DEVELOPMENT OF METHODS FOR GENERATING *PHYSCOMITRELLA PATENS* MUTANTS WITH DISABLED METHYLTRANSFERASE GENES

By

Maivy N. Le

A capstone project submitted for
Graduation with University Honors

May 28, 2020

University Honors
University of California, Riverside

APPROVED

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Dr. Eugene A. Nothnagel
Department of Botany and Plant Sciences

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Dr. Richard Cardullo, Howard H Hays Jr. Chair, University Honors

Abstract

Mosses like *Physcomitrella patens* are more desiccation tolerant than most angiosperms, the highly evolved plants. Improved desiccation tolerance of crop plants might increase food production in drought-stressed environments. *Physcomitrella* cell wall polymers contain 3-*O*-methyl-rhamnosyl residues, a methylated sugar not found in angiosperms. Moss methyltransferase genes *MT1* and *MT6* were previously found to produce 3-*O*-methyl-galactosyl residues when expressed in tobacco plants, and leaves from those transgenic plants were more desiccation tolerant than leaves from wild-type tobacco, where 3-*O*-methyl-galactosyl residues are not detected. Because 3-*O*-methyl-galactosyl residues are not detected in *Physcomitrella*, a hypothesis was formulated that MT1 and MT6 can synthesize either 3-*O*-methyl-rhamnosyl or 3-*O*-methyl-galactosyl residues, depending on conditions and available substrates. This project aims to develop methods for using homologous recombination to knock out, or disable, *MT1* or *MT6* in *Physcomitrella*. Generation of such knock-out mutants would be useful in determining whether *MT1* or *MT6*, or neither, is responsible for synthesis of 3-*O*-methyl-rhamnosyl residues in *Physcomitrella*. This report focuses on progress towards this goal, including: synthesis of a DNA construct designed to insert a Geneticin resistance gene in place of *MT1* by homologous recombination; growth of moss protonemal cultures; generation of protoplasts from protonema; use of polyethylene glycol treatment to deliver the DNA construct into the cytoplasm of protoplasts; and, in ongoing work, culturing the protoplasts back to whole cells that can be screened for resistance to Geneticin antibiotic and analyzed for 3-*O*-methyl-rhamnosyl content.
Supported by USDA NIFA grant 2008-35318-04599.

Acknowledgements

I would like to thank Dr. Eugene A. Nothnagel, my faculty mentor and principle investigator, for his continuous support throughout this project and my undergraduate career. I would also like to thank Dr. Martha L. Orozco-Cárdenas for providing guidance and laboratory space. Additionally, I would like to thank my fellow laboratory member, Anjin Huang, for the construction of the DNA knockout cassette.

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Introduction

Physcomitrella patens cell wall polymers contain 3-*O*-methyl-rhamnosyl residues, which are not found in angiosperms such as tobacco (Popper et al. 2004, Fu et al. 2007). When cDNAs from *Physcomitrella patens* methyltransferase genes *MT1* and *MT6* were expressed in tobacco plants, 3-*O*-methyl-galactosyl residues were detected in cell wall polymers of the transformed plants (Sasaninia et al. 2015, Tavernier and Nothnagel 2016, Masters et al. 2017), and leaf tissue from the transformed plants exhibited improved desiccation tolerance compared to leaf tissue from wild-type tobacco plants (Cid et al. 2015). Curiously, the abundances of 3-*O*-methyl-galactosyl residues are not above noise level and might be zero in *Physcomitrella patens* and wild-type tobacco plants. Both methylated sugars have an extra methyl group at C3 (Fig. 1), inviting the hypothesis that MT1 and MT6 might synthesize either 3-*O*-methyl-rhamnosyl residues or 3-*O*-methyl-galactosyl residues, depending on conditions.

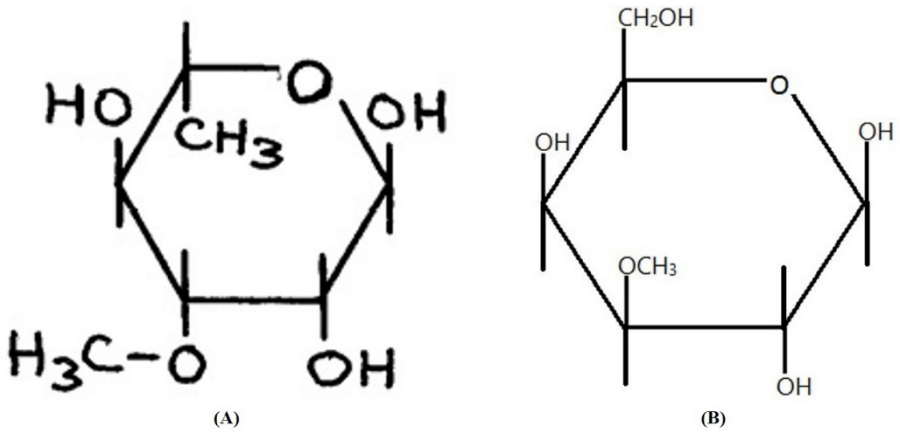


Figure 1. (A) 3-*O*-methyl-α-L-rhamnose, a 6-deoxyhexose having an extra methyl group at C3. **(B)** 3-O-methyl-β-D-galactose, a hexose having an extra methyl group at C3.

Because angiosperms evolved from simpler organisms, studying lower land plant models could enhance knowledge of angiosperm features (Fu et al. 2007). Comparing the primary cell walls of lower land plants with those of higher evolved plants indicates that methylated sugar residues became less abundant at the transition from gymnosperms to angiosperms, suggesting

that changes in cell wall composition are a factor in plant evolution (Popper et al. 2004). Mosses are more desiccation tolerant that most angiosperms, so a potential practical benefit of this project could be a pathway to improving desiccation tolerance in angiosperms, particularly crop plants, by reintroducing methylated sugar residues. Improved desiccation tolerance of crop plants might increase food production in drought-stressed environments.

Hypothesis

This project aims to develop methods to knock-out *MT1* and *MT6* in *Physcomitrella patens* to test this hypothesis: If MT1 and/or MT6 methyltransferases have active sites that can make either 3-*O*-methyl-galactosyl or 3-*O*-methyl-rhamnosyl residues, then moss *mt1* and/or *mt6* knock-out lines will have reduced or zero levels of 3-*O*-methyl-rhamnosyl residues in their cell walls.

Methods

Physcomitrella patens has been found to edit its own DNA by a process called homologous recombination (Roberts et al. 2011, Hiwatashi et al. 2012), which very rarely, if ever, occurs in most other plants. Homologous recombination will be taken advantage of to knock-out the *MT1* and/or *MT6* gene. To do this, DNA sequences from the 5' flanking region and 3' flanking region of the *MT1* or *MT6* gene in the moss chromosome were copied and inserted into the pTN182 plasmid (Fig. 2). The pTN182 plasmid (ordered from www.addgene.org) was specially made for the purpose of homologous recombination in *Physcomitrella patens* and has a G418 antibiotic resistance gene in the middle (Sakakibara et al. 2008).

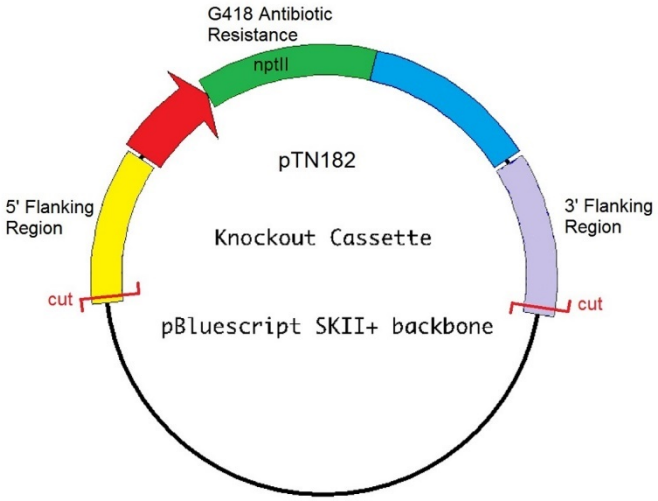


Figure 2. pTN182 Knockout cassette with the 5' flanking region (yellow) and 3' flanking region (purple) copied from *MT1* gene of the *Physcomitrella patens* chromosome. The red cuts denoted the regions the restriction endonucleases cut. In the middle of the plasmid was a G418 antibiotic resistance gene (green). Sketch modified from Zhu (2013).

The linearized DNA *MT1* knockout cassette was constructed before protoplast isolation. *E. coli* harboring the plasmid DNA construct was cultured. A QIAGEN Hi-Speed Plasmid Midi Kit, Promega Wizard Mini-prep Kit, or other similar kit was used to extract the plasmid from the *E. coli*. To excise and linearize the DNA cassette, the plasmid was digested with appropriate restriction enzymes. The digested plasmid was purified by phenol/chloroform extraction and precipitated by adding 1/20th the volume of 3 M NaOAc (pH 5.2) and 2 volumes of ethanol. The precipitated plasmid was dissolved with sterile TRIS-EDTA buffer (TE) to achieve a DNA concentration of 0.2-1.0 µg/µL. For each transformation, 30 µL (6-20 µg of DNA) of this solution was used. In the laminar flow hood, the linear DNA knockout construct was transferred into a sterile 0.22 µm GV Durapore centrifugal filter unit (Millipore) and centrifuged at 12,000 x g for 4 minutes. The sterile filtered plasmid was then transferred to a sterile tube.

If this knockout cassette can enter the cytoplasm of a single *Physcomitrella* cell, the cell might perform homologous recombination, editing its chromosome by cutting out the *MT1* gene and splicing the knockout cassette in its place (Fig. 3). The transformed cell will no longer have

the *MT1* gene and will not produce an MT1 methyltransferase. The cell will instead have a G418 resistance gene, allowing it to grow and divide in the presence of G418 antibiotics.

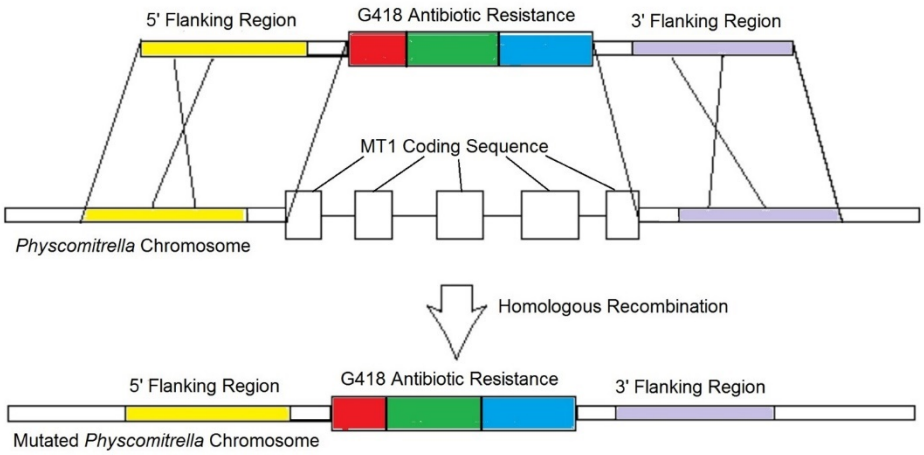


Figure 3. Schematic of the *MT1* gene in the *Physcomitrella patens* chromosome and how homologous recombination occurred to splice in the knockout cassette in place of the *MT1* gene. Compare with Figure 2. Sketch modified from Hiwatashi et al. (2012).

Protoplast isolation and transformation experimental methods were adapted from Roberts et al. (2011) and Hiwatashi and Hasebe (2012). A condensed description of the adapted methods used in this project are presented here. For a more detailed, step-by-step description of the methods used in this project, see Le and Nothnagel (2019).

All equipment and media used in protonema culturing, protoplast isolation, and transformation were sterilized by autoclaving or filtering through sterile membranes with 0.22 µm pores. Routine chemicals were ordered from Sigma-Aldrich or Fisher Scientific. Cellophane was ordered from Sigma-Aldrich. The 480 mM sucrose + 5 mM $CaCl_2$ solution was prepared and autoclaved. The MMM solution with 0.5 M Mannitol, 15 mM $MgCl_2$, and 5.1 mM MES titrated to pH 5.6 with dilute KOH was prepared and filter sterilized. The PEG buffer with 7.57% D-mannitol, 0.099 M $Ca(NO_3)_2$, and 0.0099 M Tris base titrated to pH 9.0 with dilute HCl was prepared and autoclaved. A 5 mL aliquot of the PEG buffer was added to 2.0 g of autoclaved, still warm and liquid PEG 8000 and vortexed until uniform. Media for culturing were prepared in

advance, as seen in Table 1. Mannose was present to prevent osmotic rupture of protoplasts, which are no longer supported by a cell wall. Diammonium titrate was present to promote protonema growth. G418 (Geneticin) antibiotic (from Research Products International) was added to the baby food jars of BCDAT medium to final concentrations of 0 mg/L, 25 mg/L, or 50 mg/L.

Table 1. Media for culture and transformation of Physcomitrella patens

	BCDAT (per L)	PRMB (per L)	PRMT (per L)	PRML (per L)
$MgSO_4$ heptahydrate	0.25 g	0.25 g	0.25 g	0.25 g
KH_2PO_4	0.25 g	0.25 g	0.25 g	0.25 g
KNO_3	1.0 g	1.0 g	1.0 g	1.0 g
$FeSO_4$ septahydrate	12.5 mg	12.5 mg	12.5 mg	12.5 mg
Diammonium tartrate	0.92 g	0.92	0.92 g	0.92 g
Trace element solution*	1 mL	1 mL	1 mL	1 mL
Mannitol	-	60 g	80 g	80 g
Agar	7 g	8 g	5 g	-
55.5 g/L $CaCl_2$ Solution	1 mL	10 mL	10 mL	10 mL

Notes: Dry ingredients were added to purified water, and sterilized by autoclaving. Calcium chloride ($CaCl_2$) was filtered and added after autoclaving to prevent precipitation. Adapted from Table 1 Roberts et al (2011).
*55 mg/L cupric sulfate pentahydrate, 55 mg/L zinc sulfate heptahydrate, 614 mg/L boric acid, 389 m/L manganous chloride tetrahydrate, 55 mg/L cobalt chloride hexahydrate, 28 mg/L potassium iodide, 25 mg/L sodium molybdate dehydrate.

Sterile protonema cultures were grown on BCDAT medium in preparation for protoplast isolation. When possible, protonema lines that had been recently initiated from spores were used, since multiploidy or other genetic abnormalities might arise as the number of vegetative propagations increase after spore germination. Weeks before the protoplast isolation, the protonema was transferred at 1 week intervals to promote healthy and fast-growing protonema. Healthy, green protonema cultures, where the protonema thinly covers 85%-90% of the cellophane overlay on the BCDAT medium, were selected as sources for protoplast isolation. Cultures that were too old, with leafy gametophytes starting to grow, were avoided. Optimal yield of healthy protoplasts usually resulted when protonema cultures were used at roughly 5 days after the protonema jars had been transferred (Fig. 4).

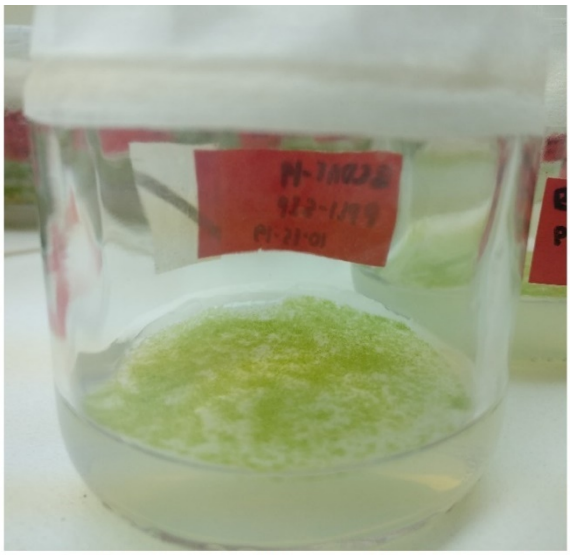


Figure 4. Protonema cultivated in a sterile cellophane-overlaid baby food jar. The protonema was healthy and green, thinly covering roughly 90% of the cellophane with no leafy gametophytes present. The protonema was fast-growing, transferred consistently at 1 week apart for several weeks and recently started from spores. At harvesting for protoplast isolation, the protonema was 5 days old since the last transfer.

Outside of the laminar hood, 60 mL of enzyme mix was prepared containing 2% (w/v) Driselase, 0.25% (w/v) bovine serum albumin, and 0.5 mM dithiothreitol in PRML liquid medium. The contents were mixed until no further Driselase solids dissolved and then centrifuged at 1000 rpm (170 xg) for 5 minutes with high brake used during slowing at the end of the run (Fig. 5). The supernatant was filtered through a sterile 150 mL polystyrene sterilizing filter unit with low protein-binding membrane with 0.22 µm pores (Corning) connected to a house vacuum. The non-sterile upper section of the filter unit was removed in the laminar flow hood. Half of the enzyme mixture was used at the start of cell wall digestion, while the other half of the enzyme solution was reserved for use in filtering steps after the incubation 1 period of wall digestion.

Figure 5. Centrifuged enzyme mix containing 2% Driselase, 0.25% bovine serum albumin, and 0.5 mM dithiothreitol in PRML liquid medium (BCDAT with 8% mannitol). The supernatant was kept and filtered, and the pellet was discarded.

A spoonula was used to scrape protonema tissue off the cellophane from 9 source protonema cultures in baby food jars and into sterile enzyme mixture. Two spoonulas were used to separate clumps of the protonema tissue. The protonema-enzyme mixture was pipetted to a 125 mL Erlenmeyer flask with a foil-covered cotton plug. The foil-covered cotton plug was again used to close the flask (Fig. 6), which was then incubated on a Gyratory Shaker-Model G2 (New Brunswick Scientific) at 30-35 rpm in the light at 25 ºC for roughly 2.5 hours (incubation 1).

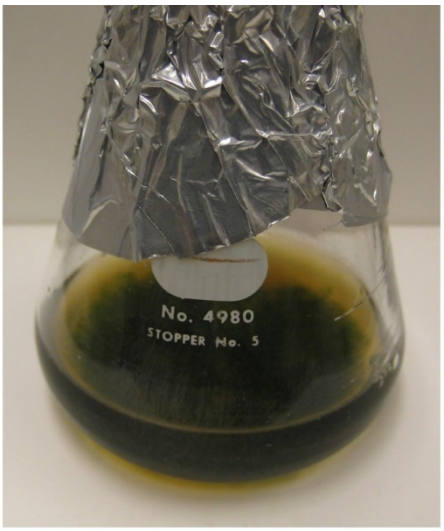


Figure 6. Incubation 1, where the enzyme mixture and protonema was shaken at 30-35 rpm at 25 ºC for 2.5 hours.

After incubation 1, the protonema-enzyme mixture was filtered through a Nitex nylon net having 350 µm pores. The fire-polished end of a glass rod and the reserved enzyme mixture were used to gently work nearly all the tissue through the net. The filtered protonema-enzyme suspension was then pipetted back into the 125 mL Erlenmeyer flask (Fig. 7) for an additional hour incubation (incubation 2). After incubation 2, the protonema-enzyme mixture was again filtered, this time through a Nitex nylon net having 64 µm pores. As before, the fire-polished end of a glass rod was used to gently work the digested tissue through the net. Instead of enzyme mixture, PRML was used to help wash the protoplasts through the net.

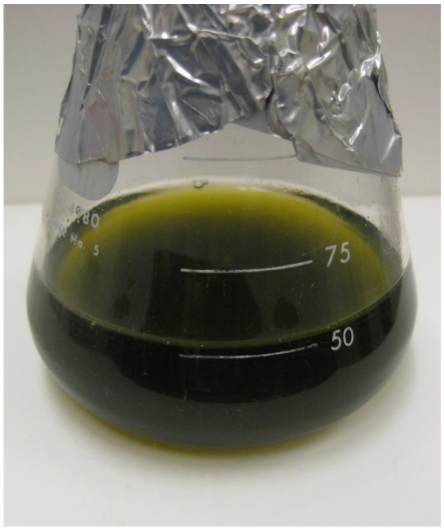


Figure 7. Incubation 2, where the enzyme mixture and protonema was shaken at 30-35 rpm at 25 ºC for1 hour after 350 µm Nitex nylon net filtering. The solution became more homogenous, taking on a clear, dark olive green appearance.

The filtrate was divided into two 50 mL screw cap centrifuge tubes. An underlayer of 5 mL 480 mM sucrose + 5 mM $CaCl_2$ was carefully added to each 50 mL tube, maintaining the layer separation. The 50 mL tubes were centrifuged at 1100 rpm (200 xg) for 15 minutes with low brake used during slowing at the end of the run.

After the centrifugation, there should be a brown upper layer, a thick green protoplast interface band, a clear 480 mM sucrose + 5 mM $CaCl_2$ underlayer, and a large green pellet. In this case, the brown supernatant was discarded first. Then, the green protoplast interface was collected from both 50 mL tubes and combined into a new 50 mL screw cap centrifuge tube. In some cases, the centrifugation instead resulted in a brown upper layer, a green layer, and a large green pellet, but there was no distinct interface between the green protoplast layer and the 480 mM sucrose + 5 mM $CaCl_2$ underlayer (Fig. 8). In this case, the brown supernatant was still discarded, but the green layer and green pellet were combined into a new 50 mL screw cap centrifuge tube. In either case, 20-25 mL of 480 mM sucrose + 5 mM $CaCl_2$ was added and the tube was gently inverted to mix. A 3 mL PRML overlayer was carefully added to the tube to

produce distinct layers. The 50 mL tube was centrifuged at 700 rpm (100 xg) for 10 minutes with low brake used during slowing at the end of the run.

Figure 8. Centrifuged 50 mL tube of 64 µm Nitex nylon net filtrate with a 5 mL 480 mM sucrose + 5 mM $CaCl_2$ underlayer. In this experiment, there was no distinct protoplast interface and sucrose + $CaCl_2$ underlayer, and a large green pellet was present. The brown supernatant was discarded. The green layer and pellet were combined into another 50 mL tube, and the procedure continued.

After the centrifugation, there should be distinct layers: a clear PRLM upper layer, a green protoplast interface band, a clear—possibly light green—480 mM sucrose + 5 mM $CaCl_2$ under layer, and a large green pellet (Fig. 9).

Figure 9. Centrifuged 50 mL tube of combined green layer and pellet from 2 previous 50 mL tubes with 20-25 mL of 480 mM sucrose + 5 mM $CaCl_2$ and a 3 mL PRML overlayer. There were three distinct layers: a PRML upper layer, a green protoplast interface, and a sucrose + $CaCl_2$ underlayer. There was a large green pellet. Only the green protoplast interface was collected.

The protoplast interface was collected and transferred to a 16 x 125 mm screw cap tube. The tube was filled to 2/3-3/4 full with 480 mM sucrose + 5 mM $CaCl_2$ and gently inverted to mix. A 2 mL PRML upper layer was carefully added, maintaining layer separation. The 16 x 125 mm tube was centrifuged at 700 rpm (100 xg) for 10 minutes with low brake used during slowing at the end of the run. After the centrifugation, there should be three distinct layers: a clear PRML upper layer, a green protoplast interface band, and a clear 480 mM sucrose + 5 mM $CaCl_2$ under layer (Fig. 10).

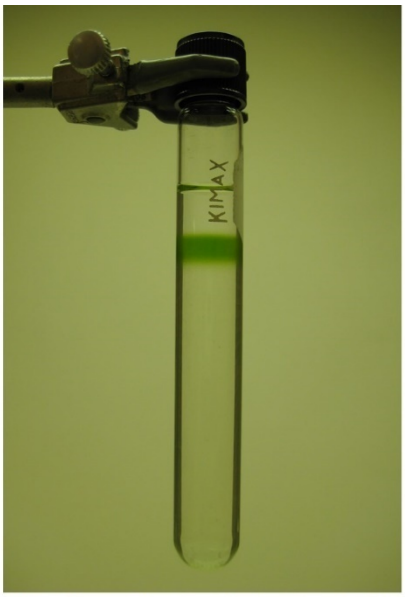


Figure 10. Centrifuged 16 x 125 mm tube of protoplast interface from the previous 50 mL tube, 2/3-3/4 full of 480 mM sucrose + 5 mM $CaCl_2$ with a 2 mL PRML overlayer. There were three distinct layers: a PRML upper layer, a green protoplast interface, and a sucrose + $CaCl_2$ underlayer. Only the green protoplast interface was collected.

The protoplast interface was collected and transferred to a 13 x 100 mm screw cap tube. It was important to not collect any of the 480 mM sucrose + 5 mM $CaCl_2$ layer at this step. The tube was filled to 3/4 full with PRML, gently inverted to mix, and centrifuged at 700 rpm (100 xg) for 7 minutes with low brake used during slowing at the end of the run. After the centrifugation, the protoplasts should be at the bottom of the tube (Fig. 11).

Figure 11. Centrifuged 13 x 100 mm tube of protoplast interface from the previous 16 x 125 mm tube, 3/4 full of PRML. In later experiments, PRML was replaced with MMM. Protoplasts should be at the bottom of the tube. The supernatant was discarded.

The supernatant was discarded, leaving roughly 1 mL at the bottom of the tube. Slowly, 1-2 mL of MMM was added to the protoplast pellet. To resuspend the protoplasts, the tube was gently rotated by hand for 2-3 minutes (Fig. 12). A small sample was removed to estimate the density with a hemocytometer.

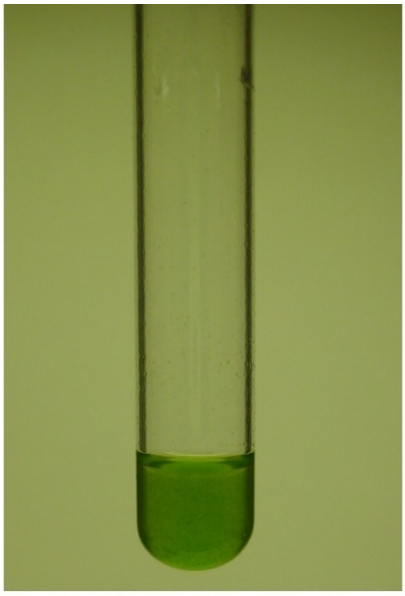


Figure 12. Protoplast pellet in 13 x 100 mm tube gently resuspended in 1-2 mL of PRML. In later experiments, PRML was replaced with MMM. Protoplast suspension was clear, dark green with many clumps.

To a 15 mL round-bottom screw cap centrifuge tube, 30 µL of sterile, linearized DNA cassette, 300 µL of protoplast suspension, and 300 µL of PEG solution were added. The mixture was incubated on a slow rotator for 10 minutes at approximately 23 ºC. The mixture was then heat shocked for 3 minutes in a 45 ºC water bath. Immediately after the heat shock, the protoplast tube was transferred to beaker with room temperature water for 10 minutes for cooling.

The cooled protoplast suspension was gradually diluted with 5 aliquots of 300 µL each of PRML at 3-minute intervals and then 5 aliquots of 1 mL each of PRML at 3-minute intervals, with gentle mixing by hand rotation of the tube after each aliquot addition. The tube was then centrifuged at 700 rpm (100 xg) for 7 minutes with low brake used during slowing at the end of the run. After centrifugation, the protoplasts should be at the bottom of the tube. The supernatant was discarded.

Slowly, 4 mL of PRMT held at 45 ºC in a water bath were added to the protoplast pellet. The tube was gently rotated by hand for 1-2 minutes to resuspend the protoplasts. To each of 8 baby jars with PRMB medium containing no antibiotic and overlayered with cellophane, 0.55 mL of the protoplast suspension were added. The baby jars were incubated for 5 days at 25 ºC and constant illumination to enable synthesis of cell walls around the protoplasts to regenerate cells.

After 5 days, sterile forceps were used to carefully lift the cellophanes with cells from the PRMB/PRMT baby food jars and place them in new baby food jars on top of BCDAT medium containing 0 mg/L, 25 mg/L, or 50 mg/L final concentration of G418 (Geneticin) antibiotic. The baby jars were then incubated for 7 days at 25 ºC and constant illumination.

Cells surviving the first 7 days on antibiotic were likely a mixture of stably and unstably transformed cells. To encourage growth of the cells to colonies, the cellophane disks with cells

were transferred to new baby food jars with BCDAT medium and no antibiotic for 7 days. To select for stably transformed colonies after the 7 days, the cellophane disks with colonies were transferred to new baby food jars with BCDAT containing antibiotic at the same concentration as during the first selection. For example, a cellophane disk with cells that was initially put on 25 mg/L of G418 will continue to be selected with 25 mg/L of G418. After this second 7 day selection with antibiotic, the cellophanes with colonies were transferred onto BCDAT medium without antibiotic for further growth. After 7 days on this medium, the cellophanes with colonies were again transferred onto BCDAT with antibiotic. In total, there should be 3 rounds of selection on G418 (Geneticin). Any colonies that grow vigorously during the third round of selection are likely to be stable transformants. After the third selection for 7 days on antibiotic, the cellophane disks with colonies were transferred and left on BCDAT without antibiotics to enable the colonies to regenerate into whole moss plants.

Results

Early in the cell wall digestion to release protoplasts, after a 2.5-hour enzyme incubation and before filtering through the Nitex nylon net having 350 μm pores, the protonema filaments were still intact and most of the cell wall was present, as seen by the rectangular shape of the cells (Fig. 13). Some protoplasts and debris were present.

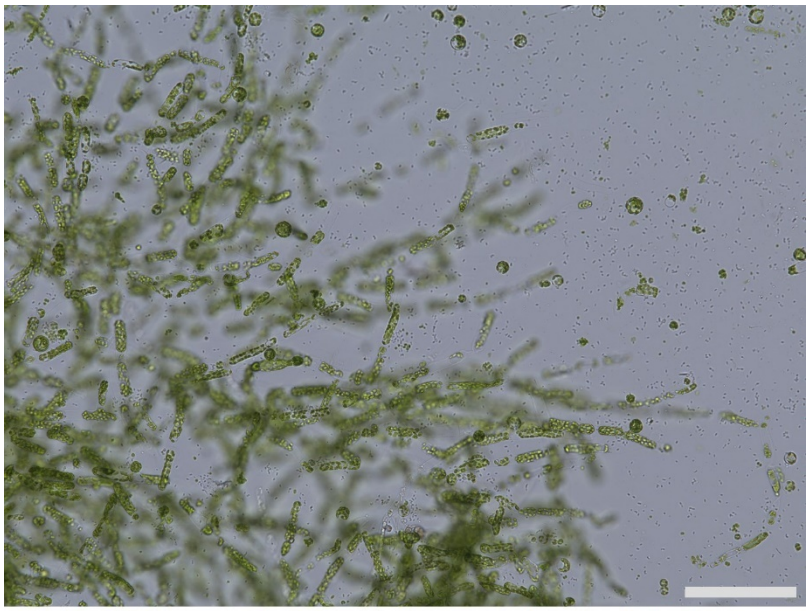


Figure 13. Protonema 2.5 hr after start of wall digestion (incubation 1) and before filtering through nylon cloth with 350 µm pores. Bar, 200 µm.

After filtering through the Nitex nylon net with 350 µm pores and before another 1-hour enzyme incubation, most of the protonema filaments were broken up (Fig. 14). Some protoplasts were present, and much more debris was observed.

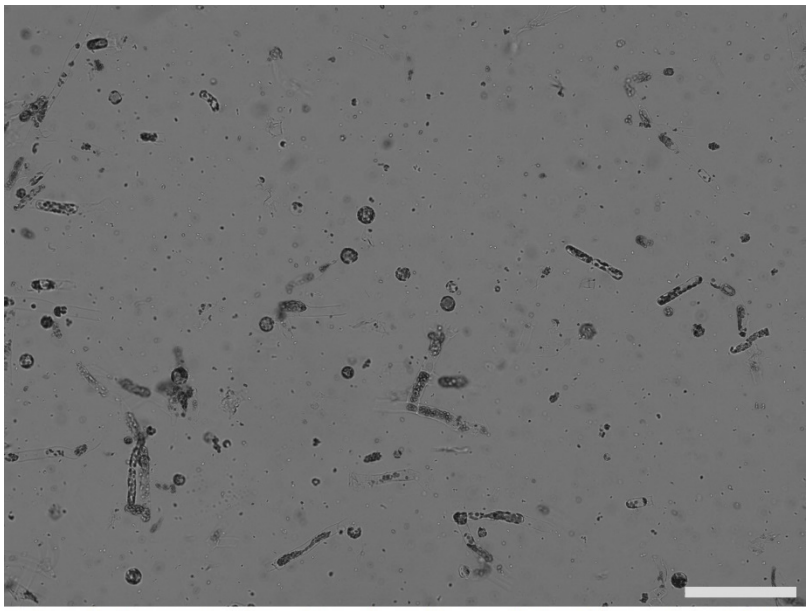
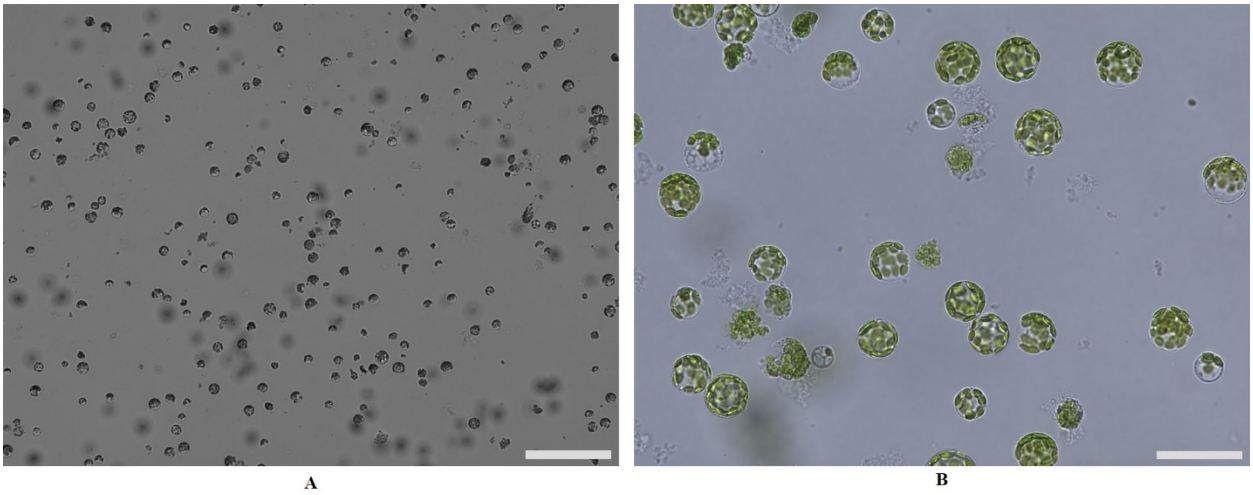


Figure 14. Protonema after 350 µm Nitex nylon net filtering and before a 1 hour incubation 2. Little intact protonema tissue was present. Some protoplast was present. More debris seen. Bar, 200 µm.

After purifying the protoplasts away from cell debris by gradient centrifugation, there were many healthy protoplasts present (Figs. 15A & B). Protoplasts are considered healthy if they are intact and spherical, with the chloroplasts uniformly distributed next to the inner face of the plasma membrane. If the chloroplasts are aggregated at the center or at one end, the protoplasts are unhealthy and unlikely to regenerate cell walls to become dividing cells. Some unhealthy protoplasts and debris were observed as well. Once isolated, the protoplasts have some tendency to clump together.



A B

Figure 15. Protoplast suspension after purification by gradient centrifugation. Many healthy protoplast present alongside some unhealthy protoplasts, debris, and clumps. **(A)** x10 objective. Bar, 200 µm. **(B)** x40 objective. Bar, 50 µm.

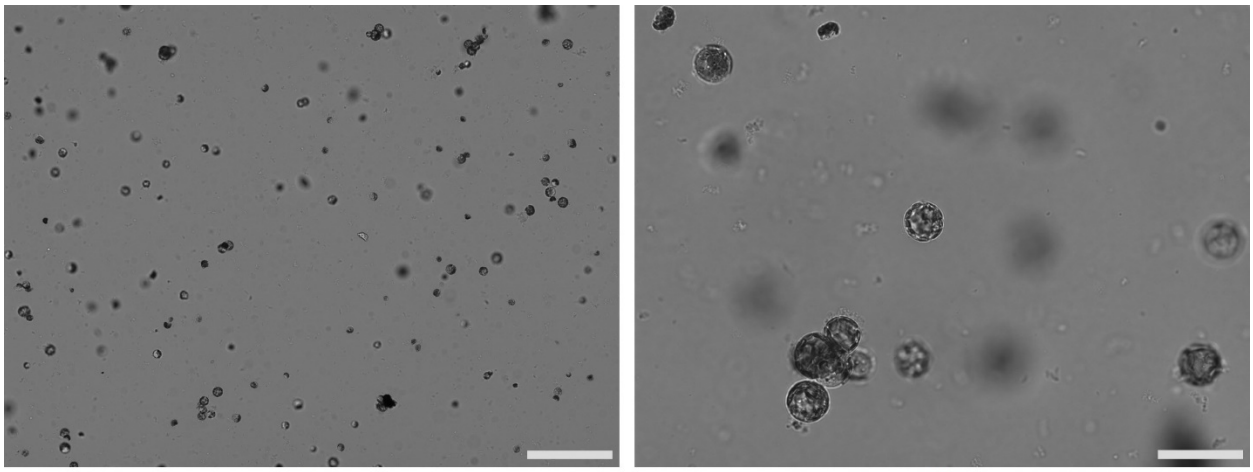
For use in homologous recombination experiments, a protoplast density of 2-4x10^5 protoplasts/mL is recommended (Roberts et al. 2011). In this project, only healthy protoplasts were counted. Starting with the protoplast isolation on October 27, 2018, the target protoplast density was reached or exceeded (Table 2). It was necessary to have many healthy protoplasts because the transformation treatment is harsh and results in low protoplast survival in even the most successful experiments. Using a larger protoplast density results in a greater chance that enough protoplasts will survive the transformation process and will grow when plated.

Table 2. Protoplast density estimated by counting on a hemocytometer

Date of Protoplast Isolation	Protoplast Density (protoplasts/mL)
6/21/2018	22,000
8/2/2018	37,500
10/27/2018	203,500
1/19/2019	520,500
10/20/2019	1,913,000
1/20/2020	803,000

Note: For homologous recombination, a protoplast density of 2-4 x 10^5 protoplasts/mL is recommended (Roberts et al. 2011).

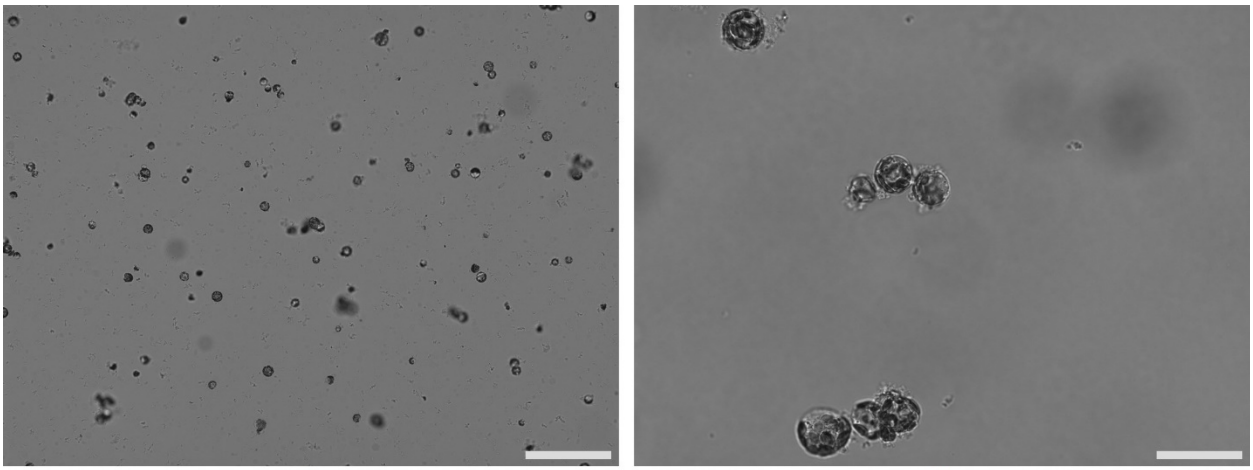
Protoplasts were examined by microscopy after the PEG treatment and after the 45 ºC heat shock treatment, the harshest steps of the procedure, to assess damage to the protoplasts. After the PEG treatment, the protoplast density decreased. Still, there were some healthy protoplasts present (Fig. 16A & B).



A B

Figure 16. Protoplasts after treatment with polyethylene glycol (PEG). Protoplast density had decreased, but some healthy protoplasts survived. **(A)** x10 objective. Bar, 200 µm. **(B)** x40 objective. Bar, 50 µm.

Similarly, after the 45 ºC heat shock treatment, some healthy protoplasts still remained (Fig. 17A & B).



A B

Figure 17. Protoplasts after after heat shock for 3 min at 45 °C. Protoplast density had decreased, but some healthy protoplasts survived. **(A)** x10 objective. Bar, 200 µm. **(B)** x40 objective. Bar, 50 µm.

In the October 20, 2019 and January 20, 2020 protoplast isolation and transformation experiments, green was visible to the eye upon plating the final protoplast suspensions in PRMT medium onto the cellophane on PRMB medium. After a few days, unfortunately, green was no longer visible. Additionally, a magnifier was used to initially visualize small, clear specks. These clear specks also seemed to disappear with time. After several weeks of observation, there was no evidence the protoplasts grew to colonies in any of the experiments.

Discussion

Despite the high protoplast density, the protoplasts did not grow to colonies in any of the experiments conducted in this project. Most likely, because the protoplasts looked very healthy just after isolation, the subsequent steps were the source of the problem. After the protoplast isolation procedures, the protoplasts tend to clump. Clumping could lead to unequal exposure of protoplasts to PEG and knockout cassette for transformation. Without transformation, the protoplasts cannot grow on antibiotic. To minimize the problem of clumping, the protoplasts were carefully resuspended at each of the latter steps in the transformation procedure. Possibly,

the repeated resuspensions broke some protoplasts. Although the checks on protoplast integrity at the treatment steps (Figs. 16A & B, 17A & B) showed that some of the protoplasts were able to survive the harsh PEG and heat shock treatments, these harsh steps of transformation process could still be reasons why the protoplasts did not grow when plated. The knockout cassette is a very large DNA molecule that does not easily pass through the plasma membrane. The PEG and heat shock treatments served to create holes in the plasma membrane large enough for the knockout cassette to enter the cytoplasm of the cell. Such large holes in the plasma membrane likely also result in leakage of cytoplasmic components, killing many protoplasts. Another issue could be some sort of deficiency of the regeneration medium, which might not be nutritive enough to allow luxurious protoplast growth.

For future continuation of this project, the next step should be to more thoroughly evaluate the later steps following the protoplast isolation to find exactly where viability is lost in the procedures. For example, after finishing the protoplast isolation, the protoplast suspension still in MMM solution could be added to PRMT and plated on PRMB. This would bypass the PEG and heat shock treatments. If the protoplasts grow to colonies of cells, then the bypassed PEG and heat shock treatments would likely be the killer steps. Successfully growing wild-type protoplasts to colonies of cells could also be used to check the adequacy of the regeneration medium, and further to check that the G418 antibiotic concentrations are strong enough to kill wild-type protoplasts. The protoplast suspension could also be taken through the PEG and heat shock treatments and, right before plating, PRMT could be added to only a small portion of the main batch. A sample of the protoplasts without PRMT and the protoplasts with PRMT could be observed with a microscope to identify if the PEG or the PRMT were the killer step.

Once the problems in the procedures have been identified and solved, another batch of DNA knockout construct must be prepared, since the available supply was all used up in the current experiments, and then the protoplast isolation and transformation could be repeated. If protoplasts can be successfully regenerated back to whole moss plants, those plants can then be screened for Geneticin resistance and analyzed for 3-*O*-methyl-rhamnosyl residue content.

This project was presented as a poster at the 2020 Undergraduate Research and Creative Activity Symposium at the University of California, Riverside (Le et al. 2020).

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Appendix

Sterile Preparation of *Physcomitrella patens* Protoplasts from Protonema and Regeneration

Version M (12-18-19)

Maivy Ngoc Le and Eugene A. Nothnagel

Department of Botany and Plant Sciences

University of California, Riverside

Equipment:

Autoclave below equipment for 30 minutes at 121 ºC (cycle 4), fast exhaust and dry, in room 3128:

+3–16 x 125 mm glass test tube with screw cap (normal strength), foil-covered
+3–13 x 100 mm glass test tube with screw cap (normal strength), foil-covered
+7–50 mL glass centrifuge tube with screw cap (normal strength), foil-covered
+2–15 mL round-bottomed glass centrifuge tube with screw cap, foil-covered
+2–125 mL Erlenmeyer flask, with cotton plug, foil-covered
1–400 mL beaker, foil-covered
+1–25 mL open-tip glass pipet, plugged with cotton at the top, in metal canister
+8–10 mL open-tip glass pipet, plugged with cotton at the top, in metal canister
+13–10 mL ordinary glass pipet, plugged with cotton at the top, in metal canister
+2–5 mL ordinary (thin) glass pipet, plugged with cotton at the top, in metal canister
+6–2 mL wide-tip ordinary glass pipet, plugged with cotton at the top, in metal canister
+3–9-inch glass Pasteur pipet, plugged with cotton, in stainless steel covered tray
1–Red pipet bulb, deflated inside a foil-covered beaker
2–P1000 open-tipped plastic tips, plugged with cotton at the top, in foil-covered beaker
1–Jar of P1000 regular-tipped plastic tips, plugged with cotton at the top
1–Jar of P200 open-tipped plastic tips, plugged with cotton at the top, in foil-covered beaker
3–Spoonula, wrapped, in stainless steel covered tray
2–Forceps, wrapped, in stainless steel covered tray (used and autoclaved x6)
7–Glass rods, wrapped, in stainless steel covered tray
1–150 mL 0.22 µm polystyrene, sterilizing, low protein binding filter unit
1–350 µm Nitex nylon net placed on top of a baby food jar with open screw cap, foiled-covered inside a foil-covered 400 mL beaker
1–64 µm Nitex nylon net placed on top of a baby food jar with open screw cap, foiled-covered inside a foil-covered 400 mL beaker
1–Square bottle, foil-covered
+56–Clear Magenta B-caps, individual foil-wrapped with autoclave tape, inside a foil-covered beaker

Non-autoclaved equipment:

2–40 mL high strength conical tube
1–5 mL ordinary glass pipet
3–10 mL ordinary glass pipet
1–125 mL Erlenmeyer flask
1–P1000 Pipetman
1–P200 Pipetman
1–Large test tube rack
1–Medium test tube rack
1–Small test tube rack

Prepared:

Autoclave the following in the liquid cycle for 20 minutes at 121 ºC (liquid 5) in room 3128:

100 mL of distilled water in 125 mL Erlenmeyer flask with a cotton plug, foil-covered
100 mL of PRML (Autoclave without added $CaCl_2$).
100 mL of 480 mM sucrose + 5 mM $CaCl_2$ solution (pH 5.60)
50 mL of warm PRMT medium (Autoclave without added $CaCl_2$. One hour before using the PRMT, melt the medium in the microwave in intervals of 10 seconds. Equilibrate the melted PRMT to 45 ºC in a nearby water bath until used).
8 Baby food jars with 30 mL of PRMB [without G418 (Geneticin) antibiotic] and cellophane overlay
30 Baby food jars with 30 mL of BCDAT [without G418 (Geneticin) antibiotic], no cellophane overlay
12 Baby food jars with 30 mL of BCDAT [with 25 mg/L of G418 (Geneticin) antibiotic], no cellophane overlay
6 Baby food jars with 30 mL of BCDAT [with 50 mg/L of G418 (Geneticin) antibiotic], no cellophane overlay
0.3 mL of PEG solution. To prepare part 1: 9 mL of 8.5% D-mannitol is combined with 1 mL of 1 M $Ca(NO_3)_2$ and 100 µL of 1 M Tris-HCl, pH 8.0, and filter sterilized; to prepare part 2: 4 g PEG 8000 (Sigma) is melted in an autoclaved 50 mL glass centrifuge tube with Teflon-lined cap by microwaving; Part 1 is added to Part 2 and vortexed until completely mixed; kept at 21–25°C for 2 h before use; stored in 1 mL aliquots at –20°C)

Alternative Preparation of PEG Solution:

Prepare 100 ml of the following PEG buffer:

7.57% = 0.0757 g/ml D-mannitol
0.099 M $Ca(NO_3)_2$
0.0099 M Tris base
Titrated to pH 8.0 with dilute HCl

Place this PEG buffer in a screw cap media bottle (Gibco, Wheaton, or similar), with the cap slightly loose and covered with aluminum foil, and autoclave. Afterward, keep sterile by always only opening and closing this bottle in the laminar flow hood, and only using sterile pipets to draw out solution.

Weigh 2.0 g of PEG 8000 (Sigma) into a 16 x 125 mm glass tube with Teflon-lined screw cap. With the cap slightly loose and covered with aluminum foil, autoclave this tube of PEG 8000. When the autoclave cycle is finished, bring

the tube immediately into the laminar flow hood. Use an autoclaved, cotton-plugged 5 ml glass pipet to immediately add 5 ml of the autoclaved PEG buffer solution to the PEG while the PEG is still warm and liquid. Put the cap back on the tube and vortex quickly (bring the vortex to the culture room so it is near the laminar flow hood) to get a uniform solution with the PEG fully dissolved. Store the resulting PEG solution at room temperature with the cap on to maintain aseptic conditions. When ready to use on the protoplasts, use an autoclaved, cotton-plugged, 1 ml glass pipet in the laminar flow hood to draw 0.3 ml of this PEG solution and add it to the protoplast suspension. If kept aseptic and tightly capped, the PEG solution can be stored at room temperature and used repeatedly, probably for several months

Non-autoclaved mediums:

130 mL of 80% ethanol
45 mL of PRML liquid medium
15 mL of PRML with 2 mM dithiothreitol
1.2 g of Driselase
0.15 g of Bovine serum albumin
45 ºC (nearby) water bath
200 mL of water in a 400 mL beaker (equilibrated to room temperature)
50 mL of MMM solution
Linear DNA knockout construct. Culture the *E. coli* harboring the plasmid DNA construct. Prepare plasmids from the *E. coli* using a QIAGEN Hi-Speed Plasmid Midi Kit, or Promega Wizard Mini-prep Kit, or other similar kit. Digest the plasmid DNA with an appropriate restriction enzyme to linearize. Purify the digested plasmid with phenol/chloroform extraction and precipitate by adding 1/10th the volume of 3M NaOAc (pH5.2) and 2 volumes of ethanol. Dissolve the precipitated plasmid with sterile TE to achieve a DNA concentration of 0.2-1.0 µg/µL. Plan to use 30 µL of this solution (6-30 µg of DNA) per transformation. Working in the laminar flow hood, filter the linear, DNA knockout construct by using a P200 Pipetman with an autoclaved and cotton plugged open tip to deliver all the DNA into a sterile 0.22µm GV Durapore centrifugal filter unit. Close the lid on the loaded filter unit, and then take it and a similar counterbalancing filter unit (labeled as balance and loaded with a similar volume of any available sterile aqueous solution) out of the hood to the Eppendorf 5418 Centrifuge to spin the filter unit at 12,000 x g for 4 minutes. Bring the centrifugal filter unit back into the laminar hood and remove the non-sterile section of the unit. Avoid closing the cap on the sterile part of the tube, as the cap is no longer sterile, and transfer to a sterile tube.

Notes on Protoplast Culture Sizes:

In plating out protoplasts, Roberts et al. use 95 mm diameter Petri dishes, but they state that the surface area of the culture is approximately 6400 mm^2, which is perhaps estimated from the surface area of a 90 mm diameter cellophane disk on top of the culture. On this disk, they layer 1.6 mL of protoplast suspension in PRMT containing a total of 2 x 10^5 protoplasts. Using baby food jars instead of Petri dishes, we will need to

scale down the Roberts conditions. With baby food jars, the diameter of the cellophane disk is 53 mm, corresponding to a surface area of 2200 mm^2. Thus, when using baby food jars, layer on 0.55 mL of protoplast suspension in PRMT containing a total of 7 x 10^4 protoplasts.

Procedure:

1. Prepare the enzyme mix of Roberts's PRML liquid medium (BCDAT with 8% mannitol), final 2% Driselase, 0.25% bovine serum albumin, and 0.5 mM dithiothreitol. To a 125 mL Erlenmeyer flask, add 15 mL of PRML with 2 mM dithiothreitol, 1.2 g of Driselase, and 0.15 g of bovine serum albumin. Add 45 mL of PRML to obtain a final volume of 60 mL. Use Para film to cover the flask, and invert until all solutes are dissolved or suspended. Divide the Driselase enzyme mix into two 40 mL high strength conical centrifuge tubes at 30 mL each. Centrifuge at 1000 rpm (170 xg) for 5 minutes (high brake). Bring the mix to the laminar hood in room 3128.

2. Remove the 150 mL 0.22µm polystyrene, sterilizing, low protein binding filter unit from the package in the laminar hood. Make sure the cap connected to the sterile section of filter is secure. Connect the filter unit to a vacuum and turn on the vacuum. Pour the supernatant (of the enzyme mix) from both 40 mL tubes into the non-sterile section of the filter and allow the enzyme mix to filter through. Pour with continuous motion to avoid agitating the pellet. Turn off the vacuum and bring the filter back into the laminar hood. Remove the top, non-sterile section of the filter.

3. Using an autoclaved ordinary 10 mL glass pipet, transfer half of the enzyme mixture (30 mL) into an empty, autoclaved 50 mL glass centrifuge tube with screw cap. Screw on the cap and set aside the mixture (inside the hood) for use during the filtering step.

4. From 9 protonema jars, using an autoclaved spoonula, scrape protonema tissue off the cellophane from the source jar. When picking out the source protonema jars, choose healthy, green jars where the protonema covers the cellophane 85%-90%. Avoid choosing jars that are too old where there are leafy gametophytes growing. Roughly 5 days after the protonema was transferred is ideal. It is best to use jars that have been transferred often (1 week apart) as the protonema will be healthy and fast-growing.

5. Transfer the protonema tissue into the enzyme mix in the sterile section of the filter. Record the time the protonema was added to the enzyme mix. Separate the protonema tissue into small pieces using two autoclaved spoonulas. Using an autoclaved 10 mL open-tip glass pipet, carefully transfer the enzyme mix and protonema tissue into an autoclaved 125 mL Erlenmeyer flask (with cotton plug, foil-covered). Work slowly to prevent protoplast breaking. Avoid air bubbles or pressing the pipet against the glass of the Petri dish. Replace the cotton plug and foil cover.

6. Incubate the covered flask on the Gyratory Shaker-Model G2 in room 3216 at 30-35 rpm (or the lowest setting the shaker works) in the light at 25 ºC for roughly 2.5 hours. Record the time the incubation begins and ends.

7. Back in the laminar flow hood, use an autoclaved ordinary 10 mL glass pipet to wet the autoclaved 350 µm Nitex nylon net placed on top of a food baby jar with an open screw cap with ~2 mL of filter-sterilized Driselase mix from the 50 mL tube set aside earlier. Using an autoclaved 10 mL open-tip glass pipet, gently draw the protoplast suspension from the flask and deliver ~5 mL of the suspension to the net. Use the tip of an autoclaved, rounded glass rod to GENTLY work the tissue through the net. If considerable tissue remains on the net, then use an autoclaved ordinary 10 mL glass pipet to add ~2 mL of filter-sterilized Driselase mix, with additional action with the glass rod, to get more of the tissue to pass through the net. Continue to deliver the suspension to the net, followed by grinding with the glass rod and washing with the Driselase mix. Progressively grind the tissue harder until nearly all the tissue has gone through the net. If appreciable tissue has remained in the 125 mL Erlenmeyer flask, then use an autoclaved clean ordinary 10 mL glass pipet to add 5 mL of filtered-sterilized Driselase mix into the 125 mL Erlenmeyer flask to facilitate picking up the remaining tissue and getting it onto the nylon net.

8. Using an autoclaved 10 mL open-tip glass pipet, carefully transfer the filtered suspension from bottle under the 350 µm Nitex nylon net back into the 125 mL Erlenmeyer flask for further incubation. Replace the cotton plug and foil cover. Incubate the covered flask on the Gyratory Shaker-Model G2 in room 3216 at 30-35 rpm in the light at 25 ºC for an additional 1 hour. Record the time the incubation begins and ends.

9. Back in the laminar flow hood, use an autoclaved ordinary 10 mL glass pipet to wet the autoclaved 64 µm Nitex nylon net placed on top of a baby food jar with an open screw cap with 2 mL of autoclaved PRML. Using an autoclaved 10 mL open-tip glass pipet, gently draw the protoplast suspension from the flask and deliver the suspension to the net. As in step 8 with the 350 µm Nitex nylon net, cycle between delivering the protoplast suspension, grinding the tissue with a glass rod (progressively use more force), and washing the protoplast through the net. Use PRML in place of the enzyme mix to wash the protoplasts through the net. Try to get nearly all the tissue through the net. With an autoclaved clean ordinary 10 mL glass pipet, add 3 mL of autoclaved PRML into the 125 mL flask to rinse the flask and to enable picking up the remaining protoplasts. Use a clean autoclaved 10 mL open-tip glass pipet to draw the solution from the flask and wash these remaining protoplasts through the net.

10. Using an autoclaved 10 mL open-tip glass pipet, equally divide the filtrate into as many autoclaved 50 mL screw cap centrifuge tubes as needed (try for just 2). Pipet close to the bottom of the glass so the protoplasts do not fall from a high height. With an autoclaved ordinary 10 mL glass pipet, pipet 5 mL of autoclaved sucrose + $CaCl_2$ solution into an autoclaved 16 x 125 mm tube. Use an autoclaved 9 in Pasteur pipette to slowly underlay the protoplast suspension in each tube with the 5 mL of autoclaved sucrose + $CaCl_2$ solution. Put the screw caps on the tubes while still in the laminar flow hood and then take the tubes out of the hood to the centrifuge to spin at 1100 rpm (200 xg) (roughly 4.25 on the dial) for 15 minutes at 24 ºC (low brake).

11. Move the tubes from the centrifuge back into the laminar flow hood. Discard the upper layer of supernatant by using an ordinary autoclaved 10 mL glass pipet to remove the scum layer (if any) and most of the brown upper layer into a 400 mL beaker. Pipet off as much of the brown layer as possible, as close to the green layer of protoplasts as possible, without pipetting off any appreciable amount of protoplasts. Use an autoclaved 10 mL open-tip glass pipet followed by a P1000 Pipetman with an autoclaved open-tip and cotton plugged tip to pick up the layer of green protoplasts sitting on top of the clear sucrose + $CaCl_2$ layer. Try to minimize the volume needed to pick up the layer of protoplasts in each tube. Remember to wipe the P1000 Pipetman with 70% ethanol before using it. Combine the protoplast layers from the 2 (or more) first centrifuge tubes into a single autoclaved clean 50 mL screw cap centrifuge tube.

12. Use an autoclaved regular 25 mL glass pipet to add 20-25 mL of autoclaved sucrose + $CaCl_2$ solution to the 50 mL tube containing the pooled protoplasts. Screw on the cap and mix 2 times by slowly inverting. Use an autoclaved ordinary 10 mL glass pipet to pipet 3 mL of autoclaved PRML into an autoclaved 13 x 100 mm tube. Use an autoclaved 9 in Pasteur pipet to add the 3 mL of PRML as an overlayer in the 50 mL tube. Work carefully and gently to create separate, distinct layers. Put the screw cap on the tube while still in the laminar flow hood and then take the tube out of the hood to the centrifuge to spin at 700 rpm (100 xg) (roughly 3.25 on the dial) for 10 minutes at 24 ºC (low brake).

13. Move the tube from the centrifuge back into the laminar flow hood. Use an autoclaved, cotton-plugged open-tip P1000 to collect the protoplasts at the interface, and transfer to an autoclaved, screw cap 16 x 125 mm tube. Use an autoclaved regular 10 mL glass pipet to add sucrose + $CaCl_2$ solution to fill the tube 2/3-3/4 full. Screw on the cap and mix 2 times by slowly inverting. After using an autoclaved regular 10 mL glass pipet to add 2 mL of autoclaved PRML to an autoclaved 13 x 100 mm tube, use an autoclaved Pasteur pipet to add an overlayer of 2 mL of PRML to the 16 x 125 mm tube. Put the screw cap on the tube while still in the laminar flow hood and then take the tube out of the hood to the centrifuge to spin at 700 rpm (100 xg) for 10 minutes at 24 ºC (low brake).

14. Move the tube from the centrifuge back into the laminar flow hood. Use an autoclaved 2 mL wide-tip pipet to collect the protoplasts at the interface. Try to get as little as possible of the sucrose + $CaCl_2$ solution. Transfer the protoplasts into a clean autoclaved 13 x 100 mm tube with screw cap. Use an autoclaved 10 mL ordinary glass pipet to add enough filter-sterilized MMM to fill the tube 3/4 full. Screw on the cap and mix 2 times by inverting slowly. Take the tube out of the hood to the centrifuge to spin at 700 rpm (100 xg) for 7 minutes (low brake).

15. Move the tube from the centrifuge back into the laminar flow hood. The protoplasts should be at the bottom of the tube. Use an ordinary autoclaved 2 mL wide-tip glass pipet to draw off and discard the supernatant into a 400 mL beaker. Use a 2 mL wide-tip autoclaved glass pipet to slowly add 1-2 mL of filtered-sterilized MMM to the protoplast pellet. Rotate the tube by hand for 2-3 minutes to gently resuspend the protoplasts. Use a P100 Pipetman with an autoclaved and cotton-plugged open tip to obtain a small sample of suspension and observe under a microscope. Estimate the density with a

hemocytometer (Number of protoplasts in triple line square/0.0002 mL in each triple line square=protoplasts/mL). The target is 2-4 x 10^5 protoplasts/mL.

16. Use a P200 Pipetman with an autoclaved and cotton plugged open tip to add 30 µL of the sterile DNA solution to a sterile 15 mL round-bottomed glass centrifuge tube with screw cap. For experiments with no DNA, use 30 µL of autoclaved distilled water. Use a 2 mL wide-tip glass pipet to transfer 0.3 mL (300 µL) of the protoplast suspension to the 15 mL tube. Use a clean 2 mL wide-tip glass pipet to add 0.3 mL (300 µL) of PEG solution. Screw the cap onto the tube. Mix the solution gently. Incubate at 21-25 ºC for 10 minutes on the slow rotator next to the other fume hood in room 3128.

17. Heat-shock the capped tube of protoplasts for 3 minutes in the 45 ºC water bath (turned off so it does not heat above 45 ºC during the 3 minute incubation) and then immediately transfer the capped tube of protoplasts to stand in 200 mL of room temperature water in a 400 mL beaker for 10 minutes.

18. Use a regular-tip P1000 to gradually dilute the cooled protoplast suspension with 5 aliquots of 300 µL of PRML at 3-minute intervals. Then use an autoclaved regular 5 mL glass pipet to add 5 aliquots of 1 mL of PRML at 3-minute intervals. Take the capped tube out of the hood to the centrifuge to spin at 700 rpm (100 xg) for 7 minutes (low brake).

19. Move the tube from the centrifuge back into the laminar flow hood. The protoplasts should be at the bottom of the tube. Use an ordinary autoclaved 2 mL wide-tip glass pipet to draw off and discard the supernatant. Use an autoclaved thin regular 5 ml glass pipet to slowly add 4 mL of PRMT held in a 45 ºC water bath. Rotate the tube for 1-2 minutes to gently resuspend the protoplasts. Use a P1000 Pipetman with an autoclaved and cotton plugged open tip to pipet 0.55 mL (550 µL) of the suspension into 8 sterile baby jar with PRMB (no antibiotic) and cellophane overlay. Incubate the baby jars for 5 days at 25 ºC and constant illumination.

20. After 5 days, use sterile forceps to carefully lift the cellophane from the PRMB/PRMT baby food jars and lay the cellophane onto BCDAT medium in new baby food jars. Aim for 2 baby jars with BCDAT and no antibiotic, at 4 jars with BCDAT and 25 mg/L of G418 (Geneticin) antibiotic, and 2 baby jars with BCDAT and 50 mg/L of G418 (Geneticin) antibiotic. Change the green organic caps into the clear Magenta caps. Incubate the baby jars for 7 days at 25 ºC and constant illumination.

21. Clones surviving after 7 days on BCDAT medium containing G418 (Geneticin) antibiotic consist of stable and unstable transformants. To select for stable transformants, the cellophane disks are transferred to new baby food jars containing BCDAT medium without antibiotic for 7 days. Change the green organic caps into the clear Magenta caps.

22. After another 7 days, transfer the cellophane disks to new baby food jars containing BCDAT medium with G418 (Geneticin) antibiotic. After 7 more days, transfer the cellophane disks to BCDAT medium without antibiotics.

23. After another 7 days, transfer the cellophane disks back onto geneticin. Total, there should be 3 rounds of geneticin selection. Typically, clones that grow vigorously during the third round of selection are stable transformants. After 7 days, transfer the cellophane disks onto BCDAT medium without antibiotics and allow to grow into protonema/leafy gametophytes.