Plant Biotechnology 36, 201–204 (2019)
DOI: 10.5511/plantbiotechnology.19.0722a

Note

A gain-of-function T-DNA insertion mutant of *Marchantia polymorpha* hyper-accumulates flavonoid riccionidin A

Noriko Hamashima†, Xiaonan Xie†, Mio Hikawa, Tomohiro Suzuki, Yutaka Kodama*

Center for Bioscience Research and Education, Utsunomiya University, Tochigi 321-8505, Japan
*E-mail: kodama@cc.utsunomiya-u.ac.jp Tel: +81-28-649-8154 Fax: +81-28-649-8651

Received May 29, 2019; accepted July 22, 2019 (Edited by S. Ogita)

Abstract *Marchantia polymorpha* is a model liverwort for which many molecular biological techniques are now available. We previously developed the S-AgarTrap method for easy genetic transformation of *M. polymorpha* using spores. In this study, we report production of a T-DNA insertion mutant library (approx. 10,000 lines) for *M. polymorpha* using the S-AgarTrap method. We further isolate and characterize a gain-of-function mutant that hyper-accumulates the flavonoid riccionidin A. The present study demonstrates that the S-AgarTrap-mediated production of a T-DNA insertion mutant library is a powerful tool for molecular biology in *M. polymorpha*.

Key words: AgarTrap, MarChantia polymorpha, MYB14, riccionidinA, T-DNA insertion mutant.

The whole-genome sequence for the model liverwort *Marchantia polymorpha* was recently reported (Bowman et al. 2017). In addition, several molecular biological techniques for *M. polymorpha* are available, including genetic transformation, genome editing, conditional gene expression/deletion, fluorescence imaging, and T-DNA insertion library (Honkanen et al. 2016; Ishizaki et al. 2008; Kodama 2016; Nishihama et al. 2016; Sugano et al. 2014; Ueda et al. 2013). We previously developed a simplified *Agrobacterium*-mediated transformation method for *M. polymorpha*, termed AgarTrap (Agar-utilized Transformation with Poring Solutions) (Tsuboyama and Kodama 2018a). To date, we have described three types of AgarTrap methods, using spores (S-AgarTrap), gemmae (G-AgarTrap), and thallus tissues (T-AgarTrap) (Tsuboyama and Kodama 2014, 2018b; Tsuboyama et al. 2018; Tsuboyama-Tanaka and Kodama 2015; Tsuboyama-Tanaka et al. 2015). Transformation efficiencies of S-, G-, and T-AgarTrap methods are approximately 20%, 100%, and 60%, respectively. Among the three, S-AgarTrap is appropriate for the large-scale production of transformants (e.g., T-DNA insertion libraries), because the abundant spores produced via sexual reproduction can be used as the starting material. In this study, we used S-AgarTrap (Tsuboyama and Kodama 2014) to produce a T-DNA insertion mutant library of *M. polymorpha*. We further report the isolation of a gain-of-function mutant as a case study.

To perform large-scale S-AgarTrap in *M. polymorpha*, we obtained spores by crossing the male strain Tak-1 with the female strain Tak-2. Before crossing, the strains were asexually maintained on M51C medium with 1% agar under continuous white light of approximately 70 μmol photons m^{-2} s^{-1} (FL40SW, NEC Corporation). The formation of sexual organs was induced with continuous white fluorescent light and fluorescent light containing the far-red spectrum (FL20S-FR-74, Toshiba Lighting & Technology Corporation). F1 spores were used for S-AgarTrap following the previously reported protocols (Tsuboyama and Kodama 2014, 2018a). The binary vector pMpGWB103-Citrine (Figure 1A) was used to prepare a T-DNA insertion mutant library for *M. polymorpha* of approximately 10,000 lines (Figure 1B). We confirmed that all mutant lines expressed Citrine yellow fluorescent protein in the cytosol and had resistance to hygromycin (Figure 1A). Among the lines, we selected a red-colored line (#3483) for further analysis (Figure 1C).

To identify the region of the genome harboring the T-DNA insertion, we sequenced the genomic DNA of line #3483 using MiSeq (Illumina). The genomic DNA was isolated using DNeasy Plant Mini Kit (Qiagen) with the following modification. An extraction using TE-saturated 1-butanol was performed between

† These authors contributed equally to this work.
This article can be found at http://www.jspcmb.jp/
Published online September 21, 2019

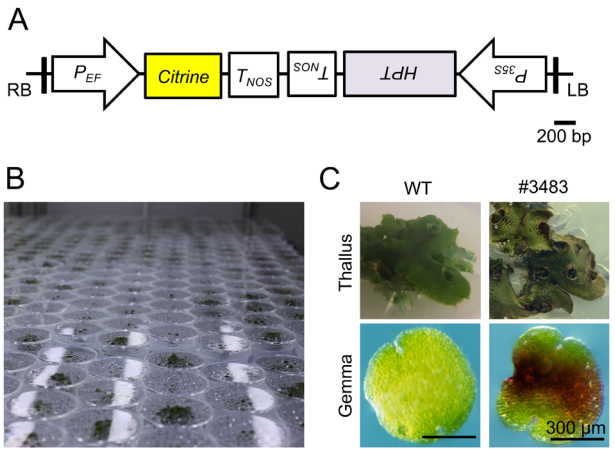

Figure 1. T-DNA insertion library and isolation of a red-colored line (line #3483). (A) Schematic illustration of the T-DNA region of the binary vector pMpGWB103-Citrine. RB, right border; P_{EF}, promoter of elongation factor; Citrine, gene for yellow fluorescent protein; T_{NOS}, terminator of nopaline synthase; HPT, gene for hygromycin phosphotransferase (hygromycin resistance gene); LB, light border. (B) Representative image of a T-DNA insertion mutant library for *M. polymorpha*. Each mutant was cultured in a 40-mm Petri dish. (C) Representative images of *M. polymorpha* thallus and gemma of wild type (WT) and the red-colored line (line #3483).

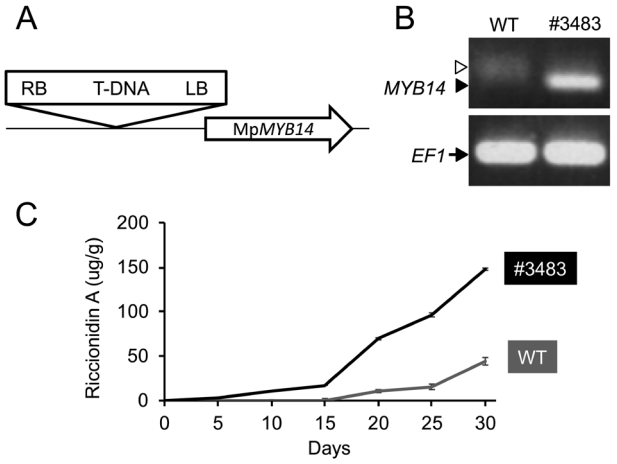

Figure 2. Gain of function of the endogenous Mp*MYB14* gene in the #3483 line. (A) Schematic illustration of the T-DNA inserted region of the #3483 genome. (B) Up-regulated gene expression of Mp*MYB14* in the #3483 line. RT-PCR experiments were performed for Mp*MYB14* and *ELONGATION FACTOR 1* (*EF1*) from *M. polymorpha* as a control. The PCR amplifications with 25 cycles are shown (Supplementary Figure S1). Closed arrowhead and arrow indicate *MYB14* and *EF1* fragments, respectively. Open arrowhead indicates non-specific faint signal. (C) Hyper-accumulation of riccionidin A in the #3483 line. Gray line, wild type (WT); black line, #3483. Bars indicate standard deviation (three technical replicates).

the QIAshredder and Buffer AW1 treatments in the Qiagen protocol: after the QIAshredder treatment, the supernatant was added to an equivalent amount of TE-saturated 1-butanol and mixed by pipetting; after centrifugation for 5 min at 15,000 rpm, the aqueous layer was mixed with Buffer AW1. The isolated genomic DNA was fragmented to approximately 550 bp using a Covaris M220 Focused-ultrasonicator (Covaris, MA). The fragmented DNA was used to prepare a library using the Illumina TruSeq® DNA PCR-Free Library Preparation Kit according to the manufacturer's instructions. The resulting library was sent to the sequencing facility at Utsunomiya University, Japan for MiSeq sequencing analysis (301 bp, paired end). The raw sequence reads were cleaned using Cutadapt ver. 1.8.1 (Martin 2011) to trim adapter sequences, low-quality ends (quality score,<30), and reads of less than 150 bp. The obtained reads were de novo assembled using SPAdes ver. 3.5.0 (Bankevich et al. 2012) with a k-mer size of 21, 33, 55, 77, 99, and 127 bp with the careful option, and contigs <200 bp were eliminated. To identify the T-DNA sequence, a local BLASTn search was performed using the assembled contigs as a database. The sequencing analysis demonstrated that the T-DNA in the #3483 line was integrated with a 6-bp deletion at 942 bp upstream from the transcription start site of the gene encoding the transcription factor MpMYB14 (Figure 2A).

Previous studies reported that transgenic *M. polymorpha* overexpressing Mp*MYB14* exhibit red-colored tissues in which flavonoids such as riccionidin A are hyper-accumulated (Albert et al. 2018; Kubo et al. 2018). Based on these studies, we hypothesized that the #3483 line might be a gain-of-function mutant overexpressing the endogenous Mp*MYB14* gene and hyperaccumulating riccionidin A.

First, using reverse-transcription PCR (RT-PCR), we tested whether Mp*MYB14* gene expression was up-regulated in the #3483 line. To synthesize a cDNA library for *M. polymorpha*, total RNA was isolated from 3-week-old gemmalings, and cDNAs were synthesized using the ReverTra Ace® qPCR RT Master Mix (TOYOBO). For RT-PCR experiments, a cDNA fragment of the Mp*MYB14* gene was amplified with EmeraldAmp® PCR Master Mix (TaKaRa) with primers: 5′-GTC CGA CAG ATT CCT GTG TAA A-3′ and 5′-GCC GCA AAC AAA CTT GTA AGA-3′. The *ELONGATION FACTOR 1*gene from *M. polymorpha* (Mp*EF1*) was used as an internal control, with primers: 5′-GCA TCT TGT CTT CTG AAA GGT TGT C-3′ and 5′-CAC GCT TGT CAA TAC CTC CCA GCT TGT AGA TAA GG-3′. The results indicated that Mp*MYB14* gene expression in 3-week-old gemmalings was up-regulated in the #3483 line, compared with the wild type (WT) (Figure 2B and Supplementary Figure S1).

Second, we checked whether riccionidin A was hyper-accumulated in the #3483 line. We quantified riccionidin A using a triple linear ion trap instrument (LIT) (QTRAP5500; AB Sciex) with an electrospray ionization (ESI) source and coupled to a UHPLC system (Nexera X2; Shimadzu) in MRM (Multiple Reaction Monitoring) analysis mood. MS/MS spectra

were recorded in product ion scan mode using LIT. The MRM analysis was performed at −35 V collision energy, and the transitions of *m*/*z* 283–255, 283–185 and 283–173 were monitored for riccionidin A in the ESI negative mode. Data acquisition and analysis were performed with the Multi Quant software (ver. 3.0.1). Quantification of riccionidin A was conducted using synthetic standards. Note that preparation of the synthetic standards was performed according to the previous study (Dyker and Bauer 1998). The exudate samples were dissolved in 50% aqueous methanol and filtered through a spin column (PVDF 0.45 μm, Millipore). An aliquot of the filtered 50% aqueous methanol sample solution was diluted with a volume of either pure 50% methanol or 50% methanol containing known amounts of riccionidin A standard. The increase in peak area on the chromatogram corresponded to the amounts of riccionidin A standards added, enabling the amount of riccionidin A in the samples to be deduced (Supplementary Figure S2). We quantified riccionidin A during a 1-month culture after detaching gemmae from gemma cups (Figure 2C). In 30-day-old gemmalings, the amount of riccionidin A in #3483 was 3-fold higher than that in the wild type (Figure 2C). Taken together, we conclude that the T-DNA insertion in line #3483 results in a gain-of-function phenotype that results from up-regulation of endogenous Mp*MYB14* gene, followed by hyperaccumulation of riccionidin A.

In the previous studies of Mp*MYB14*, overexpression of a Mp*MYB14* transgene induced hyper-accumulation of riccionidin A in transgenic *M. polymorpha* (Albert et al. 2018; Kubo et al. 2018). In our present study, we isolated a T-DNA insertion mutant overexpressing the endogenous Mp*MYB14* and hyper-accumulating riccionidin A. Although how the T-DNA insertion results in these phenotypes remains to be determined, it is likely that cauliflower mosaic virus 35S (CaMV35S) promoter elements within the T-DNA (Figure 2A) enhanced the expression of endogenous Mp*MYB14* gene. The CaMV35S promoter was inserted in the reverse direction upstream of the transcription start site of Mp*MYB14* (Figures 1A, 2A). Because CaMV35S is a unidirectional promoter (Xie et al. 2001), the reverse-oriented CaMV35S itself could not initiate the transcription of the Mp*MYB14* gene in the #3483 line. However, given that the CaMV35S promoter contains a bidirectional promoter-activation element (Kay et al. 1987), the reverse-oriented CaMV35S promoter might activate the endogenous Mp*MYB14* promoter present in the 942 bp region upstream of the Mp*MYB14* transcription start site. Because the binary vector series for *M. polymorpha* contains the CaMV35S promoter at the LB and/or RB sides within the T-DNA region (Ishizaki et al. 2015), the *M. polymorpha* T-DNA insertion library produced using these binary vectors should contain not only loss-of-function mutants, but also gain-of-function mutants such as the #3483 line.

The present study reported the S-AgarTrap-mediated production of a *M. polymorpha* T-DNA insertion library. In addition, we described the successful isolation from the library of a gain-of-function mutant overexpressing the endogenous Mp*MYB14* gene. Because S-AgarTrap is a simple transformation method suitable for large-scale production of *M. polymorpha* transformants, individual laboratories can make their own T-DNA insertion libraries and screen *M. polymorpha* mutants. Furthermore, with the recently developed techniques for cryopreservation of gemmae and spermatozoa (Tanaka et al. 2016; Togawa et al. 2018; Wu et al. 2015), these T-DNA insertion libraries could be maintained in the laboratory.

Acknowledgements

The authors thank Dr. Takayuki Kohchi (Kyoto University) and Kimitsune Ishizaki (Kobe University) for providing *M. polymorpha* and the binary vector pMpGWB103, and Dr. Rika Kitamura (Utsunomiya University) for technical support regarding MiSeq data analysis. This work was supported by the Japan Society for the Promotion of Science KAKENHI [grant No. 26840088 to Y.K.] and the Plant Transgenic Design Initiative of the University of Tsukuba to Y.K.

References

Albert NW, Thrimawithana AH, McGhie TK, Clayton WA, Deroles SC, Schwinn KE, Bowman JL, Jordan BR, Davies KM (2018) Genetic analysis of the liverwort *Marchantia polymorpha* reveals that R2R3MYB activation of flavonoid production in response to abiotic stress is an ancient character in land plants. *New Phytol* 218: 554–566

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, et al. (2012) SPAdes: A new genome assembly algorithm and Its applications to single-cell sequencing. *J Comput Biol* 19: 455–477

Bowman JL, Kohchi T, Yamato KT, Jenkins J, Shu S, Ishizaki K, Yamaoka S, Nishihama R, Nakamura Y, Berger F, et al. (2017) Insights into land plant evolution garnered from the *Marchantia polymorpha* genome. *Cell* 171: 287–304.e15

Dyker G, Bauer M (1998) Synthesis of 2,3,6,8-tetrahydroxybenzofuro [3,2-~][l]benzopyrylium chloride (*riccionidin A*). *J Prakt Chem* 340: 271–273

Honkanen S, Jones VAS, Morieri G, Champion C, Hetherington AJ, Kelly S, Proust H, Saint-Marcoux D, Prescott H, Dolan L (2016) The mechanism forming the cell surface of tip-growing rooting cells is conserved among land plants. *Curr Biol* 26: 3238–3244

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

Ishizaki K, Nishihama R, Ueda M, Inoue K, Ishida S, Nishimura Y, Shikanai T, Kohchi T (2015) Development of gateway binary vector series with four different selection markers for the liverwort *Marchantia polymorpha*. *PLoS One* 10: e0138876

Kay R, Chan A, Daly M, Mcpherson J (1987) Duplication of CaMV

35S promoter sequences creates a strong enhancer for plant genes. *Science* 236: 1299–1302

Kodama Y (2016) Time gating of chloroplast autofluorescence allows clearer fluorescence imaging *in planta*. *PLoS One* 11: e0152484

Kubo H, Nozawa S, Hiwatashi T, Kondou Y, Nakabayashi R, Mori T, Saito K, Takanashi K, Kohchi T, Ishizaki K (2018) Biosynthesis of riccionidins and marchantins is regulated by R2R3-MYB transcription factors in *Marchantia polymorpha*. *J Plant Res* 131: 849–864

Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J* 17: 10–12

Nishihama R, Ishida S, Urawa H, Kamei Y, Kohchi T (2016) Conditional gene expression/deletion systems for *Marchantia polymorpha* using its own heat-shock promoter and Cre/loxP-mediated site-specific recombination. *Plant Cell Physiol* 57: 271–280

Sugano SS, Shirakawa M, Takagi J, Matsuda Y, Shimada T, Hara-Nishimura I, Kohchi T (2014) CRISPR/Cas9-mediated targeted mutagenesis in the liverwort *Marchantia polymorpha* L. *Plant Cell Physiol* 55: 475–481

Tanaka D, Ishizaki K, Kohchi T, Yamato KT (2016) Cryopreservation of Gemmae from the Liverwort *Marchantia polymorpha* L. *Plant Cell Physiol* 57: 300–306

Togawa T, Adachi T, Harada D, Mitani T, Tanaka D, Ishizaki K, Kohchi T, Yamato KT (2018) Cryopreservation of *Marchantia polymorpha* spermatozoa. *J Plant Res* 131: 1047–1054

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 S, Kodama Y (2018a) AgarTrap protocols on your benchtop: Simple methods for *Agrobacterium*-mediated genetic transformation of the liverwort *Marchantia polymorpha*. *Plant Biotechnol* 35: 93–99

Tsuboyama S, Kodama Y (2018b) Highly efficient G-AgarTrap-mediated transformation of the *Marchantia polymorpha* model strains Tak-1 and Tak-2. *Plant Biotechnol* 35: 399–403

Tsuboyama S, Nonaka S, Ezura H, Kodama Y (2018) Improved G-AgarTrap : A highly efficient transformation method for intact gemmalings of the liverwort *Marchantia polymorpha*. *Sci Rep* 8: 10800

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

Tsuboyama-Tanaka S, Nonaka S, Kodama Y (2015) A highly efficient AgarTrap method for genetic transformation of mature thalli of the liverwort *Marchantia polymorpha* L. *Plant Biotechnol* 32: 333–336

Ueda M, Takami T, Peng L, Ishizaki K, Kohchi T, Shikanai T, Nishimura Y (2013) Subfunctionalization of sigma factors during the evolution of land plants based on mutant analysis of liverwort (*Marchantia polymorpha* L.) MpSIG1. *Genome Biol Evol* 5: 1836–1848

Wu SP, Qin ZZ, Xiao TZ, Li QP, Lu BB, Jing LJ, Wang J, Zhu RL (2015) Cryopreservation of gemmae of *Marchantia polymorpha* L. (Marchantiophyta, Marchantiaceae) without prior pretreatment. *Cryo Letters* 36: 91–96

Xie M, He Y, Gan S (2001) Bidirectionalization of polar promoters in plants. *Nat Biotechnol* 19: 677–679