# Chapter 19

# Heterologous Stable Expression of Terpenoid Biosynthetic Genes Using the Moss *Physcomitrella patens*

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## Abstract

Heterologous and stable expression of genes encoding terpenoid biosynthetic enzymes *in planta* is an important tool for functional characterization and is an attractive alternative to expression in microbial hosts for biotechnological production. Despite improvements to the procedure, such as streamlining of large scale Agrobacterium infiltration and upregulation of the upstream pathways, transient *in planta* heterologous expression quickly reaches limitations when used for production of terpenoids. Stable integration of transgenes into the nuclear genome of the moss *Physcomitrella patens* has already been widely recognized as a viable alternative for industrial-scale production of biopharmaceuticals. For expression of terpenoid biosynthetic genes, and reconstruction of heterologous pathways, *Physcomitrella* has unique attributes that makes it a very promising biotechnological host. These features include a high native tolerance to terpenoids, a simple endogenous terpenoid profile, convenient genome editing using homologous recombination, and cultivation techniques that allow up-scaling from single cells in microtiter plates to industrial photo-bioreactors. Beyond its use for functional characterization of terpenoid biosynthetic genes, engineered *Physcomitrella* can be a green biotechnological platform for production of terpenoids. Here, we describe two complementary and simple procedures for stable nuclear transformation of *Physcomitrella* with terpenoid biosynthetic genes, selection and cultivation of transgenic lines, and metabolite analysis of terpenoids produced in transgenic moss lines. We also provide tools for metabolic engineering through genome editing using homologous recombination.

**Key words** *Physcomitrella patens*, *In planta*, Functional characterization, Terpenoid biosynthesis, Terpene synthases, Heterologous expression, Metabolite analysis

## 1 Introduction

A relatively small and publicly available genome of *Physcomitrella* of just over 500 Mb, together with efficient homologous recombination in the nuclear genome, which is not feasible in any other plant system, allows convenient genome editing, including targeted removal of endogenous genes and introduction of transgenes. The higher time requirement for stable integration of genes into the genome of *Physcomitrella*, when compared to short-term

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transient systems, is clearly offset by maintenance of expression, and consequently production over time. Cost-effective and simple growth has helped establishing *Physcomitrella* as the most widely used system in plant photo-bioreactors [1]. The potential for engineering terpenoid biosynthesis was recently demonstrated by successful expression of a diterpene synthase from *Taxus brevifolia* in *Physcomitrella* which resulted in accumulation of taxadiene, the $C_{20}$ precursor diterpene of the lighthouse anticancer drug paclitaxel [2].

*Physcomitrella* represents an ancient lineage of land plants, and its metabolic and chemical diversity is low compared to higher plants. This is illustrated by the number of cytochromes P450 (P450s) and UDP glycosyltransferases (UGTs) found in the genome. The genomes of *Arabidopsis thaliana* and *Oryza sativa* contain 246 and 343 P450s respectively, while the genome of *Physcomitrella* only contains 71 [3]. Similarly the genome of *Physcomitrella* contains a low number of UGTs compared to other land plants [4]. The low number of P450s and UGTs found in *Physcomitrella* and the correspondingly lower chemical diversity reduces the risk of unspecific modifications by endogenous enzymes and products through pathways used in higher plants for detoxification of xenobiotics. In particular, in the *Nicotiana benthamiana* transient expression system, (*see* Chapter 18) those pathways can interfere with production and detection of hydroxylated terpenoids in form of conjugation of terpenoids [5]. In addition, *Physcomitrella* has a simple terpenoid profile and a genome that only contains a single diterpene synthase (TPS) [6]. This gene encodes a bifunctional copalyl-diphosphate synthase/kaurene synthase (PpCPS/KS) responsible for producing *ent*-kaurene. This product is used for the biosynthesis of the phytohormones gibberellins (GAs) in higher land plants [7]. However, GAs have not been detected in *Physcomitrella* [8, 9]. Although the role of kaurene-type molecules in *Physcomitrella* is unclear, they are produced in impressively large quantities, indicating a high native capacity to produce terpenoids [10, 11].

Gene editing by efficient homologous recombination in *Physcomitrella* provides a very powerful tool for metabolic engineering [12]. Targeted knockouts of PpCPS/KS result in viable kaurene-free moss lines, which have been created independently in our laboratory and in other laboratories [13]. In addition to having a terpenoid free-background, these transgenic moss lines may have the capacity to provide geranylgeranyl diphosphate (the precursor for the biosynthesis of kaurene-type diterpenes and other plastidial isoprenoids) that could be redirected into engineered heterologous terpenoid pathways. A clean metabolic background and a strong potential for metabolic engineering by homologous recombination are unique among plants and are key strengths of this *in planta* heterologous expression system. Additionally, stable

transgenic lines can be generated within 2–3 months and are easily maintained on solid media or grown in liquid photo-bioreactors. These features make *Physcomitrella* a very attractive system for producing high levels of terpenoids for basic research (purification and subsequent characterization of metabolites using NMR) as well as for the biotechnology industry, where this moss is currently used for large scale production of therapeutic antibodies in 300 L reactors [1, 4]. In this chapter we provide techniques for establishing *Physcomitrella* as a heterologous host for terpenoid biosynthesis. This includes two protocols for transformation and recovery of transgenic *Physcomitrella* lines as well as methods for metabolic profiling and characterization. Additionally, methods for medium-scale metabolite production using simple and inexpensive photo-bioreactors as well as the basic tools for performing gene editing by homologous recombination are provided.

## 2 Materials

### 2.1 Cultivation of Physcomitrella

1. Wild-type *Physcomitrella patens* (Gransden ecotype) can be obtained from the International Moss Stock Center at the University of Freiburg (http://www.moss-stock-center.org/).
2. Growth chamber with a 16 h light and 8 h dark cycle at 25 °C. Light intensities from 20 to 50 W/m$^2$ are used as standard growth conditions.
3. Phy B media, modified from minimal media previously described [14]. For 1 L, mix 800 mg of $Ca(NO_3)_2$, 250 mg of $MgSO_4{\cdot}7H_2O$, 12.5 mg of $FeSO_4{\cdot}7H_2O$, 0.5 g of $(NH_4)_2C_4H_4O$, 10 mL of $KH_2PO_4$ buffer (25 g of $KH_2PO_4$ per liter, adjust to pH 6.5 with 4 M KOH) and 0.25 mL of trace element solution (110 mg of $CuSO_4{\cdot}5H_2O$, 110 mg of $ZnSO_4{\cdot}7H_2O$, 1228 mg of $H_3BO_3$, 778 mg of $MnCl_2.4{\cdot}H_2O$, 110 mg of $CoCl_2{\cdot}6H_2O$, 53 mg of KI, and 50 mg of $Na_2MoO_4{\cdot}2H_2O$ per liter). The medium can be solidified with 0.7 % (w/v) agar A and is sterilized by autoclaving at 121 °C.
4. BCD media. For 1 L, mix 12.5 mg of $FeSO_4{\cdot}7H_2O$, 10 mL of solution B (25 g/L $MgSO_4{\cdot}7H_2O$), 10 mL of solution C (25 g/L $KH_2PO_4$ adjusted to pH 6.5 with 4 M KOH), 10 mL of solution D (101 g/L $KNO_3$), 1 mL of trace element solution (614 of mg $H_3BO_3$, 389 mg of $MnCl_2{\cdot}4H_2O$, 110 mg of $AlK(SO_4)_2{\cdot}12H_2O$, 55 mg of $CoCl_2{\cdot}6H_2O$, 55 mg of $CuSO_4{\cdot}5H_2O$, 55 mg of $ZnS0_4{\cdot}7H_2O$, 28 mg of KBr, 28 mg of KI, 28 mg of LiCl, and 28 mg of $SnCl_2{\cdot}2H_2O$ per liter). The medium can be solidified with 0.7 % (w/v) agar A and is sterilized by autoclaving at 121 °C. After autoclaving, add 1 mL of sterile 1 M $CaCl_2$ solution (1 mM final concentration) (*see* **Note 1**).

5. Tabletop centrifuge and spin column based plasmid purification, PCR purification and gel extraction kits.
6. PCR thermocycler and a DNA electrophoresis system for purification and PCR product verification.
7. Liquid nitrogen.
8. Sterile culturing facility (e.g., laminar air flow bench or biological safety cabinet) (*see* **Note 2**).
9. Glass bead sterilizer (250 °C) or flame for sterilization of tools.
10. Polytron (PT 1200 E, Kinematic AG) with an autoclavable Ø 12 mm dispersing aggregate (PT-DA 12/2 EC-E123, Kinematic AG) (*see* **Note 3**).
11. Common laboratory equipment: sterile petri dishes, parafilm, vortex mixer, serological pipettor and sterile pipettes (5–50 mL), 3 M surgical tape 1.25 cm (3M 1533-0).
12. Clear plastic kitchen film (e.g., Clingfilm, Vita Wrap, or Saran Wrap) cut into 2 cm wide strips.
13. Autoclaved cellophane disks (type 325P AA Packaging Ltd.) (*see* **Note 4**).
14. BugStopper (Whatman) or similar breathable flask closures for liquid culturing.

### 2.2 Protoplast Transformation

1. Isopropyl alcohol.
2. Driselase from *Basidiomycetes* sp. (Sigma-Aldrich).
3. Syringe filters (0.22 μm) for filter sterilization.
4. D-Mannitol (8.5 %, sterile).
5. MMM solution: 8.85 mL of 10.3 % mannitol, 150 μL of 1 M $MgCl_2$, 1 mL of 2-[*N*-morpholino]-ethanesulfonic acid (1 %, adjusted to pH 5.6 with HCl).
6. Polyethylene glycol (PEG) solution: 2 g of PEG (MW 6000), 118 mg of $Ca(NO_3)\cdot 4H_2O$, 5 mL of 8.5 % D-mannitol, and 50 μL of Tris–HCl pH 8.
7. Protoplast wash solution: 8.5 % D-mannitol, 10 mM $CaCl_2$.
8. Protoplast regeneration medium (bottom layer; PRMB): BCD media (*see* Subheading 2.1, **item 4**) supplemented with 6 % D-mannitol, 5 mM $(NH_4)_2C_4H_4O$, and 10 mM $CaCl_2$, and solidified with 0.7 % agarose (*see* **Note 1**).
9. Protoplast regeneration medium (top layer; PRMT): BCD media (*see* Subheading 2.1, **item 4**) supplemented with 8 % D-mannitol, 5 mM diammonium tartrate, and 10 mM $CaCl_2$, and solidified with 0.4 % agarose (*see* **Note 1**).
10. Hemocytometer.
11. Stainless steel filters (100 μm mesh).
12. Heated water bath.

### 2.3 Biolistic Transformation

1. Helios Gene Gun system (VWR) including the Helios Gene Gun kit, helium hose assembly, helium regulator, tubing prep station, syringe kit, tubing cutter, Helios Gene Gun optimization kit, and all instructions.
2. 99.8 % Ethanol.
3. Spermidine (Sigma-Aldrich). The 1 M stock solution can be kept at −20 °C.
4. 1 M $CaCl_2$ sterile solution.
5. Nitrogen and helium gas tank (grades 4.8 and 2.0, respectively).

### 2.4 Metabolite Analysis

1. Amber and silanized 2 mL GC glass vials including screw caps with Teflon-coated silicone septa.
2. 20 mL Solid-phase micro-extraction (SPME) vials with screw neck and magnetic caps with Teflon-coated silicone septa.
3. SPME fiber appropriate for volatile absorption, for example one based on divinylbenzene/carboxen/polydimethylsiloxane as carrier material (DVB/CAR/PDMS; Supelco).
4. GC-MS: Shimadzu GC-2010GC, with a 30 m Agilent HP-5MS column (250 μm i.d., 0.25 μm film) and helium as carrier gas. CTC AOC-5000 auto injector and a GCMS-QP2010 PlusMS Detector using electrical ionization mode. Similar GC-MS systems can be applied.
5. Solvent for extraction e.g., GC grade *n*-hexane or inhibitor-free diethyl ether and internal standards e.g., 1-eicosene (Sigma Aldrich).
6. 15 mL centrifugation tubes for 3,000 × *g* centrifugation.
7. Dodecane (Sigma-Aldrich) for volatile trapping.
8. 65 °C oven and an extra fine analytical balance (±0.01 mg) for dry-weight determination.

### 2.5 DNA and RNA Isolation

1. Microcentrifuge tube pestle.
2. Edwards buffer: 200 mM Tris–HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS [15].
3. TE buffer: 10 mM Tris–HCl pH 8.0, 1 mM EDTA.
4. RNAqueous kit (Ambion).

## 3 Methods

### 3.1 Cultivation of Physcomitrella

Cultivation of *Physcomitrellla* is performed according to standard protocols [16]. All work with *Physcomitrella* is done under sterile conditions using sterile materials and standard sterile techniques (*see* **Note 2**). All moss handling is performed with sterile forceps.

*3.1.1 Cultivation on Solid Media Using Petri Dishes*

1. Place a lump (approximately 2–5 mm in diameter) of *Physcomitrella* gametophyte tissue on a petri dish with Phy B solid media. Seal the plates with 3 M surgical tape (*see* **Note 5**).
2. Incubate the cultures at standard conditions in a 25 °C growth chamber.

*3.1.2 Cultivation on Solid Media Overlaid with Cellophane Disks*

By growing *Physcomitrella* on top of cellophane the tissue does not adhere to the solid agar and is therefore easier to handle.

1. Overlay a 9 mm petri dish filled with Phy B solid media with a sterilized disk of cellophane. Close the lid and let the cellophane disk absorb moisture for 10 min. Straighten the disk to avoid air bubbles and wrinkles.
2. Add a lump of approximately 1-week-old tissue (around 10 mm in diameter) to 10 mL of sterile water in a 50 mL tube and homogenize using a Polytron (*see* **Note 3**).
3. Use a serological pipette to transfer 1–3 mL of homogenized suspension to petri dishes with cellophane overlaid Phy B media and let the plates dry uncovered (*see* **Note 6**).
4. Seal the plates carefully with surgical tape and incubate the cultures 1 week in a growth chamber at standard conditions.
5. Harvest the tissue by scraping it off the cellophane.
6. For further biomass generation repeat **steps 1–5** using one plate of protonemal tissue blended in 10 mL of water.

*3.1.3 Cultivation in Liquid Medium Using Medium-Scale Photo-bioreactors*

1. Add tissue from a plate of 1-week-old protonemal tissue to 5–10 mL of sterile water and homogenize by a Polytron (*see* **Note 3**).
2. Use 5–10 mL to inoculate 50–100 mL BCD liquid media in a 500 mL Erlenmeyer flask (*see* **Note 7**).
3. Seal the flask using a sterile BugStopper and incubate for up to 3 months on a rotary shaker in a growth chamber.
4. In order to keep the tissue in the haploid stage and to enhance growth rates, the tissue should be homogenized every 1–2 weeks.

### *3.2 Protoplast Transformation*

Two approaches can be applied for integration of foreign genes into the genome of *Physcomitrella*. One is targeted integration to a specific locus via homologous recombination and the other is non-targeted or random integration. For random integration of DNA fragments into the genome of *Physcomitrella* any plant expression vector can be utilized (*see* **Note 8**). The major requirement is that the vector contains an appropriate selection marker (*see* **Note 9**). *Physcomitrella* has a uniquely high rate of homologous recombination among plants, and it is possible to perform metabolic engineering by gene editing or targeted integration. For this, 1 kb flanking regions homologous to the targeted locus

are added on each side of the DNA to be integrated (e.g., the expression cassette of a selection marker and the gene of interest) (*see* **Note 10**). Protoplast transformation is the most commonly used method for *Physcomitrella* transformation, and is well described in the literature [12]. The method requires careful handling and regeneration of fragile protoplasts and must be done under sterile conditions. This method can be very efficient and yield a large number of stable transformants. However, several attempts may be needed to successfully recover stable *Physcomitrella* lines.

### 3.2.1 Preparation of DNA for Transformation

1. Prepare plasmid DNA from *E. coli* cultures using mini- or maxiprep kits.
2. Linearize 100 μg of DNA by overnight digestion with an appropriate restriction enzyme. Inactivate restriction enzyme by heating at 60–80 °C for 20 min. Verify digestion by electrophoresis.
3. Precipitate DNA by addition of sodium acetate (pH 5.2, final concentration 0.3 M) and 0.7 volumes of isopropyl alcohol. Mix well and centrifuge at 15,000 × *g* for 15 min. Carefully decant the supernatant, and wash the pellet with 70 % ethanol. Centrifuge again at 15,000 × *g* for 5–15 min and decant the supernatant. Air-dry the pellet and redissolve the DNA in 50 μL of water. Final DNA concentration should be between 1 and 2 μg/μL.

### 3.2.2 Preparation and Transformation of Protoplasts

Protoplasts are highly sensitive to external force, so handle with great care.

1. On the day of transformation, first prepare the PEG solution, and allow it to sit for 2–3 h before use. After this, filter-sterilize the solution by passing it through a 0.22 μm syringe filter. Prepare petri dishes with PRMB, overlaid with sterile cellophane.
2. Collect 5–7-day-old moss protonema tissue by scraping it off cellophane-overlaid agar plates. Place in a sterile 50 mL plastic tube, and weigh the tissue. Prepare 1 mL of a 0.5 % driselase solution for every 40 mg tissue. Dissolve the Driselase powder in 8.5 % D-mannitol, and sterilize using a syringe filter, adding it directly to *Physcomitrella* tissue. Incubate the mixture for 30–60 min with occasional inversion of tube.
3. Pour the Driselase treated tissue through a sterile 100 μm stainless steel mesh screen, and recover the protoplasts in a sterile 100 mL beaker. Most undigested tissue and cellular debris will not pass through the mesh.
4. Centrifuge the protoplasts at 200 × *g* for 5 min, with gentle acceleration and breaking.
5. Carefully remove the supernatant using a serological pipette, being careful not to disturb the protoplast pellet.

6. Resuspend the pellet in protoplast wash solution using the same volume as driselase solution in **step 2**.
7. Repeat **steps 4** and **5**.
8. Resuspend the protoplasts in half the original volume of 8.5 % D-mannitol and estimate the protoplast density using a hemocytometer.
9. Centrifuge at 200 × *g* for 5 min, remove supernatant, and resuspend the pellet in sterile MMM solution to give a protoplast concentration of 1.5–2 × $10^6$ protoplasts/mL (*see* **Note 11**).
10. Add 20 μg of linearized DNA to the bottom of a 15 mL conical tube. Total volume of DNA should be less than 30 μL. Add 300 μL of protoplast suspension to the DNA and then add 300 μL of sterile PEG prepared in **step 1**. Mix by flicking the tube gently.
11. Incubate the mixture in a 45 °C water bath for 5 min followed by 5 min at room temperature.
12. Dilute protoplast suspension 5 times with 300 μL of 8.5 % D-mannitol, waiting for 1 min between dilutions.
13. Dilute an additional 5 times with 1 mL of 8.5 % D-mannitol.
14. Centrifuge the transformed protoplasts at 200 × *g* with gentle acceleration and braking for 5 min, and remove the supernatant with a pipette.
15. Resuspend the protoplasts in 500 μL of 8.5 % D-mannitol, and add 2.5 mL of molten PRMT, and mix (*see* **Note 12**).
16. Evenly disperse 1 mL of the protoplast suspension using cut pipet tips to fresh PRMB petri dishes, overlaid with cellophane. Each transformation will result in 3 plates.
17. Seal plates with 3 M tape and incubate in the growth chamber under standard conditions.
18. Allow protoplasts to regenerate their cell walls for 5–7 days, and transfer the cellophane with regenerated protoplasts to Phy B media containing an appropriate selection agent for 2 weeks (*see* **Note 9**). Proceed with selection of positive transformants (*see* Subheading 3.4).

### 3.3 Biolistic Transformation

Biolistic transformation is a robust alternative to PEG-mediated transformation of protoplasts. This protocol requires more specialized equipment, but it can be performed with a minimal number of tissue handling steps.

#### 3.3.1 Preparation of DNA-Coated Gold Particles

1. Mix 1.8 mg of PVP with 90 μL of 99.8 % ethanol, vortex and leave for 5 min until the PVP is fully dissolved. Transfer 4.5 μL to a 15 mL tube and add 3 mL of 99.8 % ethanol (final PVP concentration: 0.03 mg/mL).

2. Mix 25 mg of gold particles (1 μm in diameter) and 100 μL of 0.05 M spermidine. Vortex vigorously for 1 min and add 100 μL of transformation plasmid (diluted to 100 ng/μL) to the gold/spermidine solution. Vortex vigorously for 1 min.
3. To precipitate DNA onto the gold particles, add 100 μL of sterile 1 M $CaCl_2$ dropwise while vortexing. Leave at room temperature for 10 min.
4. Vortex and pellet the gold particles by a brief centrifugation. Remove the supernatant and wash with 1 mL of 99.8 % ethanol. Repeat this washing step two more times.
5. Resuspend the gold particles in 3 mL of 0.03 mg/mL PVP solution prepared in **step 1**.

### 3.3.2 Tubing Coating

1. Connect the Bio-Rad Tubing Prep Station to a nitrogen gas cylinder according to the manufacturer's manual (*see* **Note 13**). Gently insert 50 cm of Gold-Coat Tubing into the tubing support cylinder. Turn the nitrogen flow on (0.4 L/min) for 10 min. Dismount the tubing.
2. Connect the Gold-Coat Tubing to a 10 mL syringe using 5 cm of syringe adaptor tubing. Vortex the DNA–gold suspension from the previous section. Immediately after this, use the 5 mL syringe to gently suck the DNA–gold suspension into the rubber tube. Avoid air bubbles in the Gold-Coat Tubing. Mark where the DNA–gold suspension starts and stops on the gold-coat tubing.
3. Immediately following this insert the Gold-Coat Tube containing the DNA–gold suspension into the tubing support cylinder. Wait for 2 min to let the gold particles sediment in the tube.
4. Slowly suck out the PVP solution using the 10 mL syringe, leaving the sedimented gold particles in the Gold-Coat Tubing. This should take 1 min.
5. After removing the PVP solution, remove the syringe and start the rotation of the Tubing Prep Station. Rotate for 2 min to ensure even distribution of the gold particles.
6. Turn the nitrogen flow on (0.4 L/min) for 5–10 min to dry the DNA–gold particles.
7. Cut the Gold-Coat Tubing into 130 mm pieces using a BioRad Tubing Cutter. These pieces of Gold-Coat Tubing with DNA-coated gold particles on the inner wall are referred to as "cartridges" for the Helios Gene Gun. You will get around 30 cartridges from this, which should be enough for 7–8 rounds of transformation.
8. The cartridges are stored in tightly capped storage vials (e.g., a 20 mL polyethylene scintillation vial) wrapped with Parafilm and kept at 4 °C. The cartridges can be used for 12 months after date of preparation. Silica gel can be added to absorb any condensation and extend storage time.

#### 3.3.3 Particle Shooting

The following steps need to be carried under sterile conditions.

1. Attach the Helios Gene Gun to the helium regulator according to the manufacturer's guidelines and set the pressure to 120 psi.
2. Scrape 7-day-old moss protonema from cellophane-overlaid agar into a clump in the center of a petri dish. The diameter of the moss clump should be approximately 2 cm.
3. Load 4 cartridges into a sterile cartridge holder using sterile forceps, and assemble the loaded cartridge holder, Helios Gene Gun, battery, and a sterile barrel liner.
4. Hold the Helios Gene Gun vertically and aim the gun at the lump of moss. The distance between the barrel liner and the moss should be 1–2 cm. Hold in the safety interlock switch and press the trigger down. Shoot helium through the cartridge twice.
5. Repeat **step 5** three more times for the remaining 3 cartridges.
6. Close the petri dish with surgical tape and let the moss recover in a growth chamber for 2 days.
7. Transfer the moss lump to 10 mL sterile water in a 50 mL tube and blend with a Polytron.
8. Transfer the solution to three cellophane-overlaid Phy B solid media plates including appropriate antibiotics for selection of transformed cells, and let the solution dry until the tissue remains moist, but without excess water.
9. Seal the plates with surgical tape and incubate the cultures for 2 weeks in the growth chamber. To obtain stable transformants follow the selection procedure decribed below (*see* Subheading 3.4).

### 3.4 Selection of Transformants

1. After 2 weeks on Phy B selective media, transfer the cellophane disks with recovered transformants to solid Phy B media and incubate for 2 weeks under standard conditions. Unstable or transient transformants will lose the transformed DNA and the ability to survive on selective media during this period.
2. Cellophane disks with transformed *Physcomitrella* are transferred onto solid Phy B media supplemented with an appropriate selective agent and incubated for another 2 weeks (*see* **Note 9**).
3. Repeat **step 1** and **2** two more times and the moss lines obtained after three rounds of selective pressure are considered to be stably transformed moss lines. Verify transformants using metabolic profiling and PCR amplification of transgenes.

### 3.5 Metabolite Profiling

It is possible to obtain moss lines that do not express the gene of interest despite surviving the selection. We recommend performing metabolic and genetic screening in order to confirm the existence of heterologously produced metabolites before further

characterization of the moss lines. Always use glass equipment for handling solvents to avoid plasticizer contaminants.

### 3.5.1 Automated Analysis of Volatile Terpenoid Products

This method is preferred for initial screening of volatiles in stable moss lines. Many lines can be screened in parallel with minimal hands-on time using auto sampling.

1. Sterilize 20 mL GC vials, caps, and septa separately. Pipet approximately 4 mL of solid Phy B media into each vial and inoculate by placing a lump of moss on top of the agar.
2. Cap the GC vials and incubate cultures 1–4 weeks in the growth chamber.
3. Sample the headspace volatiles by incubating a Solid Phase Microextraction (SPME) fiber in the vials at room temperature for 30 min and analyze by GC-MS.

### 3.5.2 Quantification of Volatile Terpenoid Products

1. Inoculate a 500 mL Erlenmeyer flask containing 100 mL of BCD media with at least 2.5 mL of 1-week-old plate blended for 30 s in 5 mL sterile water. Seal the flasks using sterile BugStopper or other breathable flask cap.
2. Incubate the moss cultures with 70 rpm agitation under standard conditions.
3. After 4 days add 5–10 mL of dodecane, under sterile conditions, and continue cultivation for up to 3–5 weeks.
4. Transfer approximately 10 mL of culture containing dodecane to a 15 mL glass centrifuge tube and centrifuge at 4,000 rpm for 5 min.
5. Take 100 μL of dodecane from the upper phase, dilute 10 times in hexane spiked with an internal standard, and analyze by GC-MS (*see* **Note 14**).
6. The concentration of the metabolite of interest can be calculated based on the internal standard and an authentic standard run in parallel.

### 3.5.3 Analysis of Semi- or Non-volatile Terpenoid Products

1. Transfer a lump of moss into a 2 mL GC glass vial containing 500–1,000 μL of organic solvent such as n-hexane including an appropriate internal standard with a concentration of 0.2–1 mg/L.
2. Cap the vial and vortex the samples briefly.
3. Extract at room temperature for 1 h or overnight at 4 °C while mixing.
4. Transfer the extract to a new vial and analyze by GC-MS.

#### 3.5.4 Dry Weight Determination for Quantification of Terpenoid Products

Determining the dry weight is the most accurate way to quantify the biomass. When performing small-scale extractions the dry weight of the moss samples is around 5–15 mg and therefore an extra fine analytical balance is needed (±0.01 mg).

1. After extraction of terpenoid products and removal of organic solvent, the tissue is dried at 65 °C for 24 h.
2. Tare the balance using the vial containing the dried moss.
3. Remove the dry moss from the glass vial and reweigh the empty vial.

#### 3.5.5 GC-MS Analysis Conditions

The following conditions provide a typical starting point for separation of terpenoids using GC-MS. These settings and conditions allow separation of mixtures of mono-, sesqui-, and di-terpenes, as well as further oxidized products: Injection volume 1 μL, splitless injector temperature 250 °C, 30 m HP-5MS column, 2 mL/min He carrier gas, GC temperature program (50 °C for 3 min, 15 °C/min to 310 °C and hold 5 min), scan range from *m/z* 50 to *m/z* 350 with 70 eV electrical ionization. These general conditions can be modified to shorten runtime, improve resolution or sensitivity for specific compounds.

### 3.6 DNA and RNA Isolation for Further Characterization

#### 3.6.1 Rapid Small Scale DNA Isolation

1. Snap-freeze 100 mg of *Physcomitrella* tissue in a 2 mL microcentrifuge tube in liquid nitrogen.
2. Grind the frozen tissue using a microcentrifuge tube pestle, add 500 μL of Edwards buffer and vortex vigorously (*see* **Note 15**).
3. Centrifuge the sample at 15–20,000 × *g* for 10 min at room temperature.
4. Transfer 400 μL of the supernatant to a new tube and add 400 μL of isopropanol. Mix gently.
5. Centrifuge the sample 15–20,000 × *g* for 10 min at room temperature and discard the supernatant.
6. Invert the tubes and let them dry on a paper towel.
7. Once the tube is completely dry resuspend the pellet in 300 μL of TE buffer at 50 °C for 30 min and store at –20 °C until use (*see* **Note 16**).
8. For PCR use 1 μL of DNA as template.

#### 3.6.2 Rapid Small Scale RNA Isolation

1. Snap-freeze 100 mg of moss tissue in a 1.5 mL microcentrifuge tube in liquid nitrogen.
2. Add 300 μL of Lysis Solution from the RNAqueous kit and grind with a microcentrifuge pestle while the tissue is defrosting.

3. Spin the tissue at 20,000 × *g* for 3 min and grind the tissue further. Repeat this two times.
4. Add 150 µL of 100 % ethanol and vortex briefly and proceed with the extraction according to the RNAqueous kit protocol.
5. Elute in two times 15 µL of Elution Buffer, preheated to 75 °C.
6. DNase I treat and inactivate the enzyme according to the RNAqueous kit protocol.

## 4 Notes

1. Adding $CaCl_2$ after autoclaving minimizes precipitation of the media.
2. Since *Physcomitrella* is typically grown on Phy B media without supplemental antibiotics there is a significant risk of contamination by fungi or bacteria, which may not be obvious until after several weeks of growth. We recommend using a sterile facility e.g., a LAF bench dedicated for work with plant tissue culturing to limit the risk of microbial contamination.
3. Many different Polytrons or tissue homogenizers can be used. Most importantly, the blender tip needs to be autoclavable. Excessive blending will lead to poor regeneration.
4. Prevent the cellophane disks from sticking together autoclave with filter paper in between each cellophane disk.
5. Plates sealed with 3 M surgical tape have a high growth rate, but will dry up within 2–4 weeks, depending on the starting volume of media. Sealing the plates with kitchen film or Parafilm facilitates storage for 6 months or more, however, this will also slow the growth of the cultures significantly.
6. Do not overdry the plates. The cellophane will curl and the moss will not survive.
7. In addition to BCD media, liquid Phy B and KNOP media can be used for liquid growth. In order to obtain very high growth rates approximately 1.5 % of $CO_2$ can be supplemented to the moss culture and the light intensity can be increased.
8. In our lab, pCAMBIA vectors (http://www.cambia.org/daisy/cambia/585) are used as starting point for generating non-targeted *Physcomitrella* expression vectors. Since most of the vector backbone of the pCAMBIA binary vectors is nonessential for *Physcomitrella* transformation and expression, we recommend cloning the essential parts of pCAMBIA vectors (expression cassettes of the gene of interest and the selection marker) into a high copy number subcloning vector with a

smaller backbone. The use of such simpler expression vectors facilitates downstream cloning and DNA propagation. Furthermore, a viral 2A linker for expression of several genes under the control of a single promoter can be used [17].

9. We have successfully used four different selection agents for recovery of stable tranformants, namely hygromycin (30 μg/mL), G418 (kanamycin) (50 μg/mL), sulfadizine sodium (150 μg/mL), or gentamicin sulfate (100 μg/mL).
10. We recommend using at least 750 base pairs of homologous genomic sequence flanking the desired insertion site. However, transformation of DNA that does not contain targeting sequences can also lead to long-term maintenance and expression of foreign DNA. Many integration sites have successfully been established for heterologous expression in *Physcomitrella*. A very useful subset of moss vectors for targeted integration can be found at the homepage of Michael Prigge (Mark Estelle's Lab http://labs.biology.ucsd.edu/estelle/moss2.html).
11. If transformations yield few surviving lines, the protoplast concentration can be increased up to tenfold. However, this increases the risk of recovering a lawn of transformants, resulting in an inability to distinguish individual transformants due to high colony density.
12. Keep the PRMT in a 45 °C water bath. This will keep the agar melted, but cool enough to ensure protoplast survival.
13. The manual for connecting the Bio-Rad Tubing Prep Station can be found online (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_9541.pdf).
14. Depending on your metabolite abundance, the dilution can be varied from 3 to 1,000 times.
15. DNA isolation can also be performed in a 96-well high-throughput format using metal beads and a mixer mill for $2 \times 30$ s. After being frozen in liquid nitrogen the sample lids are more fragile and can break after excessive mixing in the mixer mill.
16. Small amounts of pigments might be left in the DNA extract. However, this does not typically inhibit PCR. For a cleaner DNA template include one or two steps of ethanol wash: Add 100 μL of 70 % ethanol, vortex briefly and spin the sample for 15–20,000 $\times g$ for 10 min at room temperature. Discard supernatant and dry the tubes by inverting them on a paper towel. Resuspend in 300 μL of TE buffer as described.

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