Chapter 12
Protoplast Isolation and Staining

Shiu-Cheung Lung, Sarah Schoor, Dustin Sigurdson, Makoto Yanagisawa, Kelly Yeung, Mi Qi Liu and Simon D. X. Chuong

12.1 Introduction

Protoplasts are plant cells that have had their cell wall enzymatically or mechanically removed. Protoplasts were first isolated half a century ago by Cocking using a fungal cellulase on the root tips of tomato seedlings [1]; since this initial report, numerous protocols have been optimized for isolating protoplasts from various tissues of different plant species (Table 12.1). Protoplasts have become a versatile tool in the in vivo study of plant function and development. Protoplasts readily take up small molecules, facilitating feeding experiments and improving access of fluorescent dyes. In addition to improved ease of staining, fluorescence imaging protoplasts greatly reduces background signal, allowing for higher resolution imaging of subcellular structures (see Chap. 9). Protoplasts can also be manipulated to take up larger exogenous macromolecules such as DNA, RNA and proteins [2–6]. The ability of protoplasts to take up foreign DNA is particularly valuable in transient gene expression [3, 4, 7–11], subcellular protein localization [3, 4, 12–16; Fig. 12.3f, g and h], protein–protein interaction [13, 17, 18] and cell signalling studies [2]. Finally, the totipotent nature of protoplasts also renders them invaluable to plant geneticists as a practical breeding tool for introducing genetic varieties via interspecific and intergeneric protoplast fusion and regeneration [19–23]. In this chapter, a standard protocol for protoplast isolation based on Yoo et al. [14] with minor modifications will be described. Critical factors affecting protoplast yield and viability are also discussed, with the following species highlighted (Fig. 12.1):

S. D. X. Chuong (✉)
Department of Biology, University of Waterloo, 200 University Avenue West, Waterloo N2L 3G1 Ontario, Canada
e-mail: schuong@uwaterloo.ca

S.-C. Lung · S. Schoor · D. Sigurdson · M. Yanagisawa · K. Yeung · M. Q. Liu
Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

S.-C. Lung
School of Biological Sciences, University of Hong Kong, Hong Kong SAR, China

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_12

	Plant species			
	Arabidopsis	*Kalanchoe*	*Bienertia*	*Lampranthus*
1. Vacuum infiltration	15 - 20 minutes	NA		15 - 20 minutes
2. Digestion of leaf segments to release protoplasts	1.5% cellulase, 0.4% macerozyme; 0.4 M mannitol; 3 hrs	1.5% cellulase, 0.5% macerozyme; 0.4 M mannitol; 1.5 hrs	1.5% cellulase; 0.7 M sucrose; 4 hrs	2% cellulase, 2% macerozyme; 0.8 M mannitol; 2.5 hrs
3. Filtration to remove cellular debris	100 μm nylon mesh		NA	
4. Centrifugation to collect protoplasts	100g, 2 minutes			
5. Isolation of healthy protoplasts in CS-sucrose	100g, 2 minutes		NA	

Fig. 12.1 Outline of the procedures used for isolation of protoplasts from various plant species. Species-specific adjustments are indicated at specific steps of the isolation of protoplasts from leaves of *Arabidopsis thaliana*, *Kalanchoe daigremontiana*, *B. sinuspersici* and *Lampranthus spectabilis*

Arabidopsis thaliana, *Bienertia sinuspersici, Kalanchoe daigremontiana* and *Lampranthus spectabilis*. Although protoplasts may be derived from a variety of plant tissues (Table 12.1), isolation of mesophyll protoplasts from leaves will be the main focus of this chapter.

12.1.1 Tissue Selection and Preparation

One of the most crucial factors affecting protoplast yield is the age and source of tissue. Protoplasts derived from distinct tissues often retain their tissue- or cell-specific activities or biological processes [16]. As leaf development varies by species, there is no universal age at which leaves can be sampled; however, it is usually best to avoid newly formed leaves that exhibit limited subcellular differentiation [4]. Conversely, the use of older leaves with waxy cuticle and thickened epidermal cell walls and/or high levels of secondary metabolite accumulation can also reduce protoplast yield [11, 24]. Accumulation of starch grains is one of the major factors that affect protoplast yield and viability. For example, it was shown that increasing starch levels was associated with lower protoplast viability in pea roots [25]. Similar observations have been documented in various species that starch granules are capable of disrupting protoplast integrity [26–28]. To reduce starch levels, plants can be maintained under dark conditions for 24–48 h prior to digestion. As starch levels vary in different tissue and species, pretreatment in the dark is not always required. For example, in this study we have found that a 48-h dark treatment had no significant effect on the isolation of protoplasts from *L. spectabilis,* while the same conditions greatly improved the yield and viability of protoplasts from leaves of *K. daigremontiana*.

As the leaves of terrestrial plants are protected by a waxy epidermis, the first step of a routine protoplast preparation is to expose the inner mesophyll cells to cell wall enzymes. To improve enzyme penetration, the epidermis of leaves can be peeled

Table 12.1 Examples of protoplasts isolated from various tissues of different plant species

Species	Tissues	Reference
Allium sativum	Callus	[30]
Arabidopsis thaliana	Cotyledon	[31]
A. thaliana	Leaf	[2]
A. thaliana	Suspension cultured cells	[3]
Artemisia judaica L. (Judean wormwood)	Leaf	[32]
Avena fatua (wild oat)	Leaf, aleurone layers	[17, 33]
Beta vulgaris L. (beet)	Callus	[34]
B. vulgaris L (beet)	Root	[35]
Bienertia sinuspersici	Leaf	[4]
Brachypodium distachyon	Leaf	[7]
Brassica napus (canola)	Hypocotyl	[8, 36]
Browollia speciosa (bush violet)	Cotyledon	[37]
Cucumis sativus (cucumber)	Leaf	[38]
Echinacea purpurea (purple coneflower)	Leaf	[32]
Echinops spinosissimus Turra (globe thistles)	Leaf	[39]
Euphorbia pulccherrima	Leaf	[12]
Gossypium hirsutum (cotton)	Cotyledon	[40]
Helianthus annuus (sunflower)	Cotyledon, hypocotyl	[41]
Hordeum vulgare (barley)	Leaf	[42]
Ipomoea batatas L. (sweet potato)	Stem, leaf	[9]
Jatropha curcas L.	In vitro leaf	[43]
Kalanchoe daigremontiana (Mother of Thousands)	Leaf	This study
Lactuca sativa (lettuce)	Leaf	[44]
Lampranthus spectabilis (Trailing Ice Plant)	Leaf	This study
Lupinus angustifolius (lupin)	Leaf	[45]
Manihot esculenta (cassava)	Leaf	[46]
Nicotiana tabaccum (tobacco)	Leaf	[27, 47]
N. tabaccum BY-2 cells	Suspension cultured cells	[3]
Oryza sativa (rice)	Stem and sheath	[13]
Panicum miliaceum (millet)	Leaf	[48]
Panicum virgatum (switchgrass)	Leaf	[10]
Phaseolus vulgaris L.	Cotyledons	[49]
Populas tremula x alba	Leaf	[11, 50]
Prunus dulcis (almond)	Suspension culture	[51]
Ricinus communis L.	In vitro leaf	[43]
Secale cereale L. (rye)	Leaf	[42]
Solanum lycopersicum (tomato)	Fruit, leaf and root	[1, 52]
Solanum tuberosum L. (potato)	Leaf	[53, 54]
Sorghum bicolor (sorghum)	Leaf	[55]
Triticum aestivum (wheat)	Leaf	[42]
Vitis vinifera (grape)	Leaf	[56]
Zea mays (corn)	Leaf	[2]

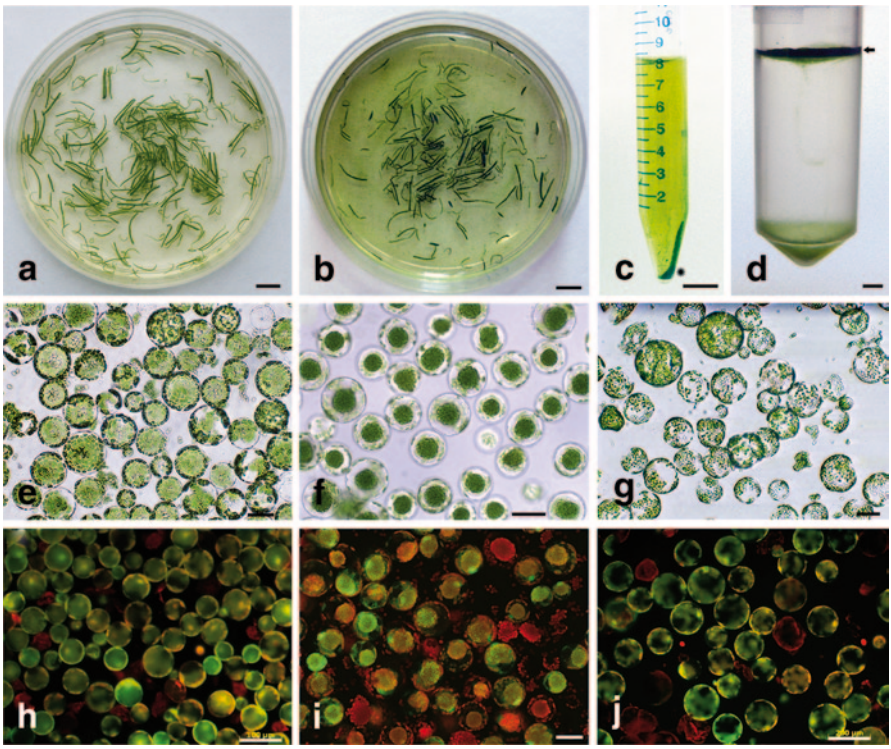

Fig. 12.2 Procedures used for isolation of mesophyll protoplasts from leaves of various dicotyledonous species. **a** Leaf sections at the beginning of cell wall removal treatment. **b** Leaf sections after 3 h in enzyme solution. **c** Protoplast pellet (*) obtained by centrifugation. **d** Healthy mesophyll protoplasts *(arrowhead)* in the floating layer of CS-sucrose buffer after centrifugation. Isolated healthy mesophyll protoplasts from **e** and **h** *Arabidopsis*; **f** and **i** *B. sinuspersici*; and **g** and **j** *K. daigremontiana* visualized under brightfield microscopy and under fluorescence microscopy after they have been stained with fluorescein diacetate (FDA) for viability assessment. Fluorescence is shown in false colours with *green* for FDA and *red* for chlorophyll autofluorescence. Overlapping signals appear as *yellow*. Scale bars **a**–**c** = 1 cm; **d** = 2 mm; **e**–**j** = 50 μm

off mechanically or removed by enzymatic digestion [4, 24, 27, 28]. Alternatively, a direct approach is to slice the leaf tissues into fine strips (Fig. 12.2a) and expose the mesophyll cells at the cut edges to the enzyme solution [14, 29]; we have found this method to be most effective for *K. daigremontiana* and *L. spectabilis*. If leaf sectioning is being performed, vacuum infiltration may be required to facilitate the penetration of the enzyme solution (Fig. 12.1). Although not all plant species require the vacuum infiltration step, such treatment can be useful in the removal of trapped air and exposure of cells in thicker tissues to the enzyme solution. Finally, mesophyll cells of succulent plant species such as *B. sinuspersici* can be directly released and exposed to the enzyme solution by gently pressing leaves with a mortar and pestle [4].

12.1.2 Cell Wall Digestion

The cell walls of plant tissues are composed of two layers: The pectin-rich middle lamella responsible for the cell–cell adhesion and the inner primary cell wall consisted mainly of cellulose microfibrils and hemicellulose that provides the structural support to the cell. Generally, the protoplast isolation procedure requires cell separation that involved treatment of plant tissue with pectinase to remove the pectin-rich matrix of the middle lamella followed by cellulase to remove the cellulosic walls. Currently, most protoplast isolation protocols routinely employ a mixture of enzymes such as cellulase and macerozyme, which consists of a mixture of pectinase and hemicellulase. The working concentrations of these enzymes vary depending on the plant species and how the tissue has been prepared (Fig. 12.1). For example, the loosely arranged spongy mesophyll cells of *Arabidopsis* require a lower enzyme concentration compared to that of the tightly packed, radially arranged mesophyll cells of *Lampranthus* (Fig. 12.1). Likewise, isolation of protoplasts from *Bienertia* leaves requires no pectinase treatment or inclusion of macerozyme in the enzyme solution as the mesophyll cells are loosely packed and can readily be released from the leaves by epidermal peel or using a mortar and pestle [4].

In addition to enzyme concentration, pH also greatly impacts the rate of digestion. As documented by Lung et al. [4], both macerozyme and cellulase are most active at a slightly acidic pH (5–5.5), however under normal conditions, the cytoplasmic pH of typical plant cells are kept at neutral; as a compromise to maintain the viability of the released protoplasts, digestion is generally performed at pH 5.7. When attempting to modify the protocol for other plant species, pH may need to be altered to ensure optimal protoplast release and viability. Furthermore, the cell wall normally provides a means of maintaining turgor pressure, thus removal of this structure can render protoplasts vulnerable to cell lysis. Thus, to counteract turgor pressure during the cell wall removal process, the presence of an osmotic solute (osmoticum) such as sorbitol, mannitol, glucose or sucrose is often required to maintain the osmotic potential. The optimum concentration and nature of an osmoticum, however, vary with different plant species (Fig. 12.1) and require empirical testing. Readers can also refer to a recent study that examined the effects of different osmotica on the yield and viability of protoplasts from a single-cell C_4 species, *B. sinuspersici*, which possesses a unique intracellular compartmentation of organelles in chlorenchyma cells [4].

The exposure time of tissue to enzyme solution also varies based on the species being studied (Fig. 12.1), but typically it should not exceed 4 h. When determining the optimal incubation time, it is not always necessary to wait for complete tissue digestion. For example, in *Arabidopsis*, portions of the undigested leaf segments are often remaining (Fig. 12.2b), when the optimal amounts of viable protoplasts have been released. To remove undigested tissue, the digest solution can be filtered using a 75–100 μm nylon mesh (Fig. 12.1). It is important to ensure the proper mesh size is used as removal of the cell wall renders the protoplasts fragile and susceptible to mechanical damage.

12.1.3 Isolation of Viable Protoplasts

The isolation of a pure population of non-stressed, healthy protoplasts is critical for maintaining reliability and reproducibility. Thus once the cell wall digestion is complete, the separation of viable and nonviable protoplasts becomes a crucial step. Depending on the osmolarity of the enzyme solution (Fig. 12.1), centrifugation will either pellet both living and dead protoplasts (Fig. 12.2c) or intact protoplasts will remain buoyant, forming a floating layer (Fig. 12.2d). With high-molecular weight solutes such as sucrose, concentrations as low as 0.4 M will allow intact protoplasts remain floating. On the other hand, no additional centrifugation steps are needed in the isolation of protoplasts from succulent plants such as *Bienertia* which requires a high concentration of osmoticum (0.7 M sucrose) [4]; Fig. 12.1). While the other solutes such as mannitol and sorbitol have nearly half the molecular weight than that of sucrose, higher concentrations of these osmotica will sufficiently increase fluid density. For example, *Arabidopsis* leaf sections are digested in 0.4 M mannitol where all protoplasts pellet, however *Lampranthus* protoplasts are released into 0.8 M mannitol, where only healthy protoplasts remain afloat at the top layer. If a floating layer is not obtained after the first centrifugation, then the pellet can be resuspended in a higher-density solution. Once the floating layer has been obtained, protoplast viability can then be assessed. Preliminary assessment of protoplast health can be performed using bright-field microscopy. Healthy protoplasts will be spherical and have uniform chloroplast distribution (Fig. 12.2e, f and g). Unhealthy protoplasts will exhibit chloroplast clumping, irregularity in shape and sometimes plasmolysis. For a more accurate assessment of protoplast viability, a small aliquot of the isolated protoplasts can be stained with FDA (Fig. 12.2h, i and j; see Chap. 9). If protoplasts that are for use in subsequent biochemical, cellular and molecular genetics studies to examine a variety of cell-specific/biological processes, viability rates of 80 % are recommended.

12.2 Materials

12.2.1 Equipment

Laboratory equipment: Controlled environmental chamber, desiccator, vacuum pump, incubators (55 and 70 °C), refrigerator, benchtop centrifuge equipped with swing-bucket rotor and a fluorescence microscope or confocal laser scanning microscope

12.2.2 Supplies for Protoplast Isolation

General supplies: Potting soil, 20:20:20 (N:P:K) fertilizers, *A. thaliana* (ecotype Col-0) seeds, Falcon tubes (15 and 50 mL), Petri dishes (90 mm), Nu-Base plate wax, flat-tip forceps, double-sided razor blades (Electron Microscopy Sciences 72000), nylon mesh (75 μm; Spectrum Scientific Inc 146490.), Pasteur pipettes, cover glasses, depression slides and hemocytometer (Neubauer).

12.2.3 Chemical Reagents

Bovine serum albumin (Sigma A7030), Cellulase "Onozuka" R-10 (Yakult Pharmaceutical Ind. Co. Ltd. L0012), Macerozyme R-10 (Yakult Pharmaceutical Ind. Co. Ltd. L0021), 2-(*N*-morpholino) ethanesulfonic acid (MES; BioShop MES503), D-mannitol (Sigma 63559), Sucrose (BioShop SUC507), Calcium chloride ($CaCl_2$; BioShop CCL444), Potassium chloride (KCl; BioShop POC888), Potassium hydroxide (KOH; BioShop PHY202)

12.2.4 Solution Preparation

1. 0.5 M MES-KOH stock solution, pH 5.7: For 500 mL, dissolve 10.65 g of MES in 400 mL of deionized water, adjust the pH to 5.7 with 1 M KOH and then add deionized water to 500 mL. Sterile by autoclaving.
2. 1 M $CaCl_2$ solution: For 500 mL, dissolve 73.51 g of $CaCl_2$ in 400 mL of deionized water. Adjust the volume to 500 mL with deionized water and sterilize by autoclaving.
3. 1 M KCl solution: For 500 mL, dissolve 37.27 g of KCl in 400 mL of deionized water. Adjust the volume to 500 mL with deionized water and sterilize by autoclaving.
4. 1 M NaCl solution: For 500 mL, dissolve 29.22 g of NaCl in 400 mL of deionized water. Adjust the volume to 500 mL with deionized water and sterilize by autoclaving.
5. Cell-stabilizing (CS)-mannitol buffer: For 50 mL, add 2 mL of 0.5 M MES-KOH, pH 5.7 (final 20 mM), 1 mL of 1 M KCl (final 20 mM) and 3.64 g of mannitol (final 400 mM). Adjust volume to 50 mL with deionized water and store at 4 °C for up to 2 weeks.
6. W5 buffer: For 50 mL, add 0.2 mL of 0.5 M MES-KOH, pH 5.7 (final 2 mM), 7.7 mL of 1 M NaCl (final 154 mM), 6.25 mL of 1 M $CaCl_2$ (final 125 mM) and 0.25 mL of 1 M KCl (final 5 mM). Adjust volume to 50 mL with water and store at 4 °C for up to 2 weeks.

7. CS-sucrose buffer: For 50 mL, add 2 mL of 0.5 M MES-KOH, pH 5.7 (final 20 mM), 1 mL of 1 M KCl (final 20 mM) and 6.84 g of sucrose (final 400 mM). Adjust volume to 50 mL with deionized water and store at 4 °C up to 2 weeks.
8. 10% (w/v) Bovine serum albumin (BSA): Dissolve 1 g of BSA in 10 mL of deionized water. Dispense in aliquots and store at −20 °C.
9. Enzyme solution: Incubate 10 mL of CS-mannitol buffer in a 15-mL falcon tube at 70 °C for 10 min. Cool it to below 55 °C before adding 150 mg of cellulase "Onozuka" R-10 (final 1.5% [w/v]) and 40 mg of macerozyme R-10 (final 0.4% [w/v]). Incubate at 55 °C for another 10 min to completely dissolve the cell wall enzymes. Cool the enzyme solution to room temperature before adding 100 µl of 10% BSA (final 0.1% [w/v]) and 100 µL of 1 M $CaCl_2$ (final 10 mM). Transfer the enzyme solution to a 90-mm Petri dish.

12.3 Methods

12.3.1 Isolation of Mesophyll Protoplasts from A. thaliana

1. Stratify *A. thaliana* seeds at 4 °C in the dark for at least 48 h.
2. Germinate the stratified seeds on potting soil covered with plastic domes during the first week in controlled environmental chambers with a day/night regime of 16/8 h at 22 °C with a photon flux density of approximately 150 µmol m^{-2} s^{-1}. Keep the plants well watered and fertilize with fertilizer regularly (*see Note 1*).
3. Harvest 20 well-expanded leaves from 3- to 4-week-old plants prior to bolting. Tissues should be processed immediately following harvesting in order to maintain cell turgor and ensure maximal healthy protoplast yield.
4. Prepare 0.5- to 1-mm strips from the middle region of a leaf using a fresh sharp double-sided razor blade on a dental wax pad (e.g. Nu-Base plate wax) (*see Note 2*).
5. Pick up the leaf strips using a pair of flat-tip forceps. Dip both sides of the strips in the enzyme solution before releasing them into the 10 mL enzyme solution in a Petri dish. (*see Note 3*)
6. Repeat steps 4 and 5 until all the leaves have been cut (*see Note 4*).
7. Vacuum infiltrate the leaf strips for 15 min in a desiccator connected to a vacuum pump (*see Note 5*).
8. Digest the leaf strips at room temperature in the dark for at least 3 h without shaking (*see Note 6*; Fig. 12.2a). Monitor the progress in cell wall digestion regularly by observing the release of protoplasts under a microscope (*see Note 7*).
9. Check the healthiness of the isolated protoplasts by their appearance under a microscope (*see Note 8*).

12.3.2 Purification of Healthy Protoplasts for Use in Cellular and Molecular Analyses

1. To remove cell debris, filter the enzyme–protoplast solution through a piece of 75-μm nylon mesh into a 50-mL falcon tube using a Pasteur pipette. Rinse the Petri dish gently by swirling with 10 mL of CS buffer and filter the content into the same falcon tube (*see Note 9*).
2. Equally divide the filtered protoplasts into two 15-mL falcon tubes and centrifuge at 100 g for 2 min using a benchtop centrifuge equipped with a swing-bucket rotor (*see Note 10*; Fig. 12.2c).
3. Remove the supernatant as much as possible without disturbing the protoplast pellets using a Pasteur pipette.
4. If the presence of any unhealthy protoplasts and/or residual cell debris matters, protoplasts can be further purified on a sucrose medium as follows (optional). Otherwise, proceed to step 6. Resuspend each protoplast pellets in 10 mL of CS-sucrose buffer (*see Note 11*). Centrifuge at 100 g for 2 min using a benchtop centrifuge equipped with a swing-bucket rotor (*see Note 12*; Fig. 12.2d).
5. Remove the pellets and the solution as much as possible without disturbing the floating layer which contains the healthy protoplasts using a Pasteur pipette. At this point, 10 μL of the healthy protoplasts can be used to determine the cell count using a hemocytometer (*see Note 13*).
6. Resuspend the protoplast pellets from step 3 or the floating layer of protoplasts from step 5 in 1 mL of W5 buffer by gentle swirling.
7. Centrifuge the protoplast suspension at 100 g for 2 min using a benchtop centrifuge equipped with a swing-bucket rotor (see Note 14).
8. Remove the supernatant as much as possible without disturbing the protoplast pellets using a Pasteur pipette. The protoplast pellets can be used for various fluorescent staining procedures (see Chap. 9) or for transfection experiments as described by Lung et al. [4].
9. For staining, resuspend the protoplast pellets by gentle swirling in CS-mannitol buffer with an appropriate amount of dye (*see Note 15*). Incubate at room temperature for 15 min (*see Note 16*).
10. Centrifuge the protoplast suspension at 100 g for 2 min using a benchtop centrifuge equipped with a swing-bucket rotor.
11. Remove the supernatant as much as possible without disturbing the protoplast pellets using a Pasteur pipette.
12. Resuspend the protoplast pellets by gentle swirling in an appropriate volume of CS-mannitol buffer to achieve the best cell density for microscopy (*see Note 17*).
13. Transfer 50 μL of the stained protoplasts to a depression slide and place a coverslip. Examine and image the protoplasts using an epifluorescence microscope or a confocal microscope (*see Note 18*; Figs. 12.2 and 12.3).

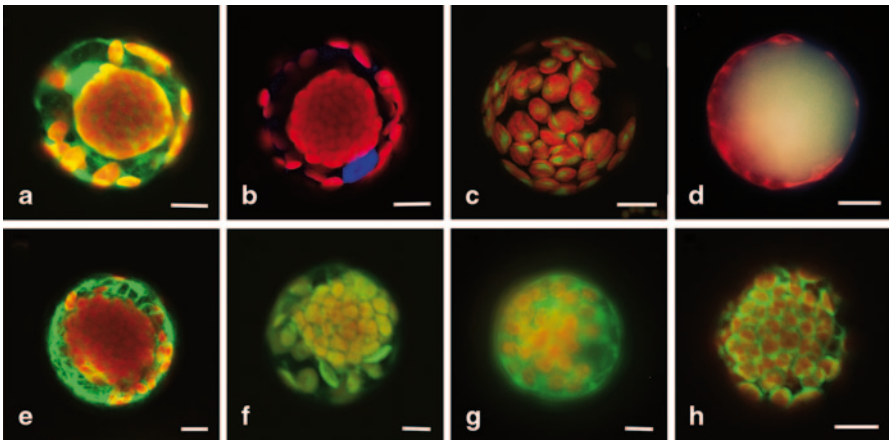

Fig. 12.3 Subcellular localization of compartment and organelles in mesophyll protoplasts stained with various fluorescent dyes **(a-d)** or transfected with chimeric constructs containing various organelle markers fused to green fluorescent protein (GFP; **e**–**h**). **a** Cytoplasm in *Bienertia sinuspersici* protoplast stained with fluorescein diacetate (FDA); **b** Nucleus in *B. sinuspersici* protoplast stained with 4′,6-diamidino-2-phenylindole (DAPI); **c** Mitochondria in *Arabidopsis* protoplast stained with rhodamine 123 (R123); **d** Vacuole in *Arabidopsis* protoplast stained with 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetae (carboxy-DCFDA); **e** Transfected *B. sinuspersici* protoplast showing the actin filaments; **f** Transfected *B. sinuspersici* protoplast with GFP targeted to the stroma of chloroplasts; **g** Transfected *Arabidopsis* protoplast with cytosolic GFP expression; **h** Transfected *Arabidopsis* protoplast with GFP targeted to the chloroplast outer membrane. Fluorescence is shown in false colours with *green* for FDA, GFP and R123, *blue* for DAPI, *greenish-white* for carboxy-DCFDA and *red* for chlorophyll autofluorescence. Overlapping signals appear *yellow*. Panels a–c, e, f, h represent confocal micrographs. Panels d, g represent epifluorescence micrographs. Scale bars = 10 μm

12.4 Notes

1. The yield and health of isolated protoplasts critically depend on the source and status of starting plant materials. For reproducible results, plants should be maintained under standard conditions.
2. A sharp razor blade is necessary to avoid tissue crushing at the cutting edge. Do not use single-edge razor blade. Instead, use a double-edge razor blade which has been snapped in half before removing the paper covering. Move to an unused area of blade or change blade after cutting 4–5 leaves. When cutting, draw the blade over the leaf surface in a single, smooth motion. Uniform leaf strips are essential for the release of protoplasts and overall yield.
3. High-quality cellulase and macerozyme should always be used. Impurities in industrial-grade enzymes will severely affect the yield and viability of isolated protoplasts.
4. Scale up the volume of enzyme solution accordingly if more leaf materials are to be used. Incubation of leaf strips in an inadequate volume of enzyme solution will prolong the digestion time or result in incomplete cell wall digestion.

5. Make sure that air bubbles are being expelled out from the cutting edges of leaf strips during vacuum infiltration. Poor contact of the enzyme solution with the mesophyll cells will affect the protoplast yield.
6. Avoid unnecessary agitation during the digestion process as the isolated protoplasts become very fragile in the medium after the removal of their cell walls.
7. Toward the end of digestion period, the medium should turn green upon a gentle swirl of the Petri dish (Fig. 12.2b).
8. The protoplast–enzyme solution contains a mixture of healthy and unhealthy protoplasts. Healthy protoplasts are spherical with uniform chloroplast distribution whereas unhealthy protoplasts often are irregularly shaped with clumped chloroplasts.
9. Be gentle and avoid splashing when filtering the isolated protoplasts by running the cell suspension down the inner wall of the falcon tube. The diameters of mesophyll protoplasts from 3- to 4-week-old *A. thaliana* plants are approximately 30–50 μm in diameters. For filtering isolated protoplasts from other plant materials, the appropriate cut-off size of nylon mesh should be chosen to minimize cell damage.
10. Centrifugation using a swing-bucket rotor rather than a fixed-angle rotor can effectively minimize the loss of isolated protoplasts by collecting them at the bottom of the falcon tubes.
11. Resuspend the protoplast pellets by gentle swirling of the falcon tube. Do not resuspend by pipetting to minimize cell damage.
12. During centrifugation, the unhealthy and broken protoplasts will move toward the bottom of the tube forming a pellet whereas the healthy protoplasts will remain afloat in the upper layer of the medium.
13. Alternatively, isolated protoplasts of some plant species are extremely fragile and cannot survive the centrifugation step, allow them to settle at the bottom of the tube by gravity. Protoplasts will readily settle in W5 solution in 20–30 min.
14. Protoplast yield will vary depending on the species being used. Generally, a volume of 10-mL digest solution can yield up to 5×10^5 protoplasts from *K. daigremontiana*, *Bienertia* and *Lampranthus*, while a yield as high as 2×10^7 can be obtained from *Arabidopsis*.
15. Stock solutions of many fluorescent dyes are insoluble in aqueous solutions but readily soluble in organic solvents, such as acetone, ethanol, dimethyl sulfoxide and methanol. Negative controls without cytochemical stains should be set up by adding the same volume of organic solvents into the CS-mannitol buffer to confirm that the organic solvents do not affect the integrity and function of the organelles.
16. The optimum concentrations, incubation time and conditions may vary with different cytochemical stains.
17. The isolated protoplasts can be pelleted by low-speed (i.e., 100 g) centrifugation in CS-mannitol buffer without a significant loss of viability. If needed, this pelleting step can be routinely incorporated into any protoplast staining procedures to adjust the cell density for imaging or to wash the stained protoplasts further if the background fluorescence is too high.

18. A depression slide has a round, concave depression well at its centre so the isolated protoplasts will not be squashed and misshaped when a coverslip is applied and the aqueous medium will not be dried out as quickly as with flat microscopic slides.

12.5 General Comments

The expected results from a protoplast isolation procedure will vary depending on the plant species or tissues being used. The procedures described in this chapter are carried out on a small scale with the intention of increasing the scale when the method becomes successful and reproducible. Often, people encountered problems when scaling up their experiments and things did not turn out as expected. One of the sources of variability arises from the ratio of enzyme solution to tissue. It is likely that in order to release more protoplasts from more tissue, a larger volume of enzyme solution will be used. Moreover, the time needed to prepare all the plant material may be longer and so the time of digestion may differ too. In addition, the protoplast yield and health from other plant species may also vary with different concentrations and types of osmoticum in the enzyme solution, optimum time of digestion and concentrations of cell wall enzymes. These parameters should be tested empirically to determine the best conditions for the plant species of interest instead of those that are described for the representative plants in this chapter. In general, when attempting to follow a published protocol, it is always advisable to carry out small-scale trials to familiarize oneself with the procedure, have a feel for any dubious steps and determine whether improvements can be made. However, it is unwise to make minor variations on the reported steps unless one is very unhappy with the overall yield, since the authors probably have already spent some time making adjustments and reported what they considered the optimum procedure.

Acknowledgments This research was supported by Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the University of Waterloo Start-Up Fund to SDXC.

References

1. Cocking EC (1960) A method for the isolation of plant protoplasts and vacuoles. Nature 187:962–963
2. Sheen J (2001) Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. Plant Physiol 127:1466–1475
3. Miao Y, Jiang L (2007) Transient expression of fluorescent fusion proteins in protoplasts of suspension cultured cells. Nat Protoc 2:2348–2353
4. Lung SC, Yanagisawa M, Chuong SDX (2011) Protoplast isolation and transient gene expression in the single-cell C_4 species, *Bienertia sinuspersici*. Plant Cell Rep 30:473–484

5. Gallie DR, Lucas WJ, Walbot V (1989) Visualizing mRNA expression in plant protoplasts: factors influencing efficient mRNA uptake and translation. Plant Cell 1:301–311
6. Cormeau J, Barthou H, Jauneau A, Petitprez M et al (2002) Cellular import of synthetic peptide using a cell-permeable sequence in plant protoplasts. Plant Physiol Biochem 40:1081–1086
7. Hong SY, Seo PJ, Cho SH, Park CM (2012) Preparation of leaf mesophyll protoplasts for transient gene expression in *Brachypodium distachyon*. J Plant Biol 55:390–397
8. Bergman P, Glimelius K (1993) Electroporation of rapeseed protoplasts: transient and stable transformation. Physiol Plant 88:604–611
9. Garcia R, Pimentel E, Somonte D, Mena J et al (1998) Isolation and transient transformation of stem and leaf protoplasts from sweet potato (*Ipomoea batatas* L). Asia Pac J Mol Biol 6:145–151
10. Mazarei M, Al-Ahmad H, Rudis MR, Stewart CN (2008) Protoplast isolation and transient gene expression in switchgrass, *Panicum virgatum* L. Biotech J 3:354–359
11. Guo J, Morrell-Falvey JL, Labbe JL, Muchero W et al (2012) Highly efficient isolation of *Populus* mesophyll protoplasts and its application in transient expression assays. PloS ONE 7:e44908
12. Pitzschke A, Persak H (2012) Poinsettia protoplasts: a simple, robust and efficient system for transient gene expression studies. Plant Methods 8:14
13. Chen S, Tao L, Zeng L, Vega-Sanchez M et al (2006) A highly efficient transient protoplast system for analyzing defence gene expression and protein-protein interactions in rice. Mol Plant Pathol 7:417–427
14. Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2:1565–1572
15. Eeckhaut T, Lakshmanan PS, Deryckere D, Bockstaele EV, Huylenbroeck JV (2013) Progress in plant protoplast research. Planta 238:991–1003
16. Faraco M, Di Sansebastiano GP, Spelt K, Koes RE, Quattrochio FM (2011) One protoplast is not the other. Plant Physiol 156 474–478
17. Galston AW, Altman A, Kaur-Sawhney R (1978) Polyamines, ribonuclease and the improvement of oat leaf protoplasts. Plant Sci Lett 11:69–79
18. Walter M, Chaban C, Schutze K, Batistic O et al. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J 40:428–438
19. Durieu P, Ochatt SJ (2000) Efficient intergeneric fusion of pea (*Pisum sativum* L.) and grass pea (*Lathyrus sativus* L.) protoplast. J Exp Bot 5:1237–1242
20. Henn HJ, Wingender R, Schnabl H (1998) Regeneration of fertile interspecific hybrids from protoplast fusions between *Helianthus annuus* L and *Helianthus* species. Plant Cell Rep 18:220–234
21. Hu Q, Andersen SB, Dixelius C, Hansen LN (2002) Production of fertile intergeneric somatic hybrids between *Brassica napus* and *Sinapis arvensis* for the enrichment of the rapeseed gene pool. Plant Cell Rep 21:147–152
22. Kisaka H, Kisaka M, Kanno A, Kameya T (1998) Intergeneric somatic hybridization of rice (*Oryza sativa* L) and barley (*Hordeum vulgare* L) by protoplast fusion. Plant Cell Rep 17:362–367
23. Orczyk W, Przetakiewicz J, Nadolska-Orczyk A (2003) Somatic hybrids of *Solanum tuberosum*: application to genetics and breeding. Plant Cell Tissue Org Cult 74:1–13
24. Sutiojono E, Nonhebel HM, Kantharajah AS (1998) Factors affecting protoplast culture of *Cucumis melo* "Green Delica". Ann Bot 81:775–777
25. Crowder AJ, Landgren CR, Rockwood LL (1979) Cultivar differences in starch content and protoplast yields from root cortical explants of *Pisum sativum*. Physiol Plant 46:85–88
26. Chang MM, Loescher WH (1991) Effects of preconditioning and isolation conditions on potato (*Solanum tuberosum* L. cv. Russet Burbank) protoplast yield for shoot regeneration and electroporation. Plant Sci 73:103–109
27. Morris P, Thain JF (1980) Comparative studies of leaf tissue and isolated mesophyll protoplasts. J Exp Bot 31:83–95

28. Rose RJ (1980) Factors that influence the yield, stability in culture and cell wall regeneration of spinach mesophyll protoplasts. Aust J Plant Physiol 7:713–725
29. Davey MR, Anthony P, Power JB, Lowe KC (2005) Plant protoplasts: status and biotechnological perspectives. Biotechnol Adv 23:131–171
30. Hasegawa H, Sato M, Suzuki M (2002) Efficient plant regeneration from protoplasts isolated from long-term, shoot primordial-derived calluses of garlic (*Allium sativum*). J Plant Physiol 159:445–449
31. Dovzhenko A, Dal Bosco C, Meurer J, Koop HU (2003) Efficient regeneration from cotyledon protoplasts in *Arabidopsis thaliana*. Protoplasma 222:107–111
32. Pan ZG, Liu CZ, Murch SJ, El-Demerdash M, Saxena PK (2003) Plant regeneration from mesophyll protoplasts of the Egyptian medicinal plants *Artemisia judaica* L. and *Echinops spinosissimus* Turra. Plant Sci 165:681–687
33. Hooley R (1982) Protoplasts isolated from aleurone layers of wild oat (*Avena fatua* L.) exhibit the classic response to gibberellic acid. Planta 154:29–40
34. Dovzhenko A, Koop HU (2003) Sugarbeet (*Beta vulgaris* L.): shoot regeneration from callus and callus protoplasts. Planta 217:374–381
35. Schmidt, R., Poole, R.J. (1980) Isolation of protoplasts and vacuoles from storage tissue of red beet. Plant Physiol 66:25–28
36. Barsby TL, Yarrow SA, Shepard JF (1988) A rapid and efficient alternative procedure for regeneration of plants from hypocotyl protoplasts of *Brassica napus*. Plant Cell Rep 5:101–103
37. Rybczyfiski JJ (1989) Plant regeneration and protoplast culture of *Browollia speciosa*. Plant Cell Rep 8:383–386
38. Coutts RHA, Wood KR. (1975) The isolation and culture of cucumber mesophyll protoplasts. Plant Sci Lett 4:189–193
39. Pan ZG, Liu CZ, Sobayed SMA, Saxena PK (2004) Plant regeneration from mesophyll protoplasts of *Echinacea purpurea*. Plant Cell Tissue Org Cult 77:251–255.
40. Firoozabady E, DeBoer DL (1986) Isolation, culture, and cell division in cotyledon protoplasts of cotton (*Gossypium hirsutum* and *G. barbadense*). Plant Cell Rep 5:127–131
41. Bohorova NE, Cocking EC, Power JB (1986) Isolation, culture and callus regeneration of protoplasts of wild and cultivated *Helianthus* species. Plant Cell Rep 5:256–258
42. Sarhan F, Cesar D (1988) High yield isolation of mesophyll protoplasts from wheat, barley and rye. Physiol Plant 72:337–342
43. Tudses N, Premjet S, Premjet D (2014) Optimal conditions for high-yield protoplast isolations of *Jatropha curcas* L and *Ricinus communis* L. Am-Eurasian J Agric Environ Sci 14:221–230
44. Engler DE, Grogan RG (1982) Isolation, culture and regeneration of lettuce leaf mesophyll protoplasts. Plant Sci Lett 2B:228–229
45. Sinha A, Wetten AC, Caligari PDS (2003) Optimization of protoplast production in white lupin. Biol Plant 47:21–25
46. Anthony P, Davey MR, Power JB, Lowe KC (1995) An improved protocol for the culture of cassava leaf protoplasts. Plant Cell Tissue Org Cult 42:299–302
47. Nagata T, Takebe I (1971) Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12–20
48. Edwards E, Lilley RM, Craig S, Hatch MD (1979) Isolation of intact and functional chloroplasts from mesophyll and bundle sheath protoplasts of the C_4 plant *Panicum miliaceum* Plant Physiol 63:821–827
49. Crepy L, Barros LM, Valente VRN (1986) Callus production from leaf protoplast of various cultivars of bean (*Phaseolus vulgaris* L.). Plant Cell Rep 5:124–126
50. Russell JA, Mccown BH (1986) Techniques for enhanced release of leaf protoplasts in populus. Plant Cell Rep 5:284–287
51. Wu SC, Kuniyuki AH (1985) Isolation and culture of almond protoplasts. Plant Sci 41:55–60
52. Gregory DW, Cocking EC (1965) The large-scale isolation of protoplasts from immature tomato fruit. J Cell Biol 24:143–146
53. Shepard JF, Totten RE (1977) Mesophyll cell protoplasts of potato. Plant Physiol 60:313–316

54. Binding H, Nehls R, Schieder O, Sopory SK, Wenzel G (1978) Regeneration of mesophyll protoplasts isolated from dihaploid clones of *Solanum tuberosum*. Physiol Plant 43:52–54
55. Sairam RV, Seetharama N, Devi PS, Verma Al et al (1999). Culture and regeneration of mesophyll-derived protoplasts of sorghum (*Sorghum bicolour* L. Moench). Plant Cell Rep 18:972–977
56. Nishimuram, Hara-Nishimura I, Robinson SP (1984) Isolation of metabolically competent protoplasts from grapevine leave. Plant Sci Lett 37:171–175