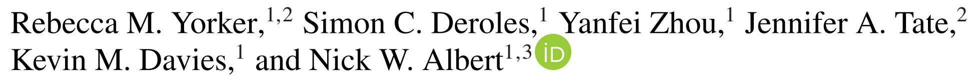
Simple and Efficient Transformation and Gene Editing of *Marchantia polymorpha* Spores



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Marchantia polymorpha (Marchantia) has become a model species for liverwort studies, owing to its rapid growth *in vitro*, ease of propagation, simple genetics, small genome, haploid-dominant life cycle, and because it is amenable to genetic transformation. Efficient transformation provides a foundation for many molecular and genetic analyses. The protocol described here is a simple and robust procedure for transforming Marchantia for a variety of applications, including gene overexpression and CRISPR genome editing. This simplified *Agrobacterium tumefaciens*-mediated transformation protocol targets spores, using common *Agrobacterium* strains GV3101 or EHA105, and overcomes challenges experienced in other methods. Spores are sterilized and distributed over sterile filter papers, which effectively retain spores and regenerating spores (known as sporelings). This approach enables the sporelings to be transferred to different agar growth media at different stages of transformation. A critical feature is preculturing the spores with acetosyringone (AS) prior to co-cultivation with *Agrobacterium*. This treatment profoundly enhances the transformation rate, particularly for *Agrobacterium* strain GV3101. GV3101 is preferred for its rapid growth rate, simple transformation, and lack of a recombinase (*recA*), stabilizing plasmids. The protocol is suitable for transforming Marchantia with constructs for CRISPR gene editing. Editing efficiency can be increased by introducing a heat-shock treatment during the transformation procedure, which increases the proportion of plants with larger edited sectors, facilitating mutant identification and propagation. Constructs and strategies for both overexpression and multiplex genome editing with sgRNA arrays using new and existing vectors are described. Using this spore transformation protocol for CRISPR gene editing, we routinely achieve 60% to 70% mutation rates, significantly reducing the effort required to generate and isolate mutants for functional analyses. © 2025 The Author(s). Current Protocols published by Wiley Periodicals LLC.

Basic Protocol: *Agrobacterium*-mediated transformation of *Marchantia polymorpha* spores
Support Protocol: Induction of spores
APPENDIX 1: Overexpression Vectors
APPENDIX 2: Multiplex CRISPR using tRNA arrays

Keywords: Marchantia • liverwort • transformation • mutagenesis • CRISPR/Cas9

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INTRODUCTION

Marchantia polymorpha (Marchantia) is a firmly established model liverwort species, with a growing set of resources and tools for genetics and molecular biology (Bowman et al., 2022). One major benefit is the capacity for genetic transformation, which enables varied molecular techniques to be performed, including gene overexpression and targeted mutagenesis using CRISPR gene editing. Yet transformation can become a bottleneck for research if procedures are inefficient, difficult, or yield inconsistent results. Several transformation protocols have been established for Marchantia that target different tissues/explants and employ different culture conditions, including biolistic (gene-gun) (Chiyoda et al., 2008) or *Agrobacterium*-mediated (Ishizaki et al., 2008; Tsuboyama & Kodama, 2014) transformation of spores (Chiyoda et al., 2008) and *Agrobacterium*-mediated transformation of thallus (Kubota et al., 2013; Seo et al., 2022) and gemmae (asexual propagules) (Tsuboyama et al., 2018). Thallus or gemmae transformations have the advantage of maintaining the genetic background (e.g., providing isogenic controls for complementation of mutants), but are relatively labor-intensive to generate, requiring precise timing and explant preparation. This can be difficult to master and is therefore not suited to students or researchers with limited transformation/tissue culture experience. Spore transformation meets researcher needs for most applications, producing high numbers of transformants for downstream analysis, and can be scaled up for applications such as tDNA mutagenesis (Honkanen et al., 2016) or activation tagging.

Several *Agrobacterium*-mediated spore transformation procedures have been reported, including in liquid culture (Ishizaki et al., 2008). In this case, the regenerating plants (sporelings) can become entangled, making it difficult to identify independent transformants. Agar-Trap methods address this issue by plating spores directly on solid agar medium, inoculating with *Agrobacterium*, and then pouring concentrated antibiotic stocks onto plates (Tsuboyama & Kodama, 2014). However, we encountered several problems when trialing this procedure, including bacterial overgrowth on the agar, spores detaching/washing away, and solutions not being sufficiently absorbed by the tissue culture medium. Furthermore, the antibiotic solutions are concentrated and must be calculated specifically based on medium volume per plate/tub, which varies if different volumes are poured into culture vessels.

In response to these challenges, we developed a simplified method that adopts some of the best features of existing methods with simple tissue culture practices (Fig. 1). Spore preparation and sterilization is simplified by using sodium dichloroisocyanurate tablets on entire desiccated archegoniophores, rather than the technically challenging sporangium dissections and surface sterilization procedures (Chiyoda et al., 2008), which also risk containment breaches if working with transgenic spores. Sterilized spores are plated onto filter papers overlying solid agar medium. The filter papers trap spores/sporelings, inhibit *Agrobacterium* overgrowth, and enable simple transfer of sporelings onto fresh selective medium. A critical feature is preculturing the spores with acetosyringone (AS) prior to co-cultivation with *Agrobacterium*. This simple

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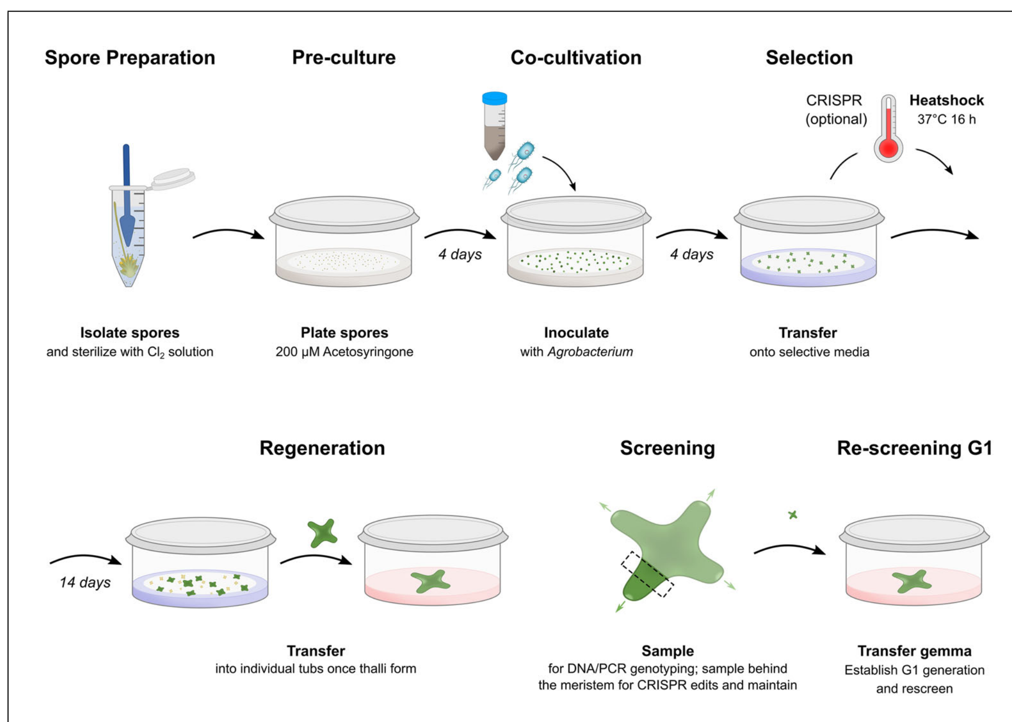

Figure 1 Overview of the *Marchantia polymorpha* spore transformation protocol.

intervention allows robust transformation, generating hundreds of transformants per tissue culture tub, using common *Agrobacterium* strains, including EHA105 and GV3101. The AS preculture is necessary even when *Agrobacterium* cultures are treated with AS to induce virulence. Marchantia produces different phenolic metabolites to natural host species of *Agrobacterium*, so the preculture may enable the accumulation of AS in sporelings to enable host identification and infection.

CRISPR gene editing methods are also well established for Marchantia, with vectors such as the pMpGE_En03/pMpGE010 series (Sugano et al., 2014, 2018). These vectors are compatible with this transformation protocol, and editing rates can be further enhanced by introducing heat-shock treatments. The initial CRISPR vectors enable expression of one guide RNA (single guide RNA, sgRNA, a fusion of crRNA and tracrRNA) and SpCas9. We have used these with synthesized tRNA-gRNA arrays to allow multiplex editing, with improved editing performance. This likely arises from improved sgRNA processing (tRNA cleavage, releasing sgRNAs from a polycistronic RNA precursor) and increased activity from multiple guides, which increases opportunities for sequence deletions.

This protocol is comprehensive, covering spore preparation, vector design, and a step-by-step transformation method. The **basic protocol** for *Agrobacterium*-mediated transformation of *Marchantia polymorpha* spores is simple and robust, using common materials and reagents. The simplicity and flexibility of timing make this protocol particularly suitable for researchers or students with limited transformation experience, enabling the production of high numbers of transformants for downstream analysis. It is suitable for a variety of applications, including overexpression or gene editing. A **support protocol** for producing spores necessary for transformation is provided. Finally, vectors for overexpressing genes of interest (APPENDIX 1) and multiplex gene editing using tRNA arrays are described (APPENDIX 2).

CAUTION: Many jurisdictions have laws and restrictions for producing or working with genetically modified organisms. Ensure you are aware of any regulations and guidelines

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that may apply to your work before commencing. All experimental procedures reported here were performed in accordance with research approvals granted in New Zealand (APP202127: GMD101562, GMD101563, GMD101566).

NOTE: Work within a laminar flow hood and use good aseptic practices. All reagents and equipment must be sterile.

STRATEGIC PLANNING

Experimental planning

It is worthwhile to plan the date to start experiments to minimize the need to do transfers outside of a normal working week. There is some flexibility during some of the cultivation steps. For example:

Day 1 – Monday: Sterilize spores/preculture
Day 3 – Wednesday: Set up *Agrobacterium* cultures
Day 5 – Friday: Inoculate spores with *Agrobacterium*/co-cultivate
Day 9 – Tuesday: Transfer to selective medium

BASIC PROTOCOL

AGROBACTERIUM-MEDIATED TRANSFORMATION OF *MARCHANTIA POLYMORPHA* SPORES

This transformation procedure is straightforward and robust, requiring only basic tissue culture and aseptic skills, as well as commonly available materials in plant molecular biology laboratories. Multiple transformation experiments can be run in parallel, and producing replicate tubs/plates is simple, providing safeguards against contamination or rare cases of low regeneration. A single tub typically results in hundreds of transformed plants. This method works successfully with the common laboratory strains of *Agrobacterium* GV3101 and EHA105. The process from spore to independent transgenic thalli takes approximately 4 weeks, with minimal hands-on time. It may be useful to use a visual marker/reporter gene, e.g., YFP derivatives (*Citrine, Venus*) or *RUBY* (He et al., 2020), when establishing this transformation protocol.

Materials

Desiccated *Marchantia polymorpha* archegoniophore (sporangia/spores) (see **Support Protocol**)
Sterilizing solution (see recipe)
Sterile water
Preculture medium (see recipe)
Agrobacterium strains containing binary vectors (e.g., GV3101, EHA105; strains can be sourced from researchers or purchased as competent cells, e.g., GoldBio, Fisher Scientific)
Selective LB-agar plates
Inoculation solution (see recipe)
Selective medium (see recipe)

1.5-ml polypropylene pestle
Vortex mixer
Pipettes and sterile filter tips (1 ml)
Sterile 1.5-2 ml tubes
Microcentrifuge
50-ml sterile tubes
15-ml sterile tubes
Laminar flow hood (equipped with forceps/scalpels)

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Sterile tissue culture tubs with lids (e.g., 98 mm/250 ml clear tubs, Alto A2113777 and A2700393)
Sterile filter papers (e.g., 70 mm Whatman grade 1 or equivalent)
28°C incubator
Sterile disposable bacterial loops (10 µl)
37°C incubator
Spectrophotometer and cuvettes (optional)
Plant tissue culture room, 25°C, 16 hr photoperiod, 30 µmol m^{-2} s^{-1} illumination
Additional reagents and equipment for plant tissue culture, DNA extraction, PCR amplification and purification, agarose gel electrophoresis, and imaging.

Work within a laminar flow hood and use good aseptic practices. All reagents and equipment must be sterile.

Spore sterilization (Day 1)

1. Add 500 µl ***sterilizing solution*** to a 2-ml tube containing a dried archegoniophore. Gently crush with a plastic pestle to break open sporangia and release spores (do not grind). Close lid and vortex to further disperse spores.

2. Aspirate the spore-containing solution with a pipette, avoiding larger pieces of tissue. This can be achieved by holding the pipette tip close to the side or bottom of the tube. Transfer to a new 1.5-ml microcentrifuge tube. Add 500 µl ***sterilizing solution*** and mix by inverting the tube several times. Leave for 15 min, then pellet spores in a benchtop centrifuge at room temperature and 17,000 × *g* for 2 min.

   Do not exceed the exposure time to the sterilizing solution.

3. Gently remove and discard the supernatant with a pipette. Wash spores with 1.5 mL sterile water. Centrifuge again, removing the supernatant. Repeat wash step.

4. Resuspend spore pellet in 1 ml sterile water and transfer to a sterile 50-ml tube. Dilute the spores with sterile water to ∼40 ml. Invert regularly to ensure spores remain suspended.

   Spores can be diluted further (e.g., to 80 ml) if lower densities of transformants are preferred.

Pre-culture spores (Day 1)

5. Place a sterile filter paper onto ***preculture medium*** in a tissue culture tub. Gently invert the diluted, sterilized spores to ensure they remain evenly suspended, and pipette 1 ml into the center of the filter paper. Immediately swirl the tub to distribute the spore/water solution over the filter paper. Transfer to a plant tissue culture growth room and preculture for 4 days.

   Delaying swirling spores/water after application to filter paper will result in poor distribution of spores because they begin to settle into the filter paper matrix.

   Preculturing Marchantia spores or sporelings in the presence of acetosyringone (200 µM for GV3101, 50–200 µM for EHA105) is crucial for achieving high-efficiency transformation (Understanding Results, Fig. 7). The preculture time can be varied between 3 and 5 days, depending on scheduling requirements.

Prepare Agrobacterium

6. (Day 3) Streak out *Agrobacterium* cultures from glycerol stocks onto ***selective LB-agar plates***. Culture the plates upside down at 28°C for 2 days.

   This will vary depending on the binary vector and strain of Agrobacterium used. See **APPENDIX 1** *for suitable Marchantia overexpression constructs, e.g., spectinomycin 100 mg L^{-1} for binary vector (pNWA98-101 or pMpGE010) + gentamycin 25 mg L^{-1} for the helper plasmid present in Agrobacterium GV3101 (MP-90).*

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7. (Day 5) Scrape a loop of confluent *Agrobacterium* culture from the agar plate into a 15-ml sterile tube containing 10 ml ***inoculation solution***. Disperse the cells by twisting the loop against the bottom of the conical tube. Close the lid and gently invert several times to assist cell suspension. Leave the cells to incubate at room temperature without agitation for 2–4 hr to induce virulence. The cell density of the culture, measured on a spectrophotometer at 600 nm (A600 nm), should be ∼0.8–1.

   Liquid Agrobacterium cultures can also be used (pelleting cells and resuspending in inoculation solution), but it is easier to grow directly from plates. Following virulence induction, Agrobacterium cultures emit a distinctive odor.

   We do not routinely check cell density on a spectrophotometer (A_{600nm}) but have included it here as a recommended benchmark for when establishing this protocol for the first time.

8. (Day 5) Dilute the *Agrobacterium* culture 10-fold with inoculation solution; the A600 nm reading should now be ∼0.1.

   Using more concentrated cultures promotes Agrobacterium overgrowth, with no improvement in transformation efficiency.

Co-cultivation

9. (Day 5) Pipette 1 ml dilute *Agrobacterium* culture onto the filter paper within each tissue culture tub. Swirl to distribute inoculum over the sporelings. Co-cultivate in a tissue culture room for 4 days.

   The co-cultivation time can be varied between 3 and 5 days, depending on scheduling requirements. Monitor for overgrowth of Agrobacterium, visible as a slimy film covering the filter paper, sporelings, and growth medium.

Selection

10. (Day 9) Transfer the filter paper/sporelings onto ***selective media***. Transfer cultures to a tissue culture room.

    Typically, $\frac{1}{2}$ Gamborg's B5 medium with either Hygromycin 8 mg L^{-1} + Ticarcillin 500 mg L^{-1} or G418 10 mg L^{-1} + Ticarcillin 500 mg L^{-1}, depending on the binary vector(s) used.

    If Agrobacterium growth is visible on the filter paper, rinse the filter paper/sporelings with 2–5 ml Ticarcillin (500 mg L^{-1}). Leave for several minutes, then swirl vigorously, before transferring the filter paper onto selective media.

    Adding food coloring to concentrated antibiotic stocks (1 ml per 10 ml, for a 1000× stock) provides a simple visual cue to identify the selection agents present in the media. This is particularly useful when running multiple transformation experiments in parallel that use different selective agents.

11. (Day 9) Optional step for gene editing. Following transfer of filter papers/sporelings onto selective media, apply heat-shock by transferring to a 37°C incubator overnight (16 hr).

    Do not exceed the heat-shock treatment time. This heat-shock treatment increases editing rates, resulting in fewer chimeric mutants and greater success identifying mutants during screening. However, it may reduce the number of viable regenerants, particularly if the treatment exceeds the recommended duration. Omit a heat-shock from one replicate tub as a backup, in case the treatment is too severe.

Regeneration (Day 10+)

12. Over the next weeks, non-transformed sporelings will die (beginning around 10 days after transferring to selective media), and transformed cells will start to regenerate. These will continue to regenerate into dark green clusters (Fig. 2A). Continue to monitor transformation tubs as plants regenerate.

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Figure 2 *Marchantia polymorpha* sporangia formation. The archegoniophore (female gametangium; top left) contains eggs, and the antheridiophore (male gametangium; bottom left) produces sperm that are released from the surface of the antheridia when wet. Fertilization of eggs by the sperm generates sporangia (right) on the archegoniophore, which rupture to release spores.

Once the selection for transformed plants has occurred and non-transformed tissues have died, the filter paper/regenerating plants can be transferred onto medium containing only Ticarcillin. This step is optional, but it will increase the rate of regeneration.

13. Regenerating plants will change from dense cell clusters into flat, differentiated thalli (Understanding Results, Fig. 7B). Regenerating thalli are most easily removed when they are small, using a sterile scalpel to gently lift the plant off the filter paper and into a separate tissue culture tub of solid $\frac{1}{2}$ B5 medium containing Ticarcillin (500 mg L^{-1}). The plants will regenerate at different rates, so remove plants once thalli form to allow others to continue to develop.

    For ease, the filters can be transferred to a sterile Petri dish while collecting plants and returned to their media/tubs to allow other plants to regenerate. Maintain Ticarcillin selection for at least one generation of plants derived from asexual gemmae (G1).

    While it is tempting to screen only the first regenerating plants, care should be taken because transgene expression or CRISPR mutations may slow regeneration. Selecting only the fastest-growing plants may select against the plants of interest.

14. Each tissue culture tub should have hundreds of regenerating transgenic plants (Understanding Results, Fig. 7B).

Screening

15. Individual plants are maintained in separate tubs, ensuring the production of independent transformants. Once sufficiently large, pieces of thallus can be removed for DNA isolation and transgene analysis.

    For CRISPR gene editing experiments, sample a section of thallus behind the meristem on a thallus branch for DNA analysis (Fig. 1). Chimeras are common with gene editing, so it is essential not to destroy mutant tissue through sampling and to retain a meristem.

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This section can be physically separated from the rest of the plant or transferred to a new culture tub. PCR amplicons of target regions can be directly sequenced (Sanger) because the haploid gametophyte will produce a single sequence, unless the tissue sampled is chimeric.

16. Transfer a single gemma (asexual propagule) from individual lines into separate tissue culture tubes with solid $\frac{1}{2}$ B5 medium and Ticarcillin, establishing the G1 generation.

    Gemmae are derived from single cells, eliminating genetic chimeras. Gemmae can be collected from gemmae cups with forceps (or entire cup collected) into water containing an organosilicone surfactant such as 0.005% (v/v) Silwet L-77® (PhytoTechnology Laboratories) or Pulse® penetrant (Yates). This solution will reduce surface tension, allowing gemmae to disperse in water. For controlled and precise distribution of gemmae, pipette a volume of water/gemmae with a 1-ml pipette, and carefully expel a single individual gemma onto the tissue culture medium. The water will be absorbed into the medium over the next day. This method is also suitable when plating multiple gemmae per tub for experiments.

    Ticarcillin selection is recommended for the G1 generation to prevent residual Agrobacterium overgrowth. Subsequent gemmae-derived generations (G2, etc.) should not require this.

17. Transgenic lines are maintained by propagating gemmae or thallus pieces on $\frac{1}{2}$ B5 medium. Transformed plants can be used as stock plants for experiments and refreshed as needed. Multiple gemmae can be used from these stock plants to produce replicated tissue for collection.

    We typically plate 3–5 gemmae per tub/biological replicate. Growing gemmae on a mixed cellulose ester (MCE) filter (on $\frac{1}{2}$ B5 medium or similar) allows plants to grow without penetrating the media, allowing simple tissue harvesting for analysis.

18. For long-term storage, cultures can be stored at 4°C with dim illumination and revived as required. This is very helpful for reducing the frequency of subculturing stock lines.

SUPPORT PROTOCOL

INDUCTION OF SPORES

Induction of reproductive structures in *Marchantia* is routinely achieved by growing male and female genotypes until they have developed a substantial thallus and then providing supplemental far-red light (Ishizaki et al., 2016). Establishing a light environment that works reliably can require some trial and error, and some labs still rely on the annual induction of reproductive structures in glasshouse-grown plants. Anecdotally, some Marchantia genotypes are more responsive to far-red light induction than others. Sporangia, which contain spores and elaters, are produced when sperm are collected from the male structures (antheridiophores) and used to fertilize eggs within the female structures (archegoniophores) (Fig. 2). The conditions used to grow Marchantia plants, induce sporophytes, and collect and store archegoniophores (containing spores) for transformation are described here.

The archegoniophore (Fig. 2, top left; female gametangium) contains eggs and the antheridiophore (Fig. 2, bottom left; male gametangium) produces sperm, ejected from the surface of the antheridia when wet. Fertilization of eggs by the sperm generates sporangia (right) on the archegoniophore, which rupture to release spores.

Materials

Male and female Marchantia genotypes, e.g., Sey-1/Aud-2; Tak-1/Tak-2; Cam-1/Cam-2, sourced from researchers.

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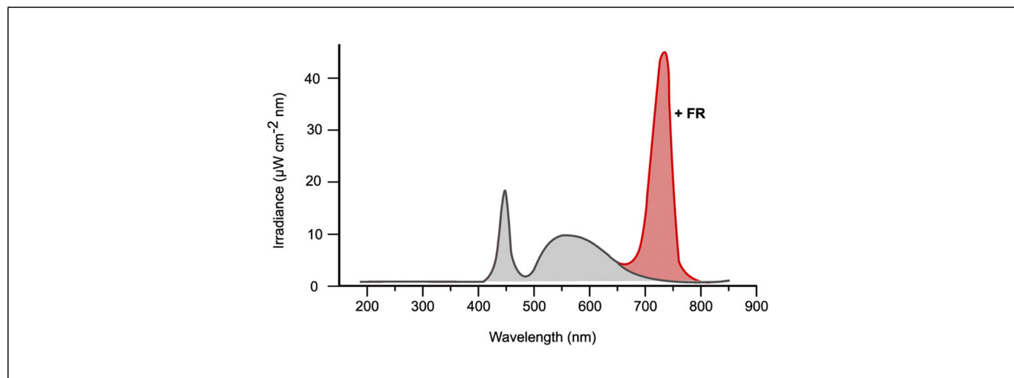

Figure 3 Spectra of light conditions used to induce *Marchantia polymorpha* reproductive structures. Grey spectrum corresponds to white light conditions, and supplemental far-red light is indicated in red. Measurements taken using International Light Technologies IT960-UV spectroradiometer and SpectrlLight III v. 3.5.4.2 software.

Jiffy-7 peat plugs (Jiffy Group), available at garden supply stores (e.g., *www.egmontseeds.co.nz*, New Zealand: SKU: 9200)
Hydroponic solution (see recipe)

Vented seed propagator (or large plastic vessel with holes for venting), available at garden supply stores (e.g., *www.egmontseeds.co.nz*, New Zealand: SKU: 9311)
Capillary matting, available at garden or hydroponic stores (e.g., Aquafelt, *www.egmontcommercial.co.nz*, or similar)
Pipette + tips (1 ml)
Water
2-ml tubes
18 G needle
Silica gel
Plant growth cabinet (e.g., Percival cabinet, model E-75L1): 25°C, 16 h photoperiod, 70 μmol m^{-2} s^{-1} intensity white light and 20 W m^{-2} intensity far-red light (λ_{max} = 730 nm). Lighting provided by LEDs. Spectra for the light conditions are shown in Figure 3.

Propagate gemmae on peat plugs

1. Hydrate peat plugs with water (~40 ml each). Add 5 ml ***hydroponic solution*** to each hydrated peat plug. Set up separate propagators for each genotype (male, female). Cover the base of a vented seed propagator with capillary matting and distribute hydrated peat plugs.

2. Collect gemmae (asexual propagules) from both male and female genotypes.

   Gemmae can be collected from gemmae cups with forceps (or entire cup collected) into water containing an organosilicone surfactant such as 0.005% (v/v) Silwet L-77® (PhytoTechnology Laboratories) or Pulse® penetrant (Yates). This will reduce surface tension, allowing gemmae to disperse in water. For controlled and precise distribution of gemmae, pipette a volume of water/gemmae with a 1000-μl pipette, and carefully expel individual gemmae onto the peat plugs. This approach can be used for plating gemmae onto tissue culture medium, provided all solutions and materials are sterile.

3. Evenly distribute 3–10 Marchantia gemmae onto each peat plug within the propagator. Repeat this for both genotypes.

4. Add sufficient water to the tray of the seed propagator to wet the capillary matting.

   Do not flood the peat plugs: excessive moisture can promote fungal contamination. Excessive hydroponic solution promotes algal growth.

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5. Transfer the propagators to a plant growth cabinet with white light (supplemental far-red light turned off). Open the vents of the propagator. Continue to water once a week or as needed to maintain moisture.

6. After 3–4 weeks of growth, turn on supplemental far-red light (20 W m^{-2}). Add another 5 ml hydroponic solution to each peat plug. Gametangia (antheridiophores and archegoniophores) will start to appear about two weeks following far-red exposure.

Produce spores by sexual crosses

The fertilization process should begin once the female archegoniophores, which are finger-like umbrella structures, have started to differentiate. This may occur over a few weeks, so additional rounds of crossing/fertilization are recommended.

7. Collect sperm by pipetting a drop of water onto the male antheridiophore and wait for it to become cloudy, indicating sperm release. Pipette up the cloudy solution and dilute with water in a 1.5-ml tube.

   If collecting sperm for bulk spore preparations, remove several antheridiophores and put them upside down in a Petri dish containing a small amount of water. Leave for several minutes for sperm to release.

8. Pipette a few drops of the diluted sperm onto the (female) archegoniophore. The liquid will wick up the finger-like structures to the archegonia, fertilizing the eggs. Repeat this process a few times over the next 2 weeks.

   Archegoniophores are ideally fertilized twice at different stages of development.

9. The archegoniophores will mature over the next 3-4 weeks, as the sporangia and spores start to develop. The finger-like structures rise, eventually exposing bright yellow sporangia.

Collection of archegoniophores/spores

10. Collect archegoniophore heads containing visible sporangia into 2-ml tubes with a needle hole in the lid. Do not wait until all sporangia are visible because this risks dehiscence. Desiccate archegoniophores/spores by placing tubes into a container with silica gel and store at −20°C.

    We have used spores stored in this manner for over 8 years with no obvious loss of viability.

    2-ml tubes are preferred for downstream spore release/sterilization. The additional volume prevents overflow or spills of spores/solution when using a plastic pestle.

11. Spores should be sufficiently desiccated after 2–3 weeks for surface sterilization and use in transformation experiments (**Basic Protocol**).

REAGENTS AND SOLUTIONS

Acetosyringone

Stocks (100 mM) are prepared by dissolving solid acetosyringone (Sigma-Aldrich) in methanol before diluting with an equal volume of sterile water (100 mM acetosyringone in 50% (v/v) methanol).

Antibiotic stocks

Antibiotic stocks are prepared by dissolving antibiotic salts in sterile water, followed by filter-sterilization (0.20 µm, e.g., Corning), and aliquots are made into sterile tubes. These are typically made at 1000× working concentrations, except for ticarcillin, which is not soluble at 500 mg ml^{-1}. Antibiotic stocks are stored frozen at −20°C until use. Working concentrations for common antibiotics used in bacterial and plant tissue culture are as follows: kanamycin sulfate (ThermoFisher)

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50 mg ml^{-1}; spectinomycin sulfate (ThermoFisher) 100 mg ml^{-1}; gentamycin sulfate (ThermoFisher) 25 mg ml^{-1}; hygromycin B (AG Scientific) 8 mg ml^{-1}; G418 sulfate (AG Scientific) 10 mg ml^{-1}; and ticarcillin disodium/clavulanate potassium (Duchefa) 100 mg ml^{-1}.

For antibiotics used in plant tissue culture media, the addition of food coloring to stock solutions provides a visual reminder of the selective agent(s) in the media. When making stock solutions, the inclusion of 1 ml liquid food coloring (Hansells, Queen, or similar) per 10 ml final volume is sufficient (e.g., 1000 mg antibiotic salt + 1 ml food coloring, made up to 10 ml with water, and filter sterilized).

1/2 B5 medium

1.58 g L^{-1} Gamborg's B5 medium with vitamins (Duchefa) (Gamborg et al., 1968), 1% (w/w) sucrose, 1% (w/w) agar, pH 6 prior to autoclaving. Pour into sterile tissue culture tubs (e.g., 98 mm clear 250-ml sterile tubs and lids, Alto: A2113777, A2700393).

Hydroponic solution

Single part hydroponic solution, diluted to manufacturer's instructions (we use one with NPK 5:2:8 + micronutrients at 5 ml per L water), available from garden or hydroponic supply stores. Alternatively, nutrient solutions such as half-strength Hoagland's solution are also effective.

Inoculation solution

10 mM $MgCl_2$ (Merck) + 10 mM MES (2-(N-morpholino) ethanesulfonic acid) (Sigma-Aldrich) solution (pH 5.6).

Preculture medium

$\frac{1}{2}$ B5 medium + 200 µM acetosyringone (Sigma-Aldrich). Allow sterile medium to cool to ~60°C before adding AS. Pour into sterile tissue culture tubs.

Selective medium

$\frac{1}{2}$ B5 medium + 500 mg L^{-1} Ticarcillin disodium/clavulanic acid (Duchefa, T0190) + selective antibiotic e.g., hygromycin 8 mg L^{-1} or G418 10 mg L^{-1} (AG Scientific). Allow sterile medium to cool to ~60°C before adding antibiotics. Pour into sterile tissue culture tubs.

Sterilizing solution

Dissolve one sterilizing tablet (500 mg sodium dichloroisocyanurate) in 1 L sterile water (e.g., Milton or other similar brands of baby bottle sterilizer containing the same active ingredient, available in supermarkets and pharmacies).

APPENDIX 1: OVEREXPRESSION VECTORS

Plant molecular genetics methods rely on the expression of genes of interest, including endogenous genes, transgenes, molecular reporters, and CRISPR gene-editing reagents such as Cas nucleases and guide RNAs. Transformation systems for Marchantia are primarily based upon hygromycin selection because Marchantia is highly sensitive to hygromycin and the selection cassette is expressed from a CaMV*35S* promoter (*$35S_{pro}$:hygromycin phosphotransferase*) (Ishizaki et al., 2008; Kubota et al., 2013; Seo et al., 2022; Tsuboyama & Kodama, 2014). In contrast, Marchantia is highly resistant to kanamycin, and common neomycin phosphotransferase (*nptII*) selection cassettes are expressed from a *nopaline synthase* (*nos*) promoter, which is not well expressed in Marchantia. However, Marchantia is sensitive to G418 (also known as geneticin), which can also be detoxified by *nptII*, allowing it to be used as an alternative selective agent if

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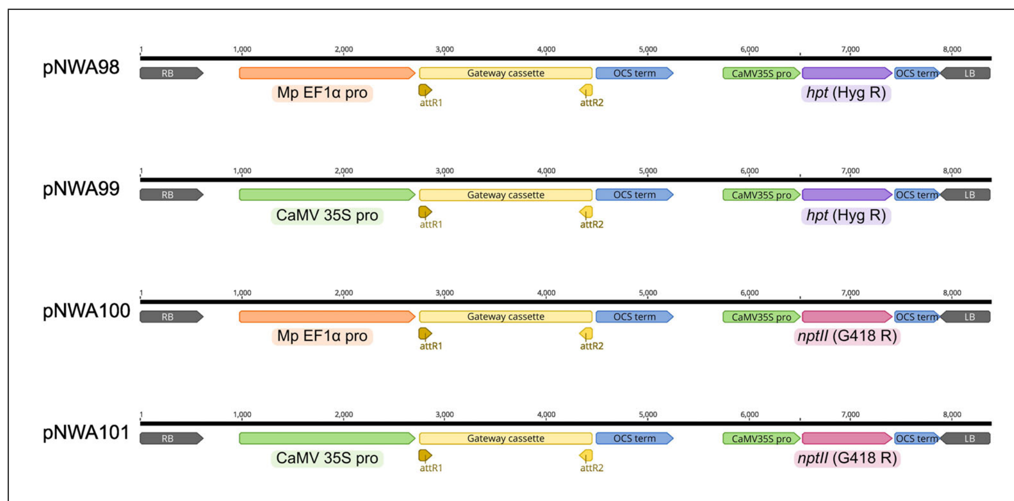

Figure 4 Gateway destination vectors for overexpression in *Marchantia polymorpha*. The T-DNA regions of pNWA98, 99, 100, and 101 are shown. The vectors express a gene of interest, introduced from an entry vector by LR recombination, from either a CaMV*35S* promoter or Mp*EF1α* promoter with either a *CaMV35S$_{pro}$:nptII:OCS* (G418) or *CaMV35S$_{pro}$:hpt:OCS* (hygromycin) selection cassette. All confer spectinomycin (100 μg ml^{-1}) resistance to bacteria (*Escherichia coli, Arobacterium tumefaciens*).

appropriate promoters are used (Albert et al., 2018; Ishizaki et al., 2015). Two promoters widely used for constitutive expression in Marchantia are Mp$EF1\alpha_{pro}$ and CaMV$35S_{pro}$, providing strong and moderate expression, respectively (Althoff et al., 2014).

A simple Gateway® destination vector series has been developed for expressing genes of interest in Marchantia, with Mp$EF1\alpha_{pro}$ or CaMV$35S_{pro}$ for expressing genes of interest, and CaMV$35S_{pro}$ for hygromycin ($35S_{pro}$:*hpt*) or G418 ($35S_{pro}$:*nptII*) selection (Fig. 4). Expression clones in binary vectors for Marchantia transformation are easily obtained by standard LR-recombination between Gateway® entry vectors containing genes of interest (e.g., attL1: Gene of Interest: attL2) and pNWA98-101 destination vectors, following standard instructions for LR clonase II (Life Technologies).

Annotated sequence files are available for download from ***Supporting Information***.

APPENDIX 2: MULTIPLEX CRISPR USING TRNA ARRAYS

Significant advances have been made in implementing CRISPR gene editing for plant molecular genetics research. CRISPR editing in plants (including Marchantia) typically involves stable transformation with vectors expressing the Cas9 nuclease and single guide RNA (sgRNA; a fusion of crRNA and tracrRNA) sequences necessary to target DNA cleavage at precise genomic locations. Subsequently, imperfect DNA repair of these DNA breaks occurs (non-homologous end joining/NHEJ), which makes CRISPR/Cas9 particularly amenable for targeted mutagenesis. CRISPR/Cas9 vectors for Marchantia were developed that express a sgRNA molecule from an Mp*U6* promoter, Cas9 from an Mp*EF1α* promoter, and hygromycin resistance (Sugano et al., 2018). The promoters are sourced from Marchantia and confer high expression of the CRISPR reagents. RNA polymerase III promoters such as Mp$U6_{pro}$ are used because the transcription start site (TSS) is precise, enabling sgRNA molecules to form without extraneous sequence. A limitation of these vectors is the ability to provide just one sgRNA (e.g., pMpGE_En02, pMpGE_En03) (Sugano et al., 2018). Furthermore, the 3' ends of the RNA molecules are not processed, because terminator sequences do not terminate transcription immediately and are often imprecise.

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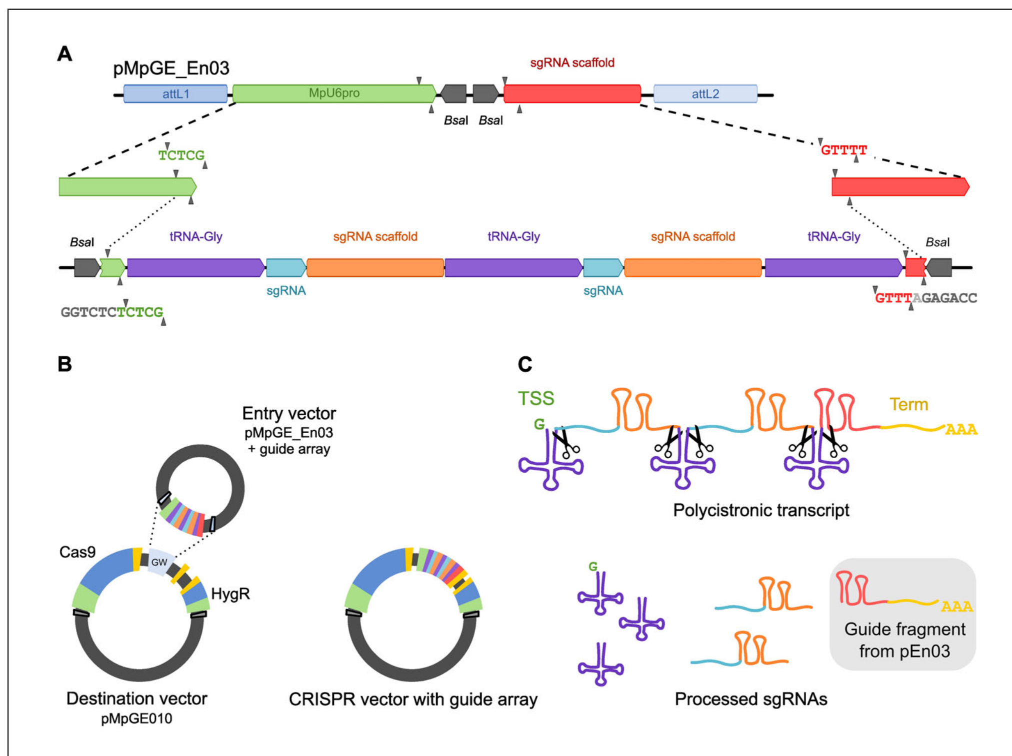

Figure 5 Multiplex CRISPR in *Marchantia polymorpha* with tRNA-sgRNA arrays and the pMpGE_En03/pMpGE010 vectors. (**A**) A tRNA/sgRNA array is designed and commercially synthesized. The *Bsa*I sites on the insert will produce unique cohesive ends corresponding to the *MpU6* promoter and the existing guide scaffold sequence of pMpGE_En03. Design of these ends is critical for success. An array of *Arabidopsis thaliana* Glycine tRNA (tRNA-Gly) and guide RNA sequences is designed. The guide RNAs consist of 18–20 bp targeting sequences (sgRNA) and scaffold sequence (sgRNA scaffold) with improved stability (Dang et al., 2015). Synthesized tRNA/sgRNA arrays are cloned into the *Bsa*I sites of pMpGE_En03. (**B**) Final CRISPR vectors for Marchantia transformation are produced by LR recombination between the entry vector and destination vector pMpGE010 (or pNWA187). (**C**) *In planta* processing of a polycistronic transcript, releasing processed sgRNAs, tRNAs, and a scaffold fragment.

CRISPR editing design strategies employing RNA processing elements have enabled the use of more typical Polymerase II promoters, and with multiple sgRNA (multiplex). This includes tRNAs, which are naturally processed by endogenous RNAses, and self-cleaving ribozymes, e.g., hammerhead (Hassan et al., 2021; Tang et al., 2016). The use of multiple sgRNAs per gene target increases the frequency of induced mutations and promotes larger sequence deletions (simplifying genotyping/mutation detection). Furthermore, guide RNAs targeting multiple gene targets can be combined within a single construct, enabling double or triple mutants to be generated (e.g., for gene families). To enable gene editing with multiple sgRNAs and improve sgRNA processing, we developed a simple sequence design to adapt existing Marchantia CRISPR/Cas9 vectors, such as the pMpGE_En03 entry vector and pMpGE010 destination vectors (Fig. 5). Instead of cloning annealed oligonucleotides corresponding to the target sequence (20 bp) and *Bsa*I cohesive ends (Sugano et al., 2018), we designed a tRNA guide array, which was commercially synthesized, and cloned this into the existing *Bsa*I sites. *Bsa*I (or its isoschizomer *Eco*31I) is a type IIS restriction enzyme, which means the cleavage site is outside the recognition sequence, producing cohesive ends with a unique sequence. Thus, particular care must be taken during the design of guide arrays. Our guide arrays use *Arabidopsis thaliana* Glycine tRNA sequences, custom 20-bp sgRNA target sequences, and an improved sgRNA scaffold sequence (Dang et al., 2015). Each sgRNA sequence is flanked by tRNAs, and the array can be repeated multiple times (we have used up to

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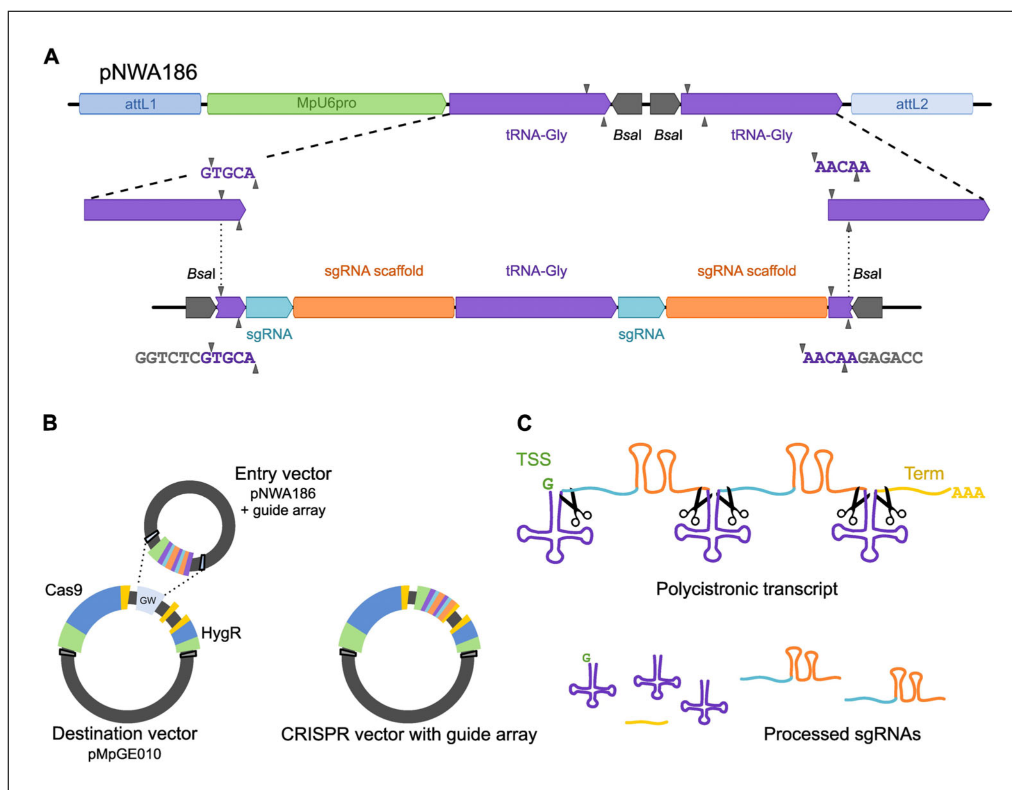

Figure 6 Multiplex entry vector pNWA186 for CRISPR/Cas9 editing in *Marchantia polymorpha*. (**A**) The pNWA186 entry vector contains *Arabidopsis thaliana* Glycine tRNAs (tRNA-Gly) that flank the *Bsa*I cloning sites. This reduces the size and complexity of the sequence for commercial synthesis. A tRNA/sgRNA array is designed and commercially synthesized. The *Bsa*I sites on the insert will produce unique cohesive ends corresponding to the tRNAs on pNWA186. Design of these ends is critical for success. The guide RNAs consist of 18-20 bp targeting sequences (sgRNA) and scaffold sequence (sgRNA scaffold) with improved stability (Dang et al., 2015). Synthesized tRNA/sgRNA arrays are cloned into the *Bsa*I sites of pNWA186. (**B**) Final CRISPR vectors for Marchantia transformation are produced by LR recombination between the entry vector and destination vector pMpGE010 (or pNWA187). (**C**) *In planta* processing of a polycistronic transcript, releasing processed sgRNAs and tRNAs.

eight sgRNAs) (Fig. 5A). Synthesis of the array by commercial suppliers is simple and overcomes challenging assembly and cloning. The synthesized sequence is sub-cloned with *Bsa*I (or *Eco*31I) into the pMpGE_En03 entry vector, followed by LR Gateway® recombination into the destination vector pMpGE010 (Fig. 5B). The construct is transformed into Marchantia with *Agrobacterium* (**Basic Protocol**), and transformed plants are screened for edits. The construct will express a polycistronic RNA molecule that will be processed to release the sgRNAs, the tRNAs, and a guide RNA scaffold from the pMpGE_En03 vector (Fig. 5C). One of the additional benefits of this approach is 3' processing of sgRNAs, which may further improve sgRNA performance. This method has enabled us to generate mutants at high frequency, reducing the effort to identify mutants for molecular analysis (Jibran et al., 2024; Zhou et al., 2025).

We have developed a modified entry vector, based on pMpGE_En02 that includes AtGly tRNA sequences flanking the *Bsa*I cloning site for the sgRNA/tRNA array (Fig. 6). This reduces the size and complexity of the sequence that is synthesized, which improves the synthesis delivery time and reduces expense. This version also removes the extraneous sgRNA scaffold sequence present within the base vector, which is not required when synthesizing a sgRNA array. The synthesized sgRNA/tRNA array must have the correct cohesive ends when digested with *Bsa*I, shown in Figure 6A. The resulting entry vector

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is then recombined with a suitable CRISPR destination vector, such as pMpGE010 (Fig. 6B). We have also generated a variant pNWA187 that replaces the existing hygromycin resistance cassette with $35S_{pro}$:*nptII*:$35S_{term}$, for G418 selection. The final constructs will express a polycistronic RNA molecule *in planta* that will be processed to release the sgRNAs and tRNAs (Fig. 6C).

It is strongly recommended to simulate cloning *in silico* when designing constructs for synthesis, using DNA analysis software such as Geneious Prime (version 2024.0.2, *https://www.geneious.com*). Annotated sequence files are available for download from ***Supporting Information***.

COMMENTARY

Background

Several *Agrobacterium*-spore transformation procedures have been reported, but we have encountered difficulty establishing these procedures within our laboratory. In response to these challenges, we developed a simplified protocol that adopts some of the best features of existing methods with simple tissue culture practices, using common lab materials and bacterial strains. The features we wanted to include were growth on solid agar medium, the ability to transfer sporelings easily between different growth media, and using *Agrobacterium* strain GV3101 for its rapid growth, ease of manipulation, and plasmid stability (*recA*). Growth on agar medium allows sporelings to regenerate and remain physically separated from one another, compared with liquid culture, which risks plants becoming entangled *via* their rhizoids. This situation presents additional challenges, such as how to transfer sporelings from one medium to another (e.g., co-cultivation to selection). We addressed this by plating spores onto sterile filter papers, which also helps inhibit *Agrobacterium* overgrowth. Despite these improvements in handling sporelings, our early transformation attempts were largely unsuccessful, but occasionally we would find that the edge of a filter paper on a single replicate tub would produce large numbers of transformants. This result suggested that small improvements may be sufficient to enable high-frequency transformation, and that transformation of Marchantia spores with GV3101 was possible. We noted that liquid culture transformation methods included acetosyringone (AS) in the co-cultivation medium, exposing both *Agrobacterium* and Marchantia sporelings to significant concentrations of AS (100–333 µM) (Ishizaki et al., 2008). Our existing practice was to induce virulence (including expression of *vir* genes) in *Agrobacterium* cultures with AS (200 µM) (Gelvin, 2000), leading us to consider that AS might also have effects upon Marchantia tissues that promote transformation. Our final protocol includes simplified spore preparation, growth of spores/sporelings on filter papers, preculture of sporelings on solid agar medium with AS, and using common *Agrobacterium* strains GV3101 and EHA105.

Critical Parameters

Acetosyringone preculture of sporelings

The most critical parameter of this protocol is to preculture spores/sporelings with 200 µM acetosyringone (AS) prior to co-cultivation with *Agrobacterium*. Omitting this step will result in very few transformants, if any. The four-day preculture period is routine, but we have had success with three to five days. This treatment is in addition to the acetosyringone induction of the *Agrobacterium* inoculum, demonstrating that this causes changes within the sporelings that promote transformation. Marchantia produces phenolic metabolites that differ from those of the natural *Agrobacterium* host species, so the effect of preculturing with AS may allow sporelings to concentrate these metabolites, which in turn enable *Agrobacteria* to identify Marchantia as a host and initiate infection. Alternatively, the growth inhibition upon sporelings may cause physical changes that make them more susceptible to infection.

CRISPR - heat shock

For transformation experiments with CRISPR gene editing constructs, the inclusion of a heat shock treatment once sporelings are transferred to selective medium increases editing efficiency. This results in both higher proportions of edited plants and reduced numbers of chimeric plants, as editing occurs at a very early stage of regeneration from transformed cells. We use a 37°C/16 hr heat shock, but a 28°C/48 hr heat shock is also effective.

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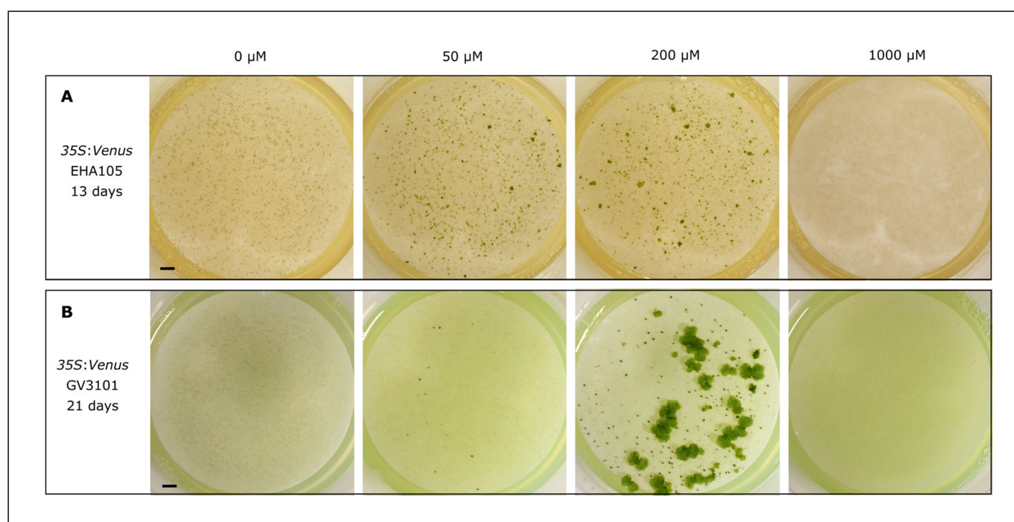

Figure 7 Pre-culturing *Marchantia polymorpha* sporelings with acetosyringone is critical for efficient transformation. Regenerating Marchantia following transformation (35*S*:*Venus* construct) with preculture medium containing 0, 50, 200 or 1000 μM acetosyringone. Two *Agrobacterium tumefaciens* strains were tested, EHA105 (**A**) and GV3101 (**B**), with images taken after 13 or 21 days since transfer to selective medium, respectively. Scale bars represent 5 mm.

Troubleshooting

Common issues are outlined in Table 1, along with their possible causes and recommended corrective actions.

Understanding Results

Spore transformation

When establishing Marchantia spore transformation, we initially used GFP-ER reporter genes. However, the fluorescent signal is strongly quenched in Marchantia thallus tissues, visible only within the meristems of $35S_{pro}$*:GFP-ER* plants or faintly visible on the thallus of $EF1\alpha_{pro}$*:GFP-ER* plants. YFP derivatives such as Citrine or Venus do not suffer from these same issues.

We assessed the effect of preculturing sporelings with varying AS concentrations (0, 50, 200, and 1000 μM) when transforming plants with a 35Spro:Venus-N7 construct (Fig. 7). Sporeling growth was inhibited by AS in a dose-dependent manner compared with the control (0 μM), and growth was completely inhibited at 1000 μM. Sporelings that were precultured without AS initially show increased biomass compared with those grown on preculture medium with AS, but these fail to transform and regenerate once antibiotic selection acts (∼10 days). However, sporelings that were precultured with either 50 or 200 μM AS contain dark green cell clusters amongst dead/dying non-transformed sporelings after 13 days on selective medium (Fig. 7A). This becomes more evident at 21 days (Fig. 7B) when differentiated transgenic thalli can be collected from the filter paper.

The optimal concentration of AS in preculture medium was 200 μM for *Agrobacterium* GV3101, yielding hundreds of transformants per tissue culture tub (Fig. 7B). Transformants were not observed if AS was either omitted or present in high concentration (1000 μM), and lower concentrations (50 μM) yielded low frequency. AS was also required for transformation with *Agrobacterium* EHA105, but similar numbers of transformants were produced with 50 and 200 μM (Fig. 7A).

We estimate the transformation efficiency for GV3101 to be 10% to 20% in our spore transformation protocol, which is similar to Agar Trap (∼10%) (Tsuboyama & Kodama, 2014), but lower than thallus (60%) (Kubota et al., 2013) or gemmae (up to 97%) (Tsuboyama et al., 2018) transformation. Notably, these other protocols are more laborious and/or require different *Agrobacterium* strains to achieve these transformation rates. GV3101 is a common strain favored for its rapid growth, ease of manipulation, and because it does not recombine plasmids. In practice, the transformation frequency is not an issue because the number of explants (spores) is high, and this method produces more transformants in a single tub than are required, with little effort. We have successfully used this method for over 50 different constructs among multiple researchers without difficulty.

CRISPR/Cas9 gene editing

We have used the spore transformation protocol for transformation with gene editing constructs for a variety of target genes.

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Table 1 Troubleshooting guide for Marchantia spore transformation

Problem	Possible cause	Solution
No/few sporelings	Non-viable spores	Test spore viability. Plate spores onto filter papers with water. They should be visible with a dissecting microscope after 5 days. A regeneration control can be included with each experiment.
	Excessive sterilization	Ensure the sterilization solution is correct. Do not exceed contact time.
Contamination	Insufficient spore sterilization	Repeat experiment, taking care to sterilize spores according to the procedure. Avoid grinding tissue or transferring larger tissue pieces into the spore prep.
	Improper aseptic technique	Ensure all solutions and equipment are sterile. Work within a laminar flow hood and adhere to aseptic practices.
No regenerants	Acetosyringone omitted from preculture medium.	Repeat experiment.
	Incorrect selective antibiotics used or incorrect concentrations.	Repeat experiment.
Agrobacterium overgrowth	Failure to dilute *Agrobacterium* culture for inoculation	Wash filter papers/sporelings with Ticarcillin 500 mg L^{-1} solution. Transfer to solid medium containing Tic 500 mg L^{-1}. If a thick biofilm is present, antibiotic solution can be pipetted vigorously over the filter to physically remove it. Sporelings should remain attached to the filter paper.
	Ticarcillin omitted from medium	Adding food coloring to antibiotic stocks helps avoid omission of antibiotics or misidentification of selective medium.
Excessive plants (lawn)	Ineffective selection for transformants	Check that the correct selective antibiotic is used at the correct concentration. Hygromycin 8 mg L^{-1} or G418 10 mg L^{-1} should be effective. Conduct a ‘kill curve’ to ensure the selective antibiotics are effective on sporelings: plate spores on $\frac{1}{2}$ B5 medium and grow for 7 days before transferring to medium containing different concentrations of selective antibiotic.
	Excessive spore density	Dilute spores further when plating for preculture.

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The first was Mp*MYB14*, a regulator of red flavonoid pigment production (riccionidin A/auronidins) (Albert et al., 2018), using the pMpGE_En02/pMpGE010 vectors (Sugano et al., 2018). This exemplar was useful because it highlighted the chimeric nature of most of the T0 mutants (red and green sectors), informing how to sample and screen plants to identify mutants more efficiently. We sampled each plant once, allowing a higher number of independent plants to be screened for a given number of samples (rather than sampling multiple branches from fewer plants). Tissue from a single branch is sampled behind the branch tip, which contains the meristem. This tissue is used for DNA extraction and amplification of the region targeted by the guide RNA(s) with PCR. PCR products can be run on agarose gels, which can identify larger insertions/deletions, and the remaining PCR product can be purified (e.g., column cleanup) or deactivated with Exonuclease I/Shrimp Alkaline Phosphatase (Exo/SAP) treatment and sequenced directly. Prior to developing multiplex sgRNA/tRNA arrays, we would generate two constructs with different sgRNAs targeting the same gene and mix *Agrobacterium* strains for transformation. Using this approach, we had a mutant frequency of ~10% to 20% and would screen 20–30 plants. Mutant tissue is physically removed into a new tissue culture tub, grown on to develop a thallus bearing gemmae cups, and a single gemma from that individual is plated in an individual tub (G1 generation) and re-screened for mutations. Gemmae are derived from a single cell, which segregates any chimeras present in the T0 tissue, establishing G1 stock plants for analysis.

As CRISPR gene editing became more widely adopted, a series of unpublished reports (blogs, etc.) suggested that gene editing with Cas nucleases (Cas9 and Cas12) could be enhanced if cultured at elevated temperatures. These temperature effects are now well established (Blomme et al., 2022; LeBlanc et al., 2018). During a transformation experiment generating CRISPR mutants of an auronidin biosynthetic gene (*ppo/aus*), we explored whether introducing a heat-shock treatment could improve gene editing and recovery of mutants. The heat shock treatment was applied early during regeneration to induce editing in founder cells, giving rise to larger mutant sectors. An overnight (16 h) incubation at 37°C reduced sporeling viability slightly, but numerous plants regenerated and were subsequently screened for edits. Mutants were identified with high frequency, and none of the plants screened were identified as chimeric when sequencing DNA. Auronidin pigments are produced with nutrient deficiency, evident in older cultures when the growth medium becomes depleted (Albert et al., 2018; Zhou et al., 2024). This allowed visual screening to occur after initial mutant identification (blind sampling) and the evaluation of chimeric sectoring (red/green). In contrast to the *myb14* mutants, which had numerous chimeric plants (identified during DNA sequencing, and sectoring observed in old plants), chimeras were not identified in the *ppo* mutants. The heat shock treatment appeared to both increase mutant identification and reduce chimeras by increasing editing activity during early stages of regeneration. This treatment was adopted within the transformation protocol for gene editing.

The effects of heat-shock treatments for enhancing CRISPR editing were explored further using sgRNA/tRNA arrays (APPENDIX 2) targeting the Marchantia orthologue of *GOLDEN 2-LIKE (GLK)*. *GLK* regulates chloroplast development (Hall et al., 1998; Hernández-Muñoz et al., 2024), such that loss-of-function provides a clear visual marker of gene editing activity. Marchantia spores transformed with the *GLK* CRISPR construct underwent different heat-shock treatments prior to regeneration. The resulting plants had a variety of thallus phenotypes, ranging from dark green (wild type), dark/pale green sectoring (chimeric *glk*), through to plants with consistent pale green thalli (*glk*) (Fig. 8). The 37°C/16 hr treatment resulted in a two-fold increase in the number of plants that were completely mutant or had large mutant sectors (compared with no treatment) and therefore might reasonably result in mutant identification from blind sampling. In contrast, a 37°C/24 hr treatment resulted in the death of most sporelings. An extended heat-shock treatment at 28°C/48 hr resulted in a three-fold increase in mutants, but with slightly lower mortality than the 37°C/16 hr treatment. In practice, we find an overnight 37°C treatment effective and simple to integrate.

Guide RNA arrays (APPENDIX 2) are now our preferred method for gene editing in Marchantia. We typically design two to three guides per gene target and apply a 37°C/16 hr heat shock during transformation. With blind sampling (no visual phenotypes) of our recent

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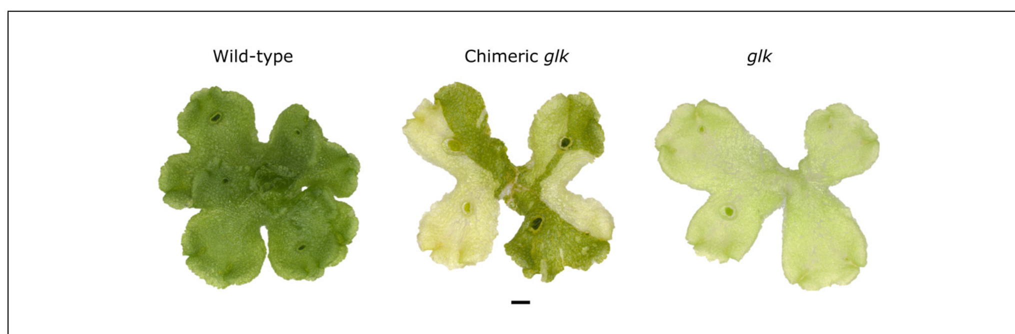

Figure 8 Phenotypes of *Marchantia polymorpha* transformed with a CRISPR construct targeting *GOLDEN 2-LIKE* (*GLK*). Images of a wild-type plant, a chimeric *glk* mutant, and a non-chimeric *glk* mutant are shown. The dark vs. light green sectoring in the chimeric *glk* mutant highlights how CRISPR editing can generate chimeras of wild-type and edited tissues. The size of the pale green sectors depends on when a cell is edited and how many descendant cells arise from this mutation. Scale bar represents 1 mm.

gene targets, mutant identification is ~60% to 70% (e.g., 10/17; 14/20; 15/20).

Time Considerations

An overview of the spore transformation protocol outlined in **Basic Protocol** is shown in Figure 1. The total time from sterilizing spores to transferring the first transformants is approximately 30 days. These T0 plants will be very small and require growth for several weeks before they can be screened for the presence of transgenes or gene edits. A breakdown of the time considerations for each day of the work plan is outlined below:

Day 1: 90 min

Day 3: 15 min

Day 5: 1 hr (excluding 2–4 hr incubation)

Day 9: 30 min

Day 10+ (Transfer individual T0 plants): 1 hr every few weeks

These are conservative time estimates for an inexperienced user. Once familiar with the protocol, steps can be completed more quickly or interspersed with other lab activities. These timings do not include the preparation of growth medium or reagents, which should be done ahead of time. For example, 1-L aliquots of autoclaved $\frac{1}{2}$ B5 medium can be prepared and stored for several weeks at room temperature. When required, it is carefully re-melted in a microwave (ensuring the lid remains attached and slightly loosened), cooled to ~60°C before the addition of antibiotics or acetosyringone, and poured into sterile tissue culture tubs.

The preparation of sporangia/spores needed for transformation outlined in the **Support Protocol** takes approximately 10–12 weeks, from establishing Marchantia cultures on peat plugs to mature sporangia. Further breakdown of the time considerations for each step in the process is outlined below:

Propagate gemmae on peat plugs: 2 hr

Fertilization: 1 hr (at least twice, several days apart)

Archegoniophore collection: 30 min

Acknowledgments

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Author Contributions

Rebecca M. Yorker: Formal analysis; validation; writing - original draft. **Simon C. Deroles**: Conceptualization; writing - review and editing. **Yanfei Zhou**: Methodology; resources; validation; writing - review and editing. **Jennifer A. Tate**: Supervision; writing - review and editing. **Kevin M. Davies**: Funding acquisition; project administration; resources; supervision; writing - review and editing. **Nick W. Albert**: Conceptualization; investigation; methodology; project

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administration; resources; supervision; writing - review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data, tools, and material (or their source) that support the protocol are available from the corresponding author upon reasonable request.

Supporting Information

Vector sequences and exemplar sgRNA/tRNA arrays are provided to assist users in designing constructs. Files are supplied as .gb format, which can be opened in text viewers or DNA analysis software (e.g., Geneious Prime), which will display annotated sequences/maps.

pNWA98.gb
Vector sequence, pNWA98

pNWA99.gb
Vector sequence, pNWA99

pNWA100.gb
Vector sequence, pNWA100

pNWA101.gb
Vector sequence, pNWA101

pNWA-186.gb
Vector sequence, pNWA186

pNWA187.gb
Vector sequence, pNWA187

sgRNAarrayexampleforpMpGEEn03.gb
Example sgRNA/tRNA array, pMpGEEn03

sgRNAarrayexampleforpNWA186.gb
Example sgRNA/tRNA array, pNWA186

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