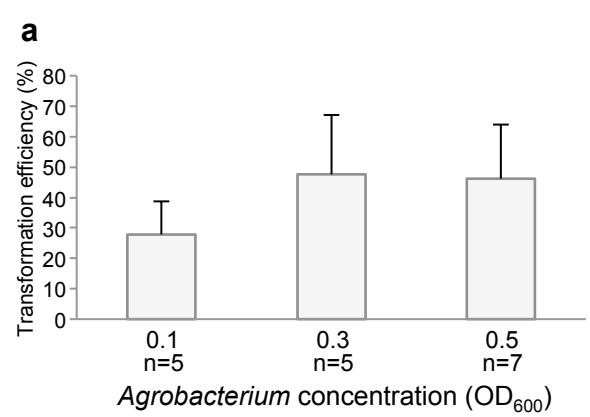


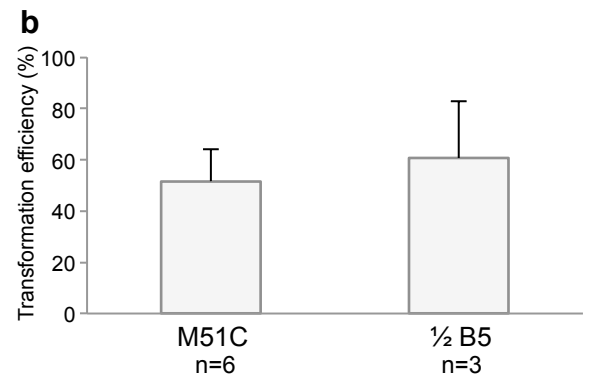
Fig. 1


a
b
Transformation efficiency (%)
0
10
20
30
40
50
60
70
80
0 n=3
1 n=6
2 n=8
3 n=3
Male
Female
Pre-culture period (day)

c
d
Transformation efficiency (%)
0
5
10
15
20
25
30
35
40
50
60
70
1 n=3
2 n=4
3 n=3
1 n=3
2 n=6
3 n=3
Male
Female
Co-culture period (day)

Fig. 2


a
Transformation efficiency (%)
80
70
60
50
40
30
20
10
0
0.1
n=5
0.3
n=5
0.5
n=7
Agrobacterium concentration (OD600)


b
Transformation efficiency (%)
100
80
60
40
20
0
M51C
n=6
½ B5
n=3

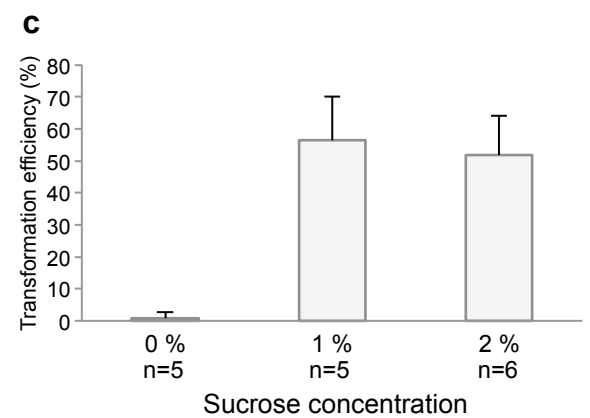

c
Transformation efficiency (%)
80
70
60
50
40
30
20
10
0
0 %
n=5
1 %
n=5
2 %
n=6
Sucrose concentration

Fig. 3

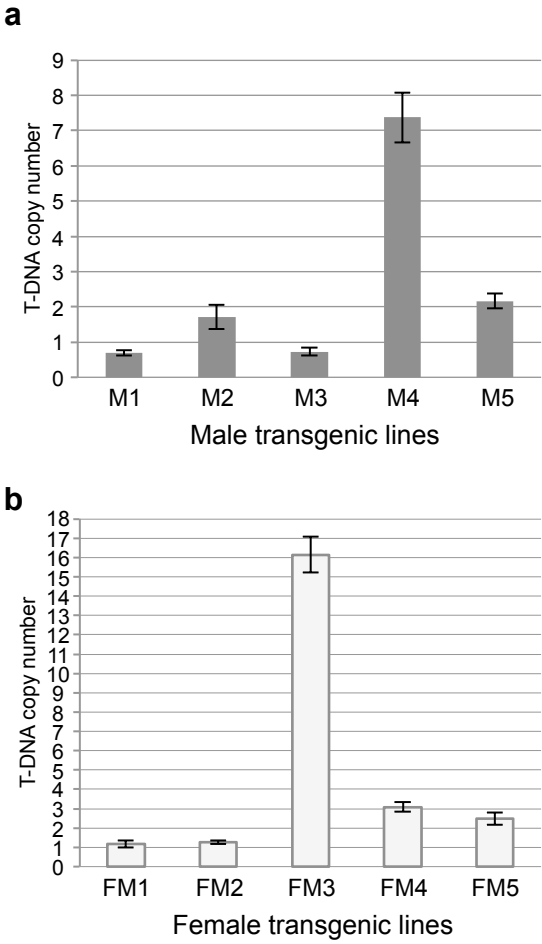

a
T-DNA copy number
0
1
2
3
4
5
6
7
8
9
M1
M2
M3
M4
M5
Male transgenic lines
b
T-DNA copy number
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
FM1
FM2
FM3
FM4
FM5
Female transgenic lines

Fig. 4

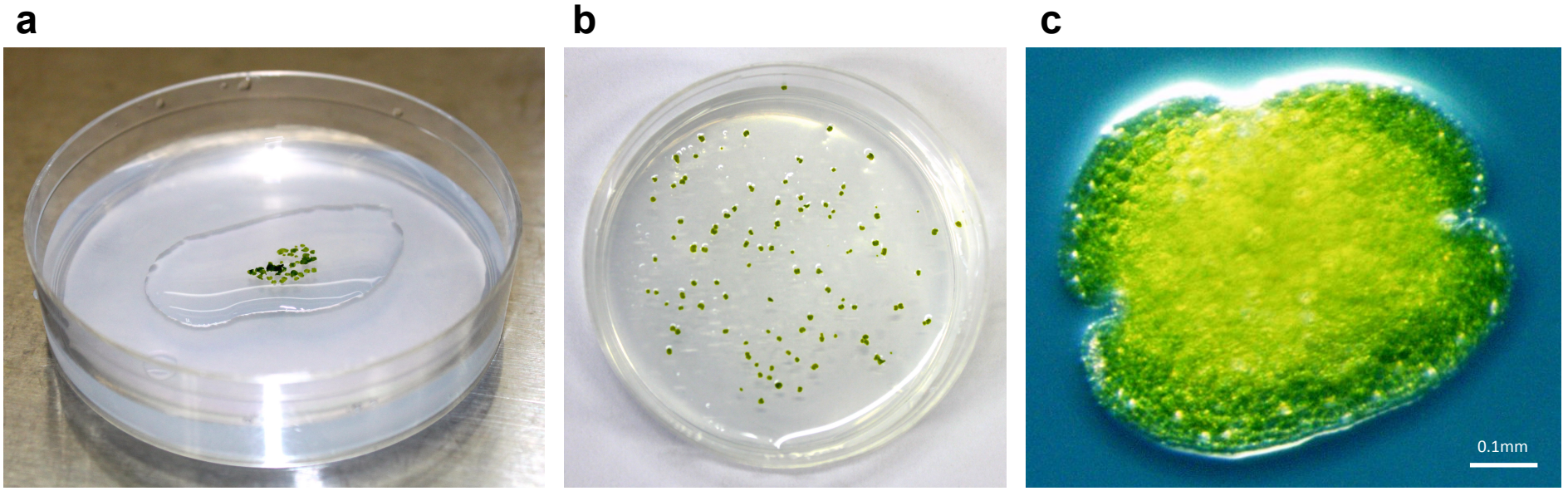


Fig. S1 Snapshots of how to sow gemmae. (a) After sterile water (approximately 500 µL) was dropped on the solid medium in 60 mm Petri dish, the gemmae were put on the drop of water. (b) The gemmae were spread individually with sterile water using tweezers. (c) After incubation for a day, the sterile water was absorbed into solid medium, and the gemmalings fixed on the medium by their rhizoids. The image was observed using a MZ16F stereo fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and taken with an Olympus DP73 digital camera (Olympus, Tokyo, Japan).

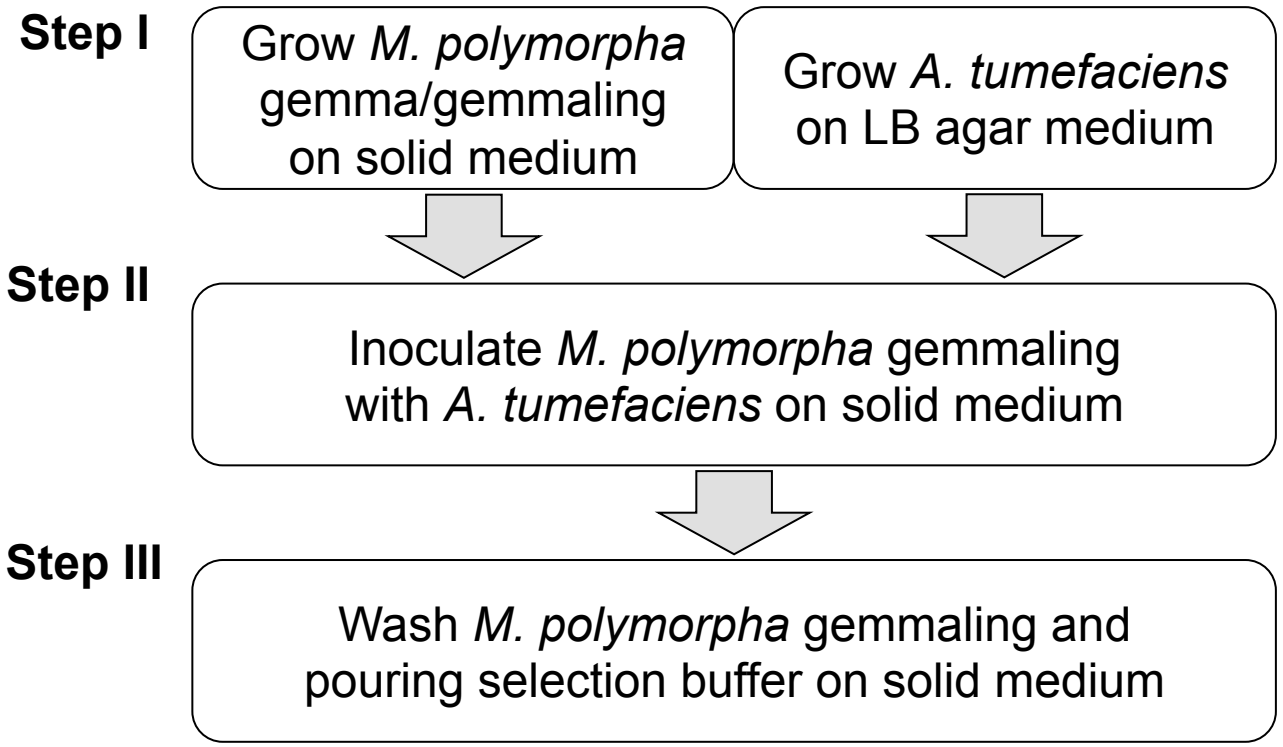


Fig. S2 Procedure of the G-AgarTrap. Step I: Growth of gemma/gemmaling from *M. polymorpha* on solid medium. *A. tumefaciens* was cultured on LB agar medium. Step II: Inoculation of the gemmaling with the *A. tumefaciens* on solid medium. Step III: Washing of the *M. polymorpha* and pouring selection buffer on solid medium. Non-transgenic cells decayed and transgenic cells grew for 2 – 4 weeks.

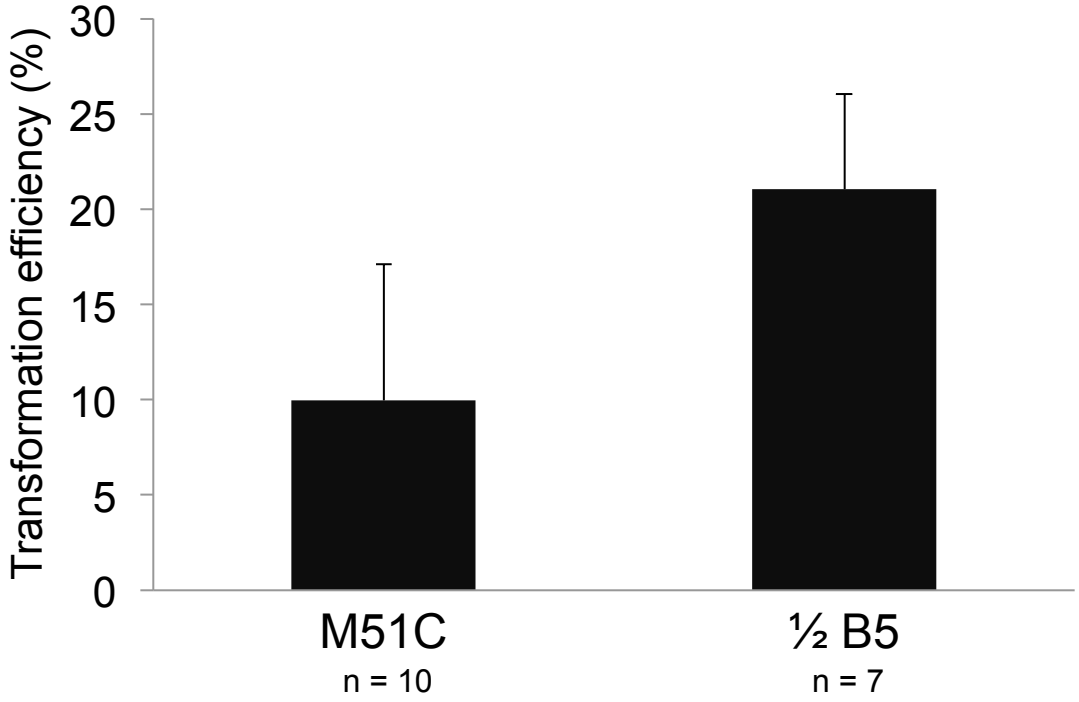


Fig. S3 Comparison of M51C and half strength Gamborg's B5 (1/2 B5) media in S-AgarTrap. Both media were supplemented with 2 % sucrose. Transformation efficiencies of M51C and 1/2 B5 media were approximately 10 % and 20 %, respectively.

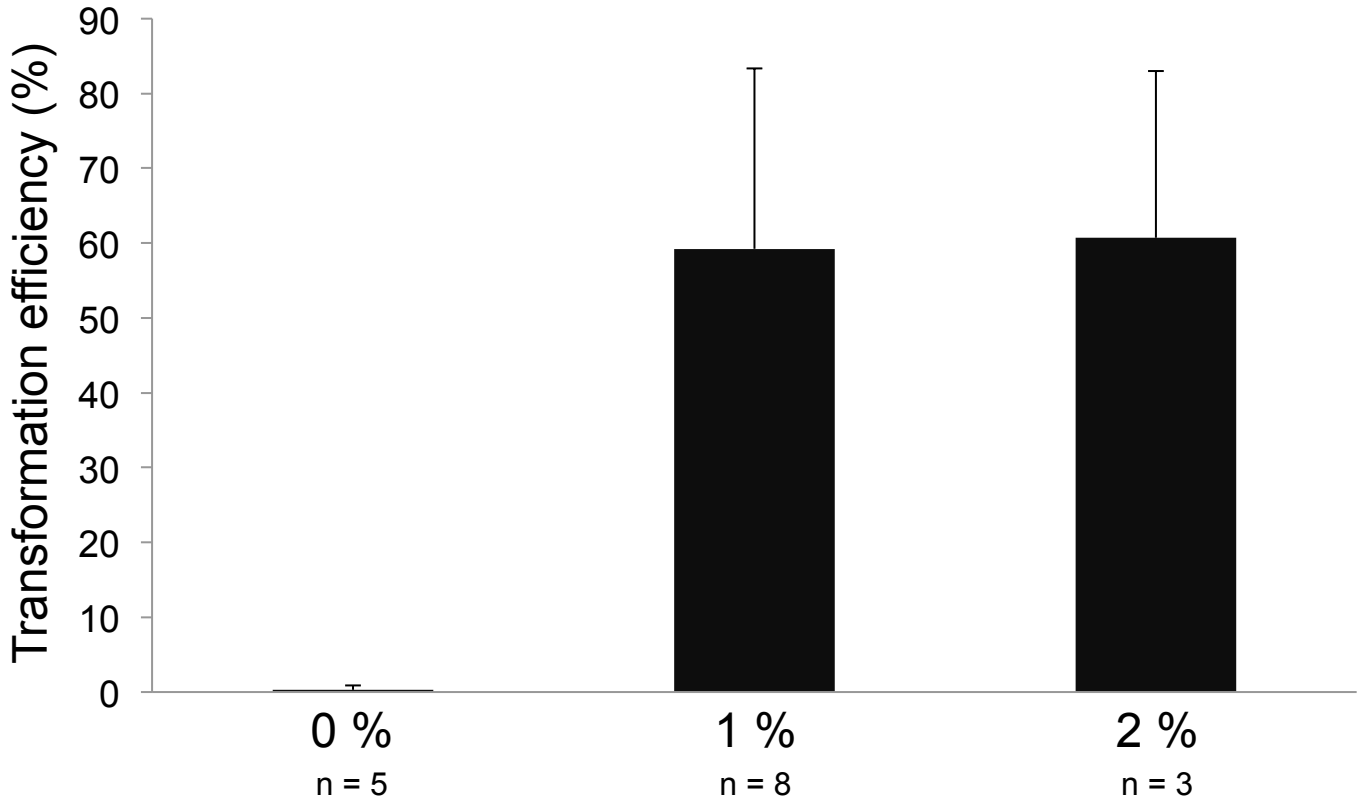


Fig. S4 Effect of sucrose in half strength Gamborg's B5 (1/2 B5) medium. Transformation efficiencies with 1/2 B5 media containing 0 %, 1 % and 2 % sucrose were approximately 0.3 %, 60 % and 60 %, respectively.

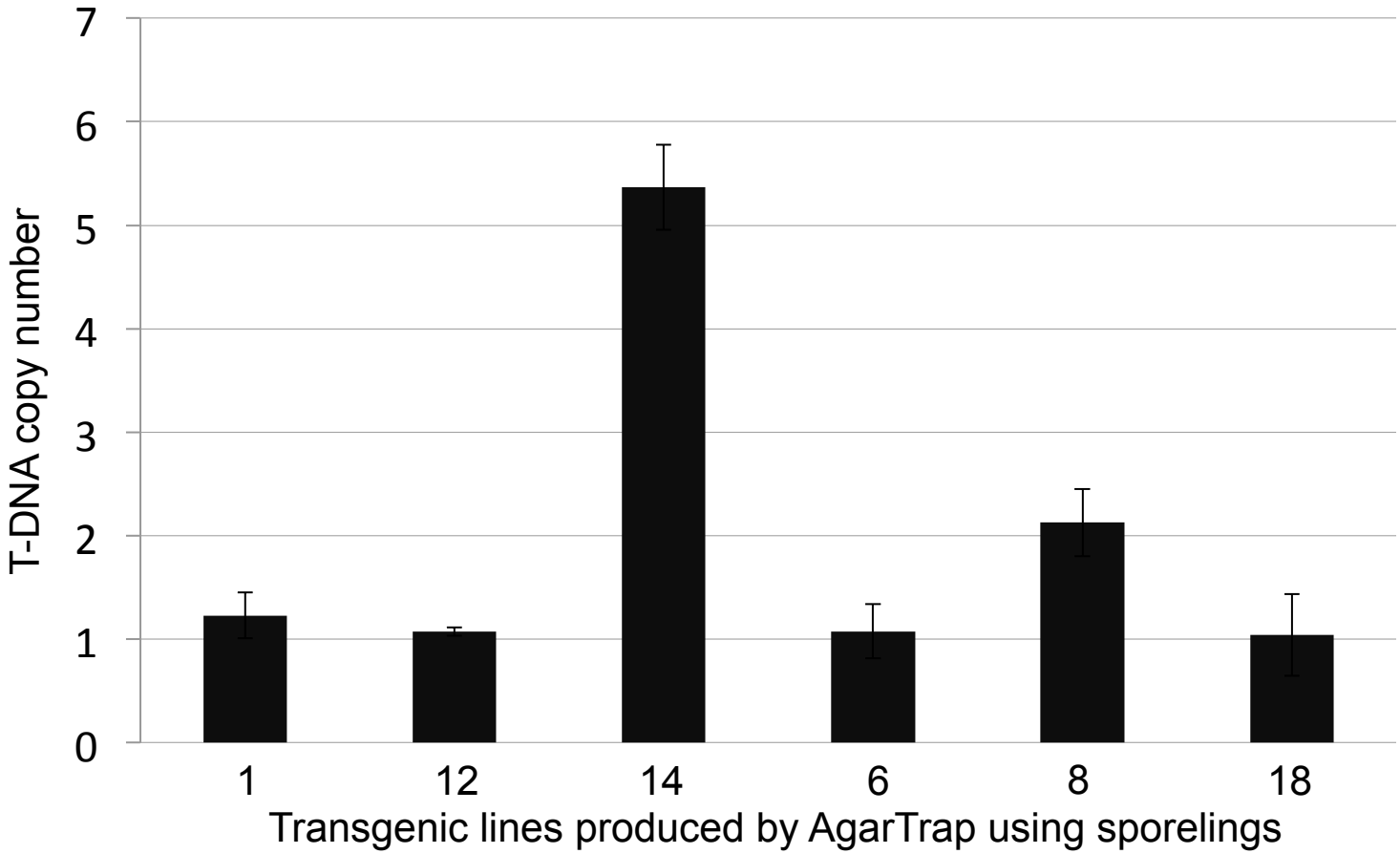


Fig. S5 Development of a method to estimate T-DNA copy number(s) using real-time PCR. The transgenic lines (No. 1, 6, 8, 12, 14 and 18) were produced by S-AgarTrap (see Tsuboyama and Kodama 2014). No. 1, 12 and 14 were male, and No. 6, 8 and 18 were female. Using real-time PCR, copy numbers of No. 1, 6, 8, 12, 14 and 18 were estimated as 1, 1, 2, 1, 5 and 1, respectively.

Table S1. Effect of pre-culture period in male gemma/gemmaling.

Pre-culture period (day)	Number of all gemmalings	Number of transformants[a]	Transformation efficiency (%)[b]
0	36	6	16.7
	31	8	25.8
	30	2	6.7
1	105	13	12.4
	79	12	15.2
	53	9	17.0
	36	14	38.9
	39	19	48.7
	43	24	55.8
2	45	4	8.9
	58	2	3.4
	50	12	24.0
	42	8	19.0
	53	0	0.0
	52	17	32.7
	44	14	31.8
	60	18	30.0
3	49	3	6.1
	38	4	10.5
	41	6	14.6

[a]Actual number of transformants with hygromycin resistance and Citrine fluorescence obtained by G-AgarTrap with 0, 1, 2 and 3 days pre-culture.

[b]Transformation efficiency was calculated as (number of transgenic gemmalings) / (number of all gemmalings sown) x 100, and rounded off to one decimal place.

Table S2. Effect of pre-culture period in female gemma/gemmaling.

Pre-culture period (day)	Number of all gemmalings	Number of transformants[a]	Transformation efficiency (%)[b]
0	37	7	18.9
	41	12	29.3
	24	3	12.5
1	86	28	32.6
	87	20	23.0
	58	15	25.9
	50	27	54.0
	38	20	52.6
	35	19	54.3
2	23	9	39.1
	65	9	13.8
	58	29	50.0
	43	21	48.8
	55	32	58.2
	39	26	66.7
	32	22	68.8
	49	25	51.0
3	46	9	19.6
	41	17	41.5
	37	20	54.1

[a]Actual number of transformants with hygromycin resistance and Citrine fluorescence obtained by G-AgarTrap with 0, 1, 2 and 3 days pre-culture.

[b]Transformation efficiency was calculated as (number of transgenic gemmalings) / (number of all gemmalings sown) x 100, and rounded off to one decimal place.

Table S3. Effect of co-culture period in male gemma/gemmaling.

Co-culture period (day)	Number of all gemmalings	Number of transformants[a]	Transformation efficiency (%)[b]
1	38	0	0.0
	58	0	0.0
	71	0	0.0
2	59	8	13.6
	59	7	11.9
	48	4	8.3
	39	2	5.1
3	55	17	30.9
	102	18	17.6
	39	8	20.5

[a]Actual number of transformants with hygromycin resistance and Citrine fluorescence obtained by G-AgarTrap with 1, 2 and 3 days co-culture.

[b]Transformation efficiency was calculated as (number of transgenic gemmalings) / (number of all gemmalings sown) x 100, and rounded off to one decimal place.

Table S4. Effect of co-culture period in female gemma/gemmaling.

Co-culture period (day)	Number of all gemmalings	Number of transformants[a]	Transformation efficiency (%)[b]
1	44	1	2.3
	42	0	0.0
	47	1	2.1
2	45	20	44.4
	39	16	41.0
	58	20	34.5
	39	17	43.6
	41	17	41.5
	29	14	48.3
3	59	21	35.6
	55	21	38.2
	60	39	65.0

[a]Actual number of transformants with hygromycin resistance and Citrine fluorescence obtained by G-AgarTrap with 1, 2 and 3 days co-culture.

[b]Transformation efficiency was calculated as (number of transgenic gemmalings) / (number of all gemmalings sown) x 100, and rounded off to one decimal place.

Table S5. Effect of *Agrobacterium* concentration (OD_{600}) in transformation buffer.

OD_{600}	Number of all gemmalings[a]	Number of transformants[b]	Transformation efficiency (%)[c]
0.1	53	21	39.6
	69	19	27.5
	47	5	10.6
	63	18	28.6
	60	20	33.3
0.3	64	22	34.4
	60	22	36.7
	46	37	80.4
	48	17	35.4
	65	33	50.8
0.5	41	14	34.1
	38	22	57.9
	58	24	41.4
	35	11	31.4
	34	21	61.8
	43	31	72.1
	40	10	25.0

[a]All experiments were investigated using female gemma/gemmaling.

[b]Actual number of transformants with hygromycin resistance and Citrine fluorescence obtained by G-AgarTrap with *Agrobacterium* concentration of OD_{600} = 0.1, 0.3 and 0.5 in transformation buffer.

[c]Transformation efficiency was calculated as (number of transgenic gemmalings) / (number of all gemmalings sown) x 100, and rounded off to one decimal place.

Table S6. Comparison of M51C and half strength Gamborg's B5 (1/2 B5) media.

Medium	Number of all gemmalings[a]	Number of transformants[b]	Transformation efficiency (%)[c]
M51C	52	26	50.0
	57	17	29.8
	35	20	57.1
	31	21	67.7
	39	20	51.3
	37	20	54.1
1/2 B5	34	29	85.3
	31	13	41.9
	42	23	54.8

[a]All experiments were investigated using female gemma/gemmaling.

[b]Actual number of transformants with hygromycin resistance and Citrine fluorescence obtained by G-AgarTrap with M51C or 1/2 B5 solid medium.

[c]Transformation efficiency was calculated as (number of transgenic gemmalings) / (number of all gemmalings sown) x 100, and rounded off to one decimal place.

Table S7. Comparison of M51C and half strength Gamborg's B5 (1/2 B5) media by S-AgarTrap.

Medium	Number of sporelings with no treatment	Number of transformants[a]	Transformation efficiency (%)[b]
M51C	311	86	27.7
	30	4	13.3
	371	24	6.5
	95	10	10.5
	54	2	3.7
	161	15	9.3
	249	9	3.6
	119	5	4.2
	125	10	8.0
	47	6	12.8
1/2 B5	332	89	26.8
	332	94	28.3
	227	32	14.1
	452	85	18.8
	402	73	18.2
	602	119	19.8
	567	121	21.3

[a]Actual number of transformants with hygromycin resistance and Citrine fluorescence obtained by S-AgarTrap with M51C or 1/2 B5 solid medium supplemented with 2 % sucrose.

[b]Transformation efficiency was calculated as (number of transgenic sporelings) / (number of sporelings with no treatment) x 100, and rounded off to one decimal place.

Table S8. Effect of sucrose concentration in M51C solid medium.

Sucrose (%)	Number of all gemmalings[a]	Number of transformants[b]	Transformation efficiency (%)[c]
0	58	0	0.0
	42	0	0.0
	37	0	0.0
	50	0	0.0
	47	2	4.3
1	50	29	58.0
	53	32	60.4
	74	24	32.4
	33	22	66.7
	53	34	64.2
2	52	26	50.0
	57	17	29.8
	35	20	57.1
	31	21	67.7
	39	20	51.3
	37	20	54.1

[a]All experiments were investigated using female gemma/gemmaling.

[b]Actual number of transformants with hygromycin resistance and Citrine fluorescence obtained by G-AgarTrap with 0, 1 and 2 % sucrose in M51C solid medium.

[c]Transformation efficiency was calculated as (number of transgenic gemmalings) / (number of all gemmalings sown) x 100, and rounded off to one decimal place.

Table S9. Effect of sucrose concentration in half strength Gamborg's B5 (1/2 B5) solid medium.

Sucrose (%)	Number of all gemmalings[a]	Number of transformants[b]	Transformation efficiency (%)[c]
0	65	0	0.0
	45	0	0.0
	39	0	0.0
	77	1	1.3
	44	0	0.0
1	42	25	59.5
	32	12	37.5
	41	30	73.2
	62	28	45.2
	33	18	54.5
	25	6	24.0
	35	33	94.3
	35	30	85.7
2	34	29	85.3
	31	13	41.9
	42	23	54.8

[a]All experiments were investigated using female gemma/gemmaling.

[b]Actual number of transformants with hygromycin resistance and Citrine fluorescence obtained by G-AgarTrap with 0, 1 and 2 % sucrose in 1/2 B5 solid medium.

[c]Transformation efficiency was calculated as (number of transgenic gemmalings) / (number of all gemmalings sown) x 100, and rounded off to one decimal place.