

Selection of a histidine auxotrophic *Marchantia polymorpha* strain with an auxotrophic selective marker

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Abstract *Marchantia polymorpha* has emerged as a model liverwort species, with molecular tools increasingly available. In the present study, we developed an auxotrophic strain of *M. polymorpha* and an auxotrophic selective marker gene as new experimental tools for this valuable model system. Using CRISPR (clustered regularly interspaced palindromic repeats)/Cas9-mediated genome editing, we mutated the genomic region for *IMIDAZOLEGLYCEROL-PHOSPHATE DEHYDRATASE* (*IGPD*) in *M. polymorpha* to disrupt the biosynthesis of histidine (*igpd*). We modified an *IGPD* gene (*IGPDm*) with silent mutations, generating a histidine auxotrophic selective marker gene that was not a target of our CRISPR/Cas9-mediated genome editing. The *M. polymorpha igpd* mutant was a histidine auxotrophic strain, growing only on medium containing histidine. The *igpd* mutant could be complemented by transformation with the *IGPDm* gene, indicating that this gene could be used as an auxotrophic selective marker. Using the *IGPDm* marker in the *igpd* mutant background, we produced transgenic lines without the need for antibiotic selection. The histidine auxotrophic strain *igpd* and auxotrophic selective marker *IGPDm* represent new molecular tools for *M. polymorpha* research.

Key words: bryophyte, CRISPR/Cas9, genome editing, *HIS3*, histidine auxotroph.

Introduction

The liverwort *Marchantia polymorpha*, a bryophyte, is rapidly being established as a model plant species. During the past several years, various molecular biological tools have been developed to analyze *M. polymorpha*. For example, its genome sequence has been released (Bowman et al. 2017), and genetic transformation (Chiyoda et al. 2008; Ishizaki et al. 2008, 2015; Kubota et al. 2013; Tsuboyama and Kodama 2014, 2018a, b; Tsuboyama et al. 2018; Tsuboyama-Tanaka and Kodama 2015; Tsuboyama-Tanaka et al. 2015), gene-targeting (Ishizaki et al. 2013), genome editing using CRISPR (clustered regularly interspaced palindromic repeats)/Cas9 or transcription activator-like effector nuclease (TALEN) (Konno et al. 2018; Kopischke et al. 2017; Sugano et al. 2014, 2018), and germplasm preservation (Takahashi and Kodama 2020, 2021; Tanaka et al. 2016; Wu et al. 2015) methods have been reported. The availability of more tools will be needed to facilitate molecular studies of *M. polymorpha*.

Auxotrophic mutant strains have been invaluable for molecular biology analyses in microorganisms such as

yeast. Auxotrophic mutants are generated by deleting genes responsible for the biosynthesis of key metabolites such as nucleotides and amino acids (Pronk 2002). Because auxotrophic mutants cannot grow in conditions lacking the key metabolite, they have been used for the biocontainment of various genetically modified organisms (Arnolds et al. 2021; Clark and Maselko 2020; Lee et al. 2018). Note that biocontainment is a technique for biorisk management to prevent the release of genetically modified organisms such as transformants into outdoor environments (outside the laboratory). Auxotrophic mutants have also been employed to select transformants via complementation of the responsible gene as an auxotrophic selective marker (Pronk 2002). Selecting transformants in an auxotrophic mutant background using an auxotrophic selective marker allows researchers to accurately select transgenic lines without obtaining non-transgenic escape lines; such lines are often obtained under selection with an antibiotic and its resistance gene.

Auxotrophic mutants have been identified in plants including *Arabidopsis* (*Arabidopsis thaliana*), ferns (*Todea barbara* and *Osmunda cinnamomea*), and moss

Abbreviations: CRISPR, clustered regularly interspaced palindromic repeats; IGPD, IMIDAZOLEGLYCEROL-PHOSPHATE DEHYDRATASE; IGPDm, modified IGPD gene; sfGFP, superfolder green fluorescent protein; sgRNA, single-guide RNA; TALEN, transcription activator-like effector nuclease.

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(*Physcomitrium patens*) via classical mutant screening, although the responsible genes have been identified only in *Arabidopsis* (Ashton and Cove 1977; Carlson 1969; Last and Fink 1988; Last et al. 1991; Muralla et al. 2007). These auxotrophic mutant plants and responsible genes have not been used as molecular biology tools. In the floating aquatic plant *Lemna minor*, isoleucine auxotrophic strains were produced via gene silencing of *THREONINE DEAMINASE* by RNA interference for the biocontainment purpose (Nguyen et al. 2012). In *P. patens*, histidine and tryptophan auxotrophic mutants have also been generated using a gene-targeting technique (Ulfstedt et al. 2017), and a histidine auxotrophic selective marker gene was developed. The *Pp3c17_23550* and *Pp3c13_21930* genes were chosen based on their homology to yeast (*Saccharomyces cerevisiae*) genes encoding PHOSPHORIBOSYLANTHRANILATE ISOMERASE and IMIDAZOLEGLYCEROL-PHOSPHATE DEHYDRATASE (IGPD), which are required for the biosynthesis of tryptophan and histidine, respectively, and were successfully knocked out (Ulfstedt et al. 2017). *Pp3c13_21930* functions as an auxotrophic selective marker when used with the histidine auxotrophic strain (Ulfstedt et al. 2017). Therefore, auxotrophic strains and auxotrophic selective markers would represent powerful new molecular biological tools for use in various plants.

In the present study, we generated a histidine auxotrophic *M. polymorpha* mutant strain using CRISPR/Cas9-mediated genome editing, which can be used for biocontainment. We also developed an auxotrophic selective marker gene that can be used in conjunction with the histidine auxotrophic *M. polymorpha* strain to select transformants without the need for antibiotic selection.

Materials and methods

Plant material and growth conditions

Marchantia polymorpha was asexually maintained on 1/2 B5 medium with 1% (w/v) agar (BOP, SSK Sales Co., Ltd.) under approximately $70 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ continuous white fluorescent light (FL40SW, NEC Corporation) in a culture room at 22°C (Ogasawara et al. 2013). The male and female accessions Tak-1 and BC3-38, respectively, were used as the wild type (WT). The histidine auxotrophic mutant line *Mpigpd* was maintained on 1/2 B5 medium with 1% (w/v) agar supplemented with $100 \mu\text{M}$ histidine (L-histidine hydrochloride hydrate). To cultivate the transformant in soil, vermiculite (Nittai Inc.), gardening soil (Nihon Hiryo Co., Ltd.), and leaf mold (Tamiya Engei) were used; the material was cultivated in oligotrophic soil (vermiculite:gardening soil=4:1) and eutrophic (leaf mold) soil under a 16-h-light/8-h-dark cycle at 22°C. The light intensity was approximately $60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ supplied by white fluorescent lights (FL40SW, NEC Corporation).

Plasmid construction

For genome editing of *MpIGPD* in *M. polymorpha*, the Gateway cloning system (Invitrogen) was employed for CRISPR/Cas9 optimized for *M. polymorpha* using the entry vector pMpGE_En03 (Addgene: #71535) and the destination vector pMpGE010 (Addgene: #71536) (Sugano et al. 2018). Five single-guide RNAs (sgRNA81–85) were designed using the sgRNA design tool CRISPR-P 2.0 (<http://crispr.hzau.edu.cn/CRISPR2/>) (Supplementary Figure S1). A pair of DNA oligos was annealed to produce dsDNAs for sgRNA81–85 (Supplementary Table S1). pMpGE_En03 was digested with *BsaI* (*BsaI*-HF; New England BioLabs), and the annealed dsDNA was ligated to the digested pMpGE_En03, producing pMpGE_En03-gR81, pMpGE_En03-gR82, pMpGE_En03-gR83, pMpGE_En03-gR84, and pMpGE_En03-gR85 (Supplementary Table S1). To construct pMpGE010-gR82 and pMpGE010-gR85, the pMpGE_En03-gR82 and pMpGE_En03-gR85 plasmids were reacted with pMpGE010 via LR reaction with the Gateway cloning system (Invitrogen).

To construct plasmids for SKLPT imaging, a pair of DNA oligos was annealed to produce dsDNAs for the targets of sgRNA81–85 (Supplementary Table S2), and pDONR/Zeo-sfGFP-SKL (Addgene: #186720) (Konno et al. 2018) was digested with *BsaI* (*BsaI*-HF; New England BioLabs). The dsDNA was ligated to the digested pDONR/Zeo-sfGFP-SKL, resulting in pDONR/Zeo-sfGFP-sgRNA81-SKL, pDONR/Zeo-sfGFP-sgRNA82-SKL, pDONR/Zeo-sfGFP-sgRNA83-SKL, pDONR/Zeo-sfGFP-sgRNA84-SKL, and pDONR/Zeo-sfGFP-sgRNA85-SKL (Supplementary Table S2). These resulting plasmids were mixed with pGWT35S (Addgene: #182790) (Fujii et al. 2018) as destination vectors, and an LR reaction was performed with the Gateway cloning system (Invitrogen) to produce pGWT35S-sfGFP-gR81-SKL, pGWT35S-sfGFP-gR82-SKL, pGWT35S-sfGFP-gR83-SKL, pGWT35S-sfGFP-gR84-SKL, and pGWT35S-sfGFP-gR85-SKL.

To clone *MpIGPD*, DNA fragments encoding *MpIGPD* were PCR amplified from Tak-1 genomic DNA as a template with the primers 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCGTGGGTGGCAGCCACAACCTGCTAACT-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATGC GCGAGATAAGACCCCTTAGAGCT-3'. The DNA fragment was reacted with pDONR207 by BP reaction with the Gateway cloning system (Invitrogen), producing pDONR207-*MpIGPD*.

To produce a modified *MpIGPD* (*MpIGPDm*) gene that is not targeted by sgRNA82 or sgRNA85, DNA fragments with silent mutations within the target sequences of sgRNA82 and sgRNA85 were produced by PCR. In the first step, two DNA fragments (F1 and F2) with the mutation for sgRNA82 were PCR amplified using pDONR207-*MpIGPD* as a template. The primers 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCGTGGGTGGCAGCCACAACCTGCTAACT-3' and 5'-CTCTGAGAAGACGATGGTACAGCAACTGATTTTAA-3' were used for the F1 fragment, and 5'-TTAAATCAGTT GCTGTACCATCGTCTTCTCAGAG-3' and 5'-GGGGAC CACTTTGTACAAGAAAGCTGGGTCTCATGCGCGAGA

TAAGACCCCTTAGAGCT-3' were used for the F2 fragment. The amplified F1 and F2 fragments were fused by joint PCR, generating a template (F3 fragment) for the second step.

In the second step, two DNA fragments (F4 and F5) with the mutation for sgRNA85 were PCR amplified using the F3 fragment from the first step as a template. The primers 5'-GGG GACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGTG GGTGGCAGCCACAACCTGCTAACT-3' and 5'-CCATTTCAC CACTGAGTTATAGCTGCTGGACTCGC-3' were used for the F4 fragment, and 5'-GCGAGTCCAGCAGCTATAACTCAG TGTGGAAATGG-3' and 5'-GGGGACCACTTTGTACAA GAAAGCTGGGTCTCATGCGGAGATAAGACCCCTT AGAGCT-3' were used for the F5 fragment. The amplified F4 and F5 fragments were fused by joint PCR, generating a DNA fragment (F6) with silent mutations within the target sequences of sgRNA82 and sgRNA85. The resulting DNA fragment (F6) was cloned into pDONR/Zeo via BP reaction with the Gateway cloning system (Invitrogen), generating pDONR/Zeo-MpIGPDm. After checking the sequence, the DNA fragment for MpIGPDm was transferred to the binary vector pMpGWB303 (Ishizaki et al. 2015) by LR reaction with the Gateway cloning system (Invitrogen) to produce pMpGWB303-MpIGPDm.

To produce a binary vector containing *Citrine* and MpIGPDm without the antibiotic resistance gene, the hygromycin resistance gene in pMpGWB103-Citrine (Tsuboyama and Kodama 2014) was replaced with MpIGPDm. Using pMpGWB103-Citrine as a PCR template, the DNA fragment without a hygromycin resistance gene was amplified by PCR with the primers 5'-TGCCACCCACGCCATATCTCA TTGCCCCC-3' and 5'-TTATCTCGCGCATGAAGTAGA TGCCGACCG-3'. The DNA fragment encoding MpIGPDm was also amplified by PCR using pDONR/Zeo-MpIGPDm as a template with the primers 5'-ATGGCGTGGGTGGCA GCCACAACG-3' and 5'-TCATGCGGAGATAAGAC CCCCTTA-3'. These two fragments were fused by In-Fusion cloning (Takara), generating pMpGWBhis03-Citrine (Addgene: #188557).

To produce the pMpGWBhis03 backbone (Addgene: #188556), pMpGWBhis03-Citrine was reacted with pDONR207 by BP reaction with the Gateway cloning system (Invitrogen), resulting in the replacement of the *Citrine* fragment with the DNA fragment for Gateway elements.

To confirm that the pMpGWBhis03 backbone is functional, we also produced another vector, pMpGWBhis03-Citrine-NLS (Addgene: #188558), using this backbone. To produce pMpGWBhis03-Citrine-NLS, pDONR207-Citrine-NLS (Addgene: #44586) (Ogasawara et al. 2013) was reacted with the pMpGWBhis03 backbone by LR reaction with the Gateway cloning system (Invitrogen).

SKLPT imaging

SKLPT imaging was performed as described previously (Konno et al. 2018). The sgRNA expression vectors pMpGE_En03-gR81, pMpGE_En03-gR82, pMpGE_En03-gR83, pMpGE_

En03-gR84, and pMpGE_En03-gR85 were used for analysis, and pGWT35S-sfGFP-gR81-SKL, pGWT35S-sfGFP-gR82-SKL, pGWT35S-sfGFP-gR83-SKL, pGWT35S-sfGFP-gR84-SKL, and pGWT35S-sfGFP-gR85-SKL were used as SKLPT plasmids. The sgRNA expression vector pMpGE_En03-gR8x (1 µg) and the SKLPT plasmid pGWT35S-sfGFP-gR8x-SKL (1 µg) were mixed with the Cas9 expression vector pGWT35S-Cas9 (Addgene: #186723; 1 µg). pGWT35S-mCherry (Addgene: #182582; 1 µg) was also used as a control to express mCherry in the cytosol. Using the mixture, gold particles (0.6 mg, 1 µm size) were coated with the plasmid DNAs. One-day-old gemmalings were subjected to particle bombardment. The bombarded gemmalings were incubated in the dark for 48 h prior to confocal microscopy.

Microscopy

Thalli were observed under an MZ16F stereomicroscope (Leica Microsystems) and photographed with an Olympus DP73 digital camera. Citrine fluorescence in the transgenic thalli was detected with a fluorescence module (excitation filter 480/40 nm and barrier filter LP 510 nm).

For SKLPT imaging, the fluorescence of sfGFP and mCherry was observed under a confocal laser scanning microscope (SP8X system, Leica Microsystems) with a white light laser and the time-gating method (Kodama 2016). sfGFP was detected at 494–530-nm emission with a 488-nm excitation laser, and mCherry was detected at 598–624-nm emission with a 587-nm excitation laser.

Transformation of *M. polymorpha*

Genetic transformation of *M. polymorpha* was performed by the G- and T-AgarTrap methods, which are *Agrobacterium*-mediated transformation methods for gemmalings and thalli, respectively (Tsuboyama and Kodama 2018a, b; Tsuboyama et al. 2018; Tsuboyama-Tanaka and Kodama 2015; Tsuboyama-Tanaka et al. 2015).

To produce the histidine auxotrophic mutant line (Mpigpd), WT gemmalings (strain BC3-38) were precultured on 1/2 B5 medium with 1% (w/v) agar supplemented with 100 µM histidine and 1% (w/v) sucrose and subjected to the G-AgarTrap method. In the co-culture steps with *Agrobacterium*, two *Agrobacterium* strains harboring binary vectors pMpGE010-gR82 and pMpGE010-gR85 were mixed before use. After co-culture, transformants that were candidate Mpigpd lines were selected by adding the antibiotic hygromycin to 1/2 B5 agar containing 100 µM histidine with 1% (w/v) sucrose, 10 µg ml⁻¹ hygromycin, and 100 µg ml⁻¹ cefotaxime.

For the complementation test of Mpigpd, Mpigpd gemmalings were precultured on 1/2 B5 agar containing 1% (w/v) sucrose with 100 µM histidine and subjected to the G-AgarTrap method with *Agrobacterium* harboring the binary vector pMpGWB303-MpIGPDm. Transformants that were candidate MpIGPDm/Mpigpd lines were selected by adding the antibiotic chlorsulfuron to 1/2 B5 agar containing 100 µM histidine with 1% (w/v) sucrose, 0.5 µM chlorsulfuron, and

100 $\mu\text{g ml}^{-1}$ cefotaxime.

To generate transformants via selection using the marker *MpIGPDm*, *Mpigpd* thalli were precultured on 1/2 B5 agar containing 1% (w/v) sucrose without histidine and subjected to the T-AgarTrap method with *Agrobacterium* harboring the binary vectors pMpGWB303-*MpIGPDm*, pMpGWBhis03-Citrine, and pMpGWBhis03-Citrine-NLS. Transformants were selected on 1/2 B5 agar containing 100 $\mu\text{g ml}^{-1}$ cefotaxime without antibiotics.

Evaluation of genome editing in *M. polymorpha*

Genomic DNA was isolated from thallus pieces (approx. 5 mm $\times$ 5 mm) from the *Mpigpd* and BC3-38 lines using a DNeasy Plant Mini Kit (QIAGEN). Total RNA was isolated from the thallus pieces using an RNeasy Plant Mini Kit (QIAGEN), and cDNA was synthesized from the isolated total RNA using ReverTra Ace[®] qPCR RT Master Mix (TOYOBO). Target DNA sites for pMpGE010-sgRNA82 and pMpGE010-sgRNA85 were PCR amplified with the following primers: for pMpGE010-sgRNA82, 5'-TGGATCGGAGCAGCAGTCGGGCGATTATC-3' and 5'-AACCTACAATCATGTACTCATCCACCTTTGT-3'; and for pMpGE010-sgRNA85, 5'-TGC ACTTGAATCTCAACTCTGAAATCTCC-3' and 5'-TCG TTCGCTTGGCCTACACCGACGGCTG-3'. The amplified DNA fragments were directly sequenced to check the target sequences.

Reverse-transcription quantitative PCR

Total RNA was isolated from 1-month-old BC3-38, *Mpigpd*, and *MpIGPDm/Mpigpd* thalli using an RNeasy Plant Mini Kit (QIAGEN). cDNA was synthesized with the isolated RNA as a template using ReverTra Ace[®] qPCR RT Master Mix (TOYOBO) and subjected to reverse-transcription quantitative PCR (RT-qPCR) on an Applied Biosystems 7500 real-time PCR system (Life Technologies) with FastStart Universal SYBR Green Master (ROX) (Roche). To quantify the expression of *MpIGPD* (or *MpIGPDm*), the cDNA fragment encoding part of the *MpIGPD* (or *MpIGPDm*) gene was amplified with the primers 5'-GAGTTTATCCCGTGGGCGA-3' and 5'-TAG TGTGAGTGTAGCGGGC-3'. The cDNA fragment encoding part of the elongation factor was also amplified as an internal control with the primers 5'-GCATCTTGTCTTCTGAAA GGTGTGTC-3' and 5'-CACGCTTGTCATACCTCCCAGCT TGTAGATAAGG-3'. *MpIGPD* (or *MpIGPDm*) transcript levels in *Mpigpd* and *MpIGPDm/Mpigpd* were calculated via comparison with transcript levels in BC3-38 using the $\Delta\Delta\text{Ct}$ method as described previously (Yong et al. 2021).

Results and discussion

Design and pre-evaluation of sgRNAs for CRISPR/Cas9

To develop a new tool for molecular biology analysis in *M. polymorpha*, we generated a histidine auxotrophic mutant using CRISPR/Cas9-mediated genome editing.

Based on a study of a histidine auxotrophic mutant in *P. patens* (Ulfstedt et al. 2017), we searched for sequences of genes encoding IGP, an enzyme required for histidine biosynthesis, in the genome database of *M. polymorpha*. We identified *Mp5g09140*, the homolog of *P. patens* IGP (*PpHIS3*), and named this gene *MpIGPD*. Within the genomic region of *MpIGPD*, five sgRNAs for CRISPR/Cas9 were designed using the sgRNA design tool CRISPR-P 2.0 (<http://crispr.hzau.edu.cn/CRISPR2/>) (Figure 1A, Supplementary Figure S1).

To determine the genome-editing efficiency of the five sgRNAs, we performed SKLPT imaging, an efficient sgRNA pre-evaluation method in vivo (Konno et al.

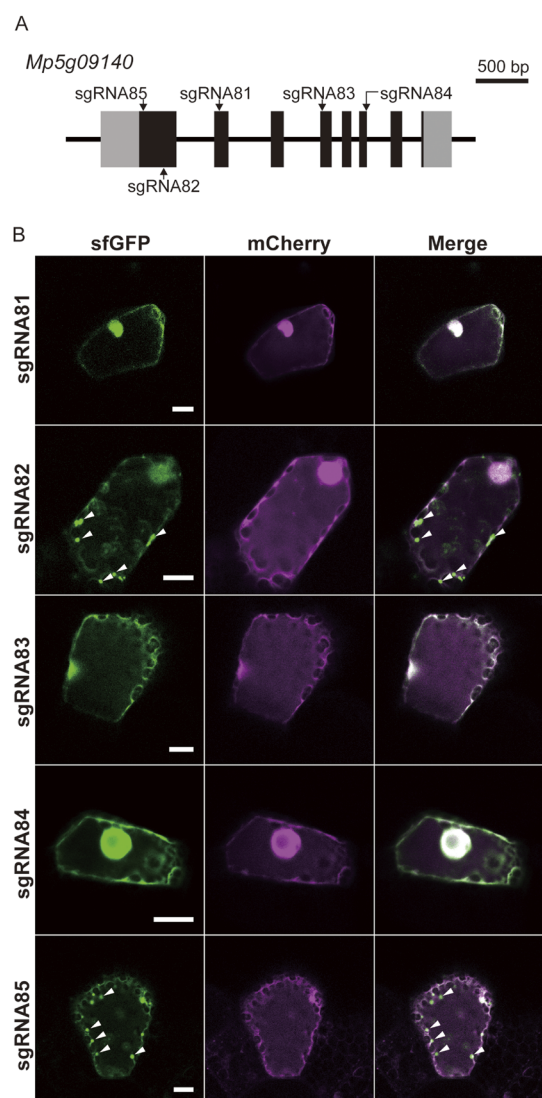


Figure 1. Design and pre-evaluation of sgRNAs for CRISPR/Cas9-mediated targeting of *MpIGPD*. (A) Positions of the sgRNAs (sgRNA81–85) in the genomic region of *MpIGPD* (*Mp5g09140*). Gray and black boxes indicate the untranslated region and exon, respectively. (B) Selection of the sgRNAs (sgRNA81–85) in *M. polymorpha* using the SKLPT imaging method. In the images for sgRNA82 and sgRNA85, sfGFP localized to peroxisomes is indicated by white arrowheads. mCherry was used as a control for particle bombardment. Bars indicate 10 μm .

2018). Briefly, the SKLPT imaging method is based on changes in the subcellular localization of superfolder green fluorescent protein (sfGFP). Successful editing causes frameshifts resulting in the fusion of sfGFP to the C-terminal tripeptide serine-lysine-leucine (SKL), the peroxisome localization signal in the sfGFP-SKL fusion protein (Konno et al. 2018), causing the fusion protein to localize to peroxisomes. Among the newly designed sgRNAs, the presence of sgRNA82 or sgRNA85 caused sfGFP to localize to peroxisomes in *M. polymorpha* cells (Figure 1B). These results suggest that the two sgRNAs can lead to mutations of the target gene *MpIGPD* in the *M. polymorpha* genome.

Generation of histidine auxotrophic *M. polymorpha* mutants using CRISPR/Cas9

To disrupt the *MpIGPD* gene of the *M. polymorpha* genome, we constructed binary vectors encoding sgRNA82 and sgRNA85 for CRISPR/Cas9 in *M. polymorpha*. We generated two *Agrobacterium* lines harboring the respective binary vectors. Using a mixture of the two *Agrobacterium* strains, we performed genetic transformation of WT strain BC3-38 by the G-AgarTrap method (Tsuboyama et al. 2018; Tsuboyama-Tanaka and Kodama 2015), as shown in Figure 2A. To isolate histidine auxotrophic mutants of *M. polymorpha*, medium supplemented with histidine must be used during genetic transformation (Figure 2A). To determine the appropriate histidine concentration to use during the G-AgarTrap procedure, we cultured *M. polymorpha*

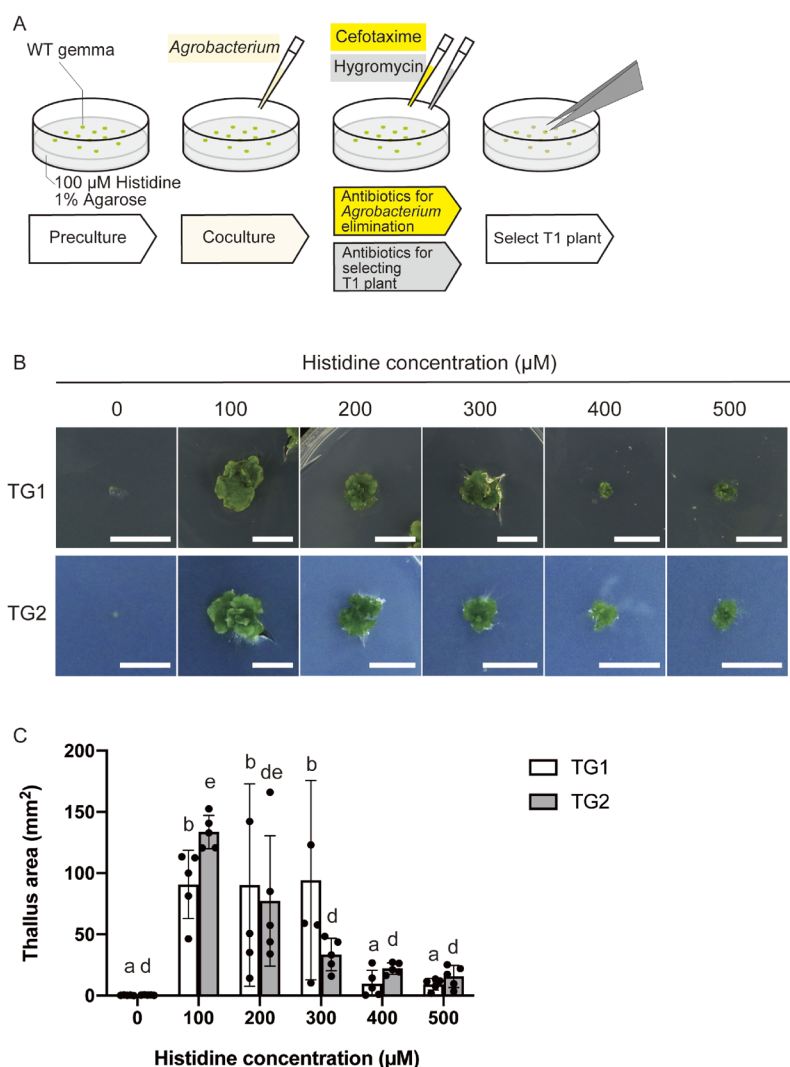


Figure 2. Generation of histidine auxotrophic *M. polymorpha* lines. (A) Diagram of genetic transformation to generate the histidine auxotrophic *M. polymorpha* lines. The BC3-38 strain was used as the wild type (WT). (B) Representative images of the histidine auxotrophic phenotype of TG1 and TG2 lines. The TG1 and TG2 lines were cultured on 1/2 B5 medium with 1% (w/v) agar and various concentrations of histidine (0, 100, 200, 300, 400, and 500 μ M) for 3 weeks. Bars indicate 1 cm. (C) Quantification of growth of the TG1 and TG2 lines under various histidine concentrations. The thallus area of plants cultured under the same conditions as in (B) was measured. Error bars indicate standard deviations (Tukey's multiple comparison test). Different lowercase letters indicate significant difference ($p < 0.05$).

gemmalings under various histidine concentrations (0, 100, 200, 300, 400, and 500 μ M; Supplementary Figure S2).

Under histidine supplementation, gemmaling growth was inhibited; the higher concentrations caused stronger growth inhibition (Supplementary Figure S2). Because abnormal morphology was also observed at histidine concentrations $>200 \mu$ M (Supplementary Figure S2), we employed 100 μ M histidine for genetic transformation. When 100 μ M histidine and hygromycin (as an antibiotic) were added to the media for gemmaling preculture, *Agrobacterium* co-culture, and transformant selection, two transformants (TG1 and TG2 lines) survived (Figure 2A). Both TG1 and TG2 grew on medium supplemented with $>100 \mu$ M histidine, but not on medium without histidine (Figure 2B, C), indicating that these lines were histidine auxotrophic mutants. Notably, similar to the WT strain (Supplementary Figure S2), the higher histidine concentrations caused stronger growth inhibition of the transformants (Figure 2B, C). Based on the histidine auxotrophic phenotype of the transgenic *M. polymorpha* strains, the *MpIGPD* gene was likely disrupted.

To test whether the histidine auxotrophic *M. polymorpha* transformants could be used for the biocontainment, we cultivated the TG1 line in two types of soil: oligotrophic (vermiculite and gardening soil mix) and eutrophic (leaf mold). When TG1 gemmae were cultured in oligotrophic soil, the gemmae did not grow (Supplementary Figure S3). Similarly, when transgenic thallus pieces (approx. 5 mm $\times$ 5 mm) from 1-week-old gemmalings were cultured in eutrophic

soil, the transgenic thalli did not grow (Supplementary Figure S3). These results suggest that the transformant cannot grow in an outdoor environment and that the auxotrophic *M. polymorpha* strains could potentially be used as biocontainment tools.

Disruption of the *MpIGPD* gene in the histidine auxotrophic *M. polymorpha* mutants

We examined whether T-DNA was integrated in the genomes of the histidine auxotrophic lines. Because a mixture of two transgenic *Agrobacterium* lines for sgRNA82 and sgRNA85 was used for transformation, we performed PCR to amplify the specific region of each T-DNA from genomic DNA of TG1 and TG2. Only T-DNA encoding sgRNA82 was detected in TG1, whereas T-DNA encoding sgRNA85 was detected in TG2 (Figure 3A). To determine whether the genomic region of *MpIGPD* was disrupted in the histidine auxotrophic mutants, we analyzed the genomic and mRNA sequences of these lines. In the genomic sequences, a large deletion of 293-bp (with a 1-bp insertion) and a 1-bp deletion (with 2-bp insertion) were found at the position targeted by sgRNA82 and sgRNA85, respectively (Figure 3B and Supplementary Figure S4). Here, the TG1 and TG2 lines were renamed *Mpigpd-1* and *Mpigpd-2*, respectively (Figure 3B and Supplementary Figure S4). We synthesized cDNAs from mRNA extracted from the *Mpigpd* mutants and amplified the cDNA sequences containing the deletions. The genomic mutations produced short sequences that do not encode any proteins in *Mpigpd-1* or *Mpigpd-2* (Figure 3C),

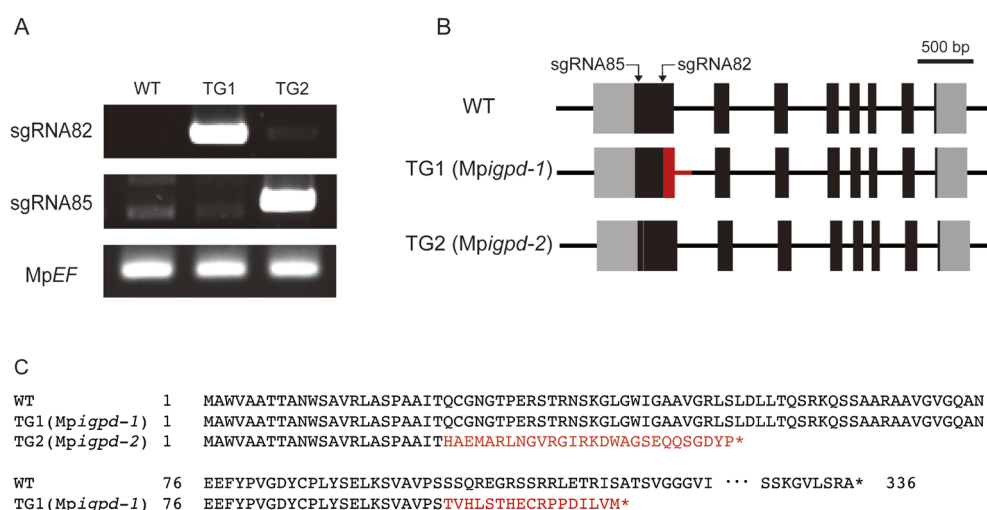


Figure 3. Evaluation of *MpIGPD* in the histidine auxotrophic *M. polymorpha* lines. (A) Identification of T-DNA integrated in the genomes of TG1 and TG2. A specific region of T-DNA encoding sgRNA82 or sgRNA85 was amplified by PCR. The elongation factor gene (*MpEF*) was also amplified by PCR as a control for the common region of both T-DNAs. The WT (BC3-38) strain was used as a control. (B) Genomic sequences of the TG1 (*Mpigpd-1*) and TG2 (*Mpigpd-2*) lines. A large 293-bp (with a 1-bp insertion) deletion and a 1-bp deletion (with 2-bp insertion) were detected at the positions targeted by sgRNA82 (TG1: *Mpigpd-1*) and sgRNA85 (TG2: *Mpigpd-2*), respectively. Gray and black boxes indicate the untranslated region and exon, respectively. The mutated DNA regions are shown in red. (C) Putative protein sequences in the TG1 (*Mpigpd-1*) and TG2 (*Mpigpd-2*) lines. The cDNA containing the target region was amplified by PCR and sequenced. The mutated regions are indicated in red. Asterisks indicate stop codons.

confirming the disruption of the *MpIGPD* gene product. As the phenotype of *Mpigpd-1* and *Mpigpd-2* was almost same (Figure 2B, C), we chose *Mpigpd-1* with the large deletion in the following experiments.

Development of a modified *MpIGPD* gene

To test the notion that disrupting *MpIGPD* caused the histidine auxotrophic phenotype in *M. polymorpha*, we carried out a complementation test by introducing cDNA from the *MpIGPD* gene into the *Mpigpd-1* mutant by transformation. Prior to transformation, we modified the cDNA of *MpIGPD* by introducing eight silent mutations not targeted by sgRNA82 or sgRNA85 (Figure 4A). The modified *MpIGPD* (*MpIGPDm*) was transformed into *Mpigpd-1* using the G-AgarTrap method (Tsuboyama et al. 2018; Tsuboyama-Tanaka and Kodama 2015), as shown in Figure 4B. The resulting transformants (*MpIGPDm/Mpigpd-1*; lines #C1–4) were selected by adding the herbicide chlorsulfuron to medium

containing histidine (Figure 4B). These lines were able to grow on medium lacking histidine (Figure 4C, D), and we confirmed the expression of *MpIGPDm* in these lines (Figure 4E). These results support the notion that disrupting *MpIGPD* caused the histidine auxotrophic phenotype in *M. polymorpha* and indicate that the *MpIGPDm* gene can rescue the histidine auxotrophic phenotype of the *Mpigpd* mutant.

Selection of transformants using the modified *MpIGPD* gene

Because the *MpIGPDm* gene rescued the histidine auxotrophic phenotype of *Mpigpd* (Figure 4C, D), we tested whether this gene could be used as a selective marker when the *Mpigpd* strain is used as material for genetic transformation. Using the binary vector shown in Figure 4A, we transformed the *Mpigpd-1* strain with *MpIGPDm* using the T-AgarTrap method (Tsuboyama-Tanaka et al. 2015), as shown in Figure

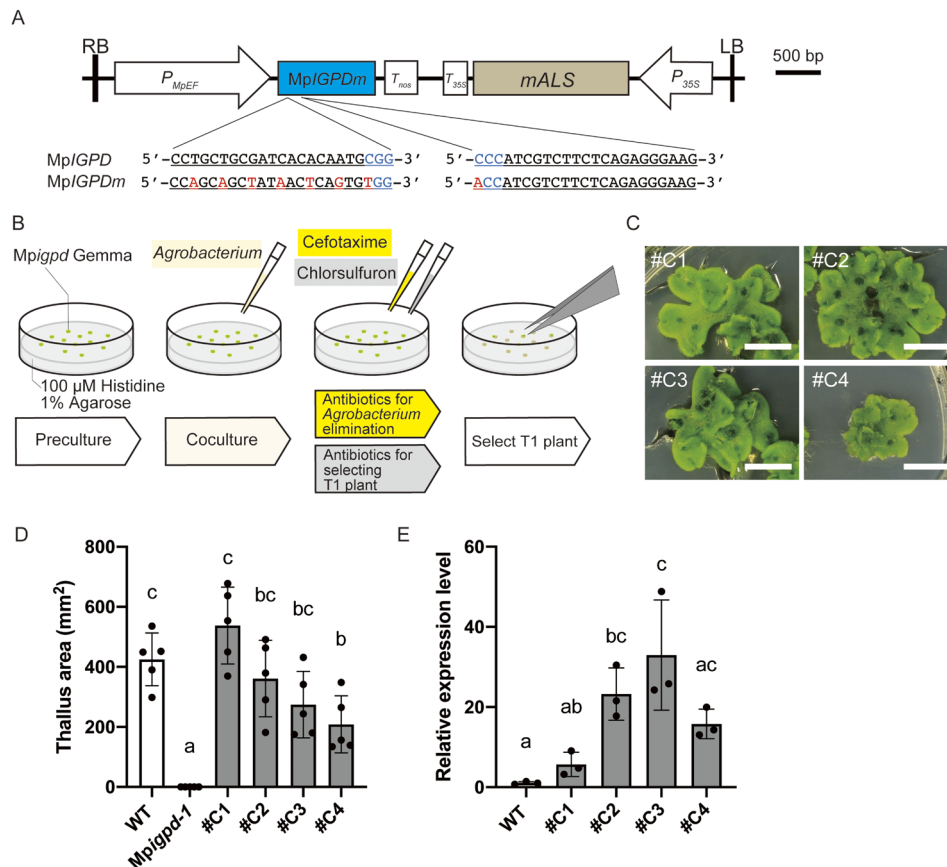


Figure 4. Complementation test using a modified *MpIGPD* gene. (A) Design of a modified *MpIGPD* (*MpIGPDm*) that is not targeted by sgRNA82 or sgRNA85. Red and blue letters indicate the silent mutation and protospacer adjacent motif (PAM) sequence, respectively. RB, right border; *P_{MpEF}*, *MpEF1a* promoter; *T_{NOS}*, NOS terminator; *T_{35S}*, 35S terminator; *mALS*, a mutated *ACETOLACTATE SYNTHASE* gene; *P_{35S}*, 35S promoter; LB, left border. (B) Diagram of genetic transformation to generate the complemented line (*MpIGPDm/Mpigpd-1*). (C) Representative images of growth of the *MpIGPDm/Mpigpd-1* lines (#C1–4). The lines were cultured on 1/2 B5 medium with 1% (w/v) agar for 3 weeks without histidine supplementation. Bars indicate 1 cm. (D) Quantification of growth of the *MpIGPDm/Mpigpd-1* lines (#C1–4). Thallus area cultured under the same conditions as in (C) was measured. Error bars indicate standard deviations (Tukey's multiple comparison test). Different lowercase letters indicate significant difference ($p < 0.05$). Data for WT (BC3-38) and *Mpigpd-1* are the same as in Supplementary Figure S2 (0 μM) and Figure 2C (0 μM), respectively. (E) *MpIGPDm* expression in the #C1–4 lines. The expression level was quantified by quantitative reverse-transcription PCR. The WT (BC3-38) strain was used as a control. Error bars indicate standard deviations (Tukey's multiple comparison test). Different lowercase letters indicate significant difference ($p < 0.05$).

5A. During all steps, media lacking histidine were used (Figure 5A). Because the *Mpigpd* strain cannot grow on medium without histidine, a 0-day preculture step was employed; this step is sometimes used in the T-AgarTrap method (Tsuboyama-Tanaka et al. 2015). When the transformants were selected on medium without chlorsulfuron or histidine, many transformants survived (Figure 5B). Therefore, *MpIGPDM* can be used as a selective marker when using the *Mpigpd* strain.

We constructed the new Gateway binary vector pMpGWBhis03, which contains the *MpIGPDM* gene as a selective marker but lacks antibiotic selective markers (Figure 5C). To test whether transformants could be produced using pMpGWBhis03, we constructed binary vectors to express the *Citrine* gene and *Citrine-NLS* (pMpGWBhis03-*Citrine* and pMpGWBhis03-*Citrine-NLS*) and performed genetic transformation of the *Mpigpd-1* strain using the T-AgarTrap method, as shown in Figure 5A. When *Mpigpd-1* was co-cultured

with *Agrobacterium* harboring pMpGWBhis03-*Citrine* or pMpGWBhis03-*Citrine-NLS* on medium without histidine, transformants exhibiting Citrine fluorescence were successfully grown (Figure 5D). These results indicate that the *MpIGPDM* gene can be used as a selective marker that rescues the histidine auxotrophic phenotype of the *Mpigpd* strain.

Four selective markers for antibiotic and herbicide resistance were previously used to produce transformants in *M. polymorpha*: hygromycin phosphotransferase (HPT), gentamicin 3'-acetyltransferase (*aacC1*), mutated acetolactate synthase (*mALS*), and neomycin phosphotransferase II (NPTII) for hygromycin, gentamycin, chlorsulfuron, and kanamycin, respectively (Ishizaki et al. 2015). Among the four markers, HPT and *mALS* have been employed in vectors for genome editing of *M. polymorpha*. In the present study, we developed *MpIGPDM* as an auxotrophic selective marker that can be used to select transformants in the auxotrophic

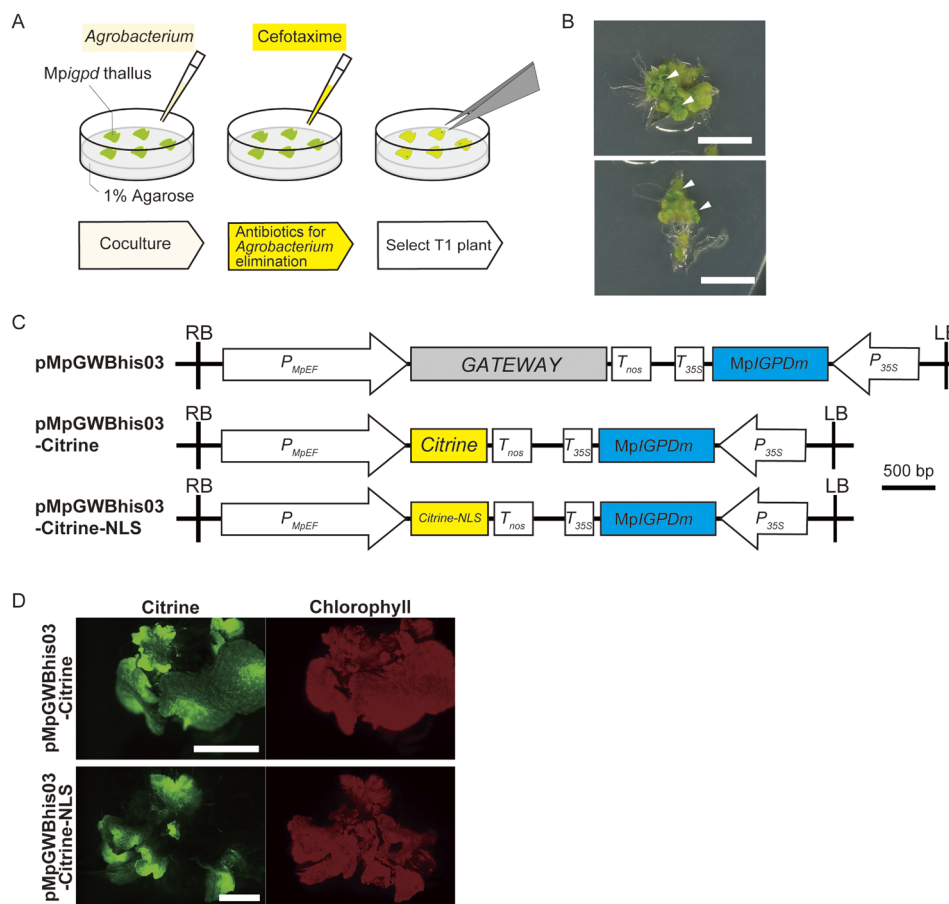


Figure 5. Use of *MpIGPDM* as a histidine auxotrophic genetic selective marker. (A) Diagram of genetic transformation using *MpIGPDM* as a genetic selective marker. (B) Representative images of surviving tissues with the *MpIGPDM* marker. Bars indicate 5 mm. Arrowheads indicate regenerated tissues. (C) Construction of Gateway binary vectors (pMpGWBhis03 backbone) containing *MpIGPDM* as a selective marker without any antibiotic selective marker. The T-DNA regions of pMpGWBhis03, pMpGWBhis03-*Citrine*, and pMpGWBhis03-*Citrine-NLS* are shown. RB, right border; *P_{MpEF}*, *MpEF1a* promoter; *GATEWAY*, Gateway cassette [attR1-CmR (*chloramphenicol* resistance gene)-*ccdB* (negative selection marker)-attR2]; *T_{nos}*, NOS terminator; *T_{35S}*, 35S terminator; *P_{35S}*, 35S promoter; LB, left border. (D) Representative images of the transformants using *Mpigpd-1* with pMpGWBhis03-*Citrine* or pMpGWBhis03-*Citrine-NLS*. Citrine fluorescence and chlorophyll fluorescence were detected by fluorescence stereomicroscopy. Bars indicate 2 mm.

Mpigpd background. Unlike selective markers for antibiotic and herbicide resistance, the use of auxotrophic selective markers and auxotrophic strains allows the desired transformants to be selected without producing non-transgenic escape lines. If *MpIGPDm* is used with the *Mpigpd* strain to produce transgenic and/or genome-edited lines, all existing genetic selective markers for antibiotics and herbicides could be used for subsequent analysis.

Concluding remarks

In the present study, we generated the histidine auxotrophic mutant *Mpigpd* in *M. polymorpha* and identified its responsible gene (*MpIGPD*). *Mpigpd* and *MpIGPDm* are the first reported auxotrophic strain and genetic selective marker in *M. polymorpha*, respectively. Notably, *Mpigpd* is the first terrestrial-plant strain that can be used for biocontainment, produced by CRISPR/Cas9-mediated genome editing, and *Mpigpd* has the potential as a material for molecular farming, which is the in planta production of recombinant proteins highly valuable to industry or medicine (Twyman et al. 2003). Using the *Mpigpd* strain and *MpIGPDm* marker, we successfully produced transgenic lines, indicating that the *Mpigpd* strain and *MpIGPDm* marker are new molecular biological tools for *M. polymorpha* research. However, as the research community for *M. polymorpha* has grown, several WT model strains have been reported. For example, Takaragaike-1 (Tak-1) and Cambridge-1 (Cam-1) are used as male strains and Tak-2, BC3-38 (a progeny of Tak-2 backcrossed three times to Tak-1), and Cam-2 are used as female strains. In this study, we produced the histidine auxotrophic mutant (*Mpigpd*) only in the BC3-38 background. To increase the utility of auxotrophic strains and genetic selective markers, the *MpIGPD* gene will need to be mutated in the other model strains. Importantly, we deposited information about the related plasmids in Addgene for the *M. polymorpha* research community (Supplementary Table S3). Histidine auxotrophic *Mpigpd* mutants of other strains could therefore be produced in various laboratories using the newly designed plasmids.

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Author contributions

Y.K. conceived and designed the study. T.F. performed experiments. T.F. and Y.K. analyzed data. Y.K. wrote the manuscript. T.F. prepared figures and tables. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no competing interest.

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