

A tRNA-gRNA multiplexing system for CRISPR genome editing in *Marchantia polymorpha*.

Eftychios Frangedakis^{1,2,*}, Nataliya E. Yelina^{1,3,4}, Satish Kumar Eeda^{1,5}, Facundo Romani¹, Alexandros Fragkidis^{1,6}, Jim Haseloff¹ and Julian M. Hibberd¹

¹Department of Plant Sciences, University of Cambridge, Cambridge, CB3 EA, UK

²Present address: School of Life Science, Rikkyo University, Nishi-Ikebukuro, Tokyo, 171-8501, Japan

³Present address: Crop Science Centre, University of Cambridge, 93 Lawrence Weaver Road, Cambridge, CB3 0LE, UK

⁴Present address: Centre for Plant Sciences, University of Leeds, Woodhouse Lane, Leeds LS2 9JT, UK

⁵Present address: Crop Transformation, NIAB, Park Farm, Villa Road, Histon, Cambridge, CB24 9NZ, UK

⁶Present address: Bristol Medical School, University of Bristol, Dorothy Hodgkin Building, Whitson Street, Bristol, BS1 3NY, UK

*correspondence: efrangedakis@gmail.com

Highlight

In this study we present a method for the generation of complex CRSIPR-mediated genome modification in the bryophyte *Marchantia polymorpha*, enabling more advanced genetic studies in this model organism.

Abstract

The liverwort *Marchantia polymorpha* is a widely used model organism for studying land plant biology, which has also proven to be a promising testbed for bioengineering. CRISPR/Cas9 technology has emerged as a transformative tool for precise genome modifications in *M. polymorpha*. However, a robust method for the simultaneous expression of multiple gRNAs, which is crucial for enhancing the versatility of CRISPR/Cas9-based genome editing, has yet to be fully developed. In this study, we introduce an adaptation from the OpenPlant kit CRISPR/Cas9 tools, that facilitates expression of multiple gRNAs from a single transcript through incorporation of tRNA sequences. The ability to deliver multiple gRNAs simultaneously, significantly improves the capacity and scalability of genome editing in *M. polymorpha*. Additionally, by combining this vector system with a simplified and optimized protocol for thallus transformation, we further streamline the generation of CRISPR/Cas9 mutants in *M. polymorpha*. The resulting gene-editing system offers a multipurpose, time-saving and straightforward tool for advancing functional genomics in *M. polymorpha*, enabling more comprehensive genetic modifications and genome engineering.

Keywords: *Marchantia*, *CRISPR*, *thallus transformation*, *tRNA*, *genome editing*

Introduction

66

67 *Marchantia polymorpha* is a powerful model organism for investigating various aspects of plant
68 biology, including genetics, cell biology, development, and evolution (Naramoto et al. 2022;
69 Bowman et al. 2022; Kohchi et al. 2021). *M. polymorpha* is also an excellent chassis for
70 bioengineering, offering a unique combination of well-established genetic modification tools,
71 simple body plan, rapid growth and remarkable regenerative capacity, as well as minimal
72 cultivation requirements (Boehm et al. 2017; Sauret-Güeto et al. 2020; Tse et al. 2024;
73 Frangedakis et al. 2021; Romani et al. 2024; Ishizaki et al. 2016). All these make it an ideal
74 platform for a wide range of synthetic biology applications, such as reprogramming cell fate,
75 engineering of biosynthetic pathways, development of biosensors, Clustered Regularly
76 Interspaced Short Palindromic Repeats (CRISPR)-based DNA-recording device or CRISPR
77 based multiplex gene regulation systems (Donà et al. 2023; Tansley, Patron, and Guiziou 2024;
78 Forestier et al. 2025; Gupta and Karkute 2021; Kocaoglan, Radhakrishnan, and Nakayama 2023).
79

80 *M. polymorpha*'s compact genome and haploid dominant life cycle make it particularly well-suited
81 for studying genetics (Kohchi et al. 2021). As a result, it is uniquely positioned to explore
82 fundamental genetic processes, with potential to enhance our understanding of key traits that are
83 conserved across land plants. Genetics has been profoundly transformed by the development of
84 CRISPR technology. CRISPR enables precise and targeted genetic modifications by inducing
85 deletions or insertions at specific loci within the nuclear genome (Ran et al. 2013; Jinek et al.
86 2012; Cong et al. 2013). This gene-editing system utilizes a guide RNA (gRNA) to direct a
87 nuclease protein such as Cas9, to a specific DNA sequence, where it induces a double-strand
88 break, allowing for accurate genome editing (Adli 2018; Ran et al. 2013). CRISPR has facilitated
89 significant advances in functional genomics, where the specific manipulation of sequence allows
90 a better understanding of gene function. CRISPR/Cas9 technology is routinely used in *M.*
91 *polymorpha* (Sugano et al. 2018, 2014; Sauret-Güeto et al. 2020). One of the challenges in
92 CRISPR-based technologies, including in *M. polymorpha*, is the expression of multiple guide
93 RNAs (gRNAs) as a single transcript, particularly for applications requiring simultaneous editing
94 of multiple genomic sites. Current CRISPR/Cas9 systems in *M. polymorpha* enable only limited
95 multiplex genome editing or require multiple plasmids with different antibiotic resistance, typically
96 allowing the expression of no more than two gRNAs simultaneously (Sugano et al. 2018, 2014;
97 Sauret-Güeto et al. 2020). Expressing multiple gRNAs as part of a tRNA-gRNA transcript is a
98 gRNA multiplexing approach and has been successfully applied in several plant species (Xie,
99 Minkenberg, and Yang 2015; Hui et al. 2019; Z. Wang et al. 2021). In this system (**Fig. 1A**), tRNA-

gRNA synthetic transcripts mimic native tRNAs^{noRNA43} transcripts in plants, allowing endogenous cellular machinery (i.e. RNase P and Z) to cleave the tRNA structure and release mature functional single gRNAs (**Fig. 1B**) (Xie, Minkenberg, and Yang 2015; Ma et al. 2019; Čermák et al. 2017). Additionally, the tRNAs tertiary triple helix structure is known to contribute to transcript stability (Wilusz *et al.*, 2012; Chery and Drouard, 2022). Moreover, tRNA sequences can act as transcriptional enhancers due to the promoter elements they contain, that can facilitate the recruitment of the RNA polymerase III (Dieci *et al.*, 2007; White, 2011). Taking advantage of this features, the tRNA-gRNA system has been used to successfully increase gRNA transcript levels in rice protoplasts (Xie et al., 2015) and to enhance the genome editing efficiency in various other plants such as maize, cotton and sweet (Qi *et al.*, 2016; Wang *et al.*, 2018b; Tang *et al.*, 2021).

The tRNA-gRNA system also reduces the complexity associated with constructing multiple independent gRNA expression cassettes. Previously, we have generated vectors as part of the OpenPlant kit (Sauret-Güeto et al. 2020) that allow the introduction of both the Cas9 protein and up to two single gRNA expression cassette into the *M. polymorpha* genome via *Agrobacterium*-mediated plant transformation. In this study, we present an updated CRISPR/Cas9 vector system for *M. polymorpha*, adapted from the OpenPlant kit, that combines the Cas9 protein with a tRNA-gRNA module as a single transcript using a simple two-step cloning strategy. Furthermore, we combine the updated CRISPR/Cas9 vector system with an optimized and simplified protocol for *M. polymorpha* thallus transformation, eliminating the need for spores, which are often time-consuming and difficult to obtain. This approach, further expands the ability to introduce multiple genetic modifications, including large DNA deletions, in *M. polymorpha* wild type or mutant backgrounds.

In summary, in this study, we outline the generation and validation of constructs for gRNA multiplexing in *M. polymorpha*, providing a straightforward and efficient pipeline for multiplex gene editing and advanced plant engineering.

Material and methods

Plant material and growth conditions

Marchantia polymorpha accessions Cam-1 (male) and Cam-2 (female) (Delmans, Pollak, and Haseloff 2017) were used in this study. Plants were grown and maintained on ½ strength

Gamborg's B5 medium plus vitamins (DUCHEFA, #G0210.0050) and 1.2 % (w/v) agar (MELFORD, #A20021), under continuous light at 21°C.

***M. polymorpha* axenic cultures for spore production**

M. polymorpha thallus fragments or gemmae were transferred into autoclaved microboxes containing 15 "Jiffy 7" pellets (JIFFY PRODUCTS INTERNATIONAL BV), as previously described (Sauret-Güeto et al. 2020). Briefly, 200 mL of water was added, and the plants were allowed to grow for one month under continuous light at 21°C. After this period, far-red light was applied to induce the development of reproductive organs. Within 3–4 weeks, mature gametophores, ready for fertilization, became visible. For fertilization, a drop of sterile water was added to the male gametophore, and the released sperm was transferred with a pipette onto the female gametophore. After an additional month, mature sporophytes were visible and ready for collection.

gRNA designed

The design of the gRNAs was performed using the CasFinder tool (<https://marchantia.info/tools/casfinder/>). The program was run with the following configurations: CasFinder.pl -D -CONFIG CASFINDER_CONFIG.txt -CONFIG_CAS9 CASFINDER_CAS9_CONFIG_20.txt -g tak1_v5.1 -i query.fa -o out -A 1 -t 4. Searches were performed against the *M. polymorpha* Tak1 v5.1 genome, for 20 nt long gRNAs and Primary/secondary PAM sequences: NGG/NAG.

In vitro gRNA testing

Primers were designed (**see Table S1**) to amplify sequences containing the gRNA target DNA sequence. PCR was performed using the Phusion DNA Polymerase (ThermoFisher Scientific #F530S), and products from 2–5 × 50 µL reactions were pooled, and then purified using the QIAquick PCR Purification Kit (QIAGEN #28104). Templates for in vitro transcription were generated by PCR using a forward primer containing the T7 promoter and crRNA sequence, a reverse primer matching the tracrRNA (**see Table S1**), and a plasmid template (pEn_Chimera, Addgene plasmid #61432). Templates were purified with the QIAquick PCR Purification Kit (QIAGEN #28104).

cr-tracrRNA was synthesized using the MEGAscript™ T7 Transcription Kit (ThermoFisher Scientific, #AM1334). Transcription reactions were assembled following the manufacturer's

protocol, with the following final volumes: 2 μ L each of ATP, CTP, GTP, and UTP; 2 μ L 10X Reaction Buffer; 2 μ L Enzyme Mix; 0.1–1 μ g of linear DNA template; and nuclease-free water to a final volume of 20 μ L. Reactions were incubated at 37°C for 16 hours.

Following transcription, 0.5 μ L of TURBO DNase (Invitrogen, #AM2238) was added to each reaction and incubated at 37°C for 15 minutes to remove the DNA template. RNA was then purified using LiCl precipitation: 15 μ L nuclease-free water and 15 μ L LiCl Precipitation Solution were added, mixed thoroughly, and incubated at –20°C for at least 30 minutes. RNA was pelleted by centrifugation at maximum speed for 15 minutes at 4°C, washed once with 70% ethanol, and resuspended in 12 μ L of nuclease-free water.

To test gRNA activity, in vitro cleavage assays were performed using recombinant Cas9 (NEB, #M0646T) and in vitro-transcribed gRNA. Reactions (30 μ L total) contained 3 μ L of 10X Cas9 Reaction Buffer, 3 μ L of gRNA (~100 ng/ μ L), 0.15 μ L of Cas9 (20 μ M), and 3 μ L of target DNA (200–300 ng), with nuclease-free water added to 30 μ L. Prior to DNA addition, Cas9 and gRNA were pre-incubated for 10 minutes at 25°C to allow complex formation. Reactions were incubated at 37°C for 1 hour, followed by addition of 1 μ L Proteinase K and incubation at 65°C for 10 minutes to degrade Cas9. Reactions were analyzed immediately on agarose gels to assess cleavage efficiency.

Single gRNA cloning into L1 vectors

Two oligos (primers) for cloning the gRNA target sequence were designed, as previously described (Sauret-Güeto et al. 2020). To prepare the annealing reaction, 1 μ L of each oligo (100 μ M) was mixed with 8 μ L of water to a final volume of 10 μ L. The mixture was then annealed in a thermocycler under the following conditions: 37 °C for 30 minutes, 95 °C for 5 minutes, followed by a gradual decrease to 25 °C at a rate of 5 °C per minute. After annealing, the gRNA target sequence was cloned into the L1_lacZgRNA-Ck2 or L1_lacZgRNA-Ck3 vectors (Addgene #136136 or Addgene #136137). L1 BbsI-mediated Loop reactions were performed with minor modifications to previous protocols (Sauret-Güeto et al. 2020). Briefly, the reaction included 7.5 nM (1 μ L) of L1_lacZgRNA-Ck2 or L1_lacZgRNA-Ck3 vectors and 2 μ L of annealed oligos. The Level 1 Loop assembly master mix contained 2 μ L of 10 $\times$ T4 DNA ligase buffer (NEB), 1.5 μ L of 1 mg/mL bovine serum albumin (NEB, #B9200S), 1.5 μ L of T4 DNA ligase 400 U/ μ L (NEB, #M0202S), 1.5 μ L of BbsI-HF 10 U/ μ L (NEB, #R3539), and nuclease-free H₂O to a final volume of 20 μ L. The cycling conditions included 26 cycles of 37 °C for 3 minutes and 16 °C for 4 minutes,

followed by enzyme inactivation at 50 °C for 5 minutes and 80 °C for 10 minutes. For transformation, 20 µL of chemically competent *E. coli* cells were incubated with 10 µL of the Loop assembly reaction and plated on LB agar containing 50 µg/mL kanamycin (MELFORD, #K22000–1.0) and 40 µg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (ThermoFisher, #15520018).

Primer design for tRNA-gRNA module amplification

The tRNA-gRNA spacer-specific primers were designed with BbsI 4 bp overlapping overhangs for Golden Gate/Loop Assembly. The sequences for these primers are as follows (**Fig. 2A**): G-primer-F (AGgaagacTACTCGAACAAAGCACCAGTGG) and "gRNA c Primer R" (GAgaagacTATAAAAC-LAST-gRNA-REVcompl-TGCACCAGCCGGGAATC). The sequences for "gRNA a Primer F" and "gRNA b Primer F" are GAgaagacAT-partial-gRNA-GTTTTAGAGCTAGAA, while "gRNA a Primer R" and "gRNA b Primer R" are GAgaagacTA-partial-gRNA-REVcompl-TGCACCAGCCGGGAA. The primer combinations used for amplification (when three gRNAs are combined, but can adapted to combine a larger number) are as follows: (1) G-primer-F with gRNA a Primer R, (2) gRNA a Primer F with gRNA b Primer R, and (3) gRNA b Primer F with gRNA c Primer R. The pGTR plasmid (Addgene #63143) should be used as the template for these reactions. For detailed instructions and an example of primer design please see **Supplemental Information**. For PCR amplification Phusion polymerase (ThermoFisher, #F534S) was used according to the manufacturer's instructions. PCR products were gel extracted using the QIAquick Gel Extraction Kit (QIAGEN, #28704).

tRNA-gRNA cloning into L1 vectors

To clone the tRNA-gRNA DNA fragments into either L1_lacZgRNA-Ck2 or L1_lacZgRNA-Ck3, the same procedure used for single gRNA cloning was followed, with the only modification being the replacement of the annealed oligos with gel-extracted fragments. Reaction volumes were adjusted accordingly (see **Supplemental Information** for detailed protocols).

L2 SapI mediated Loop Assembly

For the SapI-mediated Type IIS assembly, the following reaction was prepared: 2 µL of 10× T4 DNA ligase buffer (NEB), 1.5 µL of T4 DNA Ligase at 400 U/µL (NEB, #M0202S), 1.5 µL of SapI (10 U/µL, NEB, #R0569S), 1 µL (7.5 nM) of pCsA vector (Addgene #136067), 1 µL (15 nM) of L1 vectors, and nuclease-free H₂O to a final volume of 20 µL (please see Supplemental information

for detailed instructions). After assembly, 10 µL of the reaction mixture was transformed into *E. coli* and plated on LB agar containing 100 µg/mL spectinomycin (MERCK, #S4014-5G) and 40 µg/mL X-Gal for selection. The L1 vectors that used were: L1_Cas9-Ck4 containing the transcription unit for the Cas9 expression, (Addgene #136135 (Sauret-Güeto et al. 2020)); L1_CsR-Ck1 (Plasmid #136124) or L1_HyR-Ck1 (Addgene, #136125) containing the transcription unit for chlorsulfuron or hygromycin selection respectively; and if necessary the L1 spacer vectors, pCk2_spacer (Addgene, #136072) or pCk3_spacer (Addgene, #136073).

***Agrobacterium* culture preparation**

Five milliliters of LB medium supplemented with rifampicin 50 µg/mL (DUCHEFA, #R0146), gentamicin 25 µg/mL (DUCHEFA, #G0124), and spectinomycin 100 µg/mL (Bio Basic, #SB0901), were inoculated with 3–4 *Agrobacterium tumefaciens* colonies (GV3101). The preculture was incubated at 28°C for 2 days at 100 rpm. After incubation (without measuring OD), 5 mL of the 2-day *Agrobacterium* culture was centrifuged at 2000 × g for 7 minutes. The supernatant was removed, and the pellet was resuspended in 5 mL of KNOP co-cultivation medium (0.25 g/L KH₂PO₄, 0.25 g/L KCl, 0.25 g/L MgSO₄•7H₂O, 1 g/L Ca(NO₃)₂•4H₂O and 12.5 mg/L FeSO₄•7H₂O) containing 1% (w/v) sucrose (MELFORD, #S24060-1000.0) and 100 µM acetosyringone (4'-Hydroxy-3',5'-dimethoxyacetophenone) (MECK, #D134406). The culture was then incubated with shaking at 120 rpm at 28°C for an additional 3–5 hours.

Transformation Procedure

Approximately 20 thallus cuttings, each around 2 × 2 mm - 3 × 3 mm, were transferred into a 6-well plate containing 5 mL of liquid KNOP co-cultivation medium supplemented with 1% (w/v) sucrose, 30 mM MES (2-(N-morpholino)ethanesulfonic acid) (pH 5.5), 80 µL of *Agrobacterium* culture, and acetosyringone at a final concentration of 100 µM. The tissue was co-cultivated with *Agrobacterium* on a shaker at 110 rpm at 21–22°C under ambient light (light intensity ~ 3-6 µmol m⁻² s⁻¹) for 3 days. After co-cultivation, the liquid was removed from the wells using a sterile plastic pipette, and the cuttings were transferred onto growth medium plates containing the appropriate antibiotic (hygromycin (MELFORD, #H7502) or chlorsulfuron (SIGMA, #34322)). To facilitate even distribution of thallus fragments on the plate, 2 mL of sterile water was added to each petri dish. After 3–6 weeks, successful transformation events were observed as small green regenerating tissue on dying or dead thallus fragments.

Genotyping

For genotyping, small pieces (3 × 3 mm) of thalli from individual plants were placed in 1.5 mL Eppendorf tubes and crushed with an autoclaved micropestle in 80 µL of genotyping buffer (100 mM Tris-HCl, 1 M KCl, and 10 mM EDTA, pH 9.5). The tubes were incubated at 70°C for 25 minutes, followed by the addition of 350 µL of sterile water. A 2.5 µL aliquot of the extract was used as a template for PCR (using Phusion polymerase (ThermoFisher, #F534S) or Quick Taq HS DyeMix (TOYOBO, #DTM-101) following the manufacturer's instructions), and products were analyzed on a 1.5% (w/v) agarose gel. Primers used for MpGLK genotyping are listed in **Table S1**. For Mpr-rmyb5 genotyping we used the primers described in Frangedakis et al., 2024.

Fluorescent Microscopy

A stereo microscope with fluorescence (Leica M205 FA) was used. The Leica M205 FA was equipped with the following filters for observation of fluorescence: ET GFP (ET470/40 nm, ET525/50 nm), ET Chlorophyll LP (ET480/40 nm, ET610 nm LP), and ET GFP LP500 (ET470/40 nm, ET500 nm LP), used respectively for observing eGFP, chlorophyll, and eGFP together with chlorophyll autofluorescence.

Light microscopy

Images were captured using a KEYENCE VHX-S550E microscope (VHX-J20T lens).

Results

Synthesis of multiplex tRNA-gRNA modules using Type II S cloning

We adapted the protocol originally developed for rice (Xie et al. 2015) to clone tRNA-gRNA modules using the Loop assembly Type II S cloning (Pollak et al. 2019; Sauret-Güeto et al. 2020) to enable efficient gene editing in *M. polymorpha* (**Fig. 1 and 2**). The process begins with primer design and PCR amplification of the tRNA-gRNA parts from the pGTR plasmid (Xie et al. 2015; Addgene #63143) which contains the tRNA and gRNA sequences (**Fig. 2A**). The PCR products contain the tRNA sequence, the gRNA scaffold and a part of the gRNA protospacer sequence. These PCR products are then fused together to reconstitute multiplexed tRNA-gRNA-protospacer-gRNA-scaffold modules (up to three in our trials but the systems allows to combine a greater number) and placed under the control of the MpU6 promoter in a L1 vector (OpenPlant plasmids OP-074, Addgene #136136 or OP-075, Addgene #136137 (Sauret-Güeto et al. 2020))

via BbsI-mediated Loop assembly cloning (**Fig. 2B**). After transformation of the L1 Loop reaction into *E. coli* cells, screening 1-3 colonies was sufficient to identify correctly assembled constructs in our trials. Finally, the L1 tRNA-gRNA construct(s) is (are) transferred into the L2 pCsA (Addgene #136067 (Sauret-Güeto et al. 2020)) acceptor vector through another round of SapI-mediated Loop assembly (**Fig. 2C**), linking the tRNA-gRNA unit with the transcription unit for Cas9 expression (Addgene #136135 (Sauret-Güeto et al. 2020)) and a transcription unit containing the desired selection marker. In summary, this protocol enables simple, fast and efficient generation of constructs for targeted gene editing in *M. polymorpha*, and reduces the number of experimental steps from seven to three compared to the original protocol (Xie et al. 2015).

tRNA-gRNA multiplexing improves genome editing efficiency by enabling the simultaneous delivery of multiple gRNAs

To test efficiency of the new system, we targeted the *M. polymorpha* *GOLDEN-2 LIKE* (MpGLK) gene mutations of which lead to reduced levels of chlorophyll (Yelina et al. 2024; Frangedakis et al. 2024; Hernández-Muñoz et al. 2024), and result in an easily observable, non-lethal phenotype which is useful for the evaluation of CRISPR/Cas9 efficiency. It also allows observation of the specific regions of *M. polymorpha* thallus where mutations arise. Knowing where the mutations primarily arise, is particularly important for identifying mutated areas of the plant when a gene mutation does not produce an obvious phenotype. In all our tests the Cam-1/2 *M. polymorpha* accession was used (Delmans et al., 2017).

Using the tRNA-gRNA system, we constructed vectors to express one (gRNA3) or three gRNAs (gRNA3, gRNA7 and gRNA14) to target the MpGLK locus to generate large deletions (**Fig. 3A**). The selection of those three gRNAs was based on their *in vitro* activity in a cleavage assay (see Methods) that assessed their targeting performance (**Fig. S1A-B**). Additionally, as a control, we included a vector allowing gRNA3 expression from the commonly used MpU6 promoter without tRNA sequences (**Fig. 3A**). We transformed all three constructs into sporelings. In all cases, we observed regenerated thalli with pale green sectors, indicating successful disruption of the MpGLK gene. We classified the resulting plants into three distinct phenotypic categories: wild-type, chimeric, and fully mutant (**Fig. 3A**). For the control vector, expressing the gRNA3 without tRNA sequences, we observed approximately 88% wild-type, 10% chimeric, and 2% fully mutant plants (**Fig. 3B**). These numbers were comparable to those obtained when expressing the same gRNA3 with the tRNA sequences (**Fig. 3B**). Upon delivering the three gRNAs (gRNA3, gRNA7 and gRNA14) targeting MpGLK, approximately 55% of the primary transformants exhibited wild-

type, 33% chimeric, and 12% fully mutant phenotypes (**Fig. 3B**). Overall, the editing efficiency was 12.5%, when the gRNA was expressed without the presence of the tRNA sequences, 13.9% with the presence of tRNA sequences and a single gRNA and 45% with multiple tRNA-gRNA modules (**Fig. 3C**). It must be noted that our approach to estimate the editing efficiency has the limitation that silent mutations are not calculated thus the actual editing efficiency is likely higher. To confirm successful gene disruption, we performed Sanger sequencing, which validated the expected mutations (**Fig. 3D-E and Fig. S1C**). To estimate the frequency of large deletions, we genotyped 60 independent mutant lines and found that 10% harbored large deletions (~600-800 bp, between gRNA3&7 or gRNA7&14) (**Fig. 3F and Fig. S1C**).

To further validate our new method, we also targeted the *MpRR-MYB2* gene (**Fig. S2A**) the mutation of which does not have a phenotype that differs from the wild type plants (**Fig. S2B**) (Frangedakis et al., 2024). We generated two vectors that allow the expression of a gRNA targeting the first exon of with or without the presence of tRNA sequences (**Fig. S2C**). We sequenced 34 - 40 independent lines transformed with each vector and ~11% - 17.5% of the mutation were not altering the open reading frame (silent mutation) (**Fig. S2C-D**). The overall editing efficiency was between 73.5% and 82.5% when the gRNA was expressed without or with a tRNA sequence respectively (**Fig. S2C**), higher compared to the experiments where we targeted *MpGLK*, possibly due to the higher efficiency of the gRNA to target Cas9 to the desired loci.

Collectively, our results demonstrate a statistically significant increase in gene-editing efficiency when using tRNA-gRNA multiplexing compared to vectors expressing a single gRNA, with or without tRNA. This enhanced efficiency is likely due to the system's ability to deliver multiple gRNAs simultaneously and consequently increasing the chances of successfully targeting the Cas9 to the desired loci.

A simplified and optimized thallus transformation method for *M. polymorpha*

Stable transformation of *M. polymorpha* can be obtained using either germinating spores or thallus fragments (Iwakawa et al. 2021; Kubota et al. 2013; Ishizaki et al. 2008). Spore-based transformation offers high efficiency but requires approximately three months for spore production and can be challenging for certain *M. polymorpha* accessions. Furthermore, transformants derived from spores consist of a mix of male and female plants and are not isogenic. In contrast, thallus transformation uses thallus fragments or gemmalings as tissue material, which are more

easily obtained than the spores and yield an isogenic population of transformants. Thallus transformation also allows the introduction of additional transgenes into specific mutant strains without segregation, as it bypasses sexual reproduction.

Here, we present a simplified protocol for *M. polymorpha* thallus transformation, adapted from a method originally developed for hornwort thallus transformation (Waller et al. 2023). This protocol utilizes the 2-(N-morpholino)ethanesulfonic acid (MES) buffer to maintain a stable pH in the co-cultivation medium. Maintaining a stable pH of 5.5 during co-cultivation has been shown to significantly enhance *Agrobacterium*-mediated infection in *Arabidopsis thaliana* and also hornworts, likely by inhibiting calcium-mediated defense signaling (Y.-C. Wang et al. 2018; Waller et al. 2023). This optimized protocol omits the need for sucrose (Kubota et al. 2013) treatment of the regenerating tissue prior to co-cultivation with the *Agrobacterium* and reduces the required volumes of co-cultivation media, effectively miniaturizing the process.

The protocol consists of the following steps (**Fig. 4A&B and Fig. S3A**): Three two-weeks-old gemmalings were transferred to a well of a 6-well plate, containing 5 mL of liquid KNOP medium supplemented with 1% (w/v) sucrose and 10mM MES, pH 5.5. The gemmalings were then fragmented with a scalpel (into approximately 20-25 fragments) and acetosyringone was added. The tissue was co-cultivated with *Agrobacterium tumefaciens* carrying an *eGFP* gene fused to a cytoplasm targeting signal and hygromycin selectable marker gene, for three days on a shaker at 110 rpm, under ambient light and 21°C. After co-cultivation, thallus fragments were transferred to growth media containing hygromycin or the selectable marker of choice. Successful transformants, identifiable by *eGFP* fluorescence under a dissecting microscope, became visible from three to six weeks (**Fig. 4C-G**).

To further optimize the protocol, we evaluated the effect of MES concentration on transformation efficiency by adding it to the co-cultivation medium at concentrations ranging from 0 to 50 mM. *eGFP* fluorescence was used to estimate transformation efficiency (**Fig. 4C**). Increasing MES concentration to 30 mM resulted in more transformants, although concentrations above 40 mM led to a decrease in the total number of recovered transformants. The optimal MES concentration was therefore determined to be 30 mM. Interestingly, in many cases a single thallus fragment gave rise to more than one regenerated fragment/putative transformant (**Fig. 4C**). However not all regenerated fragments showed fluorescence potentially due to a partial T-DNA transfer or gene silencing (**Fig. 4D-H**). On average, we observed a transformation efficiency of approximately 30

transgenic plants per 20 fragments across different batches (**Fig. 4H**). All subsequent trials were performed using a MES concentration of 30 mM. The new method can be applied not only to gemmaling tissue but also to more mature thallus tissue, increasing our method's flexibility and ease of use. However, under our conditions, efficiency was reduced in older tissue (**Fig. S3B**). To address the reduced efficiency, scaling up the protocol using co-cultivation in flasks could be an alternative. Overall, our updated thallus transformation method facilitates the straight-forward generation of isogenic transformants, offering a rapid approach for genetic studies and functional analyses in *M. polymorpha*.

The optimized thallus transformation allows efficient generation of single and double mutants

Next, we tested whether our improved thallus transformation system could be used to obtain CRISPR/Cas9 mutants. To do this, we used the same vector as was used for the sporeling transformation trials to express three gRNAs targeting the MpGLK gene (**Fig. 5A**). Similarly to sporeling transformation, the plants resulting from thallus transformation fell into three distinct phenotypic categories: wild-type, chimeric, and fully mutant (**Fig. 5B**). We transformed approximately 200 thallus fragments and 74%, 23% and 3% of the primary transformants exhibited wild-type, chimeric, and fully mutant phenotypes respectively (**Fig. 5C**). Notably, chimeric plants displayed multiple sectors with the mutant phenotype, indicating that mutations in each sector arose independently (**Fig. 5B**). Mutant sectors mostly appeared close to the meristematic notch (**Fig. S4A**), possibly due to the stronger activity of the MpEf1a promoter that drives Cas9 expression in our constructs (Tse et al. 2024; Althoff et al. 2014). This observation is particularly valuable when a mutation does not result in an obvious phenotype, as tissues near the notch meristematic areas, become top candidates for identifying mutant regions. However, we also noticed that mutant sectors can arise in more mature areas of the thallus (**Fig. S4B**).

To verify successful gene disruption, we conducted Sanger sequencing, which confirmed the expected mutations (**Fig. 5D-E and Fig. S1D**). To evaluate the occurrence of large deletions, we genotyped 60 independent mutant lines and observed that 2% carried a ~765 bp deletion (gRNA3&7) (**Fig. 5E**), which was confirmed with Sanger sequencing (**Fig. 1B**).

We also assessed whether our improved thallus transformation method could be used to generate double mutants. In this approach, thalli from a mutant plant were subject to secondary transformation using an L2 construct containing a different selectable marker and a transcription

unit driving expression of a gRNA targeting a second gene (in this case *MpRR-MYB5*) (**Fig. 6A**). There was no need to include a separate transcription unit for Cas9 expression, as it was already present in the mutant background, simplifying the process. We selected the *MpRR-MYB5* gene as a target, because, similarly to *MpGLK*, its mutations lead to reduced chlorophyll, and, therefore, an easily observable, non-lethal phenotype ideal for the evaluation of gene editing efficiency (Frangedakis et al. 2024). We targeted *MpRR-MYB5* in two different mutant backgrounds, *Mpgata4* and *Mpglk* (Yelina et al. 2024) (**Fig. 6B&C**). We used two different mutant backgrounds with distinct thallus morphologies and phenotypes to test the effectiveness of our protocol across varying mutant thallus types. The resulting plants again fell into three distinct phenotypic categories: original mutant background, chimeric, and fully mutant (**Fig. 6B&C**). We transformed approximately 100 thallus fragments of *Mpgata4* or *Mpglk* mutant plants. Of the recovered transformants (n=100), 85% were wild type for *MpRR-MYB5*; 12% were chimeric and 3% were fully mutant for *Mprr-myb5* (**Fig. 6D**). Out of the transformants recovered from transforming *Mpglk* mutant thallus fragments (n=100), 78% were wild type for *MpRR-MYB5*; 20% were chimeric and 2% were fully mutant for *Mprr-myb5* (**Fig. 6E**). Successful gene disruption was confirmed by Sanger sequencing (**Fig. 6D&E**).

We also investigated whether our system could achieve simultaneous disruption of two genes using thallus tissue as the transformation material. To this end, we selected *MpGLK* and *MpRR-MYB5* and constructed a plasmid allowing the expression of two guide RNAs, each specifically targeting one of the genes (**Fig. S5A**). Following transformation, we obtained single mutants for *Mpglk* (20%) and *Mprr-myb5* (9.8%), as well as double mutants harboring disruptions in both genes (*Mpglk;Mprr-myb5*) (3.2%) (**Fig. S5B-C**). After allowing the plants to grow for an additional two weeks, we observed the emergence of several secondary mutations, predominantly localized in the meristematic regions (**Fig. S5B-D**). Approximately 17.5% of the meristematic areas contained secondary double mutations (**Fig. S5-E**).

Collectively, our results demonstrate that the optimized thallus transformation system is a reliable and efficient method for generating both single and double CRISPR/Cas9-mediated mutants, including in mutant backgrounds with altered thallus tissue morphology or development.

Conclusions

CRISPR-based genome editing tools have significantly advanced biology (Chen et al. 2019; J. Y. Wang and Doudna 2023; Pacesa, Pelea, and Jinek 2024), and their application to *M. polymorpha*

(Sugano et al. 2018; Sauret-Güeto et al. 2020; Sugano et al. 2014) has enhanced substantially this model organism's utility.

In this study, we successfully adapted gRNA multiplexing by expressing gRNAs from a single transcriptional tRNA-gRNA unit (Xie, Minkenberg, and Yang 2015) for CRISPR/Cas9 genome editing in *M. polymorpha*. By incorporating tRNA sequences into the OpenPlant CRISPR/Cas9 kit vector system, we demonstrated that tRNA-gRNA approach results in functional gRNAs. Importantly, our approach for cloning the tRNA-gRNA module into the transformation vector is significantly simplified and can also be adapted to other *M. polymorpha* CRISPR/Cas9 vector systems.

Our approach streamlines genome editing in *M. polymorpha*, allowing the introduction of multiple modifications, deletion of large DNA fragments, in a single or multiple loci, in a single transformation event. Furthermore, our new method, when combined with the optimized thallus transformation protocol we present, simplifies mutant generation by eliminating the need for spore production, which takes ~2-3 months. This is particularly useful for generating secondary mutations in mutant backgrounds, especially for those that are unable to produce spores. The targeting of genes the mutations if which generate a visible phenotype, allowed to identify the notch regions in *M. polymorpha* thallus as the areas where mutations occur more often. This observation can facilitate the identification of edited parts of the plant, when a mutation does not result in an obvious phenotype.

Beyond fundamental research, *M. polymorpha* is a promising chassis for synthetic biology applications (Sauret-Güeto et al. 2020; Frangedakis et al. 2021; Boehm et al. 2017). It offers several advantages, including a relatively fast life cycle, simpler tissue organisation compared to other plant models in addition to well characterized nuclear and plastid genomes which are both amenable to genetic manipulation and high-throughput approaches (Annese et al. 2025). To date, up to five different selectable markers have been tested in *M. polymorpha*, further expanding the capacity to introduce multiple transgenes (Ishizaki et al. 2015; Robinson et al. 2024). All these can facilitate the reprogramming of gene regulatory networks with high precision and speed. Moreover, *M. polymorpha* can potentially serve as a platform for testing and developing novel biotechnological tools, such as biosensors, bio-based production of high value compounds, and cell type specific metabolic engineering (Tansley, Patron, and Guiziou 2024; Yang and Reyna-Llorens 2023). Its ability to produce complex secondary metabolites further enhances its potential for bioengineering (Lindström Battle and Sweetlove 2025; Forestier et al. 2025). As a result,

developing improved CRISPR/Cas9 tools in *M. polymorpha* can significantly enhance the versatility of genome engineering approaches in this model system. One promising strategy involves the use of tRNA-based expression systems for gRNAs, which enables their transcription under RNA polymerase II promoters (Pol II). Unlike traditional U6 promoters, which are transcribed by RNA polymerase III and drive constitutive, ubiquitous expression, Pol II promoters offer greater regulatory flexibility. This includes the ability to achieve cell-type-specific or inducible expression of gRNAs, thereby allowing spatial and temporal control over genome editing activity leveraging on recent work on the transcription factor promoter activity landscape in *M. polymorpha* (Romani et al. 2024). It can also allow to explore CRISPR-based multiplex gene regulation systems to simultaneously control the expression of multiple genes in a coordinated way. One notable application of multiplexing is metabolic pathway optimization, where several enzymes within a biosynthetic pathway are concurrently modulated to enhance the production of valuable compounds.

In conclusion, our study establishes an improved genome editing approach for *M. polymorpha* by using the tRNA-gRNA multiplexing system. This strategy enables the simultaneous delivery of multiple gRNAs into the plant, facilitating the editing of single genes or multiple loci in parallel. Our method not only enhances editing efficiency but also expands the potential for more complex genetic modifications in *M. polymorpha*, laying the groundwork for advanced functional genomics and synthetic biology applications in this model organism.

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1: In vitro test of MgGLK gRNAs and genotyping results

Fig. S2: Generation of MpRR-MYB2 mutants

Fig. S3: Key steps of optimized thallus transformation Fig. S4:

Fig. S4: Images of chimeric Mpglk mutants

Fig. S5: Generation of Mpglk,rr-myb5 double mutants

Table S1: List of primers used in this study

Acknowledgements

We thank Keiko Sakakibara from Rikkyo University, Japan, for the support during the last stage of the project.

Author contributions

E.F. and N.Y.E. designed the work. E.F., N.Y.E., S.K.E., F.R., and A.F., carried out the work. J.H and J.M.H oversaw the project. E.F. wrote the manuscript with input from all authors.

Conflict of interest

The authors declare no competing interests.

Funding

This work was funded as part of the BBSRC/EPSRC OpenPlant Synthetic Biology Research Centre Grant BB/ L014130/1 to J.H., BBSRC BB/F011458/1 for confocal microscopy to J.H, BBP0031171 to J.M.H.

Data availability

All data supporting the conclusions of this study are contained within the manuscript and in the online supplementary data.

Fig. 1: Schematic diagram of tRNA-gRNA multiplexing for plant genome editing

(A) A plasmid is constructed containing a tRNA-gRNA cassette driven by the U6 promoter, along with a transcription unit for Cas9 expression.

(B) After transformation into the plant, the primary RNA transcript is cleaved by endogenous tRNA-processing enzymes, RNase P and RNase Z, resulting in functional guide RNAs for genome editing

Fig. 2: Schematic diagram of the workflow for generating a tRNA-mediated multiplex gRNA expression construct

(A) Top: The first primer includes a portion of the tRNA sequence along with a BbsI recognition site and four-base overhang sequences for Loop assembly. The middle primers contain BbsI recognition sites and four-base overlapping overhang sequences for Loop assembly, which can correspond to any four consecutive nucleotides (highlighted with colored rectangles) within the 20-nucleotide gRNA sequence (shown with numbers from 1 to 20). Forward middle primers also contain a portion of the gRNA scaffold sequence, whereas reverse middle primers contain a portion of the tRNA sequence. The final primer includes part of the tRNA sequence, the full reverse-complement of the last gRNA, and a BbsI recognition site plus four-base overhang sequences for Loop assembly. Overhangs for cloning into the acceptor vectors shown with pink letters. Bottom: The pGTR plasmid should be used as the template for the PCR reactions. The primer combinations used for amplification are as follows: (1) G-primer-F & gRNA a Primer R, (2) gRNA a Primer F & gRNA b Primer R, and (3) gRNA b Primer F & gRNA c Primer R, n: any nucleotide (please see **Supplemental Information** for a detailed explanation and an example of primer design).

(B) After PCR amplification and gel extraction, all fragments are combined with the OP-074 or OP-075 vector (Sauret-Gueto et al., 2020) in a Loop Assembly/Type IIS cloning reaction (see **Supplemental Information** for a detailed protocol of Loop Assembly L1 cloning). LacZ: lacZα cassette for blue-white screening of colonies (negative blue colonies contain undigested L1 vectors, while positive white colonies contain tRNA-gRNA parts inserted into the L1 vectors).

(C) Finally, the tRNA-gRNA-OP-074 or OP-075 vector is combined with the OP-073 vector, which contains the MpEF1a::Cas9 transcription unit, an appropriate L1 vector for plant selection, in a Loop Assembly/Type IIS cloning reaction (see **Supplemental Information** for a detailed protocol of Loop Assembly L2 cloning).

Fig. 3: Evaluation of mutant generation using the tRNA-mediated multiplex gRNA expression method

(A) Left: Schematic representation of the plant transformation vector for simultaneous delivery of one or three gRNAs and Cas9 into *M. polymorpha* plants (germinating spores) by *Agrobacterium*-mediated transformation, with or without the use of tRNAs. Right: The three phenotypic classes of regenerating *M. polymorpha* plants: wild-type (white arrows), chimeric (yellow arrows), and mutant (black arrows). Scale bar: 400 μ m.

(B) Comparison of the number of transformants with wild-type, chimeric, or mutant phenotypes. Graphs show values from triplicate experiments (dots) and their average (bars). Error bars represent the SEM. A pairwise t-test was conducted to compare the number of chimeric mutants (generated using either the U6 tRNA-gRNA vector or U6 tRNA-gRNA vector) against the number of plants generated using the control vector (U6 gRNA). ** for $p < 0.01$, while no significant difference (n.s) was noted for $p > 0.05$. A similar pairwise test was performed for the fully mutated plants.

(C) Summary of editing efficiency in experiments described in A and B.

(D-E) Sequence analysis of *Mpglk* mutant lines. Schematic representation of *MpGLK* gene structure showing exons as blue rectangles, untranslated regions (UTRs) as grey rectangles, and introns as grey lines. The position of the gRNA used for CRISPR/Cas9 gene editing is indicated with an arrow. The wild-type *M. polymorpha* Cam-1 sequence is shown at the top, with the 20-bp gRNA target sequence highlighted in grey. Mutations are highlighted in red.

(F) Gel electrophoresis images of PCR-based genotyping of *Mpglk* mutants obtained using the vector that allows the expression of three gRNAs. The positions of primers used are shown with red arrowheads. The expected size of PCR products when large deletions occurred is approximately ~600-800 bp (lanes 1-3). The expected size of PCR products for wild-type or mutants with small deletions is ~1648 bp (lanes 4, 5, 6 and WT).

Fig. 4: Optimized *M. polymorpha* thallus transformation protocol

(A) Outline of the thallus transformation protocol steps: Thallus tissue is transferred into a 6-well plate containing 5 mL of co-cultivation medium and then fragmented with the aid of a sterile scalpel. The tissue is co-cultivated with *Agrobacterium* for three days and then spread on antibiotic-containing growth medium. After approximately 4–6 weeks, putative transformants are visible as regenerating green tissue.

(B) Representative images of *M. polymorpha* 2-week-old gemmae, which are then transferred into a 6-well plate and fragmented prior to the addition of *Agrobacterium*.

(C) Schematic representation of constructs for the expression of two transcription units (TUs): one TU for the expression of the *hygromycin B phosphotransferase (hph)* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and one TU for the expression of eGFP under the control of the CaMV 35S promoter, targeted to the plasma membrane using the Lti6b localization signal. Representative images of *M. polymorpha* thallus fragments after 4 weeks on antibiotic-containing growth medium show regenerating tissue (white arrows) that is potentially successfully transformed. Untransformed, dying tissue is indicated with yellow arrows. Scale bar: 100 μ m.

(D-G) Images of *M. polymorpha* thallus tissue expressing p-35S::eGFP-Lti6b for plasma membrane localization. Scale bar: 1 mm.

(H) Comparison of the number of transformants (per 20 thallus fragments) under different MES concentrations in the co-cultivation medium (initial pH set to 5.5). Graphs show values from triplicate experiments (dots) and their average (bars). Error bars represent the SEM.

Fig. 5: Generation of mutants using the optimized thallus transformation

(A) Schematic representation of the plant transformation vector for simultaneous delivery of three gRNAs and Cas9 into *M. polymorpha* plants (thallus fragments) by *Agrobacterium*-mediated transformation, with or without the use of tRNAs.

(B) The three phenotypic classes of regenerating *M. polymorpha* plants: wild-type, chimeric, and mutant. Scale bar: 1 mm.

(C) Comparison of the number of transformants with wild-type, chimeric, or mutant phenotypes. n = 100.

(D) Sequence analysis of *Mpglk* mutant lines. Top: Schematic representation of *MpGLK* gene structure. The position of the gRNA used for CRISPR/Cas9 gene editing is shown with an arrow. Bottom: Mutant genotyping analysis. The wild-type *M. polymorpha* Cam-1 sequence is shown at the top, with the 20-bp gRNA target sequence highlighted in grey. Mutations are highlighted in red.

(E) Gel electrophoresis images of PCR-based genotyping of *Mpglk* mutants obtained using the vector that allows the expression of three gRNAs. Schematic representation of *MpGLK* gene structure showing exons as blue rectangles, untranslated regions (UTRs) as grey rectangles, and introns as grey lines. Positions of primers used are shown with red arrowheads. The expected size of PCR products when large deletions occur is approximately 765 bp (lanes 1 and 2). The expected size of PCR products for wild-type or mutants with small deletions is 1648 bp (lanes 3, 4, 5 and WT).

Fig. 6: Generation of double mutants using the optimised thallus transformation

(A) Schematic representation of the plant transformation vector for delivery of a gRNA for targeting *MpRR-MYB5* into *Mpgata4* or *Mpglk* mutant *M. polymorpha* plants (thallus fragments) by *Agrobacterium*-mediated transformation. mALS: Chlorsulfuron selection.

(B-C) The three phenotypic classes of regenerating *Mpgata4* and *Mpglk* mutant plants transformed with the L2 vector (A) for targeting *MpRR-MYB5*: *Mpgata4*, chimeric (yellow arrow indicates thallus part with no visible mutation, white arrows indicate thallus part with a secondary mutation), and mutant or *Mpglk*, chimeric, and mutant. Scale bar: 1 mm

(D-E) Top: Schematic representation of *MpRR-MYB5* gene structure showing exons as blue rectangles, untranslated regions (UTRs) as grey rectangles and introns as grey lines. Positions of gRNAs used for CRISPR/Cas9 gene editing are shown as arrows. Middle: Comparison of the number of transformants with *Mpglk,rr-myb5* or *Mpgata4,rr-myb5* chimeric or mutant phenotypes. n=100. Bottom: Sequence analysis of *Mpglk,rr-myb5* and *Mpgata4,rr-myb5* double mutant lines. The wild-type *M. polymorpha* Cam-1 sequence is shown at the top, with the 20 bp gRNA target sequence highlighted with grey. Mutations are highlighted in red.

651
652
653
654
655
656
657
658
659
660
661
662
663
664

665

666

667

668

669

670

671

672

673

674

REFERENCES

676 **Adli M.** 2018. The CRISPR tool kit for genome editing and beyond. Nature communications **9**,
677 1911.

678 **Althoff F, Kopischke S, Zobell O, Ide K, Ishizaki K, Kohchi T, Zachgo S.** 2014. Comparison
679 of the MpEF1 α and CaMV35 promoters for application in *Marchantia polymorpha*
680 overexpression studies. *Transgenic research* **23**, 235–244.

681 **Annese D, Romani F, Grandellis C, Ives L, Frangedakis E, Buson FX, Molloy JC, Haseloff**
682 **J.** 2025. Semi-automated workflow for high-throughput *Agrobacterium*-mediated plant
683 transformation. *The Plant journal : for cell and molecular biology* **122**, e70118.

684 **Boehm CR, Pollak B, Purswani N, Patron N, Haseloff J.** 2017. Synthetic Botany. Cold Spring
685 Harbor perspectives in biology **9**.

686 **Bowman JL, Arteaga-Vazquez M, Berger F, et al.** 2022. The renaissance and enlightenment
687 of *Marchantia* as a model system. *The Plant cell* **34**, 3512–3542.

688 **Čermák T, Curtin SJ, Gil-Humanes J, et al.** 2017. A Multipurpose Toolkit to Enable Advanced
689 Genome Engineering in Plants. *The Plant cell* **29**, 1196–1217.

690 **Chen K, Wang Y, Zhang R, Zhang H, Gao C.** 2019. CRISPR/Cas Genome Editing and
691 Precision Plant Breeding in Agriculture. *Annual review of plant biology* **70**, 667–697.

692 **Chery M, Drouard L.** 2022. Plant tRNA functions beyond their major role in translation. *Journal*
693 *of Experimental Botany* **74**, 2352–2363.

694 **Cong L, Ran FA, Cox D, et al.** 2013. Multiplex genome engineering using CRISPR/Cas
695 systems. *Science* **339**, 819–823.

696 **Delmans M, Pollak B, Haseloff J.** 2017. MarpoDB: An Open Registry for *Marchantia*
697 *Polymorpha* Genetic Parts. *Plant & cell physiology* **58**, e5.

698 **Dieci G, Fiorino G, Castelnuevo M, Teichmann M, Pagano A.** 2007. The expanding RNA
699 polymerase III transcriptome. *Trends in genetics : TIG* **23**.

700 **Donà M, Bradamante G, Bogojevic Z, Gutzat R, Streubel S, Mosiolek M, Dolan L,**
701 **Mittelsten Scheid O.** 2023. A versatile CRISPR-based system for lineage tracing in living
702 plants. *The Plant journal : for cell and molecular biology* **115**, 1169–1184.

703 **Forestier ECF, Asprilla P, Romani F, Bonter I, Frangedakis E, Haseloff J.** 2025. The
704 ABCG1 transporter facilitates sesquiterpene accumulation in *Marchantia polymorpha* oil bodies.
705 *bioRxiv*, 2025.02.05.636625.

706 **Frangedakis E, Guzman-Chavez F, Rebmann M, et al.** 2021. Construction of DNA Tools for
707 Hyperexpression in Chloroplasts. *ACS synthetic biology* **10**, 1651–1666.

708 **Frangedakis E, Yelina NE, Billakurthi K, Hua L, Schreier T, Dickinson PJ, Tomaselli M,**
709 **Haseloff J, Hibberd JM.** 2024. MYB-related transcription factors control chloroplast biogenesis.
710 *Cell* **187**, 4859–4876.e22.

711 **Gupta OP, Karkute SG.** 2021. *Genome Editing in Plants: Principles and Applications*. CRC
712 Press.

713 **Hernández-Muñoz A, Agreda-Laguna KA, Ramírez-Bernabé IE, Oltehua-López O, Arteaga-**
714 **Vázquez MA, Leon P.** 2024. *Marchantia polymorpha* GOLDEN2-LIKE transcriptional factor; a

715 central regulator of chloroplast and plant vegetative development. *The New phytologist* **243**,
716 1406–1423.

717 **Hui L, Zhao M, He J, et al.** 2019. A simple and reliable method for creating PCR-detectable
718 mutants in *Arabidopsis* with the polycistronic tRNA–gRNA CRISPR/Cas9 system. *Acta*
719 *Physiologiae Plantarum* **41**, 1–14.

720 **Ishizaki K, Chiyoda S, Yamato KT, Kohchi T.** 2008. *Agrobacterium*-mediated transformation
721 of the haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. *Plant &*
722 *cell physiology* **49**, 1084–1091.

723 **Ishizaki K, Nishihama R, Ueda M, Inoue K, Ishida S, Nishimura Y, Shikanai T, Kohchi T.**
724 2015. Development of Gateway Binary Vector Series with Four Different Selection Markers for
725 the Liverwort *Marchantia polymorpha*. *PloS one* **10**, e0138876.

726 **Ishizaki K, Nishihama R, Yamato KT, Kohchi T.** 2016. Molecular Genetic Tools and
727 Techniques for *Marchantia polymorpha* Research. *Plant & cell physiology* **57**, 262–270.

728 **Iwakawa H, Melkonian K, Schlüter T, Jeon H-W, Nishihama R, Motose H, Nakagami H.**
729 2021. *Agrobacterium*-Mediated Transient Transformation of *Marchantia* Liverworts. *Plant & cell*
730 *physiology* **62**, 1718–1727.

731 **Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E.** 2012. A
732 programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*
733 **337**, 816–821.

734 **Kocaoglan EG, Radhakrishnan D, Nakayama N.** 2023. Synthetic developmental biology:
735 molecular tools to re-design plant shoots and roots. *Journal of experimental botany* **74**, 3864–
736 3876.

737 **Kohchi T, Yamato KT, Ishizaki K, Yamaoka S, Nishihama R.** 2021. Development and
738 Molecular Genetics of. *Annual review of plant biology* **72**, 677–702.

739 **Kubota A, Ishizaki K, Hosaka M, Kohchi T.** 2013. Efficient *Agrobacterium*-mediated
740 transformation of the liverwort *Marchantia polymorpha* using regenerating thalli. *Bioscience,*
741 *biotechnology, and biochemistry* **77**, 167–172.

742 **Lindström Battle AL, Sweetlove LJ.** 2025. Bryophytes as metabolic engineering platforms.
743 *Current opinion in plant biology* **85**, 102702.

744 **Ma C, Zhu C, Zheng M, Liu M, Zhang D, Liu B, Li Q, Si J, Ren X, Song H.** 2019.
745 CRISPR/Cas9-mediated multiple gene editing in var. using the endogenous tRNA-processing
746 system. *Horticulture research* **6**, 20.

747 **Naramoto S, Hata Y, Fujita T, Kyojuka J.** 2022. The bryophytes *Physcomitrium patens* and
748 *Marchantia polymorpha* as model systems for studying evolutionary cell and developmental
749 biology in plants. *The Plant cell* **34**, 228–246.

750 **Pacesa M, Pelea O, Jinek M.** 2024. Past, present, and future of CRISPR genome editing
751 technologies. *Cell* **187**, 1076–1100.

752 **Pollak B, Cerda A, Delmans M, Álamos S, Moyano T, West A, Gutiérrez RA, Patron NJ,**
753 **Federici F, Haseloff J.** 2019. Loop assembly: a simple and open system for recursive
754 fabrication of DNA circuits. *The New phytologist* **222**, 628–640.

755 **Qi W, Zhu T, Tian Z, Li C, Zhang W, Song R.** 2016. High-efficiency CRISPR/Cas9 multiplex
756 gene editing using the glycine tRNA-processing system-based strategy in maize. *BMC*
757 *biotechnology* **16**.

758 **Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F.** 2013. Genome engineering
759 using the CRISPR-Cas9 system. *Nature protocols* **8**, 2281–2308.

760 **Robinson K, Chia K-S, Guyon A, Schornack S, Carella P.** 2024. An efficient sulfadiazine
761 selection scheme for stable transformation in the model liverwort *Marchantia polymorpha*.
762 *Journal of experimental botany* **75**, 5585–5591.

763 **Romani F, Sauret-Güeto S, Rebmann M, et al.** 2024. The landscape of transcription factor
764 promoter activity during vegetative development in *Marchantia*. *The Plant cell* **36**, 2140–2159.

765 **Sauret-Güeto S, Frangedakis E, Silvestri L, Rebmann M, Tomaselli M, Markel K, Delmans**
766 **M, West A, Patron NJ, Haseloff J.** 2020. Systematic Tools for Reprogramming Plant Gene
767 Expression in a Simple Model, *Marchantia polymorpha*. *ACS Synthetic*

768 **Sugano SS, Nishihama R, Shirakawa M, Takagi J, Matsuda Y, Ishida S, Shimada T, Hara-**
769 **Nishimura I, Osakabe K, Kohchi T.** 2018. Efficient CRISPR/Cas9-based genome editing and
770 its application to conditional genetic analysis in *Marchantia polymorpha*. *PloS one* **13**,
771 e0205117.

772 **Sugano SS, Shirakawa M, Takagi J, Matsuda Y, Shimada T, Hara-Nishimura I, Kohchi T.**
773 2014. CRISPR/Cas9-mediated targeted mutagenesis in the liverwort *Marchantia polymorpha* L.
774 *Plant & cell physiology* **55**, 475–481.

775 **Tang X, Chen S, Yu H, Zheng X, Zhang F, Deng X, Xu Q.** 2021. Development of a gRNA-
776 tRNA array of CRISPR/Cas9 in combination with grafting technique to improve gene-editing
777 efficiency of sweet orange. *Plant cell reports* **40**.

778 **Tansley C, Patron NJ, Guiziou S.** 2024. Engineering Plant Cell Fates and Functions for
779 Agriculture and Industry. *ACS synthetic biology* **13**, 998–1005.

780 **Tse SW, Annese D, Romani F, Guzman-Chavez F, Bonter I, Forestier E, Frangedakis E,**
781 **Haseloff J.** 2024. Optimizing Promoters and Subcellular Localization for Constitutive Transgene
782 Expression in *Marchantia polymorpha*. *Plant & cell physiology* **65**, 1298–1309.

783 **Waller M, Frangedakis E, Marron AO, Sauret-Güeto S, Rever J, Sabbagh CRR, Hibberd**
784 **JM, Haseloff J, Renzaglia KS, Szövényi P.** 2023. An optimized transformation protocol for
785 *Anthoceros agrestis* and three more hornwort species. *The Plant journal* **114**, 699–718.

786 **Wang JY, Doudna JA.** 2023. CRISPR technology: A decade of genome editing is only the
787 beginning. *Science* **379**, eadd8643.

788 **Wang Z, Liu X, Xie X, Deng L, Zheng H, Pan H, Li D, Li L, Zhong C.** 2021. ABE8e with
789 Polycistronic tRNA-gRNA Expression Cassette Significantly Improves Adenine Base Editing
790 Efficiency in. *International journal of molecular sciences* **22**.

791 **Wang Y-C, Yu M, Shih P-Y, Wu H-Y, Lai E-M.** 2018a. Stable pH Suppresses Defense
792 Signaling and is the Key to Enhance Agrobacterium-Mediated Transient Expression in
793 Arabidopsis Seedlings. *Scientific Reports* **8**, 1–9.

794 **Wang P, Zhang J, Sun L, et al.** 2018b. High efficient multisites genome editing in allotetraploid
795 cotton (*Gossypium hirsutum*) using CRISPR/Cas9 system. *Plant Biotechnology Journal* **16**,
796 137–150.

797 **White RJ.** 2011. Transcription by RNA polymerase III: more complex than we thought. *Nature*
798 *reviews genetics* **12**, 459–463.

799 **Wilusz JE, JnBaptiste CK, Lu LY, Kuhn C-D, Joshua-Tor L, Sharp PA.** 2012. A triple helix
800 stabilizes the 3' ends of long noncoding RNAs that lack poly(A) tails. *Genes & development* **26**,
801 2392–2407.

802 **Xie K, Minkenberg B, Yang Y.** 2015. Boosting CRISPR/Cas9 multiplex editing capability with
803 the endogenous tRNA-processing system. *Proceedings of the National Academy of Sciences of*
804 *the United States of America* **112**, 3570–3575.

805 **Yang J-S, Reyna-Llorens I.** 2023. Plant synthetic biology: exploring the frontiers of sustainable
806 agriculture and fundamental plant biology. *Journal of experimental botany* **74**, 3787–3790.

807 **Yelina NE, Frangedakis E, Wang Z, et al.** 2024. Streamlined regulation of chloroplast
808 development in the liverwort *Marchantia polymorpha*. *Cell reports* **43**, 114696.

809