METHODS BOX 3.1 Measuring PD between internode sections

(a) The size of charophyte cells allows PD measurement between different parts of the internode. A Perspex cell holder is made with two chambers A and B, connected by a groove. The cell is inserted in the groove, as shown in the figure below. Silicon grease or Vaseline is used to separate the media in each chamber. External electrodes measure the potentials $E_A$ and $E_B$ in each chamber with respect to earth, $E_A - E_B$ providing PD between the parts of the cell in each chamber. The nodal complexes are shown in black.

In steady state and with the same media in each chamber, $E_A - E_B$ is close to zero. In experiments involving mechanical stimulation, the cell section in one chamber receives a calibrated touch. Upon stimulation in chamber A, the receptor potential difference (RPD) can be measured as function of the stimulus strength. If APs are evoked, their propagation from one chamber to the other appears as negative peak (see Fig. 3.2a). For strong stimuli, variation PDs can be measured (see Fig. 3.2c).

(b) Transmembrane PD without inserted electrodes. If one of the chambers in the diagram above is filled with 50 – 100 mM KCl, the membrane PD in that section reduces to zero and $E_A - E_B$ represents the transmembrane PD. (Inserted electrodes would lead to damage upon stimulation). In the early experiments it was important to confirm that the RPDs depolarise the cell membrane PD (Fig. 3.2b). However, the osmolarity of the medium in the other chamber has to be matched with sorbitol to prevent trans-cellular osmosis (Hayama, Nakagava et al. 1979), reducing the turgor of the cell. As turgor is important variable in mechano-responses, this effect can be minimised by exposing only a small part of the cell to KCL, as shown in the figure below. If the small compartment B is filled with KCL, then $E_A - E_B$ approximates the transmembrane PD, while $E_A - E_C$ shows the PD between parts of the internode in chamber A and C.

(c) Different responses of the nodal complex and the internode (the flank) to wounding. In these experiments two adjacent cells are used and the victim (V) cell is cut. The proportion of the receptor (R) cell in chamber B, $L_{RB}$, is varied to estimate the contribution of the nodal complex and the flank.
METHODS BOX 2.3 Modifying the cell compartments

(a) Ligation If the internode is allowed to wilt in air, the nodal complex can be tied off by a silk thread (Hirono and Mitsui 1981). This technique can be used to remove one or both nodes. The ligated cells survive indefinitely. If one node is retained, new shoots and rhizoids grow from the nodal complex. Node-less internodes do not form new plants and their growth is limited. The wall reforms near the ligation after about two weeks and the thread can be removed (Beilby and Shepherd 1991). The ligation can be combined with gentle centrifugation, which gathers the cytoplasm to one end of the internode. The cytoplasmic plug is clearly visible against dark background and can be tied off, preparing cytoplasm-enriched fragments (Hirono and Mitsui 1981; Beilby and Shepherd 1989). The fragments form multiple vesicles and new vacuoles in several days, but even then offer a thick cytoplasmic layer suitable for insertion of multiple electrodes and fluorescent dye injection.

(b) Perfusion The internodal cells can be perfused by removing the cell ends and perfusing the cell with various media. In the vacuolar perfusion (see below), the vacuolar sap is replaced by artificial medium (Tazawa 1964). The cell ends are then ligated and cells survive for weeks, but modify the artificial medium with time. For greater control the cell ends can be left open and the medium can be exchanged as required. Such cells are not turgid and survive only some hours. The measurements are performed in a cell holder with three compartments (similar to that in Methods box 3.1b). The perfusion medium and the “internal electrode” are placed in the outer compartments, while the external medium and the “external electrode” are placed in the middle compartment.

If the perfusion rate is rapid or EGTA is included in the perfusion medium, the tonoplast disintegrates (Williamson 1975; Tazawa et al 1976). The cells can be ligated and impaled with electrodes (although they are very easily damaged), or the ends can be left open and measurement performed in three-chamber holder with external electrodes. In each case, the tonoplast-free cells survive only for hours.

(c) Permeabilisation In permeabilised cells the, the Ca^{2+} is removed from the outside medium (and cell wall) by EGTA. The plasma membrane disintegrates. The chloroplasts are disrupted, but the tonoplast and the vacuolar compartment is thought to be undisturbed. The external medium is made close to that of the native cytoplasm (Shimmen and Tazawa 1982). ATP can be supplied or withdrawn from the medium and the effects on cytoplasmic streaming observed.
FUTURE EXPERIMENTS BOX 3.1

(1) Dissection of RPD As it is possible to perfuse out the Ca\(^{2+}\) stores activated by pressure, perfused and ligated Chara cells should be mechano-stimulated to find out if they are capable of producing an RPD.

(2) Dissection of osmosensing If osmosensing is associated with the nodes, cells of Chara longifolia and Lamprothamnium should be subjected to node removal and ligation. Can these cells regulate turgor if challenged by hypo-osmotic or hyperosmotic media?

(3) Hunt for the turgor sensor The proton pump in salt tolerant characeae responds to turgor decrease by pumping faster. A range of structure inhibitors, such as oryzalin to inhibit the microtubule system and cytochalasin D to inhibit the actin cytoskeleton (Collings et al 1996, Foissner and Whetstone 2000) can be used and correlated with pump response.

(4) AP and cytoskeleton The association of AP with microtubules can be investigated again using range of microtubule inhibitors.

(5) H\(^+\)/OH\(^-\) channels at negative PDs The putative opening of these channels, when the cell membrane is clamped at very negative PDs needs investigation in range of media, some lacking Na\(^+\), now that salinity appears to affect these channels.

(6) The membrane PD fluctuations in saline media The transient “spiky” noise appearing in response to cell exposure to Na\(^+\) is thought to arise from cooperative opening of group of H\(^+\)/OH\(^-\) channels. The experiment that proves the identity of these channels is yet to be designed.

(7) Characeae and roots The findings from characeae can be extended to roots of land plants, both to whole plants and root protoplasts.
Fig. 3.1: (a) The experimental arrangement of Osterhout and Hill (1931) and (b) and (c) examples of their results. (b) The cell was pinched at point P, producing the response at electrode B (lower curve) and electrode A (upper curve). First pinch produced a small response at B and even smaller one at A. A harder pinch evoked an AP at B and larger response at A with subsequent propagation of the AP to A. The interval between vertical lines is 5 s. (c) In this experiment the cell was cut at Q: upper record is “death wave” PD at A, the lower record is “death wave” PD at B, both with reference to C. The interval between the vertical lines is 0.1 s.
Fig. 3.2 (a) The increase in calibrated stimulus (1 – 5) produced RPDs of increasing amplitude until AP was measured in section A and transmitted to section B. For details of the mechano-stimulating set-up see Methods box 3.1a and Shimmen (1996). (b) The K anaesthesia reveals the depolarising direction of the membrane PD response to graded mechano-stimulus (See Methods 3.1a, b and Shimmen 1996). (c) Mechanically induced AP and variation potential (Shimmen, 1996): the cell was mechanically stimulated at “a” and AP was induced. Same strength stimulus elicited RPD at “b”. After the end of refractory period another AP was stimulated at “d” and before the membrane PD repolarised, stimulation of double intensity was applied eliciting variation potential at “e”. Three spontaneous APs can be observed during PD recovery.
Fig. 3.3 (a) Reversal of the RPD direction in depolarized cells (Shimmen, 1997b). The membrane PD was depolarised by adding 100 mM KCl to both chambers. The RPDs of hyperpolarising direction were elicited by increasing stimuli (1 – 5). (b) The direction of RPD depended on the concentration of Cl⁻ in the medium. Same stimulus was used to produce all the RPDs. Cl⁻ concentration was varied from 100 mM (E_{Cl} was calculated as -39 mV by taking cytoplasmic Cl⁻ concentration as 21 mM - Tazawa et al. 1974) to 50 mM (E_{Cl} estimated as -22 mV) to 30 mM (E_{Cl} estimated as -9 mV), with K⁺ concentration kept constant. Note also a positive spike seen in some of the records as shown by a small arrow in (a), which seems to dominate the 30 mM Cl⁻ record in (b).
Fig. 3.4 AP across plasma membrane and tonoplast in different charophytes: (a) AP in *Chara australis* (Findlay and Hope 1964): the top curve is tonoplast AP, the bottom curve the plasma membrane AP. (b) The AP in *Nitella axillaris* (Barry 1968). The position of the PD-measuring electrode is not specified. The AP shows the post AP hyperpolarisation (see text). (c) The AP across both membranes (top curve), the AP across plasma membrane (middle curve) and the AP across tonoplast in *Nitella flexilis* (Shimmen and Nishikawa 1988). (d) AP across tonoplast (upper curve) and plasma membrane (bottom curve) in *Nitellopsis obtusa* (Findlay 1970).
Fig. 3.5 Comparison of AP and clamp current timing at different temperatures. (a) AP across plasma membrane of *Chara australis* as function of temperature (Beilby and Coster 1976). (b) Clamp currents across plasma membrane of *Chara australis* at ~ 20 °C (Beilby 1982). (c) Clamp currents across plasma membrane of *Chara australis* at 4 °C, 8.5 °C and 30 °C (Beilby and Coster 1979c).
Fig. 3.6 The effect of tonoplast removal in cell perfusion (a) Chara AP retains definite stimulation threshold, but the duration is greatly increased and variable. (Shimmen et al 1976). (b) There is no refractory period (Shimmen et al. 1976). (c) Excitation clamp currents across plasma membrane of tonoplast free perfused cell of Nitellopsis obtusa, compared with (d) clamp currents obtained from an intact cell (Beilby et al 1993).
Fig. 3.7 (a) Currents elicited by voltage clamps to negative levels, indicated on each curve. The clamping protocol held the membrane PD at the resting level for 2 sec, then at desired negative level for 12 sec and then returned to pre-clamp resting level for another 2 secs. The current at -500 mV became so large that the amplifier railed. The inside electrode was impaled in the vacuole. (b) The depolarisation after each negative clamp, (lasting for 16 secs), shown on this timescale as vertical line. The experiments were performed by German exchange student, Sebastian Westermann, as part of his physics project.
Fig. 3.8  (a) Currents elicited by voltage clamp to -460 mV in APW (0.1 mM Ca\textsuperscript{2+}) shown as dark line, and with Ca\textsuperscript{2+} increased to 1.0 mM (gray line). (b) Currents elicited by voltage clamp to -460 mV in APW (dark line) and APW with 0.1 mM La\textsuperscript{3+} (grey line). Same cell as in (a). (c) I/V characteristics of Chara cell in steady state in APW (filled circles, continuous dark line) and at the times of post-clamp (to -490 mV) depolarisation: 2 min (full triangles, long-dashed line), 3 min (stars, dotted line), 5 min (empty rectangles, unequally dashed line) and 10 min (filled rectangles, grey line). The I/V characteristics have been fitted with background current (see chapter 2) and H\textsuperscript{+}/OH\textsuperscript{-} current at 2 and 5 min and recovering pump current at 5 and 10 min. The “bump” between -150 and -200 mV on the 2 min I/V profile was not modelled and could originate from time dependent closure of either Cl\textsuperscript{-} or H\textsuperscript{+}/OH\textsuperscript{-} channels. The H\textsuperscript{+}/OH\textsuperscript{-} current and pump currents are shown in (d) with same types of line as in (c).
Fig. 3.9 (a) An example of the wounding response with $L_B$ of ~ 2 mm (see Methods Box 3.1c). V cell was cut at upward arrow, slow response started at “a”, AP generated in chamber B at “b”, AP propagated into chamber A at “c”, small spikes appeared at “d” and repetitive propagated APs started at “e” (from Shimmen 2002). (b) The rapid component isolated by depolarising the cell PD by adding 100 KCl (record A) or $K_2SO_4$ (record B) to both chambers (Shimmen 2002).
Fig. 3.10 Steady state turgor of *Lamprothamnium* (a) and concentrations of Cl\(^-\), K\(^+\), Na\(^+\) and sucrose in the *Lamprothamnium* vacuole (b) as a function of osmotic pressure of the external medium (Bisson and Kirst, 1980a).
Fig. 3.11 (a) I/V characteristics of *Lamprothamnium* acclimated to media of increasing salinity: 0.2 SeaWater (SW), continuous line; 0.4 SW, short-dashed line; 0.5 SW, unevenly dashed line; full SW, long-dashed line. The currents have been fitted to data from 6 – 8 cells from each medium (Beilby and Shepherd 2001). The pump (b) and background (c) current components are shown with same line types. The fit parameters are given in Table 2 of Beilby and Shepherd (2001). The fitting of background current in media 0.4 – full SW was supported by comparison with cells in background state in each medium.
**Fig. 3.12 (a)** I/V characteristics from one *Lamprothamnium* cell acclimated to 0.2 SW and challenged by 0.4 SW. Steady state I/V in 0.2 SW: 1, data points as full circles, continuous thin line; 5 min of 0.4 SW: 2, full triangles, short-dashed line; 41 min of 0.4 SW: 3, stars, long-dashed line; 2 hr 34 min of 0.4 SW: 4, empty squares, thick grey line; 3 hrs 30 min of 0.4 SW: 5, full squares, unevenly dashed line (Beilby and Shepherd 2001). The pump (b) and background (c) current components are shown with same line types. The fit parameters are given in Table 3 of Beilby and Shepherd (2001).
Fig. 3.13 Comparison of responses to increase in salinity and to equivalent increase in osmolarity (a) I/V characteristics from 9 *Lamprothamnium* cells acclimated to 1/6 SW (continuous line, triangles) and at most negative PD in 1/3 SW (dashed line, empty squares). (b) The fitted pump currents are shown with the same types of line as in (a). (c) The fitted background and rectifier currents are shown by the same types of line as in (a) and (b). The fit parameters are given in Table 2 of Al Khazaaly and Beilby (2007). (d) I/V characteristics from 10 *Lamprothamnium* cells acclimated to 1/6 SW and displaying K⁺ state characteristics (continuous line, triangles) and at most negative PD in 1/3 SW (dashed line, empty squares). (e) The fitted pump and K⁺ currents are shown with the same types of line as in (d). (f) The fitted background and rectifier currents are shown by the same types of line as in (d) and (e). The fit parameters are given in Table 5 of Al Khazaaly and Beilby (2007).
Fig. 3.14 The transporters activated at the time of hypotonic regulation in young *Lamprothamnium* cell with minimal mucilage upon transfer from 1/3 ASW to 1/6 ASW. (a) The I/V characteristics based on currents fitted to cells with Cl\(^-\) current blocked by exposure to LaCl\(_3\) or K\(^+\) current blocked by TEA at times: 3 min (continuous line); 10 min (dotted line); 15 min (long dashed line); 20 min (dash dotted line); 30 min (short dashed line) (Beilby and Shepherd 1996, Beilby and Shepherd 2001b). (b) The fitted Cl\(^-\) currents are shown with the same types of line as in (a). They appear with a slight delay after hypotonic shock at 10 and 15 min, starting to decline at 20 min. (c) The fitted K\(^+\) currents are shown by the same types of line as in (a). They appear with a greater delay at 15 min, 20 min and 30 min. (d) The fitted background currents at same times as in (a). Note the different scales in (a), (b), (c) and (d). The K\(^+\) currents are smaller than the Cl\(^-\) currents, but in some cells they persist for a longer time up to 60 min after hypotonic shock.
Fig. 2.1 The distribution of mucilage on *Lamprothamnium* plants. The cells were stained with Alcian Blue at pH 1 (Beilby et al. 1999). (a) Apical cell acclimated to $\frac{1}{2}$ ASW. Mucilage is only ~ 7 µ thick and only small clumps show staining. Bar = 50 µ. (b) Third internode from the apex from a plant acclimated to full ASW. Mucilage is ~ 28 µ. Bar = 100 µ. (c) Seventh internode of the same plant as in (b), mucilage thickness is ~ 43 µ. Bar = 100 µ.
Fig. 3.15 (a) A typical trend in the I/V characteristics from pump-dominated profile (empty rectangle, continuous line) in Sorbitol APW to background-dominated profile after 67 min in Saline APW (filled triangles, dotted line). After 117 min of Saline APW the I/V characteristics continue to change, exhibiting upwardly concave profile (filled diamonds, long-dashed line). The experimental data are fitted by the pump or OH channel models (b) and background current and inward rectifier models (c), using same line types as in part (a). The parameters are given in caption of Fig. 1 of Beilby and Al Khazaaly (2009). The thin continuous lines show the extrapolation of the models beyond the range of the data.
Fig. 3.16 Action potentials at the time of saline stress. (a) Spontaneous AP after the Chara cell was exposed to Saline APW (50 mM NaCl, 0.1 mM CaCl$_2$) for 18 min. (b) The duration of the AP in the same cell has increased considerably after 84 min of Saline APW. (c) Spontaneous repetitive APs after more than 60 min in more concentrated Saline APW (100 mM NaCl, 0.1 mM CaCl$_2$) (Shepherd et al 2008).
Fig. 3.17 The resting PD of a *Chara* cell acclimated to 90 mM Sorbitol APW and then exposed to Saline APW of same osmolarity (Al Khazaaly et al 2009). A spontaneous AP was observed in 20 sec after medium change.
Fig. 3.18 The interaction of membrane PDs and Ca$^{2+}$ signalling. The left side of the figure shows the PD profile across the main cell compartments. The filled block arrows signify electrical stress that depolarises or hyperpolarizes the membrane. This could be passage of current via voltage clamp or a current source, inhibition of the proton pump or a flow of ions. The right side of the figure outlines the cross section from the outside medium, cytoplasm and vacuole with the delimiting plasma membrane and tonoplast. The cytoplasm contains Ca$^{2+}$ stores, such as endoplasmic reticulum (ER). The empty block arrows signify mechanical stress, either due to touch or blow or movement of water due to osmotic or saline stress. The Cl$^-$ channels on both membranes are Ca$^{2+}$-activated. The Ca$^{2+}$ might pass through non selective cation channels or specific Ca$^{2+}$ channels. See text for more details.