Video Article

Measuring the Osmotic Water Permeability Coefficient (P_f) of Spherical Cells: Isolated Plant Protoplasts as an Example

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Abstract

Studying AQP regulation mechanisms is crucial for the understanding of water relations at both the cellular and the whole plant levels. Presented here is a simple and very efficient method for the determination of the osmotic water permeability coefficient (P_f) in plant protoplasts, applicable in principle also to other spherical cells such as frog oocytes. The first step of the assay is the isolation of protoplasts from the plant tissue of interest by enzymatic digestion into a chamber with an appropriate isotonic solution. The second step consists of an osmotic challenge assay: protoplasts immobilized on the bottom of the chamber are submitted to a constant perfusion starting with an isotonic solution and followed by a hypotonic solution. The cell swelling is video recorded. In the third step, the images are processed offline to yield volume changes, and the time course of the volume changes is correlated with the time course of the change in osmolarity of the chamber perfusion medium, using a curve fitting procedure written in Matlab (the 'PfFit'), to yield P_f.

Video Link

The video component of this article can be found at https://www.jove.com/video/51652/

Introduction

Water uptake and flow across cellular membranes is a fundamental requirement for plant existence at both the cellular and the whole plant levels. At the cellular level, aquaporins (AQPs) play a key role in the regulation of the osmotic water permeability coefficient (P_f) of the cell membrane[1-3].

To date, several methods have been employed in measuring the endogenous P_f of protoplast from different plant organs (*i.e.* roots, mesophyll, endodermis, *etc*., reviewed by Chaumont *et al.* [4]). One of the approaches to measure P_f is to expose the protoplasts to an osmotic challenge and to monitor the initial rate of its volume change (*i.e*., the slope of the early linear phase of the volume change). Two different methods were previously described based on this approach, both based on an instantaneous exchange of solutions. The first one consists of immobilizing the protoplast with a suction micropipette and switching the solution flow[5] and the second one of transferring the protoplast from one solution to another using a micropipette[6]. These micropipette suction and micropipette transferring methods, which allow image acquisition at the very start of the fast solution exchange (to capture the early linear phase of volume change), likely involve a physical stress to protoplasts and require specialized equipment and expert micromanipulation.

The method described here minimizes the disturbance to the cells, involves no micromanipulation and permits derivation of P_f when the bath perfusion is *not* instantaneous.

After the enzymatic digestion, the protoplasts, submerged in an isotonic solution, are immobilized on the coverslip-glass bottom of a Plexiglass (aka Lucite or perspex) chamber by charge interaction. Then, during a constant bath perfusion, the isotonic solution is flushed away by a hypotonic solution generating a hypoosmotic challenge to the protoplasts. The swelling of the protoplast is video recorded and then, by combining the information about the time course of the bath perfusion and the time course of the cell swelling, the P_f is determined by image processing and curve fitting procedures.

The advantages of this method are that the experiment is very efficient, *i.e.* it is possible to monitor a few cells simultaneously in a single assay, and that it does not require special equipment or particular micromanipulation skills. Several applications for this method are possible. For example, determination of the native P_f of a variety of cells from different tissues and plants, such as mesophyll and bundle sheath cells from Arabidopsis leaf[7], maize leaf mesophyll or root cortex cells[8-10] or suspension cultured cells[11,12]. In addition, it is possible to determine P_f of spherical animal cells such as oocyte cells[11]. Another example involves examination of AQP activity by transient expression of their gene in the protoplasts (or any other genes which may affect them; *e.g.*, genes of kinases) and determination of their contribution to P_f; for example,

expression of tomato AQP SlTIP2;2 in Arabidopsis mesophyll protoplasts by PEG transformation and determination the SlTIP2;2-related P_f[13]. Finally, examination of the effect on P_f of different molecules/substances (drugs, hormones, etc.) added to the solutions can also be examined, for example of the AQP blocker $HgCl_2$[7].

The following protocol describes the isolation of protoplasts of Arabidopsis mesophyll cells and determination of their P_f.

Protocol

1. Preparation of Solutions

1. Prepare isotonic (600 mOsm) and hypotonic (500 mOsm) solutions containing 10 mM KCl, 1 mM $CaCl_2$, and 8 M 2-(N-morpholine)-ethanesulphonic acid (MES), pH 5.7 and adjust osmolarity with the appropriate amounts of D-sorbitol: 540 mM for the isotonic and 440 mM for the hypotonic solution. Verify the osmolarity of the solution (within 3 % of the target value) using an osmometer.
2. Prepare a dry stock of 'enzymatic mix' containing the following enzymes: 0.55 g cellulase, 0.1 g pectolyase, 0.33 g polyvinylpyrrolidone K 30, 0.33 g BSA (see **Table 1** below), mix the dry powder by vortex, make 5.7 mg aliquots and store at -20 °C.

2. Isolation of Arabidopsis Mesophyll Protoplasts

1. Prepare a Petri dish (10 cm) with about 6 drops (approx. 30 µl each) of isotonic solution.
2. Peel the abaxial (lower) Arabidopsis leaf epidermis, cut the peeled leaf into squares of about 4 x 4 mm^2, then place the squares on the isotonic solution drops with the exposed abaxial side down, touching the solution.
3. Dissolve 5.7 mg of the enzyme mix in 165 µl isotonic solution (3.3 % w/w) in a 1.5 ml tube, mix gently by pipetting for a minute or so until dissolved, and place several similar drops of the enzymatic solution in the same Petri dish.
4. Transfer the leaf pieces onto the enzymatic solution drops, close the dish sealing the lid with one round of parafilm and incubate for 20 min, floating the dish in a water bath set to 28 °C.
5. Add several more drops of the *isotonic* solution to the dish (2 drops per each enzyme solution drop). Transfer each leaf piece to a new isotonic solution drop, then, sequentially, to a second drop (to wash the enzymatic solution away). Lift the piece by its edge using forceps, shake it in the second drop (like a tea bag) to release the protoplasts. Collect the drops with the protoplasts (using a clipped off 100 µl pipette tip) into a 1.5 ml tube.

3. The Hypotonic Challenge Assay: Arabidopsis Mesophyll Cell Swelling

1. Prepare the perfusion system (**Figure 1A**) by filling one column with the isotonic solution and another column with the hypotonic solution. Open the valve, let some solution flow (first the hypotonic, then the isotonic) to fill the tubing all the way down to the inlet manifold (**Figure 1B**). Ensure there are no trapped air bubbles, and then close the valve.
2. Seal a coverslip, using silicone grease (**Table 1**), to make a bottom for the chamber within the plexiglass slide (**Figure 1B**; see also the schematics of the chamber in **Figure 1C**). To make the chamber bottom (the upward facing exposed surface of the coverslip within the grease ring) "sticky" for protoplasts, coat it with positive-charge-bearing protamine sulphate (1 % in water; **Table 1**) or poly-L-Lysine (0.1 % in water; **Table 1**). Spread this 'glue' over the coverslip using a pipette tip, wait for 1 - 2 min, rinse 3 - 4 times with the isotonic solution and shake away the remaining solution.
3. Fill the chamber up with the isotonic solution. Then, add a drop of protoplasts containing solution to the chamber, using a clipped off pipette tip and wait 3 - 4 min for the protoplasts to settle. Cover the chamber with a transparent cover (**Figures 1D**, **1E**) touching the solution surface (avoid trapping air bubbles beneath).
4. Place the slide (gently!) on an inverted microscope table, connect it to the perfusion system and the pump (guarding against air bubbles in the tubing!) and turn on the isotonic solution flow for constant perfusion at 1 ml/min (faster rates can be used, up to 4 ml/min).
5. For recording volume changes, an inverted microscope is used, with a 20X objective and with a CCD video camera connected to a PC computer. Use the 'CMU 1394 Camera Driver' plugin of the ImageJ software (see the Table of Specific Materials for the download addresses of these two software pieces) to record a 60 sec video movie of selected immobile protoplasts (presumably, those stuck to the bottom) at a rate of 1 image /sec (1 Hz). Start the recording with a 15 sec wash of the isotonic solution (this constitutes the baseline), switch to the hypotonic solution for 45 sec (to complete a total 60 sec from the start of perfusion). Save the movie in TIF format. NOTE: Choose a view field with as many cells as possible, fulfilling the following criteria: spherical in shape and with a well-focused cell contour at their largest perimeter (**Figure 2A**).

4. Analysis of the Cell Volume Change Using ImageJ

NOTE: To analyze the series of images of a swelling cell, use the 'Image Explorer' and 'Protoplast Analyzer' plugins in the ImageJ software (written by Xavier Draye)[14]. Starting with the chosen protoplasts at their first time point, the 'Protoplast Analyzer' plugin will detect automatically the protoplasts edges (contours) and calculate the time course of their areas during the experiment (the plugins are available with the PfFit analysis program, below).

1. Start ImageJ. To open the movie, click 'File' on the ImageJ panel, then, consecutively on the dropdown menus as they unfold: 'Import' then 'Image Explorer'. Highlight the chosen movie, then right-click on it, then left-click on 'Protoplast Analyzer'. Browse through the movie (using a slider at the protoplast image bottom) to identify protoplasts that remain largely immobile during the experiment – these will be analyzed. Back on the first image, using the mouse, draw circles (picked from the ImageJ drawing tools) around the selected protoplasts (**Figure 2B**), then click 'OK' in the table of 'Detection parameters' that appeared.

2. To launch the protoplast detection algorithm, click 'Local' on the protoplast image top panel, then 'Process' in the dropdown menu. Examine the green circles around the selected protoplasts (**Figure 2C**) throughout the movie. Save the 'Result' in an Excel file. Quit ImageJ. NOTE: In case a red dot appears (to indicate a bad contour fit – usually due to a poor image contrast), rerun with different parameters.
3. To separate the lines belonging to each cell (which – if two or more cells were analyzed simultaneously – will be intertwined, because the analysis is done frame by frame), in Excel, sort the saved data by the cell number column ('object').
4. To determine the pixel-to-µm conversion factor for obtaining the real value of P_f, snap an image of a micrometer ruler via the same 20X microscope objective. Drag a line (picked from the ImageJ drawing tools) along the ruler image and read the pixel number equivalent to the ruler length at the bottom of the ImageJ main panel. Convert the arbitrary pixel area values in the Excel file into μm^2. Save the areas time course (for each cell separately) as a text file (two columns of numbers only). NOTE: This will be an input to the volume-fitting 'PfFit' program.

5. Modeling the Rate of Osmolarity Change in the Experimental Chamber Using ImageJ and the Matlab Program P_fFit

1. Add 2 mg xylene cyanol (**Table 1**, below) to 100 ml of the isotonic solution (to produce the 'Indicator Dye').
2. Prepare the perfusion system (as in 3.1) with the Indicator Dye and the non dyed hypotonic solution.
3. Seal a cover slip using silicon grease to the bottom of the Plexiglass chamber, then gently fill the chamber with the Indicator Dye, cover it with a cover slip (as with the protoplasts before) and place it on the microscope stage.
4. Connect the chamber to the perfusion system and the pump, and turn on the Indicator Dye flow for a constant perfusion at l ml/min.
5. Record a 60 sec movie at the rate of 1 Hz. Start the recording with 15 sec of Indicator Dye, switch to the hypotonic solution for 45 sec. Stop filming. Flush with the Indicator Dye (at least for 30 sec), then start a new movie. Repeat about 5 - 6 times and save all the movies
6. Use the ImageJ software to analyze the video images of the Indicator Dye transmittance to obtain an averaged time course of the changing transmittance.
    1. Start ImageJ, click 'File', then, 'Open', and browse for the movie. For each movie, draw a 10 pixel wide vertical rectangle anywhere on the 1st image of the movie. Click 'Image' on the ImageJ main panel, then click 'Crop' in the dropdown menu.
    2. To align the 60 frames (of the 60 sec movie) in one row, click again 'Image', then click consecutively in the dropdown menus as they unfold: 'Stacks' and 'Make Montage' (columns 60, rows 1). Draw a 1 pixel high horizontal rectangle anywhere along the whole row of images and click 'Analyze' in the ImageJ main panel, then click 'plot profile' in the dropdown menu. NOTE: A 'Plot of Montage' window will appear (not shown), and a list of transmittance data can be opened from its menu. Each image of the movie is represented in this list by 10 transmittance values originating in its 10 pixel wide rectangle and consequently the "time base" (the image sequential number) is 10 times longer.
    3. Copy the lists of the transmittance data (one list per movie) to an Excel file. Average the transmittance time courses obtained from the several movies of the Indicator Dye flushes. Generate a real time base by multiplying the image sequential number by 0.1. Save the averaged time course (two columns) to a text file. NOTE: Before averaging, if desired, plot the individual time courses, to reject any irregularities. Ensure that the movie includes at least 5 final sec of steady-state transmittance of the Indicator Dye.
7. Start the Matlab fitting program P_fFit (the 'Indicator Fit' panel, **Figure 3**) to compute the various parameters of the osmolarity time course. NOTE: based on the known initial and final concentrations of the solution in the bath, the time course of the changing osmotic concentration of the solution is calculated from the concentration time course (calculated, in turn, from the Indicator Dye transmittance), assuming it follows the same dynamics as the dye concentration. P_fFit is a program available for use free of charge. The ' PfFit_Installer_web.exe' can be downloaded from: P_fFit User Guide' with detailed explanations and definitions is accessible via Jove as a Supplemental file, which helps to familiarize the user with the P_fFit program.
8. In the 'Indicator Fit' panel, import the data of the mean time course of the Indicator Dye transmittance ('Indicator data file', **Figure 3A**) and insert manually the current experiment parameters and the initial guesses of the parameters 'width' and 't_half' describing the time course of the Indicator Dye concentration (**Figure 3B**. Click 'Run' to view the plots of the time courses of the Indicator Dye concentration (real data and fit, **Figure 4A**), and of the modeled (calculated) bath osmolarity (**Figure 4B**). NOTE: a good fit to the data is essential(a recommendation: start with the values shown in **Figure 3**).

6. Determining the P_f using the Matlab Fitting Program P_fFit

NOTE: In addition to the basic assumptions with regard to the behavior of a protoplast as a true and perfect osmometer[11], the determination of P_f rests on the presumption that P_f may change with time, that this dynamics of P_f underlies the time course of the cell volume change and that three parameters suffice to describe it: P_{fi} (the initial value of P_f), $Slope_{Pf}$ (the rate of the linear change of P_f) and Delay (the period from the start of the bath osmolarity change till the start of the cell volume change). Different models can be tested, including different combinations of these parameters and their values, including null values[11]. P_fFit searches for the best combination of these parameters to yield – by calculation – the most faithful reproduction of the experimental time course of the cell volume change[11], calculated, in turn, from the imported series of cell-contour areas (see also the Supplemental 'P_fFit User Guide').

1. Switch to the 'Volume Fit' panel (**Figure 5**). Choose for import the areas data file (the text file with the time course of the 'areas' of the analyzed protoplasts, **Figure 5A**). Choose 'Last Indicator Fitting' as the parameter source (**Figure 5B**; see the 'P_fFit User Guide' for alternatives). NOTE: These parameters (**Figure 5D**) are then used to regenerate the osmoticum change in the bath for the volumes fitting procedure.
2. In the 'Volume Fit' panel (**Figure 5C**), initialize (fill in the initial guesses for) the P_f parameters: P_f, $Slope_{Pf}$ and Delay (a recommendation: start with 1, 1, and 30, respectively), Chose the model 'Class' (a recommendation: start with II and mark 'checks' for all three parameters to be fitted). Click 'Run', then eyeball the interim figure (**Figure 5E**) and adjust the Delay parameter and the length of the record, if needed.
3. Examine the results graph (**Figure 6**) to evaluate the fit quality and record the fit error. Change the initializing parameters a few fold each, and re-'Run'. NOTE: Don't be discouraged when the program gets stuck – just restart the program!
4. Repeat this procedure several times, starting with different combinations of initialization parameters, aiming for the lowest value of the fit error.

5. Copy the list of the fit results directly from the screen, or find them in the PfFit-generated '_FIT_Vol_Results.txt' file.

Representative Results

In order to determine the P_f and compare the activity of different AQPs, mesophyll protoplasts from Arabidopsis leaf are used. These protoplasts were found to have low basal (background) P_f levels [7] and can serve as a functional-expression system to enable reproducible P_f measurements.

Protoplasts from a mature leaf from a 6 week old Arabidopsis plant were isolated and three gene constructs with AQP genes from Arabidopsis (*AtPIP2;1*) and maize (*ZmPIP1;*2 and *ZmPIP2;4*) were transiently (and separately) expressed using the PEG transformation method [15]. Assuming that the event of transformation is simultaneous for a large number of plasmids applied to the cell irrespective of their nature and based on the results which showed a 100% success rate for synchronized transient expression of two plasmids in one cell reported previously for other plant systems [15,16], they were co-transformed with a vector encoding the enhanced green fluorescent protein (eGFP) in order to label the transformed protoplasts (**Figure 7**).

For the P_f assays, protoplasts were set in the experimental chamber (**Figure 1B**) and the GFP labeled protoplasts were monitored by video while they were flushed initially with the isotonic solution (600 mOsm), then with the hypotonic solution (500 mOsm), using the perfusion system (**Figure 1A**).

The time courses of the cell volume changes (**Figure 8A)** were obtained for each cell in two stages: first, the 'Image Explorer' and 'Protoplast Analyzer' plugins were used to generate the time course of changes in the cell contour area (**Figure 2**), then, the Matlab fitting program P_fFit (**Figure 5**) was used to import these areas and convert them to cell volumes. The P_f values (**Figure 8C**) were derived for each cell using the P_fFit program (**Figure 5**), based on the time course of the cell volumes and, additionally, on the imported averaged time course of the transmittance changes of the Indicator Dye (**Figure 3**), converted to the time course of the Indicator Dye concentration change (**Figure 4A**) and then – to the time course of the bath osmolarity change (**Figures 4B**, **6A** and **8B**). It is worth noting, that delC, the difference in osmotic concentrations in the cell (Cin) and in the bath (Cout), *i.e.*, the driving force for the water influx, was due almost only to the change of Cout (**Figure 6A**). In this experiment, P_f increased during the assay (**Figure 6B**).

The P_f values of the protoplasts transformed with each of the three AQPs were significantly higher than the P_f of the control cell transformed with GFP alone (**Figure 8C**).

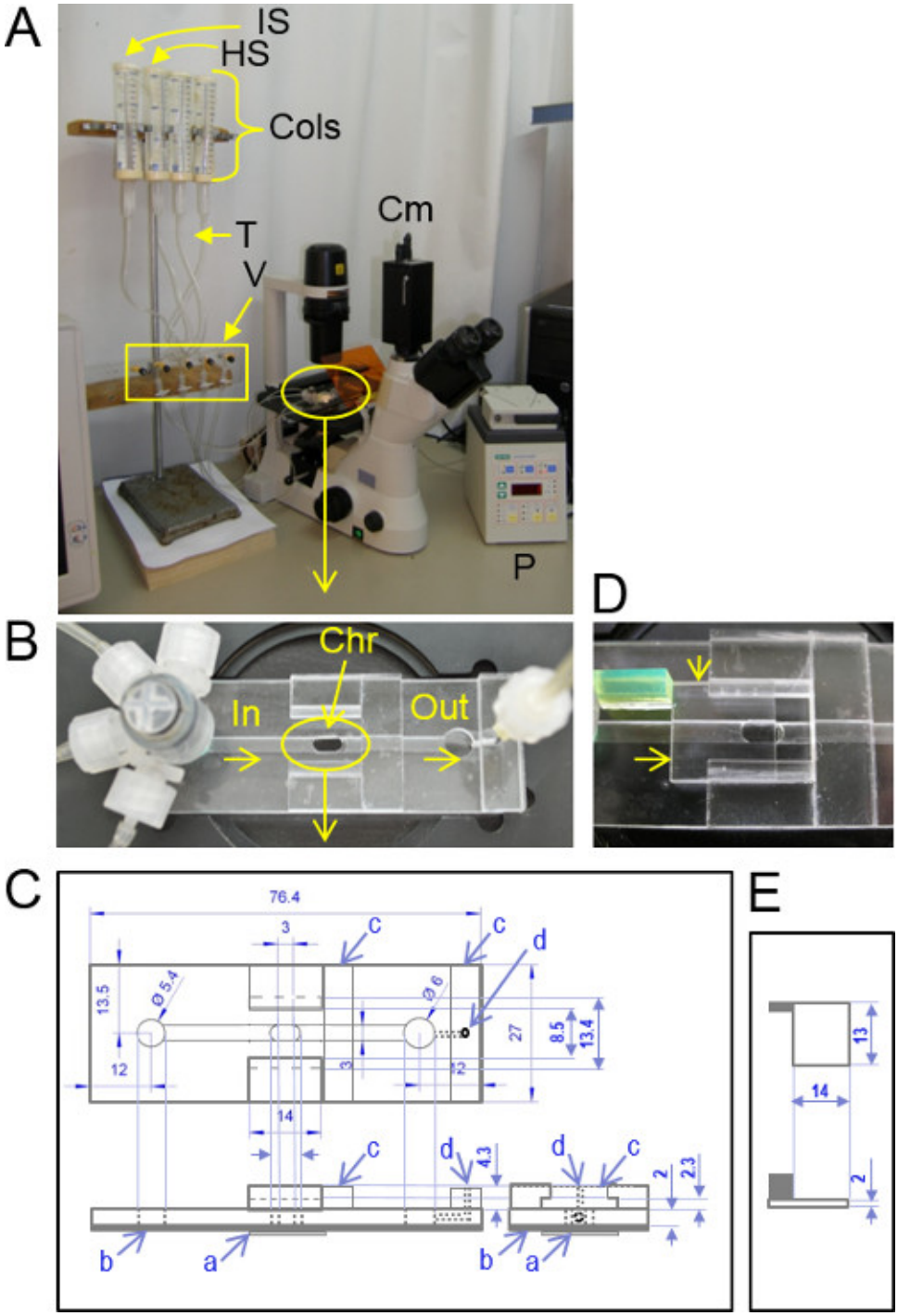

Figure 1: The volume-assay system. (A) The experimental setup: The perfusion system contains solution reservoirs (infusion columns, 'Cols'), tubing (T), valves (V) and a peristaltic pump (P) connected to the plexiglass slide set on the microscope table. HS = hypotonic solution, IS isotonic solution, Cm camera. **(B)** An enlarged view of the plexiglass slide with the experimental chamber (Chr) and the tubing attached via an inlet (In) manifold connector. The solution is sucked from the chamber via an outlet (Out) to the pump. **(C)** A schematic drawing of the plexiglass slide (counterclockwise: top view, long-side view and short-side view): a = glass cover slip, the central chamber bottom; b = clear adhesive tape (**Table 1**), serving as a bottom for the inlet and outlet solution grooves leading to and from the central chamber; when the Scotch tape is replaced (only occasionally), a hole is cut in it under the chamber; c = a plexiglass block glued to the slide; d = an outlet connector hole. Numbers are mm (but the drawing is not to scale). **(D)** An enlarged view of the center portion of the slide with the transparent cover (also plexiglass) partially covering the central chamber (arrows). **(E)** Schematic drawing (top and side views) of the transparent cover. The size of the transparent cover handle (green plastic in D) is arbitrary. Other details are as in C.

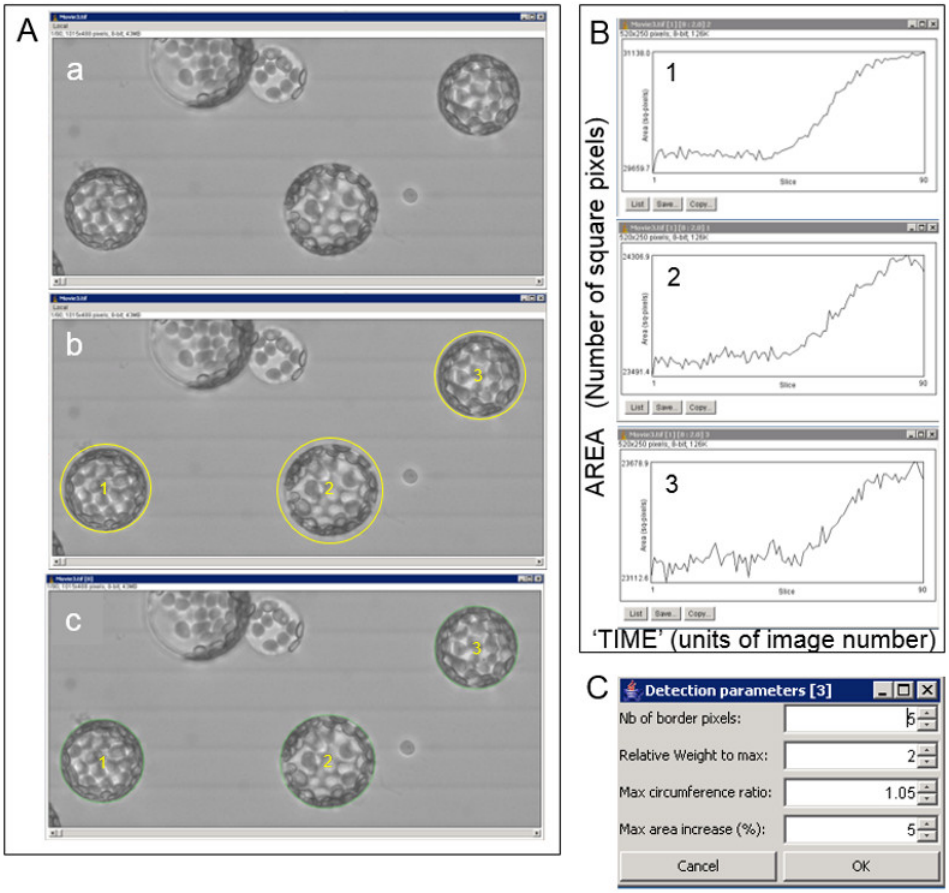

Figure 2: Analysis of swelling protoplasts images using the 'Protoplast Analyzer' plugin. (A) a, the first image of the movie with protoplasts, **b,** as in a, but yellow circles indicate the selection made after reviewing the movie, before the contours are autodetected, **c**, from the first till the last image the green circles tightly follow the contours of the "well-behaved" protoplasts undergoing analysis. **(B)** 'Time'-course plots (with units of image number on the abscissa) of the calculated areas within the protoplast contours ('Area', in square pixels), for each tracked (and numbered) protoplast. **(C)** The parameters input panel of the 'Protoplast analyzer' plugin. Four 'detection parameters' can be adjusted to fine tune the protoplast detection algorithm. The 'number of border pixels' parameter sets the minimum thickness of the protoplast contour (default value: 5). The 'relative weight' parameter influences the grey level threshold difference between the inner protoplast area and the outer border (default: 2). The 'maximum circumference ratio' defines a threshold for excluding protoplasts whenever their shape deviates from a circle. This parameter is the ratio of the protoplast circumference to the circumference of a perfect circle having the same area as the protoplast (default: 1.05). The 'maximum area increase' (% increase per time step) parameter excludes protoplasts with contour area increases above the parameter value (default value: 5%). Finally, the plugin also handles small protoplast movements but will stop tracking protoplasts that move rapidly or that disappear from the image area. The movie can be rerun as many times as necessary, and a single protoplast can be reanalyzed separately. Please click here to view a larger version of this figure.

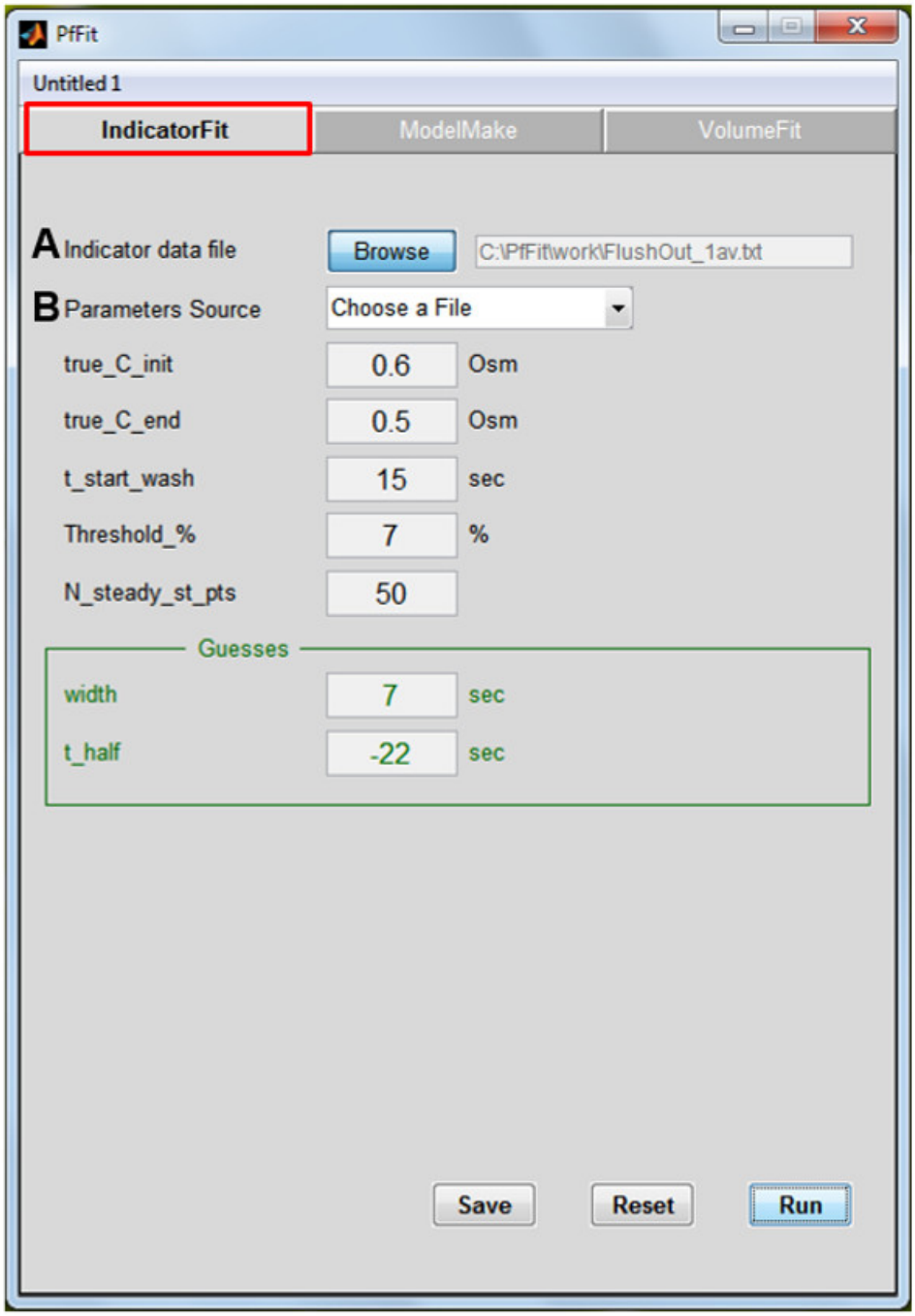

Figure 3: The 'Indicator Fit' panel of the P_fFit program. This part translates the indicator transmittance time course into bath osmolarity time course. **(A)** Browse for the saved data file containing the time course of transmittance changes of the Indicator Dye. **(B)** Either use the previously saved list of variables and parameters, or insert manually the 5 variable values of the current experiment: 'true_C_init' and 'true_C_end' (the osmolarities of the initial bath solution and the P_f-assay solution perfused via the bath), 't_start_wash' (the duration of baseline sampling at the initial Indicator Dye level), 'threshold_%' (% of baseline value, at which the program detects automatically the departure from baseline transmittance; 1 - 5% are usually the most effective), 'N_steady_st_pts' (the number of samples – with 10 samples representing every Indicator Dye image taken – to be averaged at the end steady state level of the Indicator Dye, crucial for the conversion of the Indicator Dye concentration to the osmoticum concentration) and initial guesses for two of the four parameters of the Indicator Dye transmittance sigmoidal time course, 'width' and t_{half} (roughly related to the duration of the transition part of the sigmoid, and to its midpoint, respectively; t_{half} may be negative!). Two best fit parameters, in addition to 'width' and t_{half} are obtained without the need for initial guesses: lag ('flush_lag'), the time between the valve opening to the arrival of the solution in the bath, and 'C_init', without a physical meaning, but necessary for the description of the osmolarity time course (see the Supplemental P_fFit User Guide.

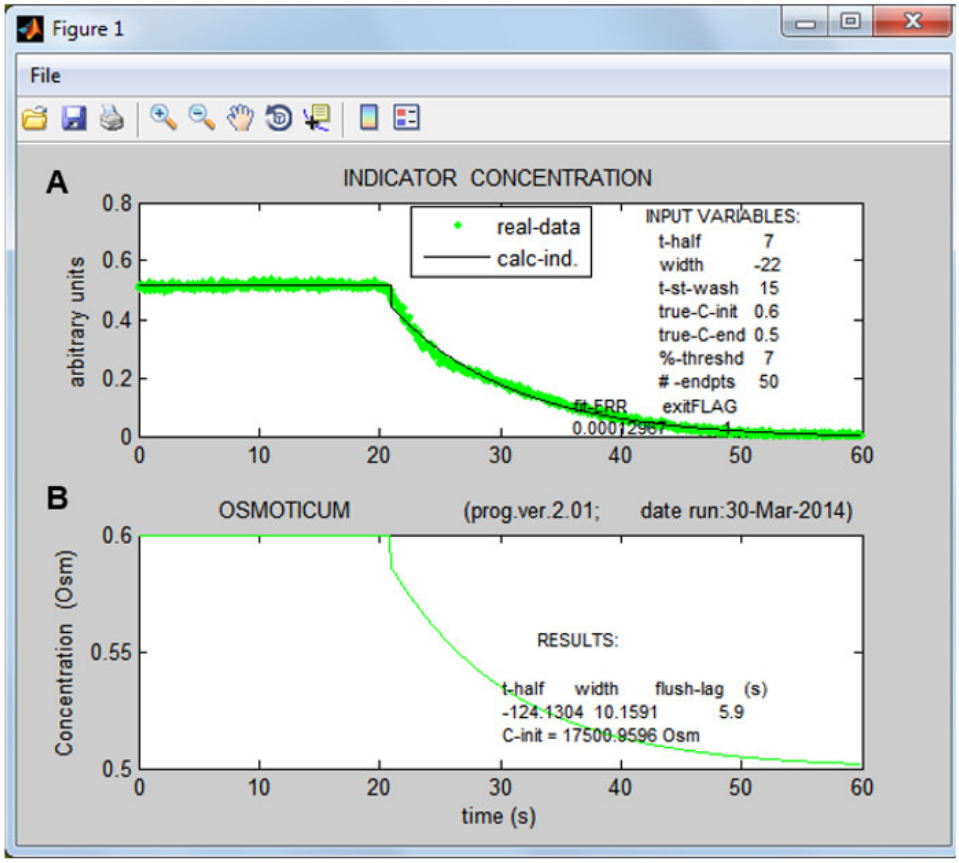

Figure 4: The Indicator Dye concentration in the bath and the osmolarity of the medium. **(A)** The time course of the Indicator Dye concentration, calculated directly from data (dots) and from the best-fit parameters (line) as it is washed away by a non dyed solution. **(B)** The calculated time course of the osmolarity change of the bath solution, assuming it follows the same dynamics as the change of the Indicator Dye concentration.

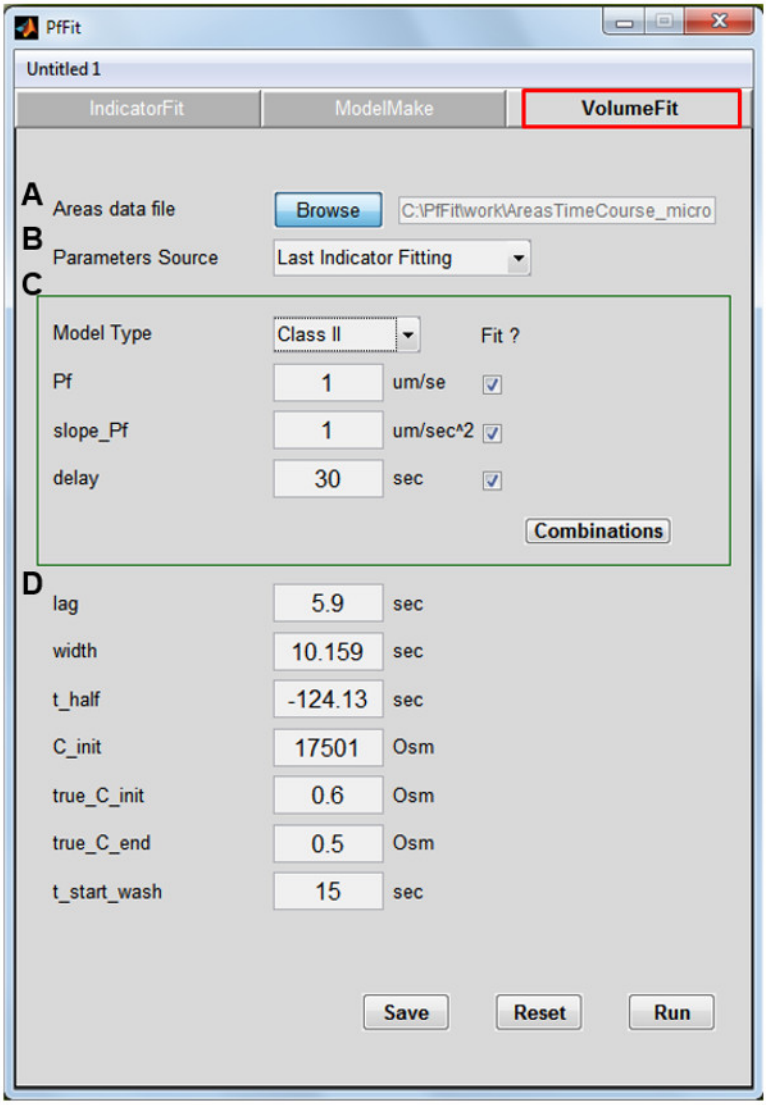

Figure 5: The 'Volume Fit' panel of the P_fFit program. **(A)** Browse for the area time course data file of the analyzed protoplast. **(B)** Choose the 'Last Indicator Fitting' option to import the experiment parameters from the last run through the 'Indicator Fit' (see the Supplemental P_fFit User Guide for alternatives). **(C)** 'Model Type' / 'Class': Class I contains the simplest model 1, Class II – models 2 - 5, class III – models 6 - 8. The models differ with respect to which parameters are being fixed and which are being adjusted (*i.e.*, freely variable) during the fitting procedure (tick the box to allow it to vary), and whether or not 'SlopePf' and/or 'Delay' are null. The models 1 - 6 are discussed at length by Moshelion *et al.*[11]. 'Combinations' lists the parameter choices dictated by the choice of 'Model Type'/'Class'. Among models with a similar fit result – choose the simplest! Initialize the 'P_f', '$Slope_{Pf}$' ('Slope_Pf') and 'Delay' parameters as shown (more details about 'Delay' in E below). (**D**) The variables and parameters describing the time course of the changing bath osmoticum are input either manually, or as described in B. **(E)** An interim plot, invoked by hitting 'RUN', of a time course of volume change (calculated from the cell contour areas) to aid in the choice of the initial value for the 'Delay' parameter. Estimate, by eyeballing, the total length of the baseline from the 1st point till the start of cell volume change (the '*inclusive* delay: the sum of 't-start-wash' + 'lag'/'flush-lag' + the "physiological" 'delay'). Insert this value as an input parameter for the 'delay' in the 'VolumeFit' panel and 'Run' again (see also the Supplemental P_fFit User Guide).

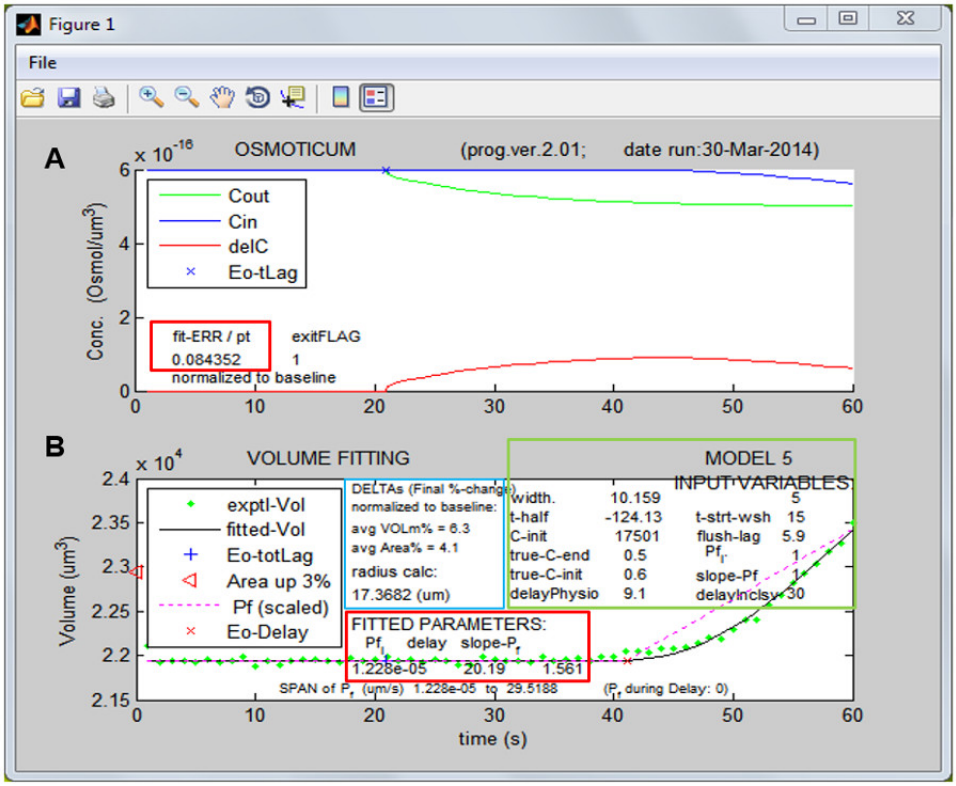

Figure 6: The results of fitting. **(A)** "Behind the scenes": the calculated ultimate time courses of the osmoticum concentrations in the two compartments: the bath (Cout, green line) and the cell (Cin, blue line; Cin is calculated based on the protoplast volume change and an assumption that the plasma membrane is permeable only to water – the "perfect and true osmometer"[11]), and the time course of the difference between them (delC, red line), which is the driving force for water flow, 'Eo-tLag' marks the end of the 'flush-lag' and the start of the hypotonic challenge (here only at about 21 sec). Red box: the error of the fit value (fit-ERR, see definition in B below). **(B)** The ultimate result of fitting the volume time course; Green box: 'INPUT VARIABLES' are the values entered via the P_fFit/'VolumeFit' panel (defined in **Figure 5A** legend). Black box: 'exptl-Vol' and 'fitted-Vol' are the experimental data and the volume calculated using the best-fit parameters, respectively, 'Eo-tLag' is the same as in A, 'Eo-Delay' marks the the start of volume change. 'Area up 3%' marks the volume at which the surface area increased by 3%, the presumed limit to the cell membrane ability to stretch without rupturing. 'Pf (scaled)' is the time course of the fitting-based calculated P_f, spanning the values indicated below the red box as 'Span of P_f'. Red box: 'FITTED PARAMETERS' are the values of the best fit parameters: 'Pf_i' (the initial P_f), 'delay' (the period between the onset of the hypotonic challenge and the start of the volume change (which, according to the model 5 used in this example, is also the start in a change in the P_f value), and 'slope-P_f' (the constant rate of change in the P_f value. 'fit_ERR' shown in A – the minimization target of the Matlab fitting procedure – is the "root-mean-square" deviation (*i.e.*, a square root of an averaged squared deviation) of a green dot from the black line), presented as % of the baseline volume. It is by this value that the relative success of repeated fitting with different parameter initialization values is judged. A NOTE OF CAUTION: As the best-fit parameter values could be the result of a local minimum found in the error minimization procedure – to verify that a global minimum has been found, several runs are required with different initialization values for these three parameters (and the lowest fit_ERR should be sought during these attempts. Blue box: DELTAs are the changes that occurred by the end of the fitted volume change period: 'avg VOLm%' is the relative extent of the calculated protoplast volume change and 'avg Area%' is the relative change of the protoplast surface area. The initial size of the cell is given by 'radius', derived from the mean value of the protoplast basal contour area.

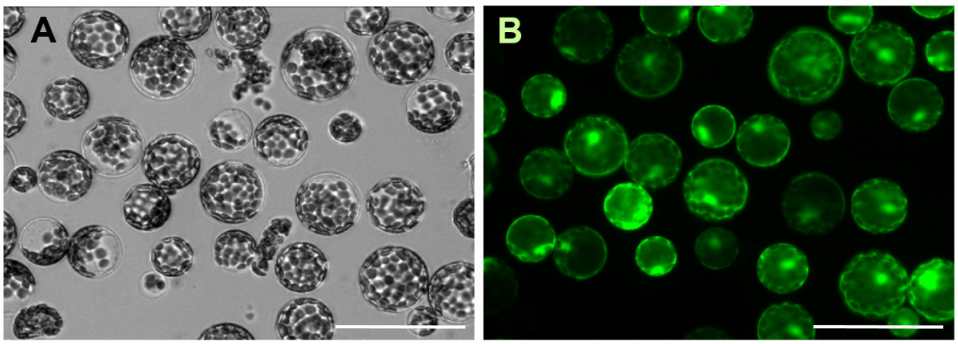

Figure 7: Epi-fluorescence microscopy view of mesophyll protoplasts from Arabidopsis leaf after PEG transformation with GFP, **(A)** under transmitted white light and **(B)** at 488 nm excitation and 520 nm emission. Scale bar: 100 µm. Please click here to view a larger version of this figure.

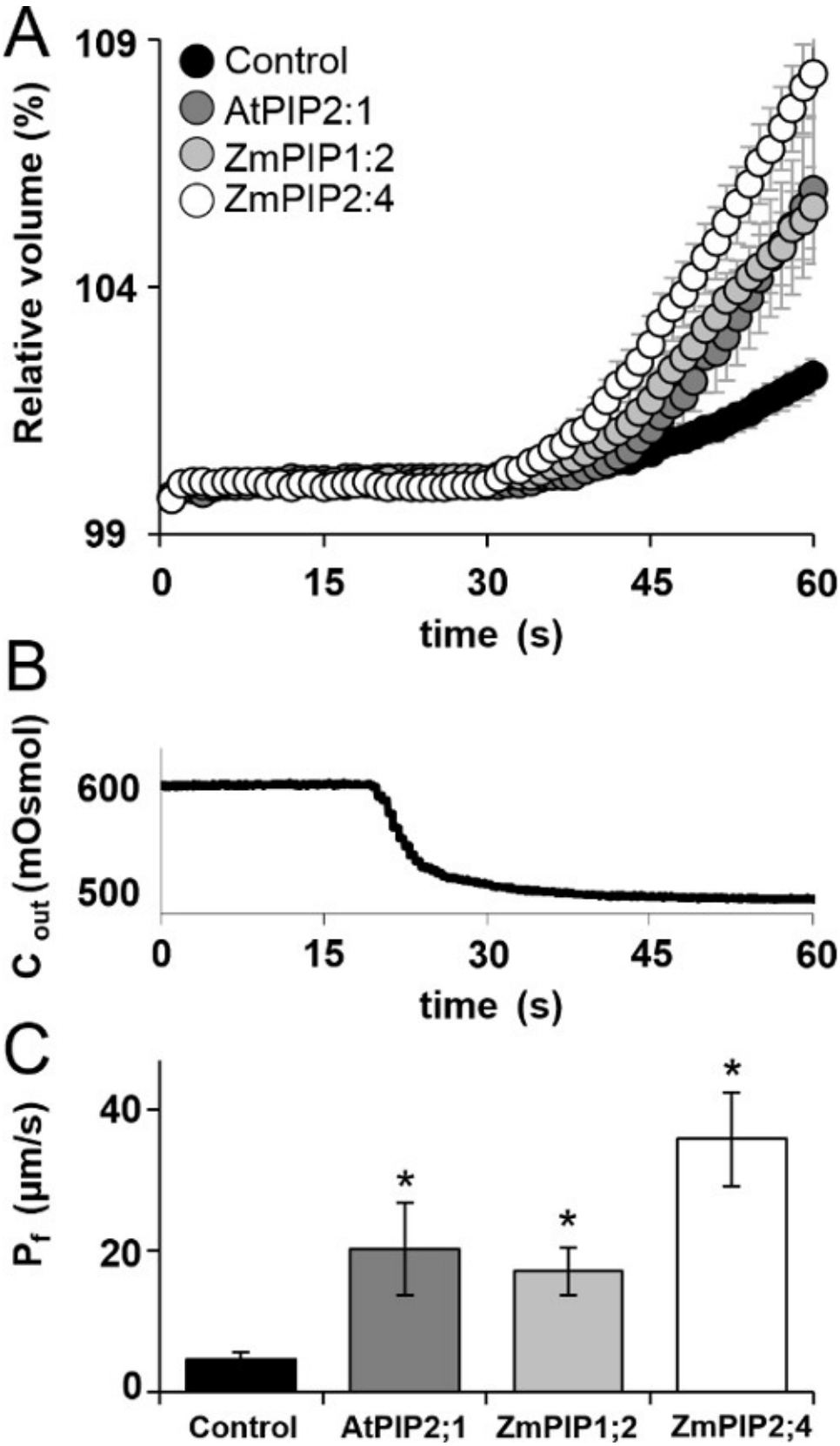

Figure 8: Volume change and the extracted osmotic water permeability, P_f. (A) Time course (60 sec) of protoplast swelling upon exposure to hypotonic challenge (mean ± SE). **(B)** The calculated osmoticum concentration in the bath during the hypotonic challenge. Note that while the hypotonic solution flow was switched on at 15 sec, it reached the bath only after a lag, here of 5.9 sec. **(C)** P_f (mean ± SE). Asterisks indicate significant differences from control ($p \leq 0.05$). Data from at least three independent experiments for each treatment with a total of n protoplasts (control: n = 52, AtPIP2;1: n = 13, ZmPIP1;2: n = 28, ZmPIP2;4: n = 34).

Discussion

Described here is a simple and very efficient procedure for measuring the P_f of isolated plant protoplasts, applicable in principle also to other spherical cells, *e.g.*, frog oocytes[11]. This method is based on measuring the P_f in response to an osmotic challenge to the cell. In contrast to the other methods based on this approach, however, the change of solutions, *i.e.*, of the osmolarity, is not instantaneous, but gradual, during a constant bath perfusion, starting with the isotonic solution, in which the baseline cell volume is established. In addition, this method does not involve a suction pipette and therefore minimizes the disturbance to the protoplasts.

The approach presented here enables measurements from a variety of protoplasts, from different plants or tissues. Yet, because of the calculations involved, only spherical cells can be analyzed. Also, the enzymatic isolation of the protoplasts and the osmolarity of the solutions need to be adjusted to the assayed cells (for example, the enzymatic isolation of tomato mesophyll protoplasts takes about an hour, considerably longer than in the case of Arabidopsis protoplasts).

The isolation of Arabidopsis mesophyll protoplasts according to the presented protocol is simple, rapid and efficient, yielding a high number of protoplasts. Notably, this, combined with their low basal P_f levels and their high transformation efficiency (**Figure 8**), makes them an attractive system for the functional expression of AQPs, to enable quantitative comparisons of P_f induced by different AQP isoforms. When expressing AQPs in these protoplasts with a marker gene (such as GFP), one can easily screen the protoplasts in the experimental chamber for fluorescing cells to analyze.

It is worthwhile to check whether this system is a viable alternative to oocytes for assaying AQPs even from animal sources (that functional animal proteins can be expressed in plant cells has been already demonstrated [17]).

Using the P_fFit program, two more parameters, beside the P_f, are obtained for the description of the protoplast responses to hypotonic challenges: delay, the time between the onset of volume change and the start of bath perfusion, and $Slope_{Pf}$, the rate of change in P_f during the osmotic challenge (described in detail in [11]).

For each experimental data set the volume fitting procedure needs to be performed several times, supplying different starting (initialization) values for these parameters, eventually choosing the fit with the lowest error. This error minimization process could be portrayed as seeking the deepest valley (a “global minimum”) in a landscape of valleys with different depths, among many hills, and attempting not be caught in a rather shallow valley (a “local minimum”).

Two types of P_f are obtained, P_f at the very beginning of the hypoosmotic swelling response (‘P_f initial’) and P_f calculated at the end of 15 sec of swelling, counting from the end of the delay (‘$P_f$final’). The difference between the two is discussed fully by Moshelion *et al.*[11], with regard to the 6 models analyzed.

There are two critical steps in the protocol: first, a good fit to the time course of the Indicator Dye concentration, second, a good fit to the time course of the volume of the swelling cell.

Disclosures

We have nothing to disclose.

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