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Methods to Study Plant Programmed Cell Death.

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Summary:

Programmed cell death (PCD) is a critical component of plant development, defence against invading pathogens and response to environmental stresses. In this chapter we provide detailed technical methods for studying PCD associated with plant development or induced by abiotic stress. A root hair assay, or electrolyte leakage assay, are excellent techniques for the quantitative determination of PCD, and cellular injury induced in response to abiotic stress, whereas the lace plant provides a unique model that facilitates the study of genetically regulated PCD during leaf development.

1. Introduction

Programmed cell death (PCD) is defined as the sequence of potentially interruptible events leading to controlled destruction of the cell [1, 2]. PCD is a fundamental process that can be activated throughout the entire plant life cycle. PCD occurs as an essential component of several highly specialized developmental programmes, such as xylogenesis [3], leaf shape remodelling [4], suspensor elimination during embryogenesis [5] or during organ senescence [6, 7]. PCD is also a facet of plant responses to biotic and abiotic stresses and can be activated in response to fungal, bacterial or viral infections. The rapid death of plant cells surrounding the site of avirulent pathogen infection, known as the hypersensitive response, is thought to restrict pathogen growth [8]. PCD also occurs in response to abiotic stresses such as cold, waterlogging, salinity and hypoxia [1]. While high levels of cell death may result in the death of whole organism it has been suggested that judicious activation of PCD during stress may be a part of an adaptation response that eliminates cells, tissues or organs that could potentially render the plant more vulnerable to adverse conditions [9].

In this chapter we present three methods to monitor and quantify PCD in plants. Firstly, we describe a system for studying a developmental PCD that sculpts the leaf morphology of the lace plant (*Aponogeton madagascariensis*). The lace plant is an aquatic monocot producing leaves with perforated lamina, formed by the genetically regulated PCD of redundant cells during leaf development [4]. Next we describe two techniques for quantitative determination of PCD induced by abiotic stress that can be used to determine death rates in practically all plant species. The root hair assay can be used for determination of PCD rates in root hairs, here used with perennial ryegrass (*Lolium perenne*) following PCD induced by salinity or cold, while electrolyte leakage is used to measure cellular damage in leaf discs, here used with Arabidopsis after PCD induced by oxidative stress.

2. Materials

2.1. Growth of lace plant and live cell imaging of developmental PCD.

2.1.1 Axenic lace plant cultures

1. Murashige and Skoog (MS) half-strength media containing 0.4 mg/l thiamine HCL, 100 mg/l myo-inositol, 30 g/l sucrose and 2.15 g/l MS basal salt mixture is dissolved in distilled water and adjusted to pH 5.7 with 1M NaOH prior to autoclaving. Half-strength solid MS is

prepared by adding 1% agar to the aforementioned mixture. Following autoclave-sterilization store excess materials at 4^{o}C and bring to room temperature before use.

2. Axenic cultures were established according to Gunawardena *et al*. (2006) and propagated in Magenta GA-7 Plant Culture boxes (bioWORLD, Dublin, OH, USA).

3. Other materials: scissors, two glass beakers, glass Petri dishes and distilled water, all of which are pre-sterilized and sealed prior to tissue culturing.

2.1.2 Aquarium lace plants

1. Filtered freshwater aquarium with gravel substrate and full spectrum LED light canopy (Fluval, Baie d'Urfé, QC, Canada).

2. Fertilizers include: monopotassium phosphate, CSM + B plantex, potassium nitrate (Aquarium Fertilizers, Napa, CA, USA).

2.1.3 Plastic and glass slides

1. Clear non-glare polystyrene (Plaskolite, Columbus, OH, USA) and glass of thicknesses of 1.25 and 1 mm, respectively are amenable to imaging whole lace plant window stage leaves. Glass provides higher resolution than the polystyrene (Fig. 1), but the polystyrene is easily customizable with common tools and the greater depth allows for larger leaves to be observed in comparison.

2.1.4 Grooved microscope slide creation

1. Lace plant leaves feature a thin lamina (4-5 cell layers thick) with parallel longitudinal and transverse veins, and a prominent central midrib. The midrib widens to its largest point at the petiole base and is smallest at the apical region of the leaf. In order to obtain adequate focal planes for long-term live cell imaging, grooves can be etched into either the polystyrene or glass material using an awl and glass etcher, respectively. The grooves should match the form of the central midrib and consequently it should become larger and deeper to one end.

2.1.5 Valap sealant

1. Valap is a biologically inert sealant consisting of Vaseline petroleum jelly, lanolin and paraffin wax is typically made in equivalent ratios (1:1:1 [w/w/w]) and has a melting point between 45-50^{o}C [10]. For this technique, the paraffin concentration is increased so that the final ratios are 1:1:2 for Vaseline, lanolin and paraffin, respectively. The mixture is placed in

a glass beaker, covered and heated to 100^{o}C. The higher concentration of paraffin increases the hardness of the valap once it cools (thereby reducing risk of smudging objective lenses), while the higher temperature facilitates its application.

2. Other recommended materials: heat source (hot plate), cotton swabs or fine paintbrush, glass beaker with cover

2.1.6 Microscopy, data collection and video processing

1. Custom slide, coverslips, valap sealant, distilled water, transfer pipette, forceps

2. Compound light microscope, for example the Nikon Eclipse 90i research microscope fitted with a DXM1200C digital camera (Nikon Canada Inc., Mississauga, ON, CAN)

3. Image and video acquisition software (e.g. Nikon NIS elements AR)

4. Video processing software such as Adobe Premiere Pro CC (Adobe Systems Inc., San Jose, CA, USA)

2.2. Growth of ryegrass seedlings and the root hair assay for quantification of rates of PCD.

2.2.1. Ryegrass seedlings

1. Sterilizing solution: 20% (v/v) commercial bleach (e.g. Domestos). The final concentration of sodium hypochlorite is approximately 1% (v/v).

2. Sterile distilled water

3. Forceps: sterilize in the autoclave

4. Filter paper (90 mm ⌀): sterilize in the autoclave

5. Sterile plastic Petri dishes (90 mm ⌀)

6. 15 ml Falcon tubes

7. Parafilm

2.2.2. AL-PCD induction in ryegrass seedlings.

1. Forceps

2. 12-well culture plates, distilled water

NaCl treatment: sterile NaCl stock solution (1M). Dilute to required concentration in distilled water directly prior to use.

Cold treatment: ice, polystyrene box, access to 4^{o}C cold room.

2.2.3. AL-PCD scoring

1. Microscope slides and cover slips

2. Surgical blades and forceps

3. Fluorescein diacetate (FDA) 0.1% w/v stock solution in acetone (store at -20^{o}C). Dilute to 100 x (final FDA concentration 0.001 % w/v) in distilled water directly prior to use.

4. Phase contrast microscope with a FITC (fluorescein isothiocynate) filter and an attached fluorescence lamp

5. Mechanical counters

2.3. Growth of Arabidopsis plants, PCD induction and leaf discs ion leakage measurements.

2.3.1. Ion leakage measurement

1. Compact Conductivity meter e.g. Horiba B-771 LAQUA twin from LAQUA Horiba Scientific (*see* **Note 1**)

2. Calibration buffer for the conductivity meter: 0.01M KCl (calibration for 1.41mS·cm^{-1} of conductivity, Horiba)

3. Absorbing paper sticks: cut blue-roll in 3cm by 2 cm pieces, fold each longitudinally to fit into the conductivity meter chamber (3cm x 0.5 cm)

4. Cell culture multi-well plate (24-well), no need for coating

5. A 3mm metal cork-borer.

2.3.2. PCD induction for positive controls

1. UVC induction using a DNA-crosslinker e.g. stratalinker 1800 (Stratagene LtD) or a CL-1000 UV crosslinker, (UVP LtD)

2. H_2O_2, 30%, Sigma

2.3.3. Plant growth

Sow Arabidopsis seeds onto moist compost in plastic pots (10 cm x 10 cm) and cover the pots with Clingfilm. To break seed dormancy, the plastic pots are placed in the cold (+4^{o}C) for three days and then transferred to short days, 8 hours light, at 22^{o}C. After one to two weeks seedlings are separated and grown either individually or five to a 10 cm x 10 cm pot or individually using Jiffy-7C coir pellet. The plants used for ion leakage test should have large leaves void of any bleaching or mottling.

3. Methods

3.1. Studying developmental PCD in lace plant leaves.

The aquatic lace plant produces leaves with perforated lamina (Fig. 3A) [4]. These perforations form as superfluous cells that are removed by means of genetically regulated PCD during leaf development (Black arrow; Fig. 3A). There are five stages of leaf development relating to perforation formation: preperforation, window, perforation formation, expansion and mature perforation [4]. Preperforation stage leaves are those that are tightly furled as they emerge from the corm and have anthocyanin pigmentation throughout the entire lamina. PCD actively occurs in window stage leaves, which exhibit a gradient of developmental PCD distinguishable by pigmentation [11]. Cells that do not undergo PCD during leaf formation are found in the region demarcated by the red anthocyanin-containing mesophyll cells (NPCD; Fig 3B, C). Adjacent to these are green cells in the early phases of PCD that have lost anthocyanin and are fated to die, but still retain abundant chlorophyll pigmentation (EPCD; Fig. 3B, D). Finally, the nearly transparent cells with little to no pigmentation remaining are nearest to death (LPCD; Fig. 3B, E). As cells are deleted a small, centralized perforation forms, which then expands as the wave of cell death continues before halting 4-5 cell layers from the veins by the time the leaf reaches maturity.

The accessibility and predictability of PCD, along with the plant's thin and nearly-transparent leaves, that are ideal for live cell imaging, make it a tractable model system for studying

developmental PCD [12-14]. The timing and intracellular dynamics of this unique form of PCD was described by Wertman *et al*. [14] using various stains and live cell imaging techniques including one that allowed for the continual observation of a whole leaf for several days. The aim of this section is to provide a detailed description of that long term live cell imaging protocol and to suggest alternative strategies that we suspect will be applicable within other systems.

3.1.1. Lace plant propagation and tissue preparation

Axenic lace plant cultures were originally provided by Dr. Michael Kane (University of Florida) and propagated according to Gunawardena *et al*. [12]. Using a UV laminar flow hood and aseptic techniques, senescent leaves are cut away using scissors and forceps. In a sterile Petri dish, the mother-corm and any small cormels produced via clonal propagation are separated and dead tissues are removed using a scalpel blade and forceps. When not in use, utensils are stored in a beaker containing 95% ethanol. Newly cleaned corms are planted in pre-autoclaved Magenta GA-7 vessels containing 50 ml of solid half-strength MS media and then 150 ml of the liquid media version is added prior to sealing with a PVC film (PhytoTechnology Laboratories). The cultures are stored at 24^{o}C and exposed to 125 µmol $m^{-2}s^{-1}$ fluorescent light on a 12 h light/dark cycle.

Alternatively, lace plant corms can be grown in filtered freshwater aquaria with a gravel substrate. The water is supplemented weekly with 1 mg/l monopotassium phosphate, 3 mg/l CSM + B plantex and 10 mg/l potassium nitrate (Aquarium Fertilizers, Napa, California, USA). Full spectrum LED canopy lights are operated on a 12 hr light/dark cycle (Fluval). Every 1-2 weeks (or as necessary), algae growths on the sides of an aquarium are removed using a brush after which 10% of the water is changed for fresh distilled water.

Plants are grown until they reach maturity and develop perforated leaves. Leaves of the window stage of development are removed at the petiole base and then rinsed thoroughly using distilled water to remove excess sucrose, debris, or possible contaminants. We recommend testing the stress limits of the tissue being used in this technique (*see* **Note 2**).

3.1.2 Tissue mounting and sealing

A window stage leaf is carefully placed into the groove of the custom slide so that the leaf blade lies flat on the slide surface. The specimen is mounted in distilled water and

coverslipped. Pre-heat valap to 100°C in a covered beaker on a hot plate. Apply the melted valap with a cotton swab to seal the slide. After an initial coating, check for air bubbles and re-apply valap where necessary to produce a complete seal.

3.1.3 Long term live cell imaging

Long term live cell imaging of lace plant window stage leaves (48-72 hr) allows for the direct observation of the cytological features of lace plant PCD during perforation formation. A window stage leaf from sterile culture or the aquarium is mounted on the custom slide and sealed as describe above. Reference images are taken for comparison to the live image in order to maintain focus. Objectives with a higher numerical aperture with smaller depth of field require monitoring and fine adjustments in order to reduce alterations of focus from sources including, but not limited to: heat, vibrations, mechanical drift, as well as leaf growth and perforation formation as PCD progresses. If done manually, this requires a great deal of labor and diligence, but technologies can be used to minimize workload (*see* **Note 3**). Tissue health must be monitored throughout the process as well (*see* **Note 2**). In order to maintain tissue integrity throughout the time series, lace plant window stage leaves are rinsed with distilled water, re-mounted in the same orientation and sealed (as described before) every 6 h. Valap can easily be removed from the custom slide. First remove the coverslip and bulk of the sealant using a razorblade then rinse the slide with hot water (> 45 °C) and wipe off any remaining valap if necessary.

3.1.4 Video preparation

Lace plant PCD is a gradual process with initial features such as the loss of anthocyanin and a reduction of chlorophyll pigmentation occurring in early window stage leaves over 48 hr prior to cell collapse (Supplementary video 1). Although many cytological features gradually unfold over this two day period, the final dramatic events including nuclear displacement, tonoplast rupture and vacuolar and plasma membrane collapse occur within the final 15-20 minutes of lace plant PCD [13] (Supplementary video 2). In order to capture these sudden changes continuous videos are recorded using Nikon NIS Elements AR software in six hour segments which correspond to the tissue re-mounting steps (*see* **Note 4**). Videos are then re-assembled, cropped and edited for playback speed and then compressed using Adobe Premiere Pro CC.

3.2. Quantitative determination of abiotic stress induced PCD in ryegrass root hairs.

The method described is adapted from Hogg *et al.* 2011 [15] and Kacprzyk and McCabe, 2015 [16]. The root hair assay is a useful system for determination of the rates of apoptosis-like PCD (AL-PCD). AL-PCD in root hair cells is induced by moderate levels of abiotic stress and is characterised by a distinct morphology: condensation of cytoplasm and retraction of the protoplast away from the cell wall [15, 17]. In contrast, higher levels of abiotic stress induce root hairs to undergo necrotic cell death, readily distinguished from AL-PCD by the lack of protoplast retraction [15, 17]. Below we describe application of a root hair assay to score the effect of two agriculturally relevant abiotic stresses (cold and salinity) in the important pasture and forage species, perennial ryegrass (*Lolium perenne*). Previously, we described a detailed protocol for application of the root hair assay in model plants *Arabidopsis thaliana* and *Brachypodium distachyon* [15, 16].

3.2.1. Growth of ryegrass seedlings

Ryegrass seeds are soaked for 2 hr in sterile distilled water and sterilized in the laminar flow hood using aseptic techniques (*see* **Note 5**). Twelve ml of sterilizing solution are added to the seeds in a 15 ml Falcon tube. Seeds are incubated in the sterilizing solution for 5 min with mixing by inversion every minute. The sterilizing solution is poured off and seeds are thoroughly washed 5 times with sterile distilled water. Single discs of filter paper are placed in 9 cm ⌀ Petri dishes and 6 ml of sterile distilled water is added to each Petri dish to moisten the filter paper. Ryegrass seeds are placed on the surface of filter paper using sterile forceps (approximately 20 seeds per Petri dish). Plates are sealed with Parafilm and seeds vernalized in the dark at 4^{o}C for 2 days. Seeds are germinated at 22^{o}C, in the dark. Seedlings are used for experiments when 4 days old.

3.2.2 Induction of AL-PCD in ryegrass seedlings.

Use of 12-well culture plates provide a convenient system for the treatment of ryegrass seedlings. Here, we present a protocol for the two cell death inducing treatments, each stress being highly relevant to agriculture: cold and salinity (NaCl).

NaCl treatment: wells of 12-well cultures plate are filled with 1 ml of NaCl solution. Four day old ryegrass seedlings are transferred to individual wells (1 plant/well) using forceps. The seedlings are incubated in the dark at 22^{o}C until scoring for PCD rates 24 hr later. Typical

rates of AL-PCD induced by 24 hr treatment with 100 mM NaCl are approximately 50 % (Fig. 4A).

Cold treatment: wells of a 12-well culture plate are filled with 1 ml of sterile distilled water and pre-chilled for 1 hr. To pre-chill, culture plates are placed inside a polystyrene box half-filled with ice. The well plates are covered with more ice to fill the whole polystyrene box, which is then closed and placed in a 4^{o}C cold room. According to our measurements, the water temperature inside the wells of 12-well plate in that system is maintained at 1^{o}C (±0.5^{o}C) for at least 24 hr. After the 12-well plates are pre-chilled, the ryegrass seedlings are transferred to the individual wells using forceps (1 plant/well) and plates are further incubated at 1^{o}C (±0.5^{o}C) using the same system until scoring for PCD rates 24 hr later. (*see* **Note 6**). Typical rates of AL-PCD induced by 24 hr treatment at 1^{o}C are approximately 50 % (Fig. 4B).

3.2.3. Fluorescein diacetate staining of ryegrass root hairs

Fluorescein diacetate (FDA) is a hydrophobic, cell-permeant compound that is cleaved by the cytoplasmic esterases in living cells, yielding a fluorescent product, fluorescein [18]. Viable cells stained with FDA therefore emit bright green fluorescence when illuminated with light of 490 nm wavelength. The FDA stock is 100 times diluted in distilled water directly prior to use. For examination of root hairs viability, the radicles of ryegrass seedlings are cut off with a blade and stained directly on the microscope slide with 100 µl of FDA solution. Roots are gently covered with the cover slip and immediately examined under a phase contrast microscope with a FITC filter and attached fluorescence lamp.

3.2.4. The root hair assay.

The root hair assay is based on observation of dying root hairs morphology. Start examining root hairs from the root tip. The FDA stained root hairs exhibiting green fluorescence under fluorescent light are categorized as viable (Fig. 5A, B). The FDA negative root hairs are categorized as PCD if they exhibit condensation of the cytoplasm and protoplast retraction away from the cell wall (Fig. 5C, D) (*see* **Note 7**). Root hairs which are FDA negative and do not present AL-PCD morphology are scored as necrotic (Fig. 5E, F). On average, 100-150 root hairs per seedling are scored and results are recorded using mechanical counters.

3.3. Determination of cellular damage using ion leakage.

Ion leakage is a convenient cell death assay using an inexpensive piece of equipment. The rationale is that as cells die in the treated tissue, they release ions into the external medium. Measuring the conductivity changes of the medium provides a quantitative measure of ion leakage from plant tissues and therefore of cell death. Depending of the experimental system used, conductivity measurements can be carried out on leaf discs [19], seedlings [20], or leaf discs punched out of a leaf at various time points after PCD induction. The protocol below is given for *Arabidopsis* leaf discs that receive a PCD-inducing treatment and are then monitored overtime. As an illustration, conditions for two PCD-inducing treatments are given. These treatments could be used as positive controls for other experimental systems.

3.3.1. Preparation of Arabidopsis leaf discs.

Use Arabidopsis plants that are 3 to 4 weeks old and select leaves of similar physiological age so to minimise variability. Leaves from bolted plants have higher leakage than non-bolted plants. The day before the experiment, water all the plants needed, because water status of the plant affects ion leakage results. Add 1100 µL of distilled water to wells of a 24-well plate. Cut suitable leaves from selected plants and place them abaxial side up, flat, on the inverted lid of a 9 cm plastic Petri dish. Punch out leaf discs using a 3 mm metal borer. Avoid the midrib for the disc tissue to be homogeneous. Use forceps to carefully handle the discs. Cut discs can be left on a piece of blue roll for a short time but do not let leaf discs dry. Float three leaf discs per well, abaxial side down. Handle discs with care using forceps, as tissue damage increases ion leakage. Use triplicate wells for each condition. Successive time-point can be taken from the same well.

3.3.2. Cell death induction and measurement of ion leakage.

Calibrate the conductivity meter using a 0.01 M KCl (calibration for 1.41mS.cm^{-1}). One hour after the discs were floated on distilled water, take a t_0 measure (blank) using 100 µl and a Horiba B-771 conductivity meter (*see* **Note 8**). The final volume in wells is now 1 ml.

PCD-induction, example 1: Leaf discs are irradiated with UV-C in an open 24-well cell culture plate using a UV crosslinker. For higher sensitivity than wildtype use 5 kJ/m^2; for resistance compared to wildtype use 15 to 20 kJ/m^2 (*see* **Note 9**).

PCD-induction, example2: For H_2O_2, 30 mM is added in wells after t_0 has been measured. For sensitivity use 20 mM with Col-0. For resistance use 30 mM with WS and 60 mM with Ler (*see* **Note 9 and 10**).

Incubate the 24-well plate in continuous light (*see* **Note 11**) and measure conductivity over time (*see* **Note 12** for examples of values obtained). To measure conductivity, take 100 µl of water from each well and add to the reading chamber of the conductivity meter (*see* **Note 13**). After each measurement, return as much of the 100 µl to the well as you can, to prevent a loss of volume. As appropriate, rinse the chamber with distilled water until a reading of zero and dry using the paper sticks before the next set of measures (*see* **Note 14 and 15**). Calculate the increase in conductivity in each well by subtracting the pre-treatment conductivity reading from the post-treatment conductivity reading.

4. Notes.

1. http://horiba.com; products; compact meters; B-771 LAQUA twin.
2. Detached leaf health should be tested prior to developing the experimental conditions for live cell imaging. Window stage lace plant leaves kept in Petri dishes will survive and continue growth for 4-5 days in distilled water. Additionally, after each long-term live cell imaging experiment the leaf blade should be visually scanned in order to ensure that the conditions did not have a cytotoxic effect in other parts of the organ.
3. The use a mechanical stage and Nikon's Perfect Focus (PFS) hardware would facilitate these observations. PFS allows for drift compensation while a mechanical stage allows for a wider area to be scanned and the images can be stitched together during post-acquisition. Additionally, virtual network computing (VNC) software can allow for remote monitoring and control of the specimen and microscope. Continuous video recording yields large data sets compared to still image time series. With our

hardware, stopping video recording periodically reduced instances of video corruption and facilitated downstream video processing.

4. Soaking ryegrass seeds in distilled water for 2 hr prior to sterilization is not an essential part of this protocol but in our experience it enhances the subsequent seeds germination.
5. Temperature controlled chamber may be used instead if available.
6. The gap between the condensed protoplast and cell wall is the hallmark of AL-PCD. Note that the extent of protoplast condensation in AL-PCD root hairs may vary. To make sure that root hairs are correctly categorized as AL-PCD carefully examine them for the presence of AL-PCD morphology by adjusting the focus, illumination and phase contrast of the microscope. See Fig. 6 for examples of different appearances of AL-PCD morphology, ranging from a less advanced cell death phenotype (Fig. 6A) to a readily recognizable gap between the condensed protoplast and cell wall present at a root tip (Fig. 6C). This gap may be also present in the middle part of the root (Fig. 6B). The condensed protoplast can also split into several units (Fig. 6D), which is more frequent in longer root hairs.
7. Untreated leaf discs leach ions due to the wounding inflicted. After one hour the ion leakage reading is stable and constitute the blank for the leaf discs in a given well.
8. The first experiment should aim at calibrating cell death induction for the plant or ecotype used. Consider that various ecotypes have different sensitivity to a given treatment.
9. **Table 1**. Calculation H_2O_2 doses in mM

Final concentration (mM) required	30% stock (µl in 1ml)
0	0.0
5	0.6
10	1.1
20	2.3
30	3.4

40	4.5
50	5.7
60	6.8

10. Some instances of PCD have been shown to be light dependent.
11. See in Fig. 7, examples of conductivity values for increasing UVC dose using Col-0 with readings taken at 18h post-treatment.
12. Pipette a few times before taking the water from each well to homogenise the liquid before measuring. Shaking the multi-well dish for a short while on a slow horizontal shaker is another possibility.
13. Measure all of the replicates for one plant or for one treatment without rinsing as values should be close, then rinse and dry the chamber using the prepared paper sticks before moving to the next set of measures.
14. If you have more than one 24-wells plate to measure, the meter will automatically switch off at some point; usually it will be near the end of the 2nd dish. To prevent this, keep pushing the on/off switch from time to time.

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pathway proteins in the Arabidopsis unfolded protein response. Proc Natl Acad Sci USA 110: 5713–5718

Figure legends:

Figure 1. Comparison of a single window stage lace plant leaf areole using custom polystyrene (A, B) and glass slides (C, D). The glass slide allows for greater resolution. All micrographs were captured using the same settings on a Nikon Eclipse 90i research microscope. Scale bars: A, C = 75 µm; B, D = 25 µm.

Figure 2. Window stage lace plant leaf mounted on a polystyrene custom slide and sealed with valap. The large central midrib of the leaf is resting in a groove that allows the leaf blade to rest squarely on the slide surface. Scale bar = 1.25 cm.

Figure 3. Programmed cell death and the lace plant (*Aponogeton madagascariensis*). (A) The lace plant produces mature leaves with perforations throughout the lamina. These holes are formed via developmentally regulated programmed cell death (PCD), which actively occurs during the window stage of development (black arrow). (B) Window stage leaf areole showing a gradient of cellular death. Non-PCD (NPCD cells; C) persist beyond leaf morphogenesis. Cells in the early phases of PCD (EPCD; D) have lost anthocyanin pigmentation and those in the late phase of PCD (LPCD; E) are nearly transparent due to chloroplast degradation. Scale bars: A = 4 cm; B = 100 µm; C, D = 30 µm.

Figure 4. Rates of AL-PCD, necrosis and viability in ryegrass root hairs following salinity and cold treatments. Four day old seedlings of perennial ryegrass cultivar Arara were subjected to (A) salinity treatment (100 and 500 mM NaCl) and (B) cold treatment (1^{o}C ±0.5^{o}C) over a period of 24 hr. Root hairs showing green fluorescence after FDA staining were scored as alive. Root hairs showing no FDA staining and characterised by retraction and condensation of the cytoplasm were scored as AL-PCD. Root hairs showing neither FDA staining nor protoplast condensation were scored as necrotic. Means (n=4) of AL-PCD, necrosis and viability rates (±SEM) are presented.

Figure 5. Cell death morphology in root hairs of ryegrass. Morphology of a viable root hair viewed under white light (A). Viable root hair fluoresces green after FDA staining when viewed under fluorescent light (B). PCD root hair from a seedling treated with 100 mM NaCl for 24 hr presents AL-PCD morphology, characterized by cytoplasmic condensation and retraction of the protoplast away from the cell wall (arrow) (C) and no fluorescence following the FDA treatment (D). Necrotic root hair from a seedling treated with 500 mM NaCl for 24 hr presents no AL-PCD morphology (E) and no fluorescence after FDA staining (F). Scale bar = 5 µm.

Figure 6. Different appearances of AL-PCD root hairs from ryegrass seedling.

Less advanced, but identifiable retraction of a protoplast at the root tip (A). Retraction of the protoplast away from the cell wall in the middle part of the root hair (B). Readily recognizable retraction of the protoplast away from the cell wall at the root tip (C). Condensed protoplast split into two units (D). Scale bar = 5 μm.

Figure 7. Ion leakage induced by UV-C treatment. Conductivity values at 18 hr for *Arabidopsis* Col-0 leaf discs treated with increasing doses of UVC delivered using a UV crosslinker. Presented values are the mean of three replicates (±SEM).

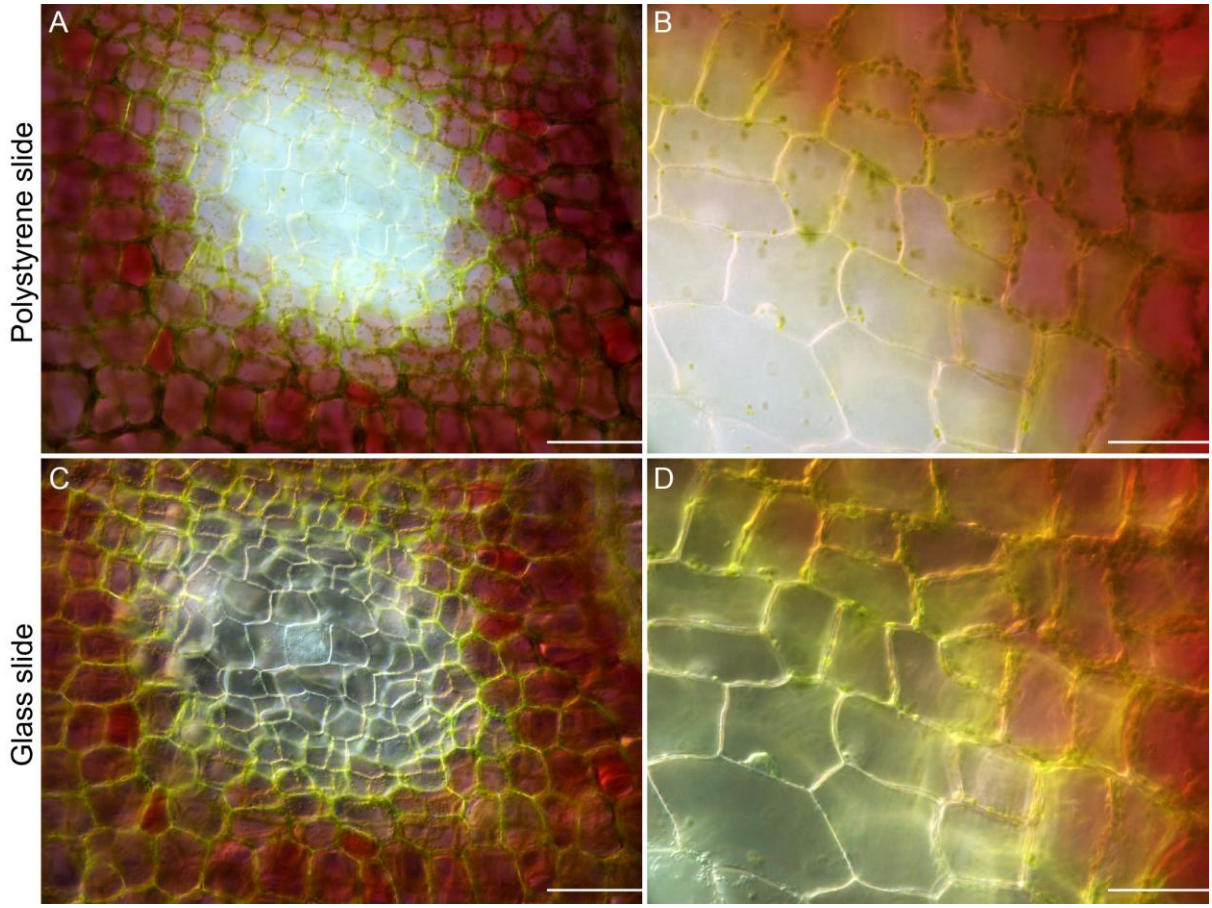

Figure 1

Figure 2

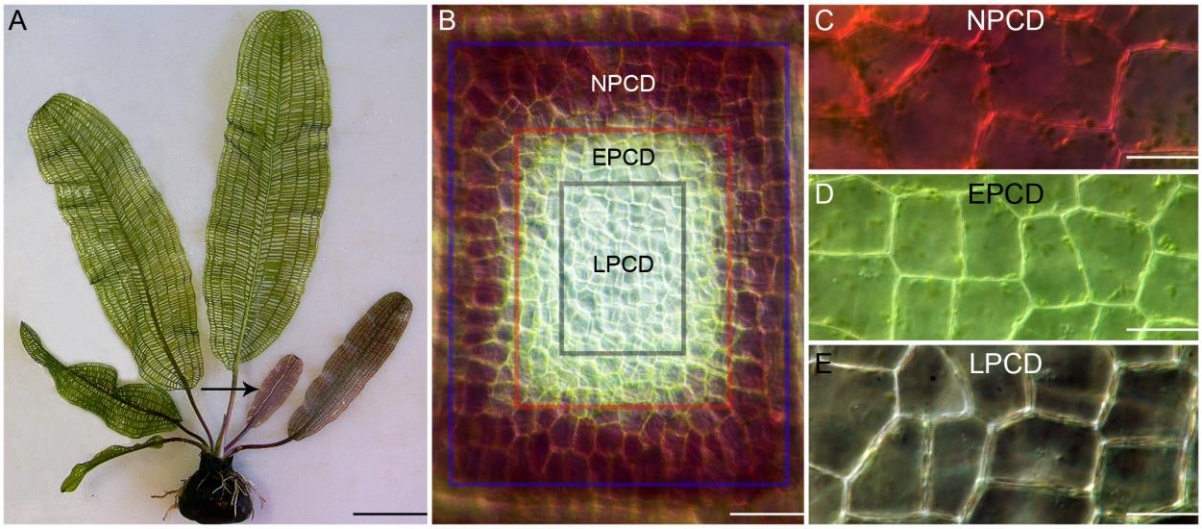

Figure 3

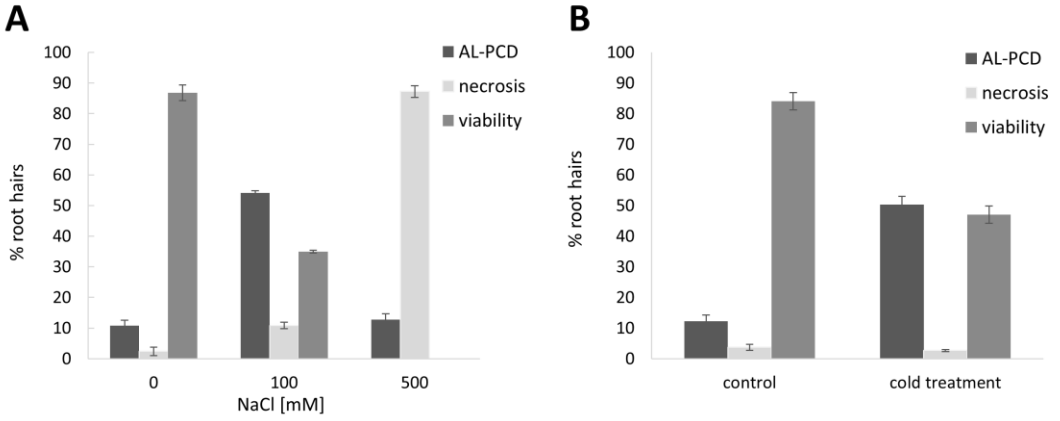

Figure 4

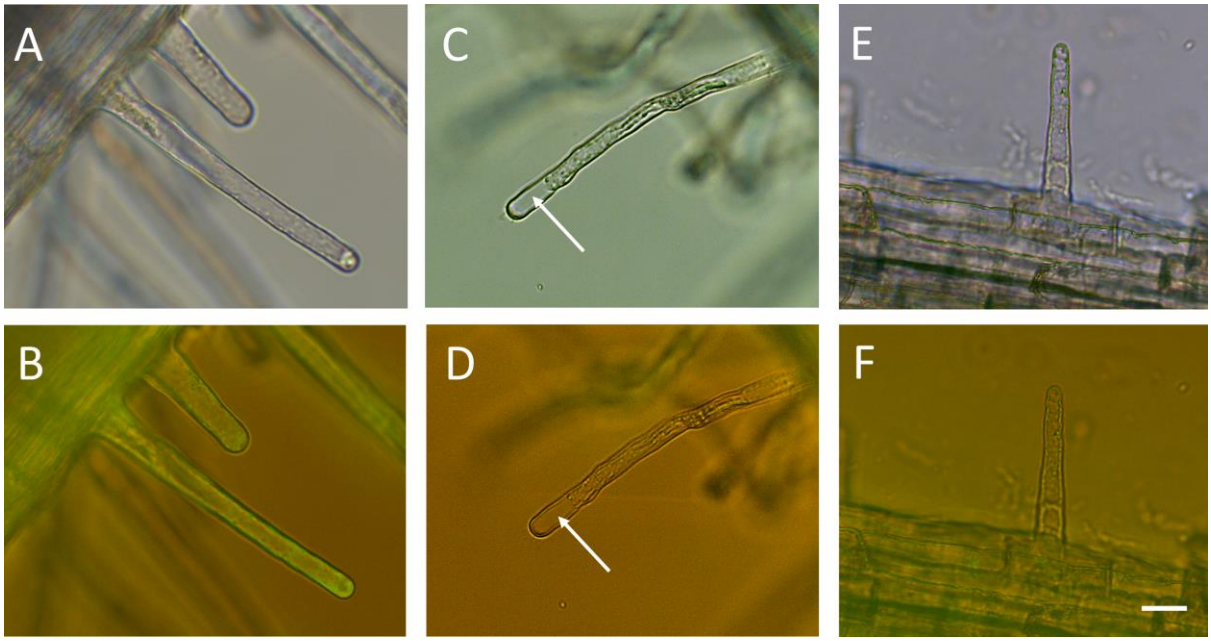

Figure 5

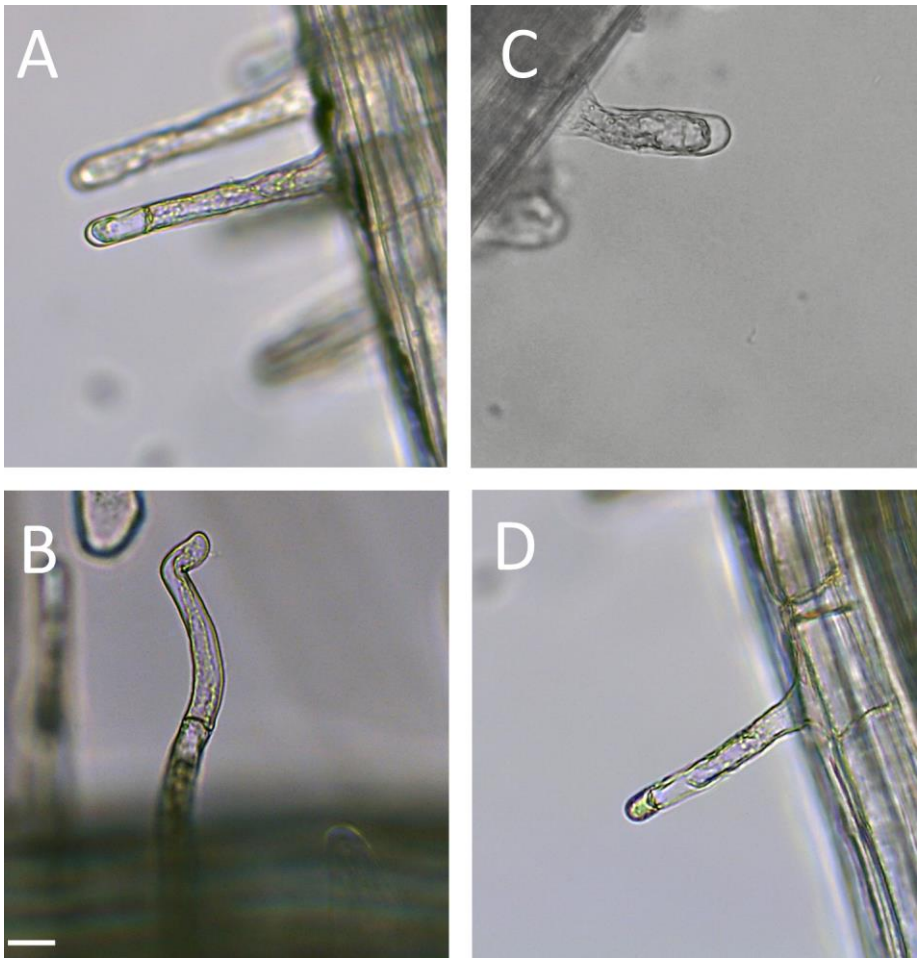

Figure 6

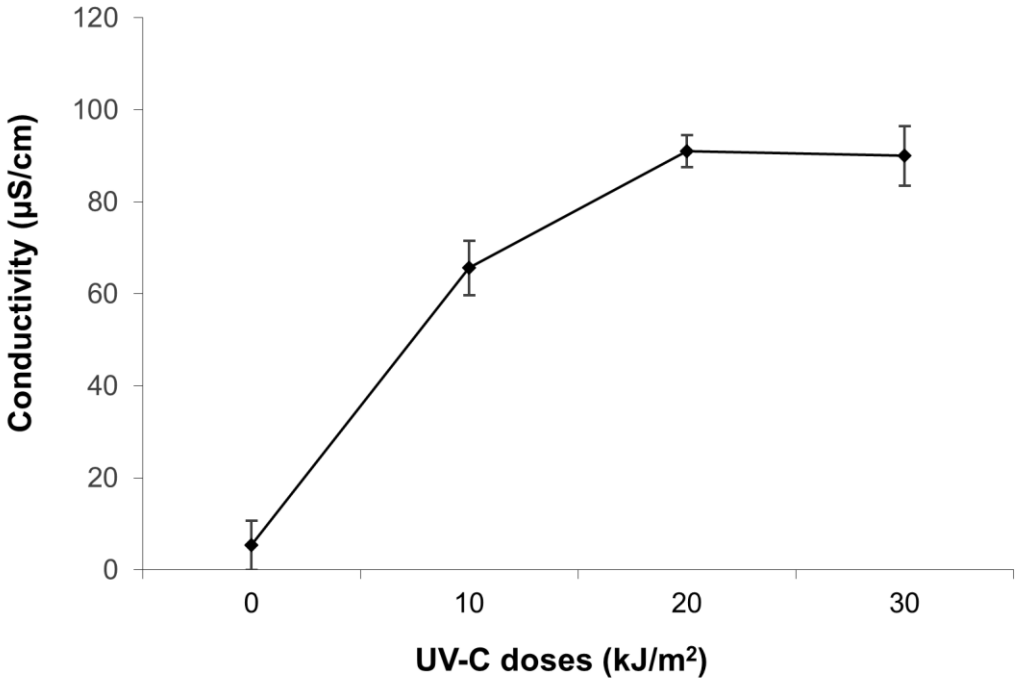
120
100
80
60
40
20
0
Conductivity (µS/cm)
0
10
20
30
UV-C doses (kJ/m²)

Figure 7