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Protocol

Isolation and Whole-Cell Patch Clamping of *Arabidopsis* Guard Cell Protoplasts

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INTRODUCTION

The flux of ions across membranes via ion channels is vital to cellular responses to internal and external stimuli, and therefore to cellular survival in changing circumstances. Patch clamping is a powerful technique for ion channel investigation, because it enables measurement of both net ion fluxes across the entire surface area of a cell and ion currents flowing through a single open channel. However, unlike animal cells, plant cells are surrounded by cell walls that prevent the physical contact between the patch pipette and the plasma membrane necessary for the patch clamp technique. To demonstrate how patch clamping can be applied to plant physiology research, we describe a protocol used to record potassium ion (K^+) channel currents in *Arabidopsis* guard cell protoplasts (a widely studied model cell type in plant biology). The protocol requires a two-step cellulase and pectinase digestion to isolate high quality *Arabidopsis* guard cell protoplasts (i.e., plant cells lacking their cell walls), preparation of suitable glass capillary microelectrodes, and formation of the whole-cell configuration with a gigohm ($G\Omega$) seal. We also describe the history of the protocol and list other types of plant cells from which successful patch clamp recordings have been obtained.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with **<!**, and recipes for reagents marked with **<R>**.

Reagents

<R>Basic solution
<R>Bath solution for K^+ current recordings
<R>Enzyme solution 1
<R>Enzyme solution 2
Ethanol (75% and 95%)
<R>MS medium (0.5X)
<R>Pipette solution for K^+ current recordings
Seeds (*Arabidopsis*)
Soil mix (Potting Mix, Miracle-Gro Inc.)

Equipment

Agar bridge (3 M KCl)
Balance (analytical, 0.01-mg; e.g., Mettler)
Blender (e.g., Waring)

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Burner (alcohol)
Capillary tubes (glass, 1.5-mm outer diameter [o.d.], 10-cm length; e.g., Beijing ZhengTianYi Science and Technology Trade Co.)
Cell chamber
Centrifuge (clinical; e.g., International Equipment Co.)
Dish (covered, with clay strip for storing pipettes)
Electrodes (Ag/AgCl wire)
Filter paper (autoclaved)
Filters (0.45-μm; Corning)
Forceps (sharp-tip)
Glass capillary pipette puller (two-step; e.g., Narishige)
Growth chamber
Hood (laminar flow)
Ice
Manipulator (three-axis coarse and fine micromanipulator; e.g., Narishige)
Mesh (nylon, 30- and 100-μm pore size, 15 cm × 15 cm; Spectra/Mesh)
Microelectrode amplifier and data acquisition system (e.g., Axon 200B with CV 201AU headstage, DigiData 1200 interface and computer with pCLAMP software installed)
Microforge (e.g., Narishige)
Micropipettors (10- and 200-μL, and 1-mL)
Microscope (bright-field; e.g., Nikon)
Microscope (inverted; e.g., Zeiss)
Osmometer (e.g., Wescor)
Pipette (1-mL)
Pots for growing *Arabidopsis*
Spatula
Squirt bottle
Syringe (plastic, disposable, 1-mL)
Tubes (centrifuge, plastic, 15- or 50-mL)
Water baths (shaking; e.g., American Scientific Products), preset to 22°C and 28°C

METHOD

Plant Growth

1. Sterilize *Arabidopsis* seeds by rinsing with 75% ethanol for 5 min, followed by a 1-min rinse in 95% ethanol.
2. Dry the seeds on autoclaved filter paper in a laminar flow hood.
3. Plate the sterilized seeds on 0.5X MS medium. Stratify in darkness for 2 d at 4°C.
4. Transfer the plates to a growth chamber. Grow for 7-10 d using an 8/16 h light/dark cycle (130 μmol/m² sec light intensity), 21°C ± 1°C light temperature/18°C ± 1°C dark temperature and 70%-80% humidity.
5. After 7-10 d, transplant the seedlings into pots filled with soil mix. Continue to grow under the conditions indicated in Step 4.

Guard Cell Protoplast Isolation

6. Harvest 5-10 fully expanded young leaves from 4-wk old *Arabidopsis* plants (Fig. 1).
7. Using sharp-tip forceps, peel the abaxial epidermis from the leaves.
This decreases mesophyll contamination.
8. Place the epidermal peels in a blender with 500 mL of cool distilled water. Blend for 30 sec.

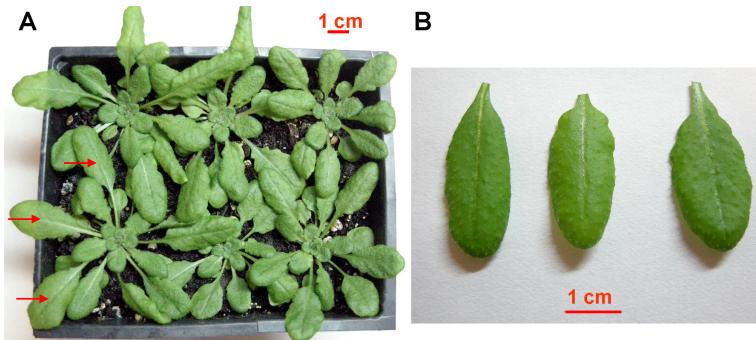


FIGURE 1. *Arabidopsis* plants and leaves selected for guard cell protoplast isolation. (A) Four-week old plants suitable for guard cell protoplast isolation. Arrows point to good leaves for guard cell protoplast isolation and patch clamping. (B) Leaves selected for guard cell protoplast isolation.

9. Filter the mixture through a 100- μm nylon mesh. Using a spatula, transfer the peels from the mesh into 10 mL of enzyme solution 1.
Peels with guard cells after blending are shown in Fig. 2A.
10. Digest the peels in a shaking water bath at 120 rpm for 30 min at 28°C.
Peels at this stage are shown in Fig. 2B).
11. Add 10 mL of basic solution. Shake for an additional 5 min.
This step reduces the osmolality difference between enzyme solutions 1 and 2.
12. Collect the partially digested peels by filtering the solution through a 100- μm nylon mesh. Using a squirt bottle, wash the peels with basic solution. Transfer the peels to 10 mL of enzyme solution 2.
13. Digest the peels by shaking at 60 rpm for at least 30 min at 22°C.

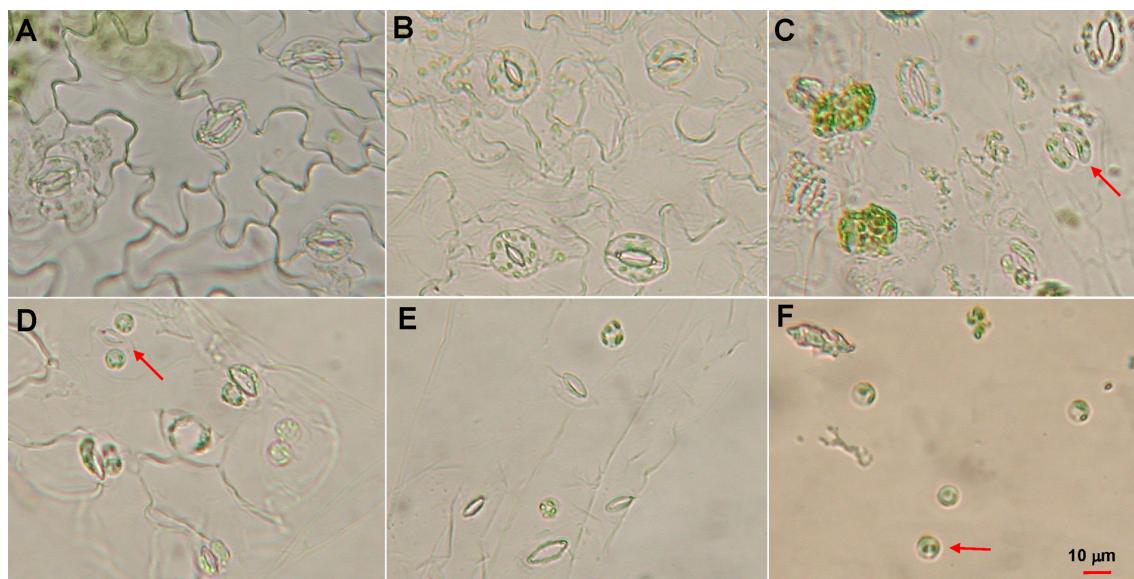


FIGURE 2. Guard cells and epidermal peels at different stages of enzymatic digestion. (A) A peel before digestion (immediately after blending). (B) A peel at the end of digestion with enzyme solution 1. (C) An underdigested peel during enzyme solution 2 digestion. The arrow indicates guard cells in the process of becoming spherical. (D) A perfectly digested peel. The arrow shows good, round protoplasts ideal for patch clamping. (E) A peel overdigested in enzyme solution 2. Most of the guard cells are detached from the peel. (F) Guard cell protoplasts in bath solution for patch clamping. The arrow indicates the best protoplast for patch clamping.

- i. After 30 min, remove an aliquot of peels at 5-min intervals.
 - ii. Using a microscope (100X and 400X total magnification), evaluate the degree of digestion.
Peels at different digestion stages are shown in Fig. 2C-E).
 - iii. Stop the digestion when 10%-20% of the guard cells are spherical (Fig. 2D).
14. Mix the peels by pipetting up and down with a 1-mL pipette.
This releases the protoplasts from the peels.
15. Filter the solution containing the protoplasts through a 30- μm nylon mesh to remove debris. Using a squirt bottle, wash the mesh with ~5 mL of basic solution. Collect both the filtrate and the wash solution into a centrifuge tube.
16. Centrifuge the solution containing the protoplasts at 800 rpm for 5 min to pellet the protoplasts. Decant the supernatant.
17. Gently resuspend the pellet in 15 mL of basic solution. Repeat Step 16.
18. Gently resuspend the pellet in 200 μL of basic solution. Place on ice in the dark for at least 1 h prior to patch clamping.
See Troubleshooting.

Glass Microelectrode Preparation

Patch clamping should always be performed with pipettes prepared on the same day.

19. Pull each glass capillary tube into two pipettes using a two-step puller. Set the temperature for each step properly to make sure the tip of the pipette is sharp and thin, with a tip opening of <1 μm .
20. Smooth the sharp tip of the pipette by polishing the tip with a microforge. Stop heating the tip when the tip opening becomes very small.
21. Store the polished pipettes mounted on a clay strip in a covered dish to avoid contamination and clogging of the pipette tip.
The seal resistance of the pipette should be 40-60 $M\Omega$ using the described patch clamping solutions.

Patch Clamping

22. Fill the cell chamber with bath solution. Add 20 μL of the protoplast solution (from Step 18). Wait 5 min to allow the protoplasts to settle to the bottom of the chamber (Fig. 2F).
23. Place the tip of the ground electrode into the bath solution via a 3 M KCl agar bridge.
24. Turn on the amplifier and computer. Load pCLAMP software (Clampex) for whole cell data acquisition. Load or edit the proper parameters to set the voltage family to evaluate guard cell K^+ channels:
 - i. A typical voltage family for inward and outward K^+ channel current recordings consists of negative and positive voltages (e.g., -200 mV to +80 mV with 20-mV increments).
See the waveform example in Fig. 3.
 - ii. Set the duration to no less than 4 sec for each voltage step to make sure the currents reach steady state, with a holding potential of -60 mV and an interpulse interval of 6 sec.
25. Heat the tip of the plastic syringe using an alcohol burner. Pull the syringe into a very thin tube with a diameter <0.5 mm.
26. Using the syringe, fill a glass pipette with pipette solution. Remove air bubbles by flicking the pipette.
Air bubbles can cause an open circuit, preventing recording of the current.
27. Tighten the pipette into the holder, making sure that only 1-2 mm of the Ag/AgCl electrode wire is submerged in the pipette solution.

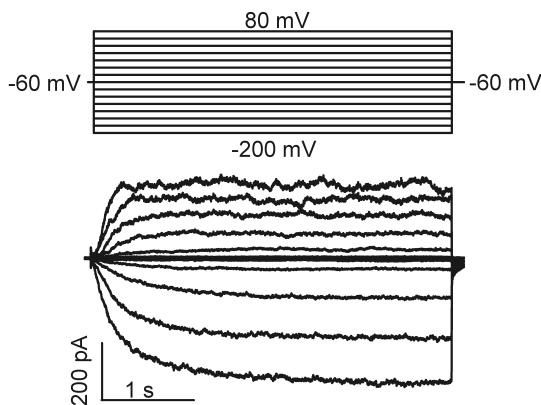


FIGURE 3. A typical whole-cell K^+ current recording from an *Arabidopsis* guard cell protoplast. The seal resistance of the recording is $2\text{ G}\Omega$. The voltage protocol is shown above the current traces; voltages were applied from -200 mV to $+80\text{ mV}$, with 20-mV increments. Scale bars for current (200 pA) and time (1 s) are also indicated.

28. Using the inverted microscope, select a round, bright, healthy protoplast with a clear membrane (as indicated by the arrow in Fig. 2F). Move it to the center of the field of view.
29. Use the coarse manipulator to move the glass pipette so that its tip is centered over the protoplast and above the surface of the bath solution.
30. Reduce the light intensity of the microscope so that it is easier to find the shadow of the pipette tip. While observing using a $10\times$ objective, use the coarse manipulator to move the pipette down until the tip is above and very close to the selected protoplast.
To avoid clogging the pipette tip, maintain positive pressure by blowing into the tube that is connected to the electrode holder.
31. While maintaining positive pressure, change to the $40\times$ objective. Use the fine micromanipulator to move the pipette so that it gently touches the protoplast, very slightly deforming it.
32. Apply gentle suction to the tube so that the tip of the glass pipette seals onto the protoplast.
See Troubleshooting.
33. Continue to apply suction. Set the pipette offset to zero. Monitor the increase in the seal resistance on the computer. Gradually apply a more negative holding potential (-10 mV for each $100\text{ M}\Omega$ increase in seal resistance) to -60 mV .
See Troubleshooting.
34. When a $\text{G}\Omega$ seal resistance is reached, give a stronger pulse of suction and/or a voltage zap from the patch clamp amplifier.
This breaks the membrane and forms the whole-cell configuration.
See Troubleshooting.
35. If the seal resistance remains $1\text{-}2\text{ G}\Omega$ or higher, apply test voltages to the cell. View whole-cell K^+ currents and record for further analysis (Fig. 3).
Seal resistances above $5\text{ G}\Omega$ yield optimal whole-cell recordings, but recordings with seal resistances between 1 and $5\text{ G}\Omega$ can also be used if leak (instantaneous) currents are not too large. For recording of single channel currents from membrane patches, seal resistances of at least $10\text{ G}\Omega$ are required in order to achieve a suitable signal-to-noise ratio.

TROUBLESHOOTING

Problem: No protoplasts are found after digestion.

[Step 18]

Solution: Consider the following:

1. Increase the digestion time of both Steps 10 and 13 to 40 min or longer.
2. If round guard cell protoplasts were observed before centrifugation, make sure the protoplast pellet was not accidentally poured off with the supernatant after the first centrifugation.

Problem: Protoplasts are dramatically swollen and break very easily when suction is applied.

[Step 32]

Solution: Consider the following:

1. Reduce the digestion time of both Steps 10 and 13. Check the peels after only 20 min of digestion (or earlier; see Step 13) to make sure that the protoplasts are not overdigested (see Fig. 2E).
2. Check the osmolality of both the basic solution and bath solution to make sure they are not hypo-osmotic relative to the guard cells.

Problem: The pipette resistance is extremely high when the pipette is dipped into the bath solution.

[Step 33]

Solution: Consider the following:

1. Make sure the pipette opening was not sealed accidentally during the polishing process.
2. Ensure that there are no air bubbles in the pipette and that the pipette solution is touching the Ag/AgCl wire.
3. Check to make sure there is no disconnect between the ground electrode and the bath solution, which would result in an open circuit.

Problem: Protoplasts are very tough during application of suction and it is very difficult to achieve a $\text{G}\Omega$ seal resistance.

[Step 34]

Solution: Consider the following:

1. Underdigested protoplasts are very tough (e.g., Fig. 2C).
2. A hyperosmotic bath solution will also make it more difficult to form a high resistance seal.
3. If the opening of the pipette is very small, a larger suction force will be needed to form the seal.

DISCUSSION

This method of patch clamping *Arabidopsis* guard cell protoplasts was adapted from one used for isolation and patch clamping of *Vicia faba* guard cell protoplasts (Schroeder et al. 1984; Assmann et al. 1985; Kruse et al. 1989); this method has been applied successfully to *Vicia* guard cell protoplasts to measure K^+ , Ca^{2+} , and anion currents at both whole-cell and single-channel levels of resolution (Wu and Assmann 1994, 1995; Li et al. 2000; Zhang et al. 2007). Pei et al. (1997) describe an alternative method to isolate *Arabidopsis* guard cell protoplasts suitable for patch clamping. The two-step method described here is more time-efficient than the method of Pei et al. (1997) (a 2-h digestion vs. overnight), yet yields sufficient healthy *Arabidopsis* guard cell protoplasts for recording of K^+ and anion currents (Wang et al. 2001; Coursol et al. 2003). By increasing the volume of enzyme solutions and the duration of the second digestion, this method can also produce large quantities of guard cell protoplasts for transcriptome and biochemical analyses (Pandey et al. 2002). Another two-step protocol for large-scale isolation of *Arabidopsis* guard cell protoplasts has also been described (Leonhardt et al. 2004).

The patch clamp technique has been applied to many other cell types from an array of plant species (Table 1) (Assmann and Romano 1999). It is important to note that different cell types require modifications to the protocol discussed here. Table 1 cites literature which can be consulted for more information on protoplast isolation from and patch clamping of other cell types. For example, since *Vicia* guard cells are much larger than *Arabidopsis* guard cells, the opening of the glass pipette should be larger, with a pipette resistance of 25–35 $\text{M}\Omega$ in the solutions listed, and weaker suction should be applied as the seal resistance increases. The cell membranes of guard cell protoplasts are more tenacious than those of other tissues, which facilitates the formation of a tight seal. Although voltage zaps can be applied to a guard cell protoplast in order to break the membrane and form the whole-cell configuration, these high voltage pulses can destroy other types of protoplasts such as those from pollen (Wang et al. 2004); for such cell types, only suction pulses are used to form the whole cell configuration.

Table 1. Protoplast isolation and patch clamping of plant cells from various species/tissues

Species	Tissue or cell type	References
<i>Arabidopsis</i>	Guard cells	Wang et al. (2001)
	Hypocotyl cells	Cho and Spalding (1996)
	Mesophyll cells	Romano et al. (1998)
	Pollen	Wang et al. (2004)
	Root hairs	Ivashikina et al. (2001)
	Pollen	Fan et al. (1999)
<i>Brassica chinensis</i>	Laticiferous vessel cells	Bouteau et al. (1993)
<i>Hevea brasiliensis</i>	Aleurone storage protein vacuoles	Jacobsen et al. (1985); Bethke and Jones (1994)
<i>Lilium longiflorum</i>	Xylem parenchyma cells	Wegner and Raschke (1994)
<i>Mimosa pudica</i>	Pollen and pollen tubes	Griessner and Obermeyer (2003)
<i>Nicotiana tabacum</i>	Pulvinar motor cells	Stoeckel and Takeda (1993)
<i>Pisum sativum</i>	Suspension culture cells	Falke et al. (1988)
<i>Triticum</i>	Mesophyll cells	Elzenga and Van Volkenburgh (1997)
	Stem and leaf epidermal cells	Elzenga et al. (1991)
	Root cells	Schachtman et al. (1991)
	Root hair cells	Gassmann and Schroeder (1994)
<i>Vicia faba</i>	Guard cells	Wu and Assmann (1994)
	Mesophyll cells	Li and Assmann (1993)
<i>Zea mays</i>	Guard cells	Fairley-Grenot and Assmann (1992)
	Root cap cells	Carroll et al. (1998)
	Root cortex cells	Roberts and Tester (1995)

(Modified from Assmann and Romano [1999].)

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