The moss *Physcomitrella patens* has been used as an experimental organism for more than 80 years. Within the last 15 years, its use as a model to explore plant functions has increased enormously. The ability to use gene targeting and RNA interference methods to study gene function, the availability of many tools for comparative and functional genomics (including a sequenced and assembled genome, physical and genetic maps, and >250,000 expressed sequence tags), and a dominant haploid phase that allows direct forward genetic analysis have all led to a surge of new activity. *P. patens* can be easily cultured and spends the majority of its life cycle in the haploid state, allowing the application of experimental techniques similar to those used in microbes and yeast. Its development is relatively simple, and it generates only a few tissues that contain a limited number of cell types. Although mosses lack vascular tissue, true roots/stems/leaves, and flowers and seeds, many signaling pathways found in angiosperms are intact in moss. For example, the phytohormones auxin, cytokinin, and abscisic acid, as well as the photomorphogenic pigments phytochrome and cryptochrome, are all interwoven into distinct but overlapping pathways and linked to clear developmental phenotypes. In addition, about one quarter of the moss genome contains genes with no known function based on sequence motifs, raising the likelihood of successful discovery efforts to identify new and novel gene functions. The methods outlined in this chapter will enhance the use of the *P. patens* model system in many laboratories throughout the world.
The moss *P. patens* (Hedw.) Bruch & Schimp was first established as a laboratory experimental system in the 1920s by Fritz von Wettstein (1924), who studied the effects of ploidy variation and inheritance patterns in interspecific and interfamilial crosses within the moss family Funariaceae. The modern era of *Physcomitrella* research dates to the work of Paulinus Engel (1968), who generated the first biochemical and morphological mutants in the species.

Like all land plants, the moss life cycle consists of a multicellular haploid gametophyte generation that alternates with a morphologically distinct diploid sporophyte generation. But unlike vascular plants, the gametophyte (Fig. 1C) is the dominant portion of the moss life cycle. Haploid spores germinate to produce a filamentous protonemal stage (Fig. 1D). Protonemata are initially

**FIGURE 1.** *P. patens* cultures. (A) Six-day-old *P. patens* protonemata grown on cellophane over solid BCD medium supplemented with diammonium tartrate. (B) Easy harvesting, with a spatula, of protonemata grown on solid media overlaid with cellophane. (C) Four-week-old inoculum grown on BCD supplemented with diammonium tartrate. Note the presence of the two major tissues that are characteristic of the haploid growth phase of *P. patens* development: the filamentous protonemata and the leafy gametophore. (D) *P. patens* protonemata displaying the characteristic branching pattern. (E) Six-week-old *P. patens* gametophores. (F) Three 3-day-old filaments regenerating from protoplast on PRMB medium. (G) Transformation plate after 2 weeks on antibiotic selection. Transformants surviving selection are easily identified as individual growing plants. (H) Individual spot-inoculums of *P. patens* strains on a 9-cm Petri dish after 2 weeks’ growth. Up to 32 independent isolates can be grown and stored this way. (I) Multiple plates can be easily stored after growth in an incubator with 2 hours of light per day at 10°C.

AU: Changed “at 8°C” to “at 10°C” because that’s the temp used in Protocol 1. OK?
composed of chloronemal cells that are full of large chloroplasts. Chloronemal cells extend by serial division of the apical cell, and subapical cells branch to form new apices. Some apical chloronemal cells develop into a second cell type, caulonemata. Caulonemal filaments contain fewer and less-well-developed chloroplasts. But they extend more rapidly than chloronema; the division times of the apical cells of caulonema and chloronema are about 6 and 24 hours, respectively. The subapical cells of caulonemal filaments branch to form more filaments and leafy stems, called gametophores (Fig. 1E), on which gametes are produced. Moss is monoecious: Both male and female gametes are produced on the same gametophore. Although self-fertilization is common, cross-fertilization can occur when two strains are grown adjacent to each other. Fertilized zygotes develop into sporophytes that remain attached to the gametophore. Within the sporophyte, spore mother cells give rise to spores meiotically.

*P. patens* is small, and in nature, the gametophores seldom reach more than 5 mm in height. It is mostly found on wet soil and, in particular, on sites that are exposed to seasonal flooding, such as the banks of lakes, ponds, rivers, and drainage ditches (Crum and Anderson 1981). Although it is distributed widely in the northern hemisphere, it is uncommon throughout its range. Natural populations produce spores from September to March, depending on the locality. Although *P. patens* itself is restricted to North America and Europe, other morphologically similar species are found in Africa, Asia, South America, and Australia. Many variants of *P. patens* have been given species or subspecies rank, although the degree to which the morphological features that distinguish these taxa have a genetic basis has not been established experimentally. Natural hybrids between closely related species in the family Funariaceae, similar to those produced in culture by von Wettstein, have been documented in several localities (Pettet 1964).

### SOURCES AND HUSBANDRY

The strain of *P. patens* used by Engel was generated from a single spore that was obtained in 1962 from a plant in Gransden Wood, Huntingdonshire, England by Dr. H.W.K. Whitehouse. This strain has since been used by many laboratories, but it is routinely taken through its sexual cycle about every year, during which time cultures are reestablished from individual spores. Therefore, Gransden strains will often be identified by the laboratory and the year in which a spore was used to start a new culture (e.g., the material used to sequence the *P. patens* genome came from the Gransden St. Louis 2004 strain). Because of gametophytic haploidy, all such strains differ only as a result of mutation or epigenetic variation. More recently, additional collections of *P. patens* have been made from Europe and North America, and these are now being genetically and morphologically characterized (von Stackelberg et al. 2006). This new collection is curated and distributed from the University of Freiburg, Germany (see http://www.cosmoss.org).

*P. patens* can be grown on either solid (agar-based) or liquid media (for details, see Protocol 1). Temperatures between 24°C and 26°C are used for routine culture, although little difference in growth rate is observed from 20°C to 26°C. Growth is slower but still satisfactory at 15°C, and this has been used as the permissive temperature when temperature-sensitive mutants are sought. For routine culture, continuous light from fluorescent tubes at an intensity of between 5 and 20 Wm⁻² is generally satisfactory, although the exact quality of light is not critical. Many laboratories use intermittent light, most commonly a 16-hour light/8-hour dark cycle. This regime entrains the cell cycle of chloronemata. Development is slower under intermittent light regimes; developmental landmarks are achieved in response to the total hours of illumination experienced.

### RELATED SPECIES

Two additional moss species are currently used for experimental research: *Ceratodon purpureus* and *Tortula ruralis*. *C. purpureus* is one of the most common mosses in exposed rock and soil in temperate regions of the northern and southern hemispheres. In the spring, it is easily recognized by
the purple seta that elevates the diploid sporophyte. This species also has a long history in experimental biology; the term “heterochromatin” was coined for the dark-staining sex chromosomes of this and other moss species (Heitz 1928). A genetic map of *C. purpureus* has been constructed, and several natural isolates have been extensively characterized (for review, see McDaniel et al. 2008). Cultures of *C. purpureus*, isolated by E. Hartmann in Germany and by D.J. Cove in Austria, are used by several labs worldwide, principally to study phototropism and gravitropism. *C. purpureus* is so abundant that the isolation of additional cultures is fairly straightforward; isolates currently in use are available from D.J. Cove (Washington University, St. Louis). All of the experimental procedures that are described for *P. patens* are also used for *C. purpureus* with few modifications.

*T. ruralis* has been used principally to study water stress because it is able to withstand complete desiccation, similar to seeds. Although a modest collection of expressed sequence tags (ESTs) is available for *T. ruralis* (Oliver et al. 2004), it is less amenable to growth in culture than either *C. purpureus* or *P. patens* and has not been shown to be easily transformable or to undergo efficient gene targeting.

### USES OF THE *P. PATENS* MODEL SYSTEM

The common ancestor of mosses, such as *P. patens*, and seed plants, such as *Arabidopsis thaliana*, pines, and other species, lived approximately 480 million years ago (Mya). Comparative studies including members of both of these lineages allow us to infer biological properties of this common ancestor, giving us a richer understanding of the diversity of plant life. This may have practical value to the extent that understanding diverse plant systems yields novel solutions to problems in crop breeding, for example.

During the last several years, *P. patens* has been used as a model to study various components of cell, developmental, and evolutionary plant biology. Its development is relatively simple, and it generates only a few tissues that contain a limited number of cell types. Although mosses lack vascular tissue, true roots/stems/leaves, and flowers and seeds, many signaling pathways found in angiosperms are intact in moss. For example, the phytohormones auxin, cytokinin, and abscisic acid, as well as the photomorphogenic pigments phytochrome and cryptochrome, are all interwoven into distinct but overlapping pathways and linked to clear developmental phenotypes (Quatrano et al. 2007; Rensing et al. 2008).

RNA interference (RNAi) methods (Bezanilla et al. 2005) have been used to analyze the role of ARPC1 (Harries et al. 2005), a member of the Arp2/3 complex, and profilin (*Vidal et al. 2007*) in tip growth. Khandelwal et al. (2007) studied the role of the *P. patens* presenilin protein using RNAi. Presenilin possesses γ-secretase activity, is involved in Alzheimer’s disease (AD), and is an intermediate in the NOTCH signaling pathway of animal cells; however, unlike animal cells, *P. patens* and other plants do not contain this pathway although the protein is present. The observed mutant phenotype indicates a possible role for presenilin that is independent of γ-secretase activity and the NOTCH pathway, thus raising the possibility of using *P. patens* as a novel system for studying the off-target effects of AD therapy and drug discovery.

Targeted gene deletion and replacement methods have been used to study the role of another member of the Arp2/3 complex, ARPC4 (Perroud and Quatrano 2006), and BRICK1, a member of the Scar/Wave family (Perroud and Quatrano 2008). Like the other papers referenced above, the excellent cell biology of *P. patens* was used to localize ARPC4 and BRICK1 in growing tip filaments. Transcriptome (Nishiyama et al. 2003; Cuming et al. 2007) and metabolic studies (Thelander et al. 2005; Kaewsuwann et al. 2006; Schulte et al. 2006), as well as detailed analyses of microRNAs (Axtell et al. 2006, 2007), have now appeared using *P. patens*. Finally, comparative genomic studies have elucidated the role of the transcriptional regulators LEAFY (Maizel et al. 2005), ABI3 (Marella et al. 2006), and transcription factors involved in rooting function (Menand et al. 2007) in *P. patens* as well as in *A. thaliana*. 

AU: Verify ref.
GENETICS, GENOMICS, AND ASSOCIATED RESOURCES

*P. patens* is amenable to classical genetics studies, with the haploidy of the gametophyte allowing straightforward analysis (Cove 2005). Efforts to identify polymorphisms among isolates of *P. patens* are proceeding and will enable map-based cloning of both ethylmethanesulfonate (EMS) - and UV-generated mutants (for a method to generate such mutants, see Protocol 4), as well as quantitative trait locus (QTL) mapping of natural variants (von Stackelberg et al. 2006). A number of mutant strains are available from different laboratories (http://www.cosmoss.org; http://biology4.wustl.edu/moss), including those having requirements for the vitamins *p*-aminobenzoic acid, nicotinic acid, and thiamine. Vitamin requirements have been exploited to increase the frequency of cross-fertilization. When two complementary *p*-aminobenzoic acid or nicotinic acid auxotrophs are grown together on a medium with only a limited level of supplementation, the sporophytes produced are the result of cross-fertilization (Courtice and Cove 1978).

No universally accepted system of gene nomenclature has been adopted, but there has been general agreement that annotated genes will be identified by numbers. Trivial names can then be added as synonyms. Originally, the system used for trivial names was similar to that used for many bacteria and fungi: Each symbol was composed of a three-letter lowercase code to designate the mutant gene family, an uppercase letter to designate the family member, and a number to designate the allele (e.g., *pab* A4). More recently, some laboratories have adopted the system used by the yeast and *A. thaliana* communities, designating the family member by a number rather than by an uppercase letter (e.g., *pab* 1-4). But no general agreement has yet been reached as to which system should be adopted.

The assembled *P. patens* genome (~487 Mb), representing eight times coverage, has been released by the Joint Genome Institute (http://shake.jgi-psf.org/Phyta1/Phyta1.home.html; Rensing et al. 2008). In parallel, sequences of full-length cDNAs, additional ESTs, and bacterial artificial chromosomes (BAC) ends are being developed, and updates can be accessed through the *Physcomitrella* Genome Consortium website (http://www.mossgenome.org). Various libraries and vectors are available (see links at http://biology4.wustl.edu/moss/links.html), as is an Agilent microarray (MO gene), which contains 44,000 features (~28,000 gene models) based on all of the open reading frames (ORFs) in the draft genome (Fig. 2).

Several tools are available for the functional analysis of genes in *P. patens*. For example, the dexamethasone- (Chakhparonian 2001), heat-shock- (Saidi et al. 2005), and homoserine-lactone- (You et al. 2006) inducible promoter systems have all been successfully used in this system. Forward genetics can be used to dissect gene function using a shuttle-mutagenesis library (Nishiyama et al. 2000; Hayashida et al. 2005). A targeted deletion library that was created using ESTs (Schween et al. 2005) has also been used for functional analysis (Schulte et al. 2006). Transformation can be performed via polyethylene glycol (PEG)–mediated DNA uptake by isolated protoplasts (see Protocol 5), via *Agrobacterium* (see Protocol 6), or via a gene gun (see Protocol 7), and somatic hybridization has been used to analyze mutants genetically (see Protocol 3) (Cove and Quatrano 2006). Reverse genetics using gene targeting is a tool of choice for manipulating individual genes in *P. patens*, and RNAi allows the down-regulation of gene families. An RNAi system has been developed in *P. patens* that silences the nucleus-localized green fluorescent protein::β-glucuronidase (GFP::GUS) fusion protein at the same time that it silences the gene(s) of interest (Bezanilla et al. 2005).

TECHNICAL APPROACHES

The following eight protocols outline techniques for manipulating *P. patens* in the laboratory and include three transformation methods (Protocols 5–7). *P. patens* (and *C. purpureus*) have a high frequency of gene targeting (Kamisugi et al. 2005, 2006); when a transforming construct contains
a genomic sequence, the construct is targeted to the corresponding sequence in the genome. This can be exploited to knock out or modify a gene.

For knockout, aim to replace the coding sequence with a selection cassette and to border this on each side by about 1000 bp of genomic sequence. Linear DNA fragments generated by polymerase chain reaction (PCR) give the highest rates of targeting. It is convenient to perform a number of transformations at the same time (ten is not difficult). For each experiment, make sure to include a minus DNA control to assess protoplast viability.

Figure 1G shows a plate of transformants that have been growing for 2 weeks on selective media. Following transformation, the regenerants are of three types:

- **Transient**: These do not retain resistance upon subculture.
- **Unstable**: These exhibit slow growth on selective medium. Resistance is probably not transmitted through meiosis and is rapidly lost when selection is relaxed.
- **Stable**: These grow on selective medium almost as fast as on nonselective medium. Resistance is transmitted regularly through meiosis and is retained even when selection is absent.

AU: Fig 1G citation OK?
Culturing the Moss Physcomitrella patens

Here, we present a series of methods for culturing the moss *P. patens* at all stages of its life cycle. Gametophytes are axenically cultured on solid agar-based media (Methods IA and IB, adapted from Grimsley et al. 1979) and in shaken liquid cultures (Method IC). Growth rates in shaken liquid cultures are not as great as those obtained on solid media or in bioreactors (Boyd et al. 1988), especially if these are supplied with CO₂. AU: See query. For long-term storage of gametophytes, cultures are maintained on solid medium at 10°C in a very short day (see Method IIA; Fig. 1H, I), but cryopreservation (Method IIB, adapted from Grimsley and Withers 1983) may also be used. Finally, sporophytes are generated by self-fertilization and sexual crossing (Method III, adapted from Ashton and Cove 1977).

**MATERIALS**

The recipes for items marked with <R> are on page XX.

**CAUTION:** See Appendix XX for appropriate handling of materials marked with <!>.

### Reagents

- **BCD medium** (liquid and solid) <R>, containing appropriate supplements
  - A common moss media supplement <R> is diammonium tartrate, which is added to BCD medium <R> at a final concentration of 5 mM. For cryopreservation, also include mannitol (500 mM) in the appropriately supplemented liquid BCD medium (see Step 1 of Method IIB).
- Dimethylsulfoxide (DMSO)–glucose solution <R>
- Ethanol (70%) <!>
- Liquid nitrogen <!>
- Nitrogen-free medium <R> containing 400 µM of KNO₃ <!>
- Somatic tissue or spores from *P. patens*
  - To establish a new culture, a fragment of tissue 1–2 mm in diameter is sufficient. For routine subculture, it is best to use protonemal tissue from a vigorously growing culture that is no more than 20 days old, which will be composed mostly of chloronemal tissue. Chloronemal tissue, the growth of which is enhanced when ammonium is provided as nitrogen source, is easiest to subculture. Tissue other than chloronemata (e.g., leaf cells) may take a long time to regenerate. For sexual crosses, it is best that at least one of the two strains to be crossed is self-sterile. Strains containing vitamin auxotrophies (e.g., for thiamine, *p*-aminobenzoic acid, or nicotinic acid) are normally self-sterile but cross-fertile (Courtice and Cove 1978).

### Equipment

- **Blender** (e.g., Fisher Scientific PowerGen Model 125 Homogenizer)
  - Different tissue blenders are used in different laboratories, and some have been made specifically for blending moss tissue. Provided the blending assembly can be sterilized, it appears that there is little difference among blenders in the end result—a tissue inoculum that will grow rapidly.
- **Cellophane discs** (type 325P), sterile (Cannings)
  - These are most conveniently sterilized dry, by autoclaving interleaved with disks of filter paper.
- **Erlenmeyer flasks** (1 liter, sterile)
- **Ethanol bath** (controlled, low temperature) <!>
Forceps (fine)
Incubator with temperature control and white light at intensities between 5 and 20 Wm\(^{-2}\) (e.g., Percival Scientific Model CU-36L5)

*Because of the radiant heat from light sources, it may be necessary to keep the air temperature below the desired culture temperature. In this protocol, the temperatures given are those of the medium in which the cultures are grown.*

Liquid nitrogen storage facility
Magentajars (optional; see Method III)
Microcentrifuge tubes (1.5 ml)
Micropore surgical tape (3M, 1530-0)
Parafilm (optional; see Step 4 of Method IIA)
Petridishes (sterile)

*Most work uses 90-mm-diameter, presterilized disposable plastic Petri dishes, but glass Petri dishes are also suitable. Ideally, the dishes should be vent-free to slow evaporation and limit contamination.*

Shaker (platform)
Spatula (sterile)
Test tubes (25 x 150 mm, sterile) with caps
Tubes (2 ml, plastic) containing 1.5 ml of agar medium (see Step 1 of Method IIA)

*If space is not at a premium, 15-ml tubes containing 10 ml of medium provide a better resource for long-term storage.*

Vials (2 ml, plastic)
Water bath, set at 30°C

METHODS

I. Growth of Gametophytes

*Using Petri Dishes Containing Solid Medium*

1. Inoculate a Petridish containing appropriately supplemented solid BCD medium as follows:
   - For spores, use standard microbiological procedures to spread them on the agar. If individual plants are required, add about 100–200 spores per 90-mm-diameter Petri dish.
   - For somatic tissue, place a clump of gametophyte tissue (usually 1–2 mm in diameter) on the agar. The size of the clump is not critical. However, for growth tests, the clumps should be as uniform as possible, and uniformity is best achieved by picking the tissue while looking through a microscope.

2. Seal the Petridishes with Micropore surgical tape.
   
   *This reduces the risk of contamination without affecting the growth or development of the cultures. Do not seal cultures with Parafilm; this slows growth and prevents regeneration.*

3. Incubate the cultures in an incubator at 25°C with constant white light at intensities between 5 and 20 Wm\(^{-2}\).
   
   *Depending on the purpose of the procedure, growth under these conditions may range from a few days (e.g., to score antibiotic resistance) to several weeks (e.g., to score the requirements of some vitamins). For an image of 6-day-old protonemata grown under these conditions, see Figure 1A.*

*AU: Fig 1A cite OK?*

B. *Using Petri Dishes Containing Solid Medium Overlaid with Cellophane*

1. Overlay a Petridish containing appropriately supplemented solid BCD medium with a sterilized cellophane disc. Allow the dish to stand for at least 10 minutes to allow the cellophane to hydrate and then, if necessary, straighten the disc while maintaining sterility.
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2. Obtain one dish of protonemal tissue from plants that have developed for about 10 days from tissue-clump inocula growing on appropriately supplemented BCD medium (see Method IA). Harvest the tissue with a spatula as shown in Figure 1B.

3. Add the tissue to 10 ml of H₂O, and blend it for about 2 minutes. The exact procedure will depend on the type of blender used. **Blending should result in an easily pipettable suspension, but it should still consist of tissue clumps containing 20–50 cells. Overblending leads to poor regeneration.**

4. Pipette 1–2 ml of the protonemal suspension from Step 3 onto each Petri dish from Step 1. Spread the suspension evenly. **There should be sufficient tissue to inoculate 5–10 Petri dishes.**

5. Incubate the culture for 7 days at 25°C under a 16-hour light/8-hour dark cycle (with white light at intensities between 5 and 20 Wm⁻²). **For a wild-type culture, each 90-mm-diameter Petri dish will yield about 200 mg (fresh weight) of vigorously growing protonemal tissue, consisting mainly of chloronemata.**

6. Harvest the tissue by scraping it from the cellophane using a sterile spatula. **Once tissue is growing on cellophane, it is convenient to use this tissue (instead of the tissue described in Step 2) for further inoculation by repeating Steps 3–6. However, do not repeat this cycle more than three times.**

C. Using Liquid Medium

1. Add tissue from one cellophane-overlay plate (see Method IB) to 10 ml of H₂O and blend as described in Step 3 of Method IB.

2. Inoculate 200 ml of appropriately supplemented liquid BCD medium (in a 1-liter Erlenmeyer flask) with 2 ml of the tissue suspension from Step 1. **Other quantities of the liquid BCD medium and/or the tissue suspension may be used instead.**

3. Shake the cultures on a platform shaker. **Vigorous agitation is not necessary for growth.**

II. Long-term Storage of Gametophyte Tissue

A. Storage on Solid Medium

1. Place a clump of gametophyte tissue (usually 1–2 mm in diameter) in each plastic tube containing agar medium (usually, appropriately supplemented solid BCD medium).

2. Make sure that the lids are not tightly sealed. For screw-cap lids, tighten the cap and then release it about one quarter of a turn.

3. Grow the cultures for about 3 weeks in an incubator at 25°C with continuous white light at intensities between 5 and 20 Wm⁻².

4. After 3 weeks, tightly seal each tube. If the tube has an air-tight seal, this is sufficient; if it does not, seal the tube with Parafilm.

5. Transfer the tubes to an incubator at 10°C with a 2-hour light/22-hour dark cycle (with white light at intensities between 5 and 20 Wm⁻²) for long-term storage. **Cultures can be kept in a healthy state for a considerable period of time (at least 3 years) under these conditions.**
B. Cryopreservation

1. For each strain to be preserved, grow gametophyte tissue as described in Steps 1–5 of Method IB. After 7 days of incubation (Method IB, Step 5), transfer 100 mg of tissue onto a Petri dish overlaid with fresh cellophane as described in Step 1 of Method IB. Pipette 1 ml of appropriately supplemented liquid BCD containing 500 mM of mannitol onto the surface of the tissue.

2. Incubate the culture for an additional 7 days at 25°C under a 16-hour light/8-hour dark cycle (with white light at intensities between 5 and 20 Wm\(^{-2}\)).

3. To each of ten 2-ml sterile plastic vials, add 2 ml of DMSO–glucose solution. Then add one tenth of the tissue on the plate to each vial. Incubate the vials for 1 hour at 20°C.

4. Freeze the vials at a rate of 1°C per minute to −35°C using a controlled low-temperature ethanol bath.

5. Place the vials in liquid nitrogen for storage.

   *It is advisable to freeze multiple aliquots of tissue. Recovery from cryopreservation is usually good for vigorous strains, but some mutant strains recover poorly. To thaw the cultures, proceed with Steps 6–8.*

6. Retrieve the vials from the liquid nitrogen and place them in a water bath at 30°C until thawed.

7. After thawing, add tissue from one vial to 10 ml of H\(_2\)O. Allow the mixture to stand for 30 minutes at room temperature.

8. Inoculate the tissue suspension from Step 7 onto appropriate solid medium as described in Method IA (use 1–2 ml of the tissue suspension per plate).

III. Production of Sporophytes and Isolation of Spores

1. Prepare test tubes (25 x 150 mm) with between 15 and 20 ml of solid nitrogen-free medium containing 400 \( \mu \)M of KNO\(_3\).

   *Alternatively, fill Magenta jars two-thirds full with the same medium.*

2. Inoculate the medium with protonemata by placing a clump of tissue (usually 1–2 mm in diameter) on the agar.

   - If sporophytes resulting from self-fertilization are required, place a single inoculum into a test tube. Alternatively, place four inocula into a Magenta jar.

   - To establish a sexual cross, place one or more inocula of each strain into a tube (or a Magenta jar).

   - Harvest and test the sporophytes produced from sexual crosses individually because they may result from either self- or cross-fertilization. Strains that have been kept for prolonged periods in vegetative culture may lose fertility.

3. Place the cap on the tube, but do not completely tighten it.

4. Culture for about 4 weeks at 25°C in continuous white light at intensities between 5 and 20 Wm\(^{-2}\).

   *Continuous white light accelerates sporophyte production somewhat, but sporophyte yield may be reduced.*

5. Transfer the culture to 15°C for an additional 3 weeks under an 8-hour light/16-hour dark cycle.

6. Irrigate the culture with H\(_2\)O to facilitate fertilization. Make sure to thoroughly wet but not submerge the culture. Allow it to stand for 24 hours.
7. After 24 hours, decant excess H₂O. Place the culture for 1 week at 15°C under an 8-hour light/16-hour dark cycle.

8. Repeat the irrigation procedure as described in Step 6.

9. After 24 hours, decant excess H₂O. Place the culture for 2–5 weeks at 15°C under an 8-hour light/16-hour dark cycle. 

After this incubation period, sporophytes will have developed.

10. Use fine forceps to harvest pale brown sporophytes by separating them from the gametophytic material. Do not take the green or yellow sporophytes (these are immature) or the dark-brown sporophytes (these burst easily).

11. Pinch the base of the seta, which can be identified by a zone of pigmentation, to release the spore capsule.

Each capsule of a mature sporophyte contains $1 \times 10^3$ to $4 \times 10^3$ viable, haploid, uninucleate spores.

12. Place one or more sporophyte capsules in a sterile 1.5-ml microcentrifuge tube. Sterilize the capsules by adding 1 ml of 70% ethanol and incubating for 4 minutes at room temperature.

13. Remove the ethanol. Gently rinse the sporophyte capsules three times with 1 ml of H₂O at room temperature.

14. Add 1 ml of H₂O and transfer the tube to 4°C.

This step increases spore germination, but some spores will germinate if it is omitted.

15. Crush the sporophyte capsules and mix to produce a spore suspension.

Any residual sporophytic tissue will not regenerate and can be ignored. The spore suspension may be kept for several weeks at 4°C.

16. If desired, dry the spores.

Dry spores may be stored for several years.
Protocol 2

Isolation and Regeneration of Protoplasts

This protocol describes how to isolate individual protoplasts from young gametophyte tissue (Method I) and how to regenerate them into plants (Method II) if desired. It is adapted from a protocol described by Grimsley et al. (1977).

MATERIALS

The recipes for items marked with <R> are on page XX.

Reagents

Driselase solution (sterile) <R>
Protonemal tissue from P. patens

This tissue is grown on cellophane-overlay plates as described in Method IB of Protocol 1.

Protoplast regeneration medium for the bottom layer (PRMB) <R>

Prepare Petri dishes (50 mm or 90 mm) containing PRMB and overlay with sterile cellophane.

Protoplast regeneration medium for the top layer (PRMT) <R>

This medium should be melted and kept at 45°C in a water bath.

Protoplast wash (PW) solution <R>

Equipment

Centrifuge
Filters (pore sizes of 50 µm and 100 µm, sterile)

Two filters are needed for each protoplast isolation. Filters can be made of stainless steel or nylon.

Hemocytometer
Incubator with temperature control and white light at intensities between 5 and 20 Wm⁻² (e.g., Percival Scientific Model CU-36L5)

METHODS

I. Protoplast Isolation

1. After culturing protonemal tissue for 7 days (Method IB, Steps 1–5), harvest the tissue as described in Step 6 of Method IB. Weigh the tissue and add 1 ml of sterile driselase solution for every 100 mg of tissue (fresh weight) (i.e., use 2 ml of driselase solution per 90-mm-diameter plate of tissue).

   It is difficult to obtain protoplasts from tissue other than gametophytes growing on cellophane-overlay plates. Use young protonemal tissue; older tissue may leave clumps of undigested tissue, which can act like glue and stick protoplasts together.

2. Incubate the tissue-driselase mixture for 30–60 minutes at room temperature with occasional gentle shaking. Monitor the breakdown of the tissue (no microscope is necessary).

   For some strains, the incubation time may need to be longer. Protoplast viability is not greatly affected by exposure to driselase for up to 2 hours, but it will decrease as the incubation time increases.
3. To isolate protoplasts from the digested tissue, steriley filter the tissue-driselase mixture through mesh with a pore size of 100 µm.

4. Sterilely refilter the filtrate from Step 3 through mesh with a pore size of 50 µm.

5. Sediment the protoplasts by centrifuging the filtrate from Step 4 at 100–200g for 4 minutes at room temperature with no braking.

6. Discard the supernatant.

7. Resuspend the protoplast pellet in PW. Use about the same volume as the volume of driselase used in Step 1.


   This should yield about $10^6$ protoplasts per 90-mm-diameter plate of tissue cultured for 7 days as described in Method IB. Protoplasts can be used in a number of techniques (e.g., Protocols 3, 5, and 6). If protoplasts are to be plated without further manipulation, proceed to Method II.

II. Protoplast Regeneration

1. Estimate the density of protoplasts using a hemocytometer. Adjust the volume of the protoplast suspension with XXXX so that the density of protoplasts is approximately XXXXX.

2. Determine the quantity of molten PRMT needed (use 1 volume of protoplast suspension for every 2 volumes of molten PRMT). Gently add the protoplast suspension from Step 1 to the appropriate volume of molten PRMT.

3. Pipette 1 ml of the protoplast-PRMT mixture gently but quickly onto a 90-mm PRMB plate overlaid with cellophane.

   Alternatively, use 400 µl of the protoplast-PRMT mixture to cover a 50-mm dish.

4. Incubate the plate for 3 or 4 days at 25°C with strong, constant light (intensities >5 Wm⁻²).

   For images of 3-day-old filaments regenerating from protoplasts, see Figure 1F.

5. After about 4 days, transfer the protoplasts to appropriately supplemented, fresh BCD medium as described in Method XX of Protocol 1.
Protocol 3

Somatic Hybridization in *P. patens* Using PEG-induced Protoplast Fusion

As an alternative to sexual crossing (see Protocol 1), protoplasts from two strains of moss (*P. patens*) can be hybridized using polyethylene glycol (PEG). This protocol for PEG-induced protoplast fusion was adapted from the protocol described by Grimsley et al. (1977). Although the efficiency is low, it requires no sophisticated apparatus. Hybrids are readily obtained using complementary auxotrophic mutants or strains with transgenic antibiotic resistance markers. It is now routine to obtain hybrids using transgenic strains that are hygromycin- or G418-resistant by selecting hybrids on medium containing both antibiotics.

**MATERIALS**

The recipes for items marked with <R> are on page XX.

**Reagents**

BCD medium (solid) <R>, containing common moss media supplements <R> and antibiotics <R> as necessary

PEG solution for protoplast fusion (PEG/F) <R>

*For each hybridization, 750 μl of PEG/F is required.*

Protoplast regeneration medium for the bottom layer (PRMB) <R>

*For each hybridization, prepare eight plates containing PRMB and overlay with sterile cellophane.*

Protoplast regeneration medium for the top layer (PRMT) <R>

*This medium should be melted and kept at 45°C in a water bath.*

Protoplast wash (PW) solution <R>

*For each hybridization, prepare 30 ml of PW solution.*

Protoplasts from two different *P. patens* strains

*These protoplasts are isolated as described in Protocol 2, Method I.*

**Equipment**

Centrifuge

Hemocytometer (optional; see Step 2)

Incubator with temperature control and white light at intensities between 5 and 20 Wm⁻² (e.g., Percival Scientific Model CU-36L5)

Petri dishes (90 mm), sterile

**METHOD**

1. For each strain to be hybridized, resuspend 10⁶ protoplasts (approximately the number produced on one 90-mm plate in Protocol 2, Method I) in 2.5 ml of PW.
2. Combine 2.5 ml of each of the two strains to be hybridized. 

Judge the density of protoplasts by eye (if preferred, density can be determined using a hemocytometer). If the yield of protoplasts from one strain is poor, the yield of hybrids involving that strain can be maximized by mixing with an excess of the other strain.

3. Sediment the mixed protoplasts by centrifugation at 100–200g for 4 minutes at room temperature with no braking.

4. Discard the supernatant. Resuspend the protoplasts in 250 µl of PW.

5. Hybridize the two strains of protoplasts by performing the following steps on schedule:
   i. At 0 minutes, add 750 µl of PEG/F and mix gently.
   ii. At 40 minutes, add 1.5 ml of PW and mix gently.
   iii. At 50 minutes, add 10 ml of PW and mix gently.
   iv. At 60 minutes, add 10 ml of PW and mix gently.
   v. At 70 minutes, sediment the protoplasts by centrifugation at 100–200g for 4 minutes at room temperature with no braking. Discard the supernatant and resuspend the pellet in 1 ml of PW.

   Multiple hybridizations can be performed in parallel. It should be possible to do these at 30-second intervals.

6. Add the protoplast suspension to 7 ml of molten PRMT and plate 2 ml onto each of four plates of PRMB overlaid with cellophane. In addition, to estimate the survival of the individual component strains, add 50 µl of the protoplast suspension to 8 ml of molten PRMT and plate 2 ml onto each of four plates of PRMB overlaid with cellophane. Incubate all plates at 25°C with strong, constant light (intensities >5 Wm⁻²).

7. After 5–6 days (when protoplasts have regenerated, but growth is still not great), transfer the protoplasts from Step 6 to Petri dishes containing the appropriate BCD selective medium to select for hybrids. In addition, to estimate the survival of the individual component strains, transfer the cellophane overlays from Step 6 onto the appropriate BCD media to select for one or the other component strain. Incubate all plates under the conditions described in Step 6.

   Selection of hybrids using vitamin auxotrophies usually takes about 3 weeks; using transgenic antibiotic resistances, hybrids can usually be identified after only 7 days.
Chemical and UV Mutagenesis of Spores and Protonemal Tissue

This protocol describes how to mutagenize spores and protonemal tissue from moss (P. patens) using chemicals or UV light. Spores are mutagenized using the alkylating agents N-methyl-N′-nitro-N-nitrosoguanidine (NTG) and ethylmethanesulfonate (EMS) (Methods IA and IB, respectively), and protonemal tissue is mutagenized with NTG and UV light (Methods IIA and IIB, respectively). The chemical mutagenesis protocols were adapted from those described by Ashton and Cove (1977) and Boyd et al. (1988). Compared to alkylating agents, UV is less effective as a mutagen, but it may be advantageous because it is less hazardous and may not lead to clustered lesions.

MATERIALS

The recipes for items marked with <R> are on page XX.

CAUTION: See Appendix XX for appropriate handling of materials marked with <!>.

Reagents

BCD medium (solid) <R>, containing common moss media supplements <R> as necessary
Ethylmethanesulfonate (EMS) <!>
N-methyl-N′-nitro-N-nitrosoguanidine (NTG) <!>
Moss (P. patens) spores (see Protocol 1, Method III) or protonemal tissue on a cellophane-over-
lay plate (see Protocol 1, Method IB)
Orthophosphoric acid (H₃PO₄; 100 mM, pH 7.0; sterile) <!>
Sodium thiosulfate (Na₂S₂O₃; 400 mM, sterile )
Tris-maleate buffer (sterile) <R>

Equipment

Centrifuge
Filter (pore size 100 µm, sterile)
Petri dishes (90 mm)
UV light source <!>

Any standard mutagenic UV source is likely to be effective, but this will need to be calibrated.

METHODS

Perform all chemical mutagenesis procedures in safe handling facilities for mutagens.

I. Mutagenesis of Spores

A. Using NTG

1. Prepare 10 ml of a spore suspension containing about 10⁶ spores in sterile Tris-maleate buffer.
2. Observing strict safety procedures, carefully add 1.2 mg of NTG to a separate tube containing
10 ml of Tris-maleate buffer. Incubate the tube for 30 minutes at 25°C.

AU: In Step 1 of Method IIA below, you say
“Incubate the mixture for
30 minutes at 25°C.” Is
that necessary here, too?
Please check.
3. Mix the spore suspension with the NTG solution and shake it gently for an additional 30 minutes at 25°C.

4. Terminate the treatment by sedimenting the spores by centrifugation (XXXg for XX minutes at room temperature) and dispose of the supernatant safely.

   Usually about 10% of the originally viable spores survive this treatment.

5. Wash the spores three times as follows:
   i. Add 10 ml of H₂O to the tube.
   ii. Centrifuge the tube at XXXg for XX minutes at room temperature.
   iii. Discard the supernatant.

6. After the final wash, resuspend the spores in XXX. Spread about 100 surviving spores onto the surface of appropriately supplemented BCD medium in 90-mm Petri dishes.

7. Incubate the plate under the desired conditions. For standard cultivating procedures and conditions, see Protocol 1.

   The mutagenic treatment results in an initial delay in development, but once germinated, the sporelings grow as usual.

B. Using EMS

1. Prepare 9.5 ml of a spore suspension containing about 10⁶ spores in sterile 100 mM orthophosphoric acid (pH 7.0).

2. Observing strict safety procedures, add 500 µl of EMS.

3. Incubate the mixture for 45 minutes at 25°C.

4. Terminate the treatment by adding 10 ml of sterile 400 mM sodium thiosulfate.

5. Sediment the spores by centrifugation (XXXg for XX minutes at room temperature) and dispose of the supernatant safely.

   Usually 35–40% of the originally viable spores survive this treatment.

6. Wash the spores three times as follows:
   i. Add 10 ml of H₂O to the tube.
   ii. Centrifuge the tube at XXXg for XX minutes at room temperature.
   iii. Discard the supernatant.

7. After the final wash, resuspend the spores in XXX. Spread about 100 surviving spores onto the surface of appropriately supplemented BCD medium in 90-mm Petri dishes.

8. Incubate the plate under the desired conditions. For standard cultivating procedures and conditions, see Protocol 1.

   The mutagenic treatment results in an initial delay in development, but once germinated, the sporelings grow as usual.

II. Mutagenesis of Protonemal Tissue

A. Using NTG

1. Add 1 g (fresh weight) of young protonemal tissue from a cellophane-overlay plate to 10 ml of sterile Tris-maleate buffer. Incubate the mixture for 30 minutes at 25°C.

2. Observing strict safety procedures, carefully add 1.2 mg of NTG to a separate tube containing 10 ml of Tris-maleate buffer. Incubate the tube for 30 minutes at 25°C.
3. Mix the tissue with the NTG solution and shake it gently for an additional 30 minutes at 25°C.

4. Terminate the treatment by filtering the mixture through a sterile filter.
   
   The filter retains the protonema tissue. This procedure results in a cell survival rate of about 10%.

5. Wash the tissue thoroughly, with at least 100 ml of H₂O.
   
   If individual cells are required, these may be obtained by protoplasting the tissue as described in Method I of Protocol 2.

B. Using UV Light

1. Remove the lid from a cellophane-overlay plate and irradiate the tissue with UV light. The kill level is difficult to determine, but a dose of 2000 Jm⁻² should give about 10% survival. It is best to perform a number of experiments to calibrate the UV source in order to get a feel for the correct exposure. Petri dishes are opaque to UV light, so make sure to remove the lid.

2. Immediately after irradiation, transfer the plate to darkness for at least 24 hours to prevent photoreactivation.
   
   Following incubation in the dark, the tissue can be protoplasted (see Protocol 2, Method I), but very few protoplasts regenerate following UV irradiation. Yields are better if the tissue is allowed to grow following incubation (see Protocol 1, Method XX), but this may yield mutant clones.
Protocol 5

Transformation Using Direct DNA Uptake by Protoplasts

This protocol describes how to transform moss (P. patens) protoplasts using PEG-mediated DNA uptake. The transformation rates for direct uptake by protoplasts of DNA with and without genomic sequence (a targeting construct) are typically $10^{-5}$ and $10^{-3}$, respectively (these are the frequencies of stable transformants among regenerants surviving the transformation procedure).

MATERIALS

The recipes for items marked with <R> are on page XX.

CAUTION: See Appendix XX for appropriate handling of materials marked with <!>.

Reagents

BCD medium (solid) <R>, containing common moss media supplements <R> and antibiotics <R> as necessary (optional; see Step 15)

DNA to be used in transformation

Use 15–30 µg of DNA in a volume no greater than 30 µl.

D-Mannitol (8.5%, w/v) <R>

MMM solution <R>

Prepare 10 ml of MMM solution; this is sufficient for 10–15 transformations.

PEG solution for transformation (PEG/T)

To prepare sufficient PEG/T for 15 transformations, melt 2 g of PEG (MW 6000) in a microwave on the day of transformation. Add 5 ml of MCT solution <R>, mix well, and allow the mixture to stand for 2–3 hours at room temperature before use.

Protoplast regeneration medium for the bottom layer (PRMB) <R>

For each transformation, prepare four plates containing PRMB and overlay with sterile cellophane.

Protoplast regeneration medium for the top layer (PRMT) <R>

This medium should be melted and kept at 45°C in a water bath.

Protoplasts, isolated as described in Method I of Protocol 2

Equipment

Centrifuge
Hemocytometer
Tube (10 ml, sterile)
Water bath, set at 45°C

METHOD

Except where stated otherwise, perform all procedures at room temperature, providing this is between 20°C and 25°C. Otherwise, use a water bath. The ten-step dilution described in Steps 8–11 maximizes the recovery rate; however, the dilution can be made in fewer steps if maximum recovery is not required.
1. Resuspend the protoplasts in 8.5% (w/v) D-mannitol solution. Use 2.5 ml for each plate of tissue digested.

2. Use a hemocytometer to estimate protoplast density.

3. Centrifuge **Speed (in g), for how long, and at what temp?** and discard the supernatant. Resuspend the protoplasts in MMM solution at a concentration of $1.6 \times 10^6$ protoplasts/ml.

4. Prepare the DNA to be used in transformation by dispensing 15–30 µg of DNA into a sterile 10-ml tube. Centrifuge briefly to bring the DNA to the bottom of the tube.

5. Add 300 µl of protoplast suspension from Step 3 and 300 µl of PEG/T solution to the DNA from Step 4. Gently invert the tube to mix.

6. Heat the mixture in a water bath for 5 minutes at 45°C.

7. Transfer the tube to room temperature (20°C) and leave it for 5 minutes.

8. Add 300 µl of 8.5% D-mannitol solution. Gently invert the tube to mix. Wait at least 1 minute.

9. Repeat Step 8 four times.

10. Add 1 ml of 8.5% D-mannitol solution. Invert gently to mix. Wait at least 1 minute.

11. Repeat Step 10 four times.

12. Centrifuge at 100–200 g for 5 minutes at room temperature.

13. Discard the supernatant and resuspend the pellet in 500 µl of 8.5% D-mannitol solution.

14. Add 2.5 ml of molten PRMT. Dispense 1 ml of the mixture to each of three 90-mm plates containing PRMB and overlaid with cellophane.

15. Incubate the plates in continuous white light for 5 days at 25°C.

   *If appropriate, transfer the protoplasts on the top layer to the appropriate BCD selective medium.*
Transformation Using T-DNA Mutagenesis

In this protocol, the transformation of moss (*P. patens*) protoplasts is performed via *Agrobacterium*-mediated transfer of T-DNA. The transformation rate for this protocol is typically $10^{-4}$ (expressed as the frequency of stable transformants among regenerants surviving the transformation procedure).

**MATERIALS**

The recipes for items marked with <R> are on page XX.

**CAUTION:** See Appendix XX for appropriate handling of materials marked with <!>.

**Reagents**

- Acetoseringone (3′5′-dimethoxy-4′-hydroxyacetophenone) (100 mM) <!>, prepared in ethanol <!>
- *Agrobacterium tumefaciens* culture (strain AGL-1)
  - About 1 ml of a mid-log-phase culture ($OD_{600}$ = 0.4–0.6) is needed.
- BCD medium (modified)
  - Prepare Petri dishes with solid BCD medium <R> containing 5 mM diammonium tartrate (920 mg/liter of BCD) and 0.05 mg/ml vancomycin <!> (1 ml of 1000 stock per liter of BCD).
- Protoplast regeneration medium for the bottom layer (PRMB, modified)
  - After autoclaving 1 liter of PRMB <R>, add 1 ml of 1000x cefotaxime <!> and 1 ml of 1000x vancomycin <!> (for final concentrations of 0.2 mg/ml and 0.05 mg/ml, respectively). Pour the solution into Petri dishes and overlay each with a sterile cellophane disc. Four dishes are required per transformation.
- Protoplast regeneration medium for the top layer (PRMT, modified)
  - Melt 2.5 ml of PRMT <R> for each transformation and keep it at 45°C in a water bath. Add 1 µl of 1000x cefotaxime <!> and 1 µl of 1000x vancomycin <!> per milliliter of PRMT (for final concentrations of 0.2 mg/ml and 0.05 mg/ml, respectively).
- Protoplast regeneration medium (PRML, liquid) <R>
  - Protoplast wash solution for *Agrobacterium* transformation (APW), maintained at 25°C <R>
  - Prepare 35 ml of APW foreach transformation.
- Protoplasts, isolated as described in Method I of Protocol 2
  - Selection medium
    - For the selection medium, prepare Petri dishes with solid BCD medium <R> containing 5 mM diammonium tartrate (920 mg/liter of BCD), 0.2 mg/ml cefotaxime (1 ml of 1000x stock per liter of BCD), 0.05 mg/ml vancomycin (1 ml of 1000x stock per liter of BCD), and an appropriate antibiotic <R> used to select for T-DNA insertion.

**Equipment**

- Centrifuge
- Hemocytometer
- Incubator with temperature control and white light at intensities between 5 and 20 Wm⁻² (e.g., Percival Scientific Model CU-36L5)
**Method**

1. Use a hemocytometer to estimate the density of protoplasts.
2. Centrifuge the protoplasts at 100–200 g for 4 minutes at room temperature with no braking and discard the supernatant. Resuspend the protoplasts in PRML at a concentration of $5 \times 10^5$ protoplasts/ml.
3. For each milliliter of protoplast suspension, prepare a Petri dish containing 9 ml of PRML.
4. Add 1 ml of protoplast suspension to each prepared Petri dish.
5. Calculate the quantity of *Agrobacterium* culture to add by determining the OD$_{600}$ with a spectrophotometer. Add 110 µl of culture per 1.0 OD$_{600}$ to each Petri dish.
6. Add 20 µl of 100 mM acetoseringone to each Petri dish.
7. Wrap each dish with Micropore surgical tape and incubate for 48 hours at 25°C under a 16-hour light/8-hour dark cycle. The protoplasts will tend to adhere to the Petri dish surface during incubation.
8. Use a pipette to carefully resuspend the protoplasts.
9. Centrifuge the protoplasts at 100–200 g for 4 minutes at room temperature with no braking and discard the supernatant. Resuspend the pellet in 10 ml of APW.
10. Repeat Step 9 twice, but after the last centrifugation step, resuspend the pellet in 2 ml of APW and add 2.4 ml of modified PRMT.
11. Dispense 1.1 ml of the protoplast suspension into each of four Petri dishes containing modified PRMB and overlaid with sterile cellophane discs. Incubate the dishes for 7 days at 25°C under continuous white light.
12. Transfer the cellophane overlays onto plates containing selection medium and incubate for 7 days at 25°C under a 16-hour light/8-hour dark cycle.
13. Transfer the cellophane overlays to plates containing modified BCD medium and incubate for 7 days at 25°C under continuous white light. **AU: incubation conditions OK in Steps 13, 15?**
14. Transfer the cellophane overlays to plates containing selection medium and incubate for 7–10 days at 25°C under a 16-hour light/8-hour dark cycle.
15. Pick small clumps of tissue from each vigorously growing plant and place them on plates containing modified BCD medium. Incubate the plates for 7 days at 25°C under continuous white light. These should be stable transformants. However, to confirm that these are transformants, test them once more for resistance by growing small clumps of tissue on selection medium for 7–10 days under a 16-hour light/8-hour dark cycle.
Transformation of Gametophytes Using a Biolistic Projectile Delivery System

This method is especially suitable for transient gene expression studies, but it can be used to obtain stable transformants.

**MATERIALS**

**CAUTION:** See Appendix XX for appropriate handling of materials marked with <!>.

**Reagents**

- CaCl₂ (2.5 M) <!>
- Ethanol (70% and 100%) <!>
- Gold beads (1 μM)  
  *Prepare in 100% ethanol and resuspend by vigorous mixing for 5 minutes.*

**Macrocarriers**

- Wash in 100% ethanol and allow to dry on sterile filter paper.

**Plasmid DNA**

- Use 2.5 μg of plasmid DNA contained in less than 4 μl.

**Protonemal tissue from P. patens**

- *This tissue is grown as described in Protocol 1, Method IB.*

**Spermidine (0.1 M)**

**Equipment**

- Biolistic particle delivery system (Bio-Rad, PDS-1000/He)  
  *Before use, clean the chamber with H₂O followed by 100% ethanol and allow it to dry. The disruption disks (900 psi) should be immersed in 100% isopropanol <!> until bombardment.*

- Centrifuge

- Incubator with temperature control and white light at intensities between 5 and 20 Wm⁻² (e.g., Percival Scientific Model CU-36L5)

**METHOD**

1. While mixing vigorously, add the following solutions to 2.5 μg of plasmid DNA:
   - Gold beads (1 μM) 25 μl
   - CaCl₂ (2.5 M) 25 μl
   - Spermidine (0.1 M) 10 μl

2. Mix for an additional minute. Allow the beads to settle and then centrifuge the tube for 2 minutes.
3. Discard the supernatant. Wash the pellet with 70% ethanol by adding \( XX \) µl of 70% ethanol and centrifuging at \( XXX \).  

   *Do not resuspend the gold particles during this step.*

4. Discard the supernatant. Resuspend the gold particles in 24 µl of 100% ethanol and apply 6 µl to each macrocarrier. Allow the ethanol to evaporate.

5. Place the protonemal tissue so that it is about 150 mm below the stopper plate of the Bio-Rad PDS-1000/He biolistic particle delivery system. Make two discharges into each dish, moving the position of the dish between discharges.

6. Following bombardment, incubate the tissue for 48 hours at 25°C under continuous white light at intensities between 5 and 20 Wm\(^{-2}\).

7. After the 48-hour incubation, harvest the tissue and blend and plate it onto selective medium as described in Method IB of Protocol 1.

   *Alternatively, if clones derived from single cells are required, isolate protoplasts from the tissue as described in Method I of Protocol 2.*
This protocol describes a series of procedures for isolating nucleic acids (DNA and RNA) and proteins from moss (*P. patens*) gametophyte tissue. Method IA, a rapid small-scale procedure for isolating DNA, results in genomic DNA that is suitable for PCR only. However, larger amounts of genomic DNA suitable for Southern analysis may be obtained using Method IB (adapted from Luo and Wing 2003) together with the Nucleon PhytoPure Genomic DNA Extraction Kit (GE Healthcare). The RNA isolation procedure (Method II) uses the Plant RNA Isolation MiniKit (Agilent) with some modifications. Proteins can be obtained from moss gametophytes using Method III.

**MATERIALS**

The recipes for items marked with <R> are on page XX.

CAUTION: See Appendix XX for appropriate handling of materials marked with <!>.

**Reagents**

- Isopropanol <!> cold?
- Liquid nitrogen <!>
- Nuclei isolation buffer containing β-mercaptoethanol (NIBM) <R> <!>
- Nuclei isolation buffer containing Triton X-100 (NIBT) <R> <!>
- Protein extraction solution, prechilled to 4°C <R>
- Protein wash solution, prechilled to 4°C <R>
- 1x Shorty buffer <R> prechilled?
- Tris-Cl (10 mM, pH XX) containing EDTA (1 mM, pH XX) (TE buffer)
- Tissue of interest from *P. patens* gametophytes (see Protocol 1)

*For DNA isolation (Method I), use protonemal tissue grown for 6–7 days on cellophane-overlay plates as described in Method IB of Protocol 1.*

**Equipment**

- Centrifuge, at 4°C
- Centrifuge tubes (50 ml)
- Cheesecloth (pore size of 1.4 mm × 2.4 mm)
- Erlenmeyer flask (1 liter)
- Filter paper
- Incubator, set at 65°C
- Microcentrifuge
- Microcentrifuge tube pestles (prechilled; Pellet Pestles, Kontes)
- Microcentrifuge tubes (1.5 ml and 2 ml, prechilled)
- Miracloth (pore size of 25 µm; Calbiochem)
- Mortar and pestle (prechilled)
- Nucleon PhytoPure Genomic DNA Extraction Kit (GE Healthcare)

*The pellet that results from Step 15 of Method IB is the starting material for the large sample of the kit (RPN8511).*
Paintbrush (small)
Paper towels
Plant RNA Isolation Mini Kit (Agilent)

Before performing the procedure, prepare the Extraction Solution from the kit by adding 10 µl of β-mercaptoethanol <!> per milliliter of Extraction Buffer and store at 4°C.

Rotator, at 4°C
Spatula (prechilled)

METHODS

1. DNA Isolation

A. Rapid Small-scale Preparation of DNA

1. Blot the tissue of moisture by sandwiching the tissue between sheets of filter paper and firmly compressing the tissue with the thumb.

2. Weigh 100 mg of tissue and freeze it in liquid nitrogen. Then, using a prechilled spatula, transfer the frozen tissue to a prechilled 2-ml microcentrifuge tube.

3. Grind the frozen tissue in liquid nitrogen using a prechilled microcentrifuge tube pestle. Make sure the tissue remains frozen.

4. Add 400 µl of prechilled? 1x Shorty buffer to the tube and mix well.

5. Centrifuge the sample in a microcentrifuge at maximum speed for 5 minutes.

6. Transfer 300 µl of the supernatant to a fresh 2-ml microcentrifuge tube containing 300 µl of cold?? isopropanol. Mix by inverting the tube.

7. Centrifuge the sample at 16,000 g for 10 minutes temp??.

8. Discard the supernatant and dry the pellet by inverting the tube on a paper towel.

   The pellet may not be obvious, but DNA is there—don’t worry!

9. Once the tube is dry, add 300 µl of TE. Resuspend the pellet by incubating for 30 minutes at 65°C or overnight at 4°C.

10. Store the DNA at 4°C for up to ??.

   For PCR, use 1 µl of DNA per reaction.

B. Isolation of High-molecular-weight DNA

1. Blot the tissue of moisture by sandwiching the tissue between sheets of filter paper and firmly compressing the tissue with the thumb.

2. Weigh the blotted tissue and record the mass in grams.

3. Freeze the tissue in liquid nitrogen. Then, using a liquid-nitrogen-chilled mortar and pestle, grind the frozen tissue to a coarse powder. Transfer the ground tissue to a 1-liter Erlenmeyer flask.

4. To the flask, add 13.3 ml of cold NIBM per gram of tissue.

5. Gently shake the flask by slowly rotating it for 15 minutes at 4°C.

6. Filter the ground tissue through four layers of cheesecloth plus one layer of Miracloth.

   The filtrate will contain nuclei.

7. Squeeze the pellet to allow maximum recovery of nucleus-containing solution.
8. Filter the filtrate again through one layer of Miracloth.
9. To the filtrate, add 1 ml of NIBT for every 30 ml of filtrate. Gently shake the mixture by slowly rotating it for 15 minutes at 4°C.
10. Transfer the mixture to 50-ml centrifuge tube and centrifuge at 2400g for 15 minutes at 4°C.
11. Discard the supernatant and gently resuspend the pellets in the residual buffer by tapping the tubes or by mixing with a small paintbrush.
12. Dilute the nuclei suspensions with NIBM and combine them into two 50-ml centrifuge tubes. Adjust the total volume in each tube to 50 ml with NIBM and centrifuge at 2400g for 15 minutes at 4°C.
13. Discard the supernatant and gently resuspend the pellets in the residual buffer by tapping the tubes or by mixing with a small paintbrush.
14. Dilute the nuclei suspensions with NIBM and combine them into one 50-ml centrifuge tube. Adjust the total volume to 50 ml with NIBM and centrifuge at 2400g for 15 minutes at 4°C.
15. Discard the supernatant and resuspended the pellet in the residual buffer (1 ml). Use the resuspended pellet, which is enriched in nuclei, as the starting material for genomic DNA extraction with the Nucleon PhytoPure Genomic DNA Extraction Kit (GE Healthcare).

II. RNA Isolation

1. Blot the tissue of moisture by sandwiching the tissue between sheets of filter paper and firmly compressing the tissue with the thumb.
2. Weigh 100 mg of the tissue and, without delay, freeze the tissue in liquid nitrogen. Use a prechilled spatula to transfer the frozen tissue to a prechilled 1.5-ml microcentrifuge tube.
3. Grind the frozen tissue in liquid nitrogen using a prechilled microcentrifuge tube pestle. Make sure that the tissue remains frozen.
4. Add 500 µl of Extraction buffer (containing β-mercaptoethanol) from the Plant RNA Isolation Mini Kit (Agilent) and grind continuously for at least 7 minutes to maximize yield.
   *This and all subsequent steps can be performed at room temperature.*
5. Centrifuge the sample at 12,000g for 1.5 minutes and transfer the supernatant to the prefiltration column that is supplied with the Plant RNA Isolation Mini Kit (Agilent). Proceed per the kit protocol. Store all RNA samples at –80°C.

III. Protein Isolation

1. Blot the tissue of moisture by sandwiching the tissue between sheets of filter paper and firmly compressing the tissue with the thumb.
2. Weigh about 700 mg of the blotted tissue and freeze it in liquid nitrogen.
3. Using a liquid-nitrogen-chilled mortar and pestle, grind the frozen tissue to a fine powder.
4. Divide the tissue powder into 100-µl aliquots in 1.5-ml microcentrifuge tube and store them at –80°C until needed for protein extraction.
5. When required, suspend about 100 µl of powder in 1 ml of prechilled protein extraction solution. Incubate the mixture for 2 hours at –20°C.
6. Centrifuge the mixture at 20,000g for 30 minutes at 4°C and discard the supernatant.
7. To remove pigments, lipids, and nucleic acids, add 1 ml of prechilled protein wash solution, mix, and incubate for 1 hour at –20°C.
8. Centrifuge the mixture at 20,000g for 30 minutes at 4°C and discard the supernatant.

9. Repeat Steps 7 and 8 until the supernatant is completely clear and uncolored.

10. Dry the pellet by leaving the centrifuge tubes open at room temperature. Store the pellets at –80°C.
BCD MEDIUM, LIQUID OR SOLID

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar (Sigma-Aldrich A9799)</td>
<td>7 g</td>
<td>0.7% (w/v)</td>
</tr>
<tr>
<td>CaCl₂ &lt;!&gt;</td>
<td>111 mg</td>
<td>1 mM</td>
</tr>
<tr>
<td>FeSO₄ · 7H₂O &lt;!&gt;</td>
<td>12.5 mg</td>
<td>45 μM</td>
</tr>
<tr>
<td>Solution B &lt;R&gt;</td>
<td>10 ml</td>
<td>1 mM MgSO₄</td>
</tr>
<tr>
<td>Solution C &lt;R&gt;</td>
<td>10 ml</td>
<td>1.84 mM KH₂PO₄</td>
</tr>
<tr>
<td>Solution D &lt;R&gt;</td>
<td>10 ml</td>
<td>10 mM KNO₃</td>
</tr>
<tr>
<td>TES &lt;R&gt;</td>
<td>1 ml</td>
<td>trace</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Do not include CaCl₂ if the medium is to be used to prepare protoplast regeneration media (PRMB, PRMT, or PRML). Include agar for solid media only. Most high-grade agars can be used, but some may affect the pH of the medium. If an agar other than Sigma-Aldrich A9799 is used, the quantity (7 g) may need to be altered. Sterilize the medium by autoclaving for 40 minutes at 121°C. Add common moss media supplements <R> as necessary. Store at XX°C for up to XXX.

COMMON ANTIBIOTICS INCLUDED WITH MOSS MEDIA

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Quantity (per liter of H₂O) for stock solution</th>
<th>Concentration in stock solution</th>
<th>Concentration in medium (1x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G418 &lt;!&gt;</td>
<td></td>
<td></td>
<td>50 μg/ml</td>
</tr>
<tr>
<td>Hygromycin &lt;!&gt;</td>
<td></td>
<td></td>
<td>30 μg/ml</td>
</tr>
<tr>
<td>Sulfadiazine &lt;!&gt;</td>
<td></td>
<td></td>
<td>150 μg/ml</td>
</tr>
</tbody>
</table>

COMMON MOSS MEDIA SUPPLEMENTS

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Quantity (per liter of H₂O) for stock solution</th>
<th>Concentration in stock solution</th>
<th>Concentration in medium (1x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000x p-Amino-</td>
<td>247 mg</td>
<td>1.8 mM</td>
<td>1.8 μM</td>
</tr>
<tr>
<td>benzoic acid &lt;!&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100x Diammonium</td>
<td>92 g</td>
<td>500 mM</td>
<td>5 mM</td>
</tr>
<tr>
<td>tartrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000x Nicotinic acid</td>
<td>1 g</td>
<td>8 mM</td>
<td>8 μM</td>
</tr>
<tr>
<td>100x D-Sucrose</td>
<td>500 g</td>
<td>1.5 M</td>
<td>15 mM</td>
</tr>
<tr>
<td>1000x Thiamine-HCl</td>
<td>500 mg</td>
<td>1.5 mM</td>
<td>1.5 μM</td>
</tr>
</tbody>
</table>

Stock solutions may be either autoclaved or filter-sterilized and added to growth media as required to give the concentrations listed above. For crossing thiA1 strains, add thiamine-HCl to a final concentration of 15 nM (0.01x), not 1.5 μM (1x).
DMSO–GLUCOSE SOLUTION

Reagent | Quantity (for XX ml) | Final concentration
--- | --- | ---
Dimethylsulfoxide (DMSO) <!> | XX g | 5% (v/v)
Glucose | XX g | 10% (w/v)
H₂O | to XX ml |

AU: Do you need to prepare this solution fresh? If so, please indicate here. If not, please indicate at what temp you should store this solution and how long it will last.

DRISELASE SOLUTION

Reagent | Quantity (for 100 ml) | Final concentration
--- | --- | ---
Driselase | 2 g | 2%
D-Mannitol (8.5% w/v) | to 100 ml |

Stir to mix (but do not shake vigorously) for at least 15 minutes. Centrifuge at 2500g for 5 minutes and filter-sterilize the clear supernatant. There may be variation among batches of driselase, so adjustment to the driselase quantity (2 g) may be necessary.

HOAGLAND’S A-Z TRACE ELEMENT SOLUTION (TES)

Reagent | Quantity (for 1 liter) | Final concentration
--- | --- | ---
Al₂(SO₄)₃ • K₂SO₄ • 24H₂O <!> | 55 mg | 0.006% (w/v)
CoCl₂ • 6H₂O <!> | 55 mg | 0.006% (w/v)
CuSO₄ • 5H₂O <!> | 55 mg | 0.006% (w/v)
H₃BO₃ <!> | 614 mg | 0.061% (w/v)
KBr <!> | 28 mg | 0.003% (w/v)
KI <!> | 28 mg | 0.003% (w/v)
LiCl <!> | 28 mg | 0.003% (w/v)
MnCl₂ • 4H₂O <!> | 389 mg | 0.039% (w/v)
SnCl₂ • 2H₂O <!> | 28 mg | 0.003% (w/v)
ZnSO₄ • 7H₂O <!> | 55 mg | 0.006% (w/v)
H₂O | to 1 liter |

The exact composition of this solution is probably not important.

MCT SOLUTION

Reagent | Quantity (for 10 ml) | Final concentration
--- | --- | ---
Ca(NO₃)₂ (1 M) <!> | 1 ml | 100 mM
D-Mannitol (8.5% w/v) | 9 ml | 7.65% (w/v)
Tris-Cl (1 M, pH 8.0) | 100 µl | 10 mM

Prepare fresh on day of use and filter-sterilize.
### MMM SOLUTION

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 10 ml)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-Mannitol</td>
<td>910 mg</td>
<td>9.1%</td>
</tr>
<tr>
<td>2-[N-morpholino] ethanesulfonic acid (MES) (1% w/v, pH 5.6)</td>
<td>1 ml</td>
<td>10%</td>
</tr>
<tr>
<td>MgCl₂ (1 M)</td>
<td>150 μl</td>
<td>15 mM</td>
</tr>
<tr>
<td>H₂O</td>
<td>8.85 ml</td>
<td></td>
</tr>
</tbody>
</table>

Dissolve the D-mannitol in the H₂O, sterilize by autoclaving, and store at room temperature if necessary. On the day of use, add the MES and MgCl₂ to the D-mannitol solution and filter-sterilize.

### NITROGEN-FREE MEDIUM

**AU: For the Nitrogen-free medium, added CaCl₂ to the list of ingredients and deleted the note about “except for protoplast regeneration media” because Nitrogen-free medium is not used to prepare any of the protoplast regeneration media. OK?**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar (Sigma-Aldrich A9799)</td>
<td>7 g</td>
<td>0.7% (w/v)</td>
</tr>
<tr>
<td>CaCl₂ (1 M)</td>
<td>111 mg</td>
<td>1 mM</td>
</tr>
<tr>
<td>FeSO₄ • 7H₂O</td>
<td>12.5 mg</td>
<td>45 μM</td>
</tr>
<tr>
<td>Solution B &lt;R&gt;</td>
<td>10 ml</td>
<td>1 mM MgSO₄</td>
</tr>
<tr>
<td>Solution C &lt;R&gt;</td>
<td>10 ml</td>
<td>1.84 mM KH₂PO₄</td>
</tr>
<tr>
<td>TES &lt;R&gt;</td>
<td>1 ml</td>
<td>trace</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Include the agar for solid media only. Most high-grade agars can be used, but some may affect the pH of the medium. If an agar other than Sigma-Aldrich A9799 is used, the quantity (7 g) may need to be altered.

### NUCLEI ISOLATION BUFFER CONTAINING β-MERCAPTOETHANOL (NIBM)

**AU: for up to how long?**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (500 mM)</td>
<td>20 ml</td>
<td>10 mM</td>
</tr>
<tr>
<td>KCl (1 M)</td>
<td>100 ml</td>
<td>100 mM</td>
</tr>
<tr>
<td>β-Mercaptoethanol (1%)</td>
<td>20 μl</td>
<td>2% (v/v)</td>
</tr>
<tr>
<td>Spermidine (1 M)</td>
<td>4 ml</td>
<td>4 mM</td>
</tr>
<tr>
<td>Spermine (1 M)</td>
<td>1 ml</td>
<td>1 mM</td>
</tr>
<tr>
<td>D-Sucrose</td>
<td>171 g</td>
<td>17.1% (w/v)</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Mix all ingredients except for the β-mercaptoethanol and filter-sterilize. Store at 4°C **how long?**. Add the β-mercaptoethanol immediately before use.

### NUCLEI ISOLATION BUFFER CONTAINING TRITON X-100 (NIBT)

**AU: for up to how long?**

For NIBT, assumed a 20% stock concentration of Triton X-100, for a final concentration of 2% in the solution. Are these values correct?

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (500 mM)</td>
<td>20 ml</td>
<td>10 mM</td>
</tr>
<tr>
<td>KCl (1 M)</td>
<td>100 ml</td>
<td>100 mM</td>
</tr>
<tr>
<td>Spermidine (1 M)</td>
<td>4 ml</td>
<td>4 mM</td>
</tr>
<tr>
<td>Spermine (1 M)</td>
<td>1 ml</td>
<td>1 mM</td>
</tr>
<tr>
<td>D-Sucrose</td>
<td>171 g</td>
<td>17.1% (w/v)</td>
</tr>
<tr>
<td>Triton X-100 (20%)</td>
<td>100 ml</td>
<td>2%</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Filter-sterilize. Store at 4°C **how long?**.
PEG SOLUTION FOR PROTOPLAST FUSION (PEG/F)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for XX ml)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ • 6H₂O</td>
<td>109 mg</td>
<td>XX</td>
</tr>
<tr>
<td>H₂O</td>
<td>10 ml</td>
<td>XX</td>
</tr>
<tr>
<td>Polyethylene glycol (PEG) (MW 6000)</td>
<td>5 g</td>
<td>XX</td>
</tr>
</tbody>
</table>

Melt the PEG in a microwave oven or water bath. Dissolve the CaCl₂ • 6H₂O in H₂O and then mix the solution with the melted PEG. Dispense into appropriate aliquots (750 µl is needed for each fusion).

PROTEIN EXTRACTION SOLUTION

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 100 ml)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Mercaptoethanol (!)</td>
<td>70 µl</td>
<td>0.07% (v/v)</td>
</tr>
<tr>
<td>Trichloroacetic acid (TCA) (10% w/v) (!)</td>
<td>10 ml</td>
<td>1% (w/v)</td>
</tr>
<tr>
<td>Ethanol (!)</td>
<td>to 100 ml</td>
<td></td>
</tr>
</tbody>
</table>

Filter-sterilize. Store at 4°C how long?

PROTEIN WASH SOLUTION

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 100 ml)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (500 mM)</td>
<td>400 µl</td>
<td>2 mM</td>
</tr>
<tr>
<td>β-Mercaptoethanol (!)</td>
<td>70 µl</td>
<td>0.07% (v/v)</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF) (1 M) (!)</td>
<td>100 µl</td>
<td>100 mM</td>
</tr>
<tr>
<td>Acetone (!)</td>
<td>to 100 ml</td>
<td></td>
</tr>
</tbody>
</table>

Filter-sterilize. Store at 4°C how long?

PROTOPLAST REGENERATION MEDIUM FOR THE BOTTOM LAYER (PRMB)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar (Sigma-Aldrich A9799)</td>
<td>7 g</td>
<td>0.7% (w/v)</td>
</tr>
<tr>
<td>CaCl₂ (!)</td>
<td>1.1 g</td>
<td>10 mM</td>
</tr>
<tr>
<td>Diammonium tartrate</td>
<td>920 mg</td>
<td>5 mM</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>60 g</td>
<td>6% (w/v)</td>
</tr>
<tr>
<td>BCD medium, liquid &lt;!&gt;</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Most high-grade agars can be used, but some may affect the pH of the medium. If an agar other than Sigma-Aldrich A9799 is used, the quantity (7 g) may need to be altered. Sterilize the medium by autoclaving for 40 minutes at 121°C.
### PROTOPLAST REGENERATION MEDIUM FOR THE TOP LAYER (PRMT)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for XX ml)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar (Sigma-Aldrich A9799)</td>
<td>4 g</td>
<td>0.4% (w/v)</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.1 g</td>
<td>10 mM</td>
</tr>
<tr>
<td>Diammonium tartrate</td>
<td>920 mg</td>
<td>5 mM</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>80 g</td>
<td>8% (w/v)</td>
</tr>
<tr>
<td>BCD medium, liquid &lt;R&gt;</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Most high-grade agars can be used, but some may affect the pH of the medium. If an agar other than Sigma-Aldrich A9799 is used, the quantity (4 g) may need to be altered. Sterilize the medium by autoclaving for 40 minutes at 121°C.

### PROTOPLAST REGENERATION MEDIUM, LIQUID (PRML)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>1.1 g</td>
<td>10 mM</td>
</tr>
<tr>
<td>Diammonium tartrate</td>
<td>920 mg</td>
<td>5 mM</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>85 g</td>
<td>8.5% (w/v)</td>
</tr>
<tr>
<td>BCD medium, liquid &lt;R&gt;</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Most high-grade agars can be used, but some may affect the pH of the medium. If an agar other than Sigma-Aldrich A9799 is used, the quantity (1.1 g) may need to be altered. Sterilize the medium by autoclaving for 40 minutes at 121°C.

### PROTOPLAST WASH (PW) SOLUTION

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ • 6H₂O</td>
<td>2.19 g</td>
<td>10 mM</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>85 g</td>
<td>8.5% (w/v)</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Sterilize by autoclaving for 40 minutes at 121°C. This recipe contains CaCl₂ at 10 mM, but different laboratories use concentrations between 0 mM and 50 mM with no apparent differences in protoplast viability.

### PROTOPLAST WASH SOLUTION FOR AGROBACTERIUM TRANSFORMATION (APW)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 100 ml)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ • 6H₂O</td>
<td>219 mg</td>
<td>10 mM</td>
</tr>
<tr>
<td>1000x Vancomycin stock solution</td>
<td>100 µl</td>
<td>1x</td>
</tr>
<tr>
<td>1000x Cefotaxime stock solution</td>
<td>100 µl</td>
<td>1x</td>
</tr>
<tr>
<td>D-Mannitol solution (8.5% w/v)</td>
<td>100 ml</td>
<td></td>
</tr>
</tbody>
</table>
5x SHORTY BUFFER STOCK SOLUTION

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 500 ml)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (500 mM)</td>
<td>25 ml</td>
<td>25 mM</td>
</tr>
<tr>
<td>LiCl (2 M)</td>
<td>100 ml</td>
<td>400 mM</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS) (10% w/v)</td>
<td>50 ml</td>
<td>1% (w/v)</td>
</tr>
<tr>
<td>Tris-Cl (1 M, pH 9.0)</td>
<td>100 ml</td>
<td>200 mM</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>225 ml</td>
<td></td>
</tr>
</tbody>
</table>

Store at 4°C **AU: for up to how long?**. Dilute fivefold with H$_2$O as required.

**SOLUTION B**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO$_4$ • 7H$_2$O</td>
<td>25 g</td>
<td>0.1 M</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

**SOLUTION C**

**AU: FOR SOLUTIONS B, C, AND D:** If you should sterilize this solution, please specify how. Do you need to prepare this solution fresh? If so, please indicate here. If not, please indicate at what temp you should store this solution and how long it will last.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>25 g</td>
<td>184 mM</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Dissolve the KH$_2$PO$_4$ in 500 ml of H$_2$O and adjust the pH to 6.5 with a minimal volume of 4 M KOH. Bring the final volume to 1 liter with additional H$_2$O.

**SOLUTION D**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO$_3$</td>
<td>101 g</td>
<td>1 M</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

**TRIS-MALEATE BUFFER**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity (for 1 liter)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maleic acid</td>
<td>6 g</td>
<td>50 mM</td>
</tr>
<tr>
<td>Tris-(hydroxymethyl)amino-methane</td>
<td>6 g</td>
<td>50 mM</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>to 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Adjust the pH to 6.0 with 10 M NaOH or KOH. Store at 4°C **AU: for up to how long?**.
REFERENCES


