Candum canales: Exploring ion channels in chloroplast membranes of the alga *Eremosphaera viridis*[1].

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OBJECTIVE

To characterize the diversity of ion channels present in chloroplast membranes using the bilayer lipid membrane technique.

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INTRODUCTION

Much of the theoretical underpinnings of membrane transport begin with the Nernst equation which describes the electric potential difference across a concentration gradient of ions outlined below

$$V_N = \frac{RT}{zF}\ln\left(\frac{C_o}{C_i}\right) \qquad (eqn\ 1.0)$$

where V_N is the Nernst potential, R the gas constant, T the temperature in Kelvins, z the ion charge, F the Faraday constant, C_o the concentration of the ion outside and C_i the concentration of the ion inside. For a monovalent ion, RT/F is equal to about 25 mV at room temperature (295 K).

For cells, maintaining and utilizing concentration gradients across their membranes is crucial for survival. There are many methods exploited by the cell to establish and vary the gradient, one is through ion channels. Ion channels provide a mechanism to relieve the concentration gradient, carrying ions from high to low concentration past a membrane barrier. The amount of current passing through the channel is proportional to the Nernst Potential via Ohm's Law

$$I = V_N \cdot G \qquad (eqn\ 1.1)$$

where I is the current through the channel, V_N is the Nernst Potential, and G is the conductance of the channel. The units commonly used for ion channels are pA (I), mV (V_N) and pico- or nanoSiemens (G)

Channels that are important in various fields of biology commonly exhibit selectivity for one ion. One example of an ion-selective channel (outlined by Alberts *et al*. 2008) is the potassium channels of nerve cells, which are used to return the excited cell back to its resting potential after an action potential has been triggered. Szabo *et al*. (2008) discuss a second potassium channel in mitochondria that plays a role in inducing apoptosis. This second example is drawing interest because of its potential applications in cancer treatments.

There has been a significant amount of research done with mitochondria and plasma membrane channels, but little has been done to characterize their electrical properties in chloroplasts of photosynthetic organisms. In the chloroplast, the behaviour of ion channels must be intertwined with the photosynthetic process, in which light energy is captured and transformed to chemical energy to produce carbohydrate. To investigate chloroplast ion channels, we used *Eremosphaera viridis* as the model organism because of the ease with which relatively pure and concentrated chloroplasts can be isolated.

MATERIALS AND METHODS

Chloroplast Harvest. *Eremosphaera viridis* cell suspensions were harvested after 16–28 days growth in Bold's medium. Details of culturing are available in previous reports on the growth of *E. viridis* (Khine and Lew, 2010) and its plasma membrane ion transport (Lew, 2010). The cells were first centrifuged at 50×g for two minutes to separate the cells from the culture medium. The supernatant was discarded and the pellet was re-suspended in grinding medium (0.6 M sucrose, 0.05 M HEPES, 0.02 M KCl; 2.4 mL of 5 N NaOH per 200 mL of medium was required to adjust the pH to 7.5) to a final volume of 36 mL. A glass 'mortar and pestle' (a Glass/PTFE Potter Elvehjem Tissue Grinder, Figure 1) was then used to gently disrupt the cells. By plunging the Teflon pestle four times with a twisting motion, the cells were disrupted sufficiently, with practically complete release of chloroplasts in an intact state, as determined by observing them under bright field and phase contrast (Figure 2, top panel). The homogenate was filtered through four layers of cheesecloth and centrifuged for 1.5 minutes at 50×g to remove cell walls.

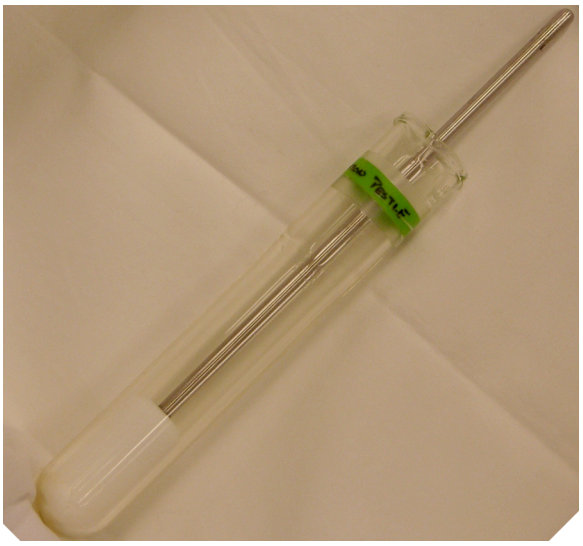

Figure 1: The Glass/PTFE Potter Elvehjem Tissue Grinder used to gently disrupt cells of the alga *Eremosphaera viridis*. The cell suspension was added to the pestle, and the Teflon plunger pushed to the bottom with a twisting motion so that the cells were forced between the Teflon pestle and the glass wall of the mortar. Because of the large size of the cells, only four strokes were required to disrupt the cells and release intact chloroplasts. For scale, the length of the glass mortar is 20 cm.

Several different protocols were used to purify the chloroplasts.

Protocol 1. The first protocol was used in preliminary experiments to see whether we could fuse chloroplast membranes with the bilayer lipid membrane (BLM). The cell homogenate was topped up to 36 mL with grinding medium. This filled the centrifuge tube nearly to its top to prevent collapse of the centrifuge tubes during high g-force centrifugation. The homogenate was centrifuged for 10 minutes at 80 000×g. The supernatant was discarded and the pellet was re-suspended with 10 mL of grinding medium. Aliquots (1 mL each) were transferred to Eppendorf tubes and stored frozen at –20°C until they were used for BLM experiments.

Protocol 2. The second chloroplast separation technique used sucrose step gradients to improve the purity of the isolated chloroplasts. The chloroplast solution was underlain with 5 mL of 45% (w/v) sucrose, which was in turn underlain with 5 mL of 50% sucrose (Stabenau *et al.*, 1984). The tube was topped up with grinding medium to bring the total volume to 36 mL. The tubes were than centrifuged at 80 000×g for 1.5 hours. The chloroplasts were found to have pelleted through the sucrose step gradient, which meant that their density was higher than the density of 50% sucrose (1.24 gm ml^{-1}). The supernatent was discarded and the pellet was re-suspended with 10 mL of grinding medium, aliquoted into ten samples and stored at –20ºC.

Protocol 3. Protocol 3 was similar to protocol 2 but with a 45 and 60% step gradient (60% w/v has a density of 1.29 gm ml^{-1}). Material was observed at the 45/60% interface under phase which showed fewer refractile organelles than the pellet suggesting that there were more intact chloroplast in the pellet and fewer thylakoids (resulting from breakage of the intact chloroplasts).

Protocol 4. Due to mechanical failure of the ultracentrifuge used for 80 000×g spins, the step gradient was abandoned and a lower rpm centrifuge was used. The cell homogenates were centrifuged at 1200×g for 15 minutes. The pellet was re-suspended in 35 mL of grinding medium and centrifuged at 1200×g for 15 minutes. The pellet was then re-suspended in 10 mL of grinding medium and aliquoted into ten samples and stored at –20ºC. The chloroplasts in the aliquot are shown in figure 2 (bottom panels).

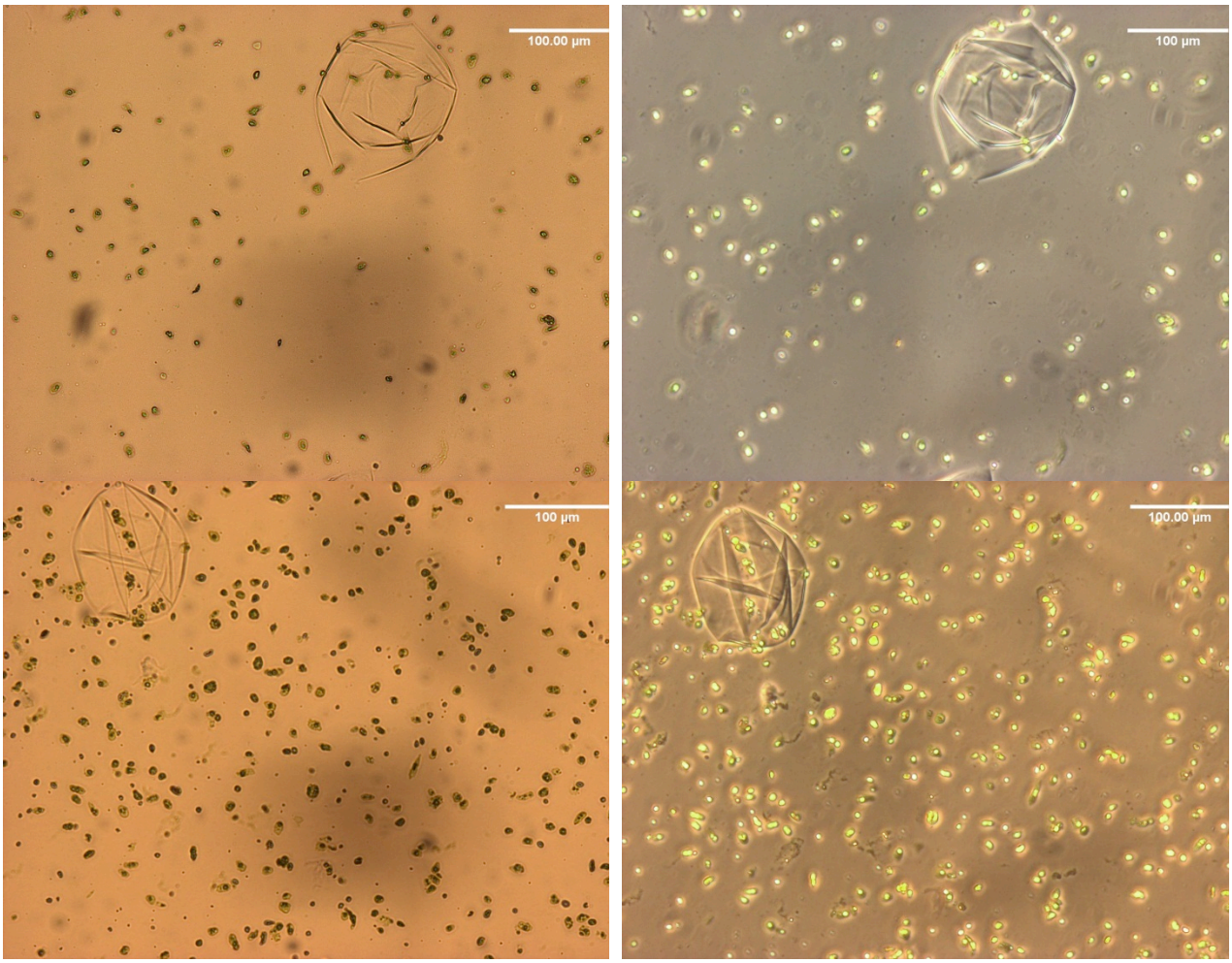

Figure 2: Microscope views of cell disruption. The chloroplasts after homogenization can be seen as green orbs in the top two panels (left, bright field; right, phase contrast; ×10 objective). The resulting chloroplast aliquot using protocol 4 is shown in the bottom panels. Most of the chloroplasts were refractile under phase contrast, indicating that they were intact. Some cell walls can be observed in the top left of the bottom panels indicating that protocol 4 was not successful in creating a perfectly pure solution.

Phospholipid Preparation. Based on previous work by Silverman-Gavrila and Lew (2002), stable phospholipid membranes for the BLM apparatus were formed from a mixture (by weight) 50:30:10:10 of L-α Phosphatidylcholine (Soybean), L-α Phosphatidylserine (Soybean), L-α Phosphatidylethanolamine (Soybean) and cholesterol. All lipids were obtained from Avanti Polar Lipids, Inc. The mixture was dissolved in benzene and stored at –20ºC. Small aliquots (equal to 1 mg total lipid) were added to a small glass vial with a positive displacement pipette. A gentle stream of nitrogen gas was applied to evaporate the benzene solvent. The lipids were than suspended in 40 μL of decane, and stored at room temperature until use. Lipids were prepared fresh daily.

BLM Apparatus Set Up. The main apparatus used was a Warner Instruments' BLM workstation which included a small chamber with accompanying cuvette, silver wires, capacitive feedback operational amplifier, and an 8-pole Bessel filter.

Figure 3 illustrates the BLM chamber used. The cuvette was placed inside the *trans* compartment of the chamber and filled with 40 mM KCl solution. The *cis* compartment as well as the half cells were filled with 200 mM KCl solution and 1 M KCl bridges electrically joined the half cells to both the *cis* and *trans* compartment. Silver wires were coated with chloride and inserted into the headstage and their tips submerged in the half cells. The headstage and chamber were housed inside a grounded Faraday cage on top of a vibration isolation table (a granite slab resting on tennis balls) that shielded the system from background electromagnetic radiation and mechanical vibrations. The cage was lined with soft foam to minimize any vibration caused by ambient noise in the room.

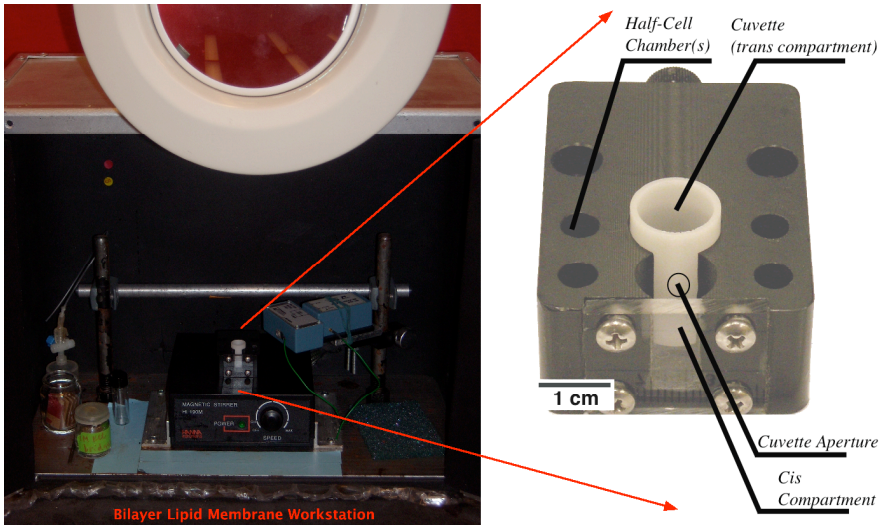

Figure 3: The chamber used during the BLM set up. The electrically and sound shielded workstation is shown in the left panel and the BLM cuvette is shown in the right panel.

The headstage was connected to an amplifier that produced a user controlled voltage across the chambers and amplified the resulting current (known as voltage clamping). The current output from the operational amplifier was than fed into an 8 pole Bessel filter with an initial cut off frequency of 2.51 kHz. We eventually reduced the cut off frequency to 500 Hz to improve noise reduction so that we could resolve smaller channels. The voltage output of the operational amplifier was connected to a Tektronix TDS 2001C digital oscilloscope to observe the voltage being applied across the chamber's compartments at a gain of 10. The second channel on the oscilloscope was connected to the Bessel filter output to observe the clamping current (the channel opening and closing events). In the absence of a membrane, the chambers were subjected to a capacitance check. This was a triangular waveform voltage with a peak-to-peak amplitude of 2.7 mV and rise time 4.8 ms. The resulting current and voltage outputs were observed on the oscilloscope with the current manifesting as a clipped square waveform signal (in the absence of a membrane). The clipping is due to the chambers behaving similarly to a short circuit resulting in large currents for applied small voltages. This changes when a membrane is successfully formed as described below.

Membrane Addition. Figure 4 shows the small aperture on the cuvette (200 μm in diameter) across which the bilayer membrane would be formed. 4 μL of the prepared lipids (in decane) were placed on the aperture using a positive displacement pipette that was slowly swept over the aperture dispensing the lipids in the process. The chambers were than subjected to a second capacitance check. The current output was observed to be a square wave between 24-28 pA (equal to a membrane capacitance of 24 to 28 pF) if a stable membrane had formed, else the clipped waveform would persist.

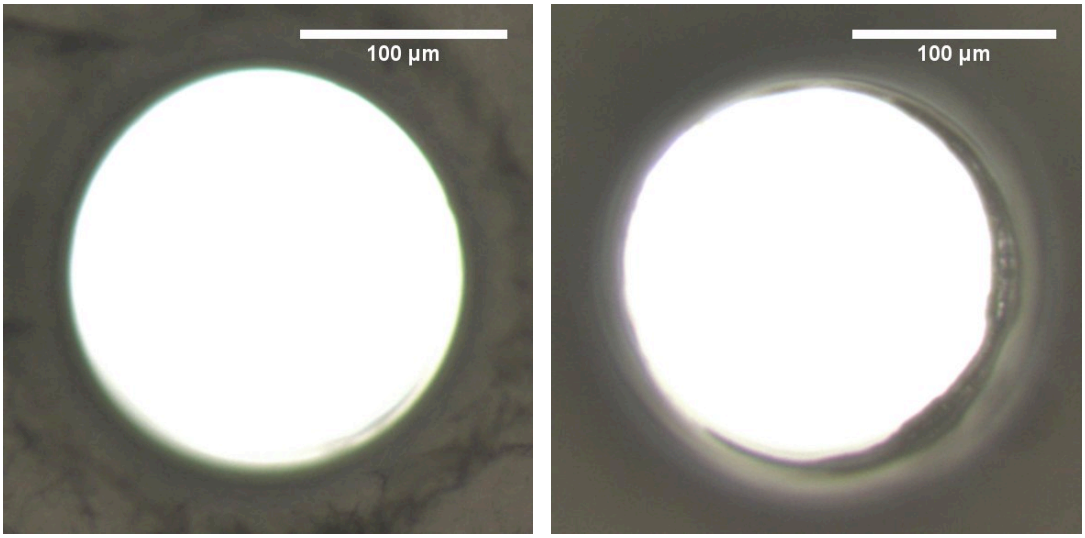

Figure 4: Cuvette aperture. The outer (left) and inner (right) images of the cuvette aperture. Residue can be seen accumulating on the inner ring. Imaged with a ×10 objective.

Chloroplast Addition. For experiments in which pH was not balanced in the two compartments, 45-60 μL of the chloroplast suspension was applied to the *cis* chamber just above the aperture. Increments of 15 μL every 5-8 minutes of solution were added and stirred with a metallic probe until channel activity was observed on the oscilloscope due to fusion of the membranes to the bilayer. The balanced pH trials were similar to unbalanced pH trials but grinding medium was added to the *trans* compartment equal to the amount of chloroplasts added to the *cis* compartment. By adding the grinding medium, it was found that the *cis* and *trans* compartments had a pH of 7.5 after the addition of 40 μL of grinding medium/chloroplast suspension and remained at 7.5 when additional grinding medium/chloroplasts were added.

Recording. Channel recordings were digitized at 1 kHz with a National Instruments digitizer using NiDataLogger software (National Instruments). The oscilloscope was monitored during the recording to ensure no alias frequencies were recorded. Further filtering of the data was completed in Matlab using a digital filter that implemented the recursive function $y(k) = 0.8 \cdot y(k-1) + 0.2 \cdot x(k)$ where x(k) is the k^{th} unfiltered data point, y(k-1) is the $k\text{-}1^{th}$ filtered data point and y(k) is the k^{th} filtered data point.

RESULTS

Unbalanced pH. When a channel formed on the bilayer, we observed random square pulses on the oscilloscope due to the opening and closing of the channel. Figure 9 illustrates several different channels that were observed including a porin of 670 pS (bottom panel). Outlined in figures 10 and 11 are recordings that were used to arrive at the current voltage relations (also included in figures 10 and 11) for a highly selective anion channel and a low selectivity cation channel respectively. From the linear fit to the current voltage relation, we obtain the reversal potential as the voltage intercept and the conductance as the slope. Figure 12 (top panel) shows the reversal potential and conductances for all 63 trials. There appeared to be a large number of trials around the 20 mV range while the conductances seemed to show a main spread across 20 to 40 pS range. We used a smooth Gaussian kernel to estimate the exact location of these high frequency areas, which fits a 2-D Gaussian to the data. The fit can be seen in figure 12 (bottom panel) showing the high clustering around a reversal potential of 18.7 mV and conductance 22.4 pS.

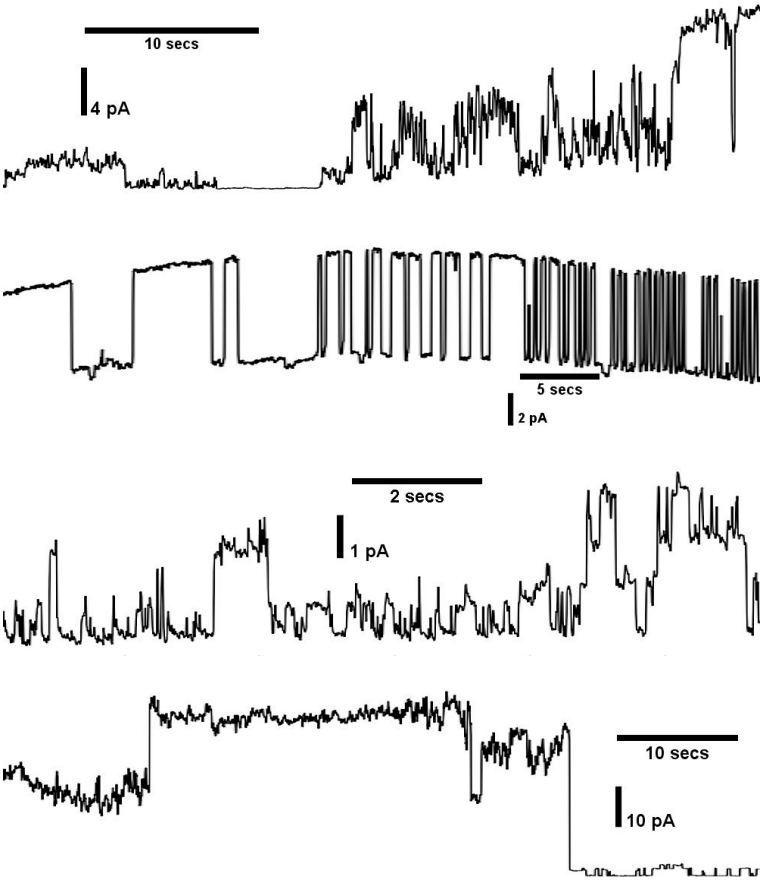

Figure 9: Various channel recording showing the kinetics of the many different channels observed. The top two panels show flickering channels that opened and closed very rapidly. The third panel shows a recording with multiple channels present and the last panel shows a porin with small sub conductance states at the tail end of the recording (data from this trial was not included during the analysis).

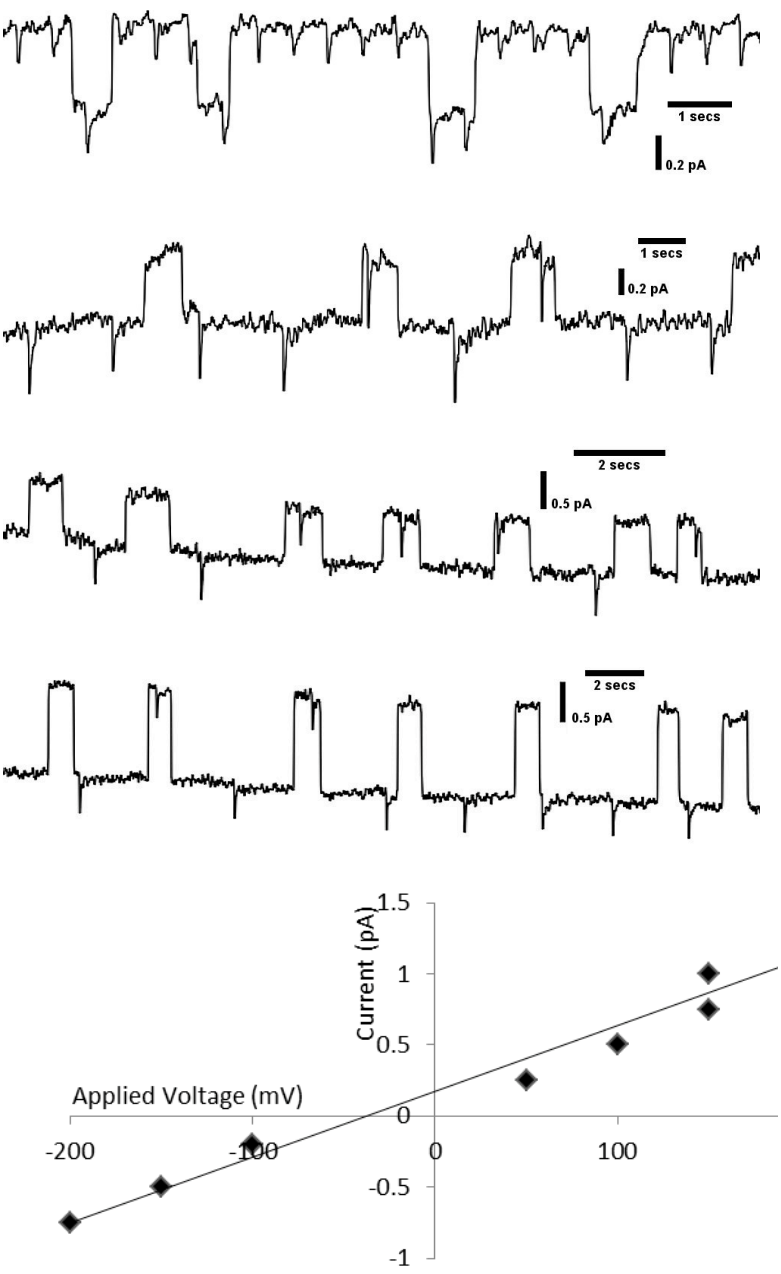

Figure 10: Data of an anion selective channel. The top panel shows channel recording with an applied voltage of -150 followed by the next panel at 100, 150 mV and 200 mV. Observed periodic spiking is the result of the reset of the capactior in the operational amplifier. The bottom panel is the voltage current relation showing the reversal potential at -37 mV and conductance at 4.6 pS.

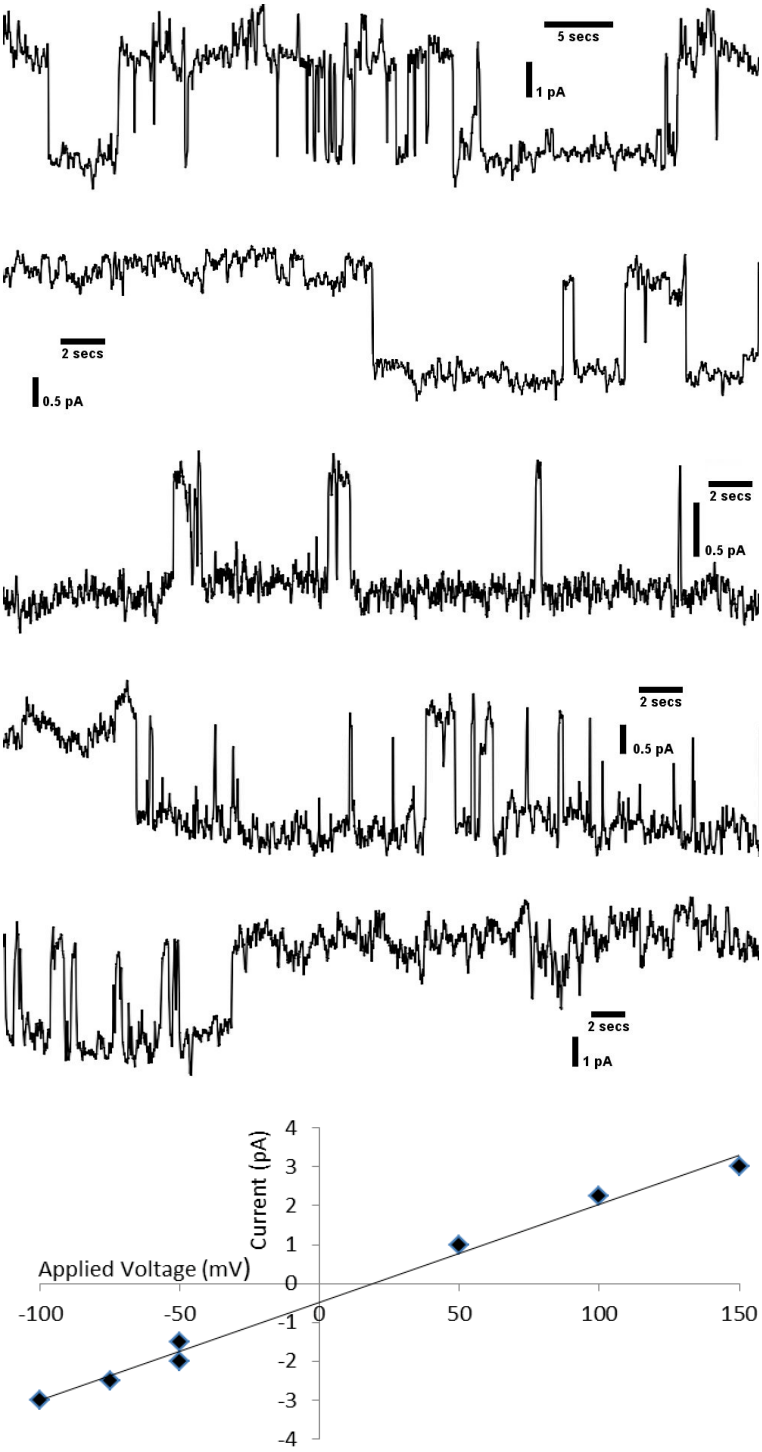

Figure 11: Data for a cation selective channel. The top shows a channel recording with an applied voltage of-100 mV, the next at -50 mV followed by 50 mV, 100 mV and 150 mV. Observed periodic spiking is due to resetting the capactior in the operational amplifier. The bottom panel is the voltage current relation showing a reversal potential at 19 mV and conductance at 25.2 pS.

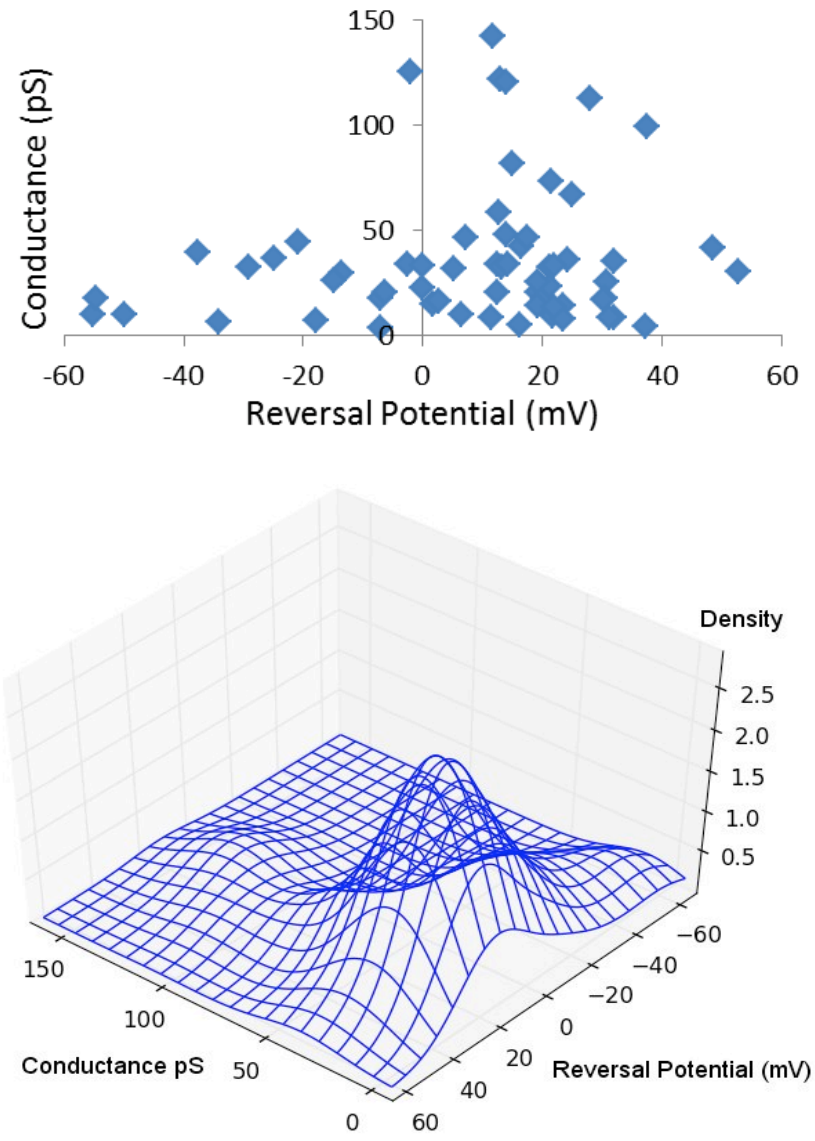

Figure 12: The reversal potential and conductance of the observed chloroplast channel with unequal pH between the cis and trans compartments is shown in the top panel. The kernel density for the reversal potential and conductance is shown in the bottom panel. The kernel peak resides over a conductance of 22.4 pS and 18.7 mV. Note: density is used as a relative measure, the exact value is meaningless.

Balanced pH. During our experiments, we discovered that the pH was 7.4 in the *cis* chamber and 5.8 in the *trans* chamber. This is due to the addition of chloroplasts to the *cis* compartment that contained a buffer from the grinding medium. By adding grinding medium to the *trans* compartment, a change to the KCl concentration is expected, producing a new Nernst potential. The new Nernst potential was found to be no less than 37 mV assuming 0.3 mL was applied on both sides of the membrane. Figure 13 (top panel) shows the channel data for when the pH was equalized using grinding medium for 13 trials. Kernel density estimate for the channel data is shown in the bottom panel of figure 13 showing a clustering around reversal potential of -1.7 mV and conductance 29.1 pS.

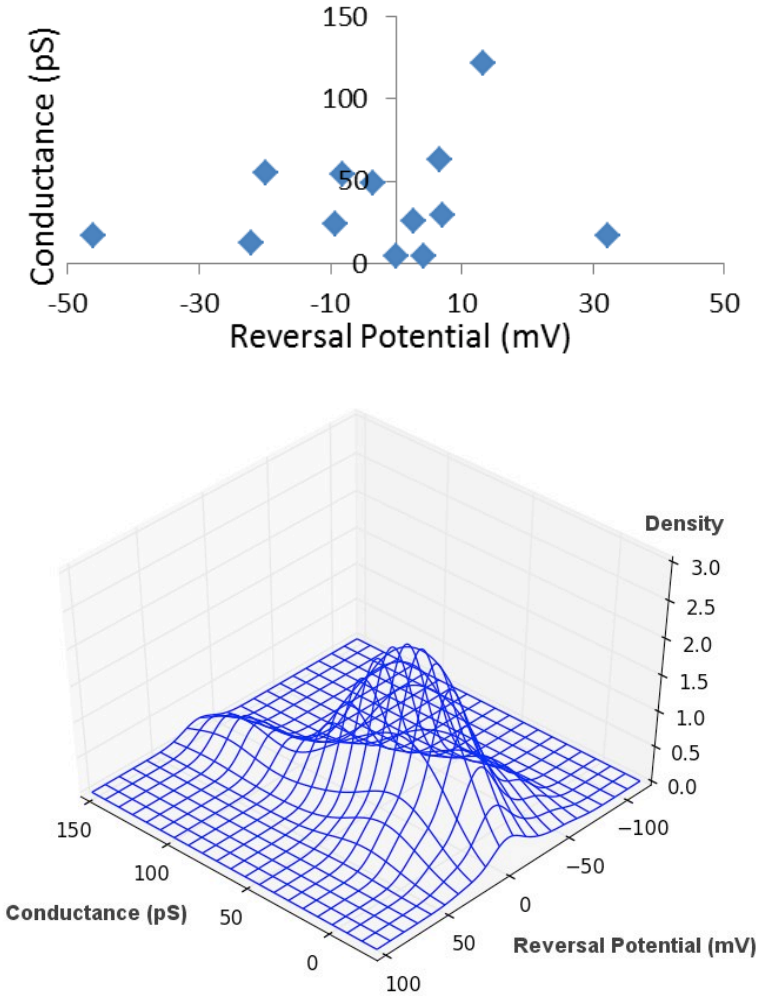

Figure 13: The reversal potential and conductance of the observed chloroplast channel with equal pH between the cis and trans compartments is shown in the top panel. The kernel density for the reversal potential and conductance is shown in the bottom panel. The kernel peak resides over a conductance of 29.1 pS and -1.7 mV. Note: density is used as a relative measure, the exact value is meaningless.

DISCUSSION

We observed a large variability in the kinetics of potassium and chloride channels for chloroplast membranes isolated from *Eremosphaera viridis* (figure 9). Some channels flickered rapidly between the open and close states while others stayed open for long durations. Channels could be seen to have various conductance states and other recordings showed the presence of multiple channels in the bilayer. The variable nature of the channel data is well exhibited in figure 12 (top) as a large spread across many conductances and reversal potentials. To observe any clustering in the data a kernel density was used (figure 12 bottom). Most of the channels cluster at a reversal potential of 18.7 mV and a conductance 22.4 pS, consistent with a small potassium selective channel. This suggests that, while many ion channels may be present at low density, the small conductance potassium channel is the major one in the chloroplast membranes.

For a conductance of 22.4 pS, we can estimate the size of the pore using a simplistic model from Hille (1992) that assumes the channel is a hollow cylinder. The equation is:

$$d = \frac{\rho g}{\pi}\left(\frac{\pi}{2} + \sqrt{\frac{\pi^2}{4} + \frac{4\pi l}{\rho g}}\right) \qquad (eqn\ 1.3)$$

where d is the pore diameter, ρ is the resistivity of the solution, l is the length of the channel and g is the conductance. Using values supplied by Cruickshank *et al* (1997) for the resistivity of KCl (49.7 Ω cm) and the length of the bilayer (42 Å), the channel pore diameter is estimated to be 2.5 Å. This is similar to the van der Waal radii of ions, including potassium (about 2.5 to 3.0 Å) (Batsanov, 2001). Note that the pore diameter is a rough value; the true merit of the estimate is as an order of magnitude approximation, the exact value is difficult to ascertain.

For the reversal potential, the expected value should have been close to the Nernst potential for potassium, which for our 200 mM to 40 mM gradient is 41 mV. The measured value is about 22 mV, about 2–fold less than the Nernst potential. The lower experimentally measured potential could be due to the channel conducting an oppositely charged ion such as chloride (the only other ion present in the solutions (and/or a significant selectivity for protons [H^+] or hydronium ions [H_3O^+])). The selectivity ratio of the channel for potassium *versus* chloride can be estimated from the Goldman equation, which describes the electric potential difference across a medium with several concentration gradients and varying selectivity for each ion:

$$V = \frac{k_B T}{e}\ln\left(\frac{P_K[K]_c + P_{Cl}[Cl]_t}{P_K[K]_t + P_{Cl}[Cl]_c}\right) \qquad (eqn\ 1.4)$$

where V is the membrane potential, k_B is the Boltzmann constant, T is the temperature, e the electron charge, P_K is the selectivity of the channel to potassium, P_{Cl} is the selectivity of the channel to chloride, $[K]_t$ is the potassium concentration on the *trans* compartment, $[K]_c$ is the

potassium concentration on the *cis* compartment, $[Cl]_t$ is the chloride concentration in the *trans* compartment and $[Cl]_c$ is the chloride concentration on the *cis* compartment. Re-arranging for the selectivity ratio, we obtain

$$\frac{P_K}{P_{Cl}} = \frac{[Cl]_t - e^{\frac{Ve}{k_B T}}[Cl]_c}{e^{\frac{Ve}{k_B T}}[K]_t - [K]_c} \qquad (eqn\ 1.5)$$

For our data the selectivity ratio is 3.5. The low selectivity ratio of the channel seems unusual. Potassium and chloride ions have atomic diameters of ≈ 2.5Å. For these ions to pass through a channel with a pore size of about 2.5 Å, they will come very close to the amino acids lining the channel. The amino acids often are electrostatically charged and will repel or attract ions depending on the Coulombic forces between them. For potassium channels, many negative charges will line the inner channel (for example, electronegative oxygens on carbonyl groups), acting as a selectivity filter and promoting the flow of potassium ions through the channel, while repelling chloride ions (Alberts *et al*. 2008). It is unexpected to see a channel that would be able to pass both cations and anions. This would imply the channel may have an overall electrostatic charge of zero or is only weakly negative to account for the slight favouring of potassium ions.

A cation/anion channel may not be unreasonable, as small channels with low selectivity have been observed. Van den Wijngaard *et al*. (1999) found an anion channel in the chloroplast of spinach with a conductance of 42 pS and selectivity ratio of 6.6 of P_{Cl}/P_K. Their channel exhibited no opening events above the reversal potential —unlike the channels we observed— making it an unlikely match. It does confirm that small channels may demonstrate selectivity to both cation and anions.

A possible cause for the low reversal potential maybe due to a counteracting hydrogen current due to a variance in pH across the membrane. To observe this, we equalized the pH across the membrane. Shown in figure 14 (top) we can see the reversal potential is now at –1.7 mV and the conductance is 29.1 pS for the kernel densities. This lowered reversal potential is not just related to the pH as these trials were completed using protocol 4, which for the unbalanced trials had a reversal potential of 6.9 mV according to the kernel in figure 14 (bottom panel). To determine if there was any significance difference, we employed a two tailed t-test, to measure whether the two sets of data were statistically significant. Before the t-test was administered, we parsed each set of data to values that resided within the yellow ring shown in the kernel densities (figure 14) to remove any additional channels and outliers. The resulting t-test value between the balanced pH and all the unbalanced trials (excluding the removed values) was 0.0013 —which is statistically significant— and for the unbalanced trials with protocol 4 was 0.064, which is not significant. The t-test value for the unbalanced trials using protocol 4 is so close to being significant that removal of one data set close to the yellow ring's edge caused it to become less than 0.05 (the threshold value for statistical significance). It would then be reasonable to claim that pH does have an impact on the value of the reversal potential. This

would suggest that the channel is permeable to hydrogen. Using the values from the total unbalanced trials and assuming the channel is impermeable to chloride, the permeability ratios of hydrogen to potassium is 30 000 to 1.

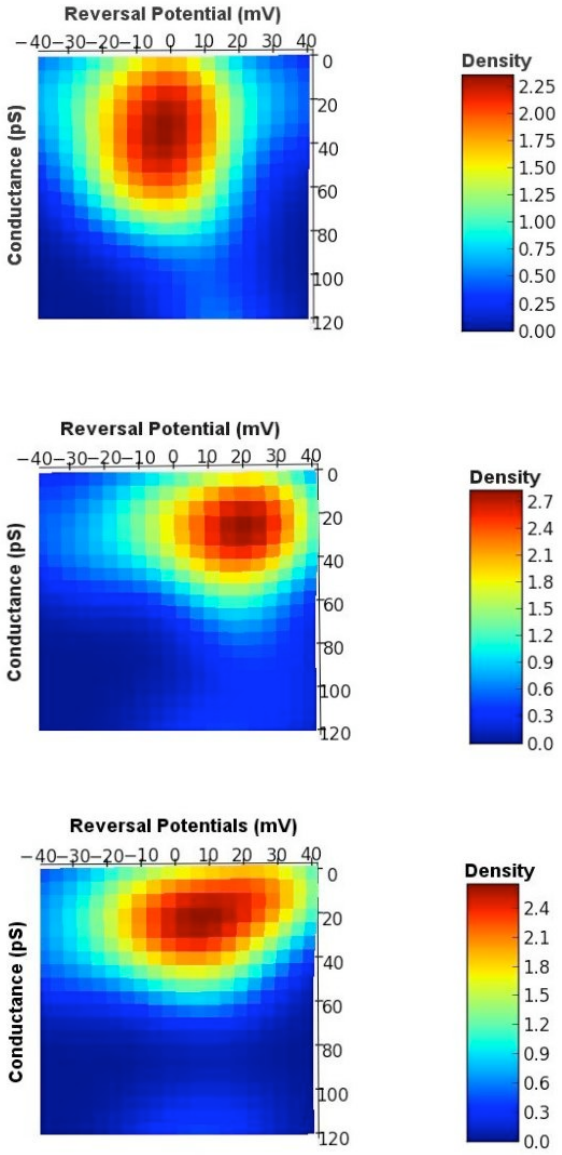

Figure 14: The kernel densities from a top down view. Balanced pH trials are in the top panel followed by all the unbalanced trials in the middle panel and the bottom panel shows the unbalanced trials using chloroplast separated by protocol 4. Two tailed t-tests were applied against the balanced pH trial after parsing the data to values within the yellow band of the kernel density. For the reduced unbalanced trials the t-test value was 0.0013 and for the unbalanced trials with protocol 4 the value was 0.064.

We cannot know for certain which of the three chloroplast membranes (outer envelope, inner envelope and thylakoid) fused with the planar bilayer, we can however draw inferences from available literature. Flugge and Benz (1984) reported a porin of 720 pS in the outer envelope of spinach chloroplast which is similar to the porin in figure 9 that we observed having a conductance of 670 pS. We observed this porin in only one of 63 successful experiments suggesting that the outer envelope fuses very rarely to our planar bilayers. For the inner

envelope, Feng *et al*. (1994) reported a potassium channel of 160 pS with a selectivity ratio very close to the Nernst potential indicating a high selectivity. This is similar to a second channel we observed for 6 trials at 120 pS, indicated as a second peak in the kernel for unbalanced trials (figure 12 (bottom)). The channel though, showed a selectivity ratio of only 2.3, making it an unlikely match. The inner membrane has also been characterized with a porin of 525 pS by Heiber *et al*. (1995), which as we stated earlier were rarely recorded. Thylakoid membranes are possibly the most frequently forming membrane as Tester and Blanche (1989) reported two potassium channels, one of 14 pS (no reversal potential given) and a second at 120 pS with a selectivity ratio of 4.5 making them both suitable matches to our channels.

Further investigation of the experiment is certainly warranted. Future experiments can include: establishing a larger concentration gradient to observe how the measured reversal potential will change, using sodium in place of potassium to ensure the non-selectivity of the channels, using pH-buffered solutions with an impermeant counterion, and use of the perfusion apparatus to ensure observations of only one ion channel in the bilayer at a time.

Conclusion

We identified a low selectivity potassium channel in chloroplasts of *Eremosphaera viridis* that may show permeability to hydrogen and a possible second higher conductance channel. Our research provides a baseline for further investigations of chloroplast ion channels.

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