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4
5 **Methods to Study Plant Programmed Cell Death.**

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17 **Running head** Plant Programmed Cell Death Methods

18 **Keywords:** programmed cell death, root hair assay, electrolyte leakage, developmental
19 programmed cell death, leaf morphogenesis, abiotic stress

20 **Summary:**

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

28 **1. Introduction**

29 Programmed cell death (PCD) is defined as the sequence of potentially interruptible events
30 leading to controlled destruction of the cell [1, 2]. PCD is a fundamental process that can be
31 activated throughout the entire plant life cycle. PCD occurs as an essential component of
32 several highly specialized developmental programmes, such as xylogenesis [3], leaf shape
33 remodelling [4], suspensor elimination during embryogenesis [5] or during organ senescence
34 [6, 7]. PCD is also a facet of plant responses to biotic and abiotic stresses and can be
35 activated in response to fungal, bacterial or viral infections. The rapid death of plant cells
36 surrounding the site of avirulent pathogen infection, known as the hypersensitive response, is
37 thought to restrict pathogen growth [8]. PCD also occurs in response to abiotic stresses such
38 as cold, waterlogging, salinity and hypoxia [1]. While high levels of cell death may result in
39 the death of whole organism it has been suggested that judicious activation of PCD during
40 stress may be a part of an adaptation response that eliminates cells, tissues or organs that
41 could potentially render the plant more vulnerable to adverse conditions [9].

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

52

53 **2. Materials**

54 **2.1. Growth of lace plant and live cell imaging of developmental PCD.**

55 **2.1.1 Axenic lace plant cultures**

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

59 prepared by adding 1% agar to the aforementioned mixture. Following autoclave-sterilization
60 store excess materials at 4°C and bring to room temperature before use.

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

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

65 **2.1.2 Aquarium lace plants**

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

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

70 **2.1.3 Plastic and glass slides**

71 1. Clear non-glare polystyrene (Plaskolite, Columbus, OH, USA) and glass of thicknesses of
72 1.25 and 1 mm, respectively are amenable to imaging whole lace plant window stage leaves.
73 Glass provides higher resolution than the polystyrene (Fig. 1), but the polystyrene is easily
74 customizable with common tools and the greater depth allows for larger leaves to be observed
75 in comparison.

76 **2.1.4 Grooved microscope slide creation**

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

83 **2.1.5 Valap sealant**

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

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

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

93 **2.1.6 Microscopy, data collection and video processing**

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

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

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

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

100

101 **2.2. Growth of ryegrass seedlings and the root hair assay for quantification of rates of** 102 **PCD.**

103 **2.2.1. Ryegrass seedlings**

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

106 2. Sterile distilled water

107 3. Forceps: sterilize in the autoclave

108 4. Filter paper (90 mm \varnothing): sterilize in the autoclave

109 5. Sterile plastic Petri dishes (90 mm \varnothing)

110 6. 15 ml Falcon tubes

111 7. Parafilm

112 **2.2.2. AL-PCD induction in ryegrass seedlings.**

113 1. Forceps

- 114 2. 12-well culture plates, distilled water
- 115 **NaCl treatment:** sterile NaCl stock solution (1M). Dilute to required concentration in
- 116 distilled water directly prior to use.
- 117 **Cold treatment:** ice, polystyrene box, access to 4°C cold room.

118

119 **2.2.3. AL-PCD scoring**

- 120 1. Microscope slides and cover slips
- 121 2. Surgical blades and forceps
- 122 3. Fluorescein diacetate (FDA) 0.1% w/v stock solution in acetone (store at -20°C). Dilute to
- 123 100 x (final FDA concentration 0.001 % w/v) in distilled water directly prior to use.
- 124 4. Phase contrast microscope with a FITC (fluorescein isothiocyanate) filter and an attached
- 125 fluorescence lamp
- 126 5. Mechanical counters

127

128 **2.3. Growth of Arabidopsis plants, PCD induction and leaf discs ion leakage**

129 **measurements.**

130 **2.3.1. Ion leakage measurement**

- 131 1. Compact Conductivity meter e.g. Horiba B-771 LAQUA twin from LAQUA Horiba
- 132 Scientific (*see Note 1*)
- 133 2. Calibration buffer for the conductivity meter: 0.01M KCl (calibration for 1.41mS·cm⁻¹ of
- 134 conductivity, Horiba)
- 135 3. Absorbing paper sticks: cut blue-roll in 3cm by 2 cm pieces, fold each longitudinally to fit
- 136 into the conductivity meter chamber (3cm x 0.5 cm)
- 137 4. Cell culture multi-well plate (24-well), no need for coating
- 138 5. A 3mm metal cork-borer.

139 **2.3.2. PCD induction for positive controls**

140 1. UVC induction using a DNA-crosslinker e.g. stratalinker 1800 (Stratagene Ltd) or a CL-
141 1000 UV crosslinker, (UVP Ltd)

142 2. H₂O₂, 30%, Sigma

143 **2.3.3. Plant growth**

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

150

151 **3. Methods**

152 **3.1. Studying developmental PCD in lace plant leaves.**

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

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

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

176

177 **3.1.1. Lace plant propagation and tissue preparation**

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

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

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

198 **3.1.2 Tissue mounting and sealing**

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

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

204 **3.1.3 Long term live cell imaging**

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

220 **3.1.4 Video preparation**

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

231

232 **3.2. Quantitative determination of abiotic stress induced PCD in ryegrass root hairs.**

233 The method described is adapted from Hogg *et al.* 2011 [15] and Kacprzyk and McCabe,
234 2015 [16]. The root hair assay is a useful system for determination of the rates of apoptosis-
235 like PCD (AL-PCD). AL-PCD in root hair cells is induced by moderate levels of abiotic
236 stress and is characterised by a distinct morphology: condensation of cytoplasm and
237 retraction of the protoplast away from the cell wall [15, 17]. In contrast, higher levels of
238 abiotic stress induce root hairs to undergo necrotic cell death, readily distinguished from AL-
239 PCD by the lack of protoplast retraction [15, 17]. Below we describe application of a root
240 hair assay to score the effect of two agriculturally relevant abiotic stresses (cold and salinity)
241 in the important pasture and forage species, perennial ryegrass (*Lolium perenne*). Previously,
242 we described a detailed protocol for application of the root hair assay in model plants
243 *Arabidopsis thaliana* and *Brachypodium distachyon* [15, 16].

244 **3.2.1. Growth of ryegrass seedlings**

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

255 **3.2.2 Induction of AL-PCD in ryegrass seedlings.**

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

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

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

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

274

275 **3.2.3. Fluorescein diacetate staining of ryegrass root hairs**

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

284 **3.2.4. The root hair assay.**

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

292

293 **3.3. Determination of cellular damage using ion leakage.**

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

303 **3.3.1. Preparation of Arabidopsis leaf discs.**

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

315

316 **3.3.2. Cell death induction and measurement of ion leakage.**

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

320

321 **PCD-induction, example 1:** Leaf discs are irradiated with UV-C in an open 24-well cell
322 culture plate using a UV crosslinker. For higher sensitivity than wildtype use 5 kJ/m²; for
323 resistance compared to wildtype use 15 to 20 kJ/m² (*see Note 9*).

324

325 **PCD-induction, example2:** For H₂O₂, 30 mM is added in wells after t₀ has been measured.
326 For sensitivity use 20 mM with Col-0. For resistance use 30 mM with WS and 60 mM with
327 Ler (*see Note 9 and 10*).

328

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

337

338 **4. Notes.**

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

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

353 4. Soaking ryegrass seeds in distilled water for 2 hr prior to sterilization is not an
354 essential part of this protocol but in our experience it enhances the subsequent seeds
355 germination.

356 5. Temperature controlled chamber may be used instead if available.

357 6. The gap between the condensed protoplast and cell wall is the hallmark of AL-PCD.
358 Note that the extent of protoplast condensation in AL-PCD root hairs may vary. To
359 make sure that root hairs are correctly categorized as AL-PCD carefully examine them
360 for the presence of AL-PCD morphology by adjusting the focus, illumination and phase
361 contrast of the microscope. See Fig. 6 for examples of different appearances of AL-
362 PCD morphology, ranging from a less advanced cell death phenotype (Fig. 6A) to a
363 readily recognizable gap between the condensed protoplast and cell wall present at a
364 root tip (Fig. 6C). This gap may be also present in the middle part of the root (Fig. 6B).
365 The condensed protoplast can also split into several units (Fig. 6D), which is more
366 frequent in longer root hairs.

367 7. Untreated leaf discs leach ions due to the wounding inflicted. After one hour the ion
368 leakage reading is stable and constitute the blank for the leaf discs in a given well.

369 8. The first experiment should aim at calibrating cell death induction for the plant or
370 ecotype used. Consider that various ecotypes have different sensitivity to a given
371 treatment.

372 9. **Table 1.** Calculation H₂O₂ 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

373

374

375 10. Some instances of PCD have been shown to be light dependent.

376 11. See in Fig. 7, examples of conductivity values for increasing UVC dose using Col-0
377 with readings taken at 18h post-treatment.

378 12. Pipette a few times before taking the water from each well to homogenise the liquid
379 before measuring. Shaking the multi-well dish for a short while on a slow horizontal
380 shaker is another possibility.

381 13. Measure all of the replicates for one plant or for one treatment without rinsing as
382 values should be close, then rinse and dry the chamber using the prepared paper sticks
383 before moving to the next set of measures.

384 14. If you have more than one 24-wells plate to measure, the meter will automatically
385 switch off at some point; usually it will be near the end of the 2nd dish. To prevent
386 this, keep pushing the on/off switch from time to time.

387

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395

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448

449 **Figure legends:**

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

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

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

465 **Figure 4. Rates of AL-PCD, necrosis and viability in ryegrass root hairs following**
466 **salinity and cold treatments.** Four day old seedlings of perennial ryegrass cultivar Arara
467 were subjected to (A) salinity treatment (100 and 500 mM NaCl) and (B) cold treatment (1°C
468 $\pm 0.5^{\circ}\text{C}$) over a period of 24 hr. Root hairs showing green fluorescence after FDA staining
469 were scored as alive. Root hairs showing no FDA staining and characterised by retraction and
470 condensation of the cytoplasm were scored as AL-PCD. Root hairs showing neither FDA
471 staining nor protoplast condensation were scored as necrotic. Means (n=4) of AL-PCD,
472 necrosis and viability rates ($\pm\text{SEM}$) are presented.

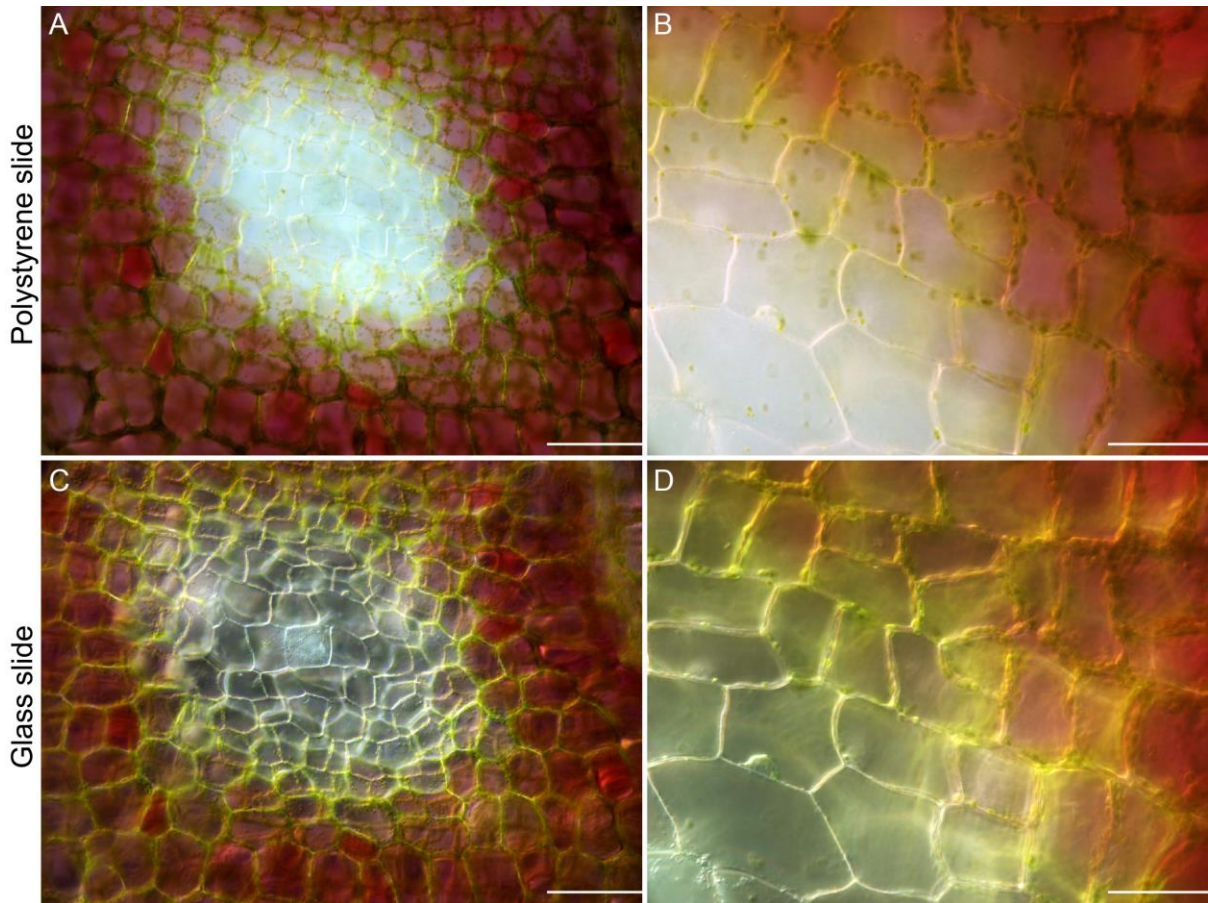
473 **Figure 5. Cell death morphology in root hairs of ryegrass.** Morphology of a viable root
474 hair viewed under white light (A). Viable root hair fluoresces green after FDA staining when
475 viewed under fluorescent light (B). PCD root hair from a seedling treated with 100 mM NaCl
476 for 24 hr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

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

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

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

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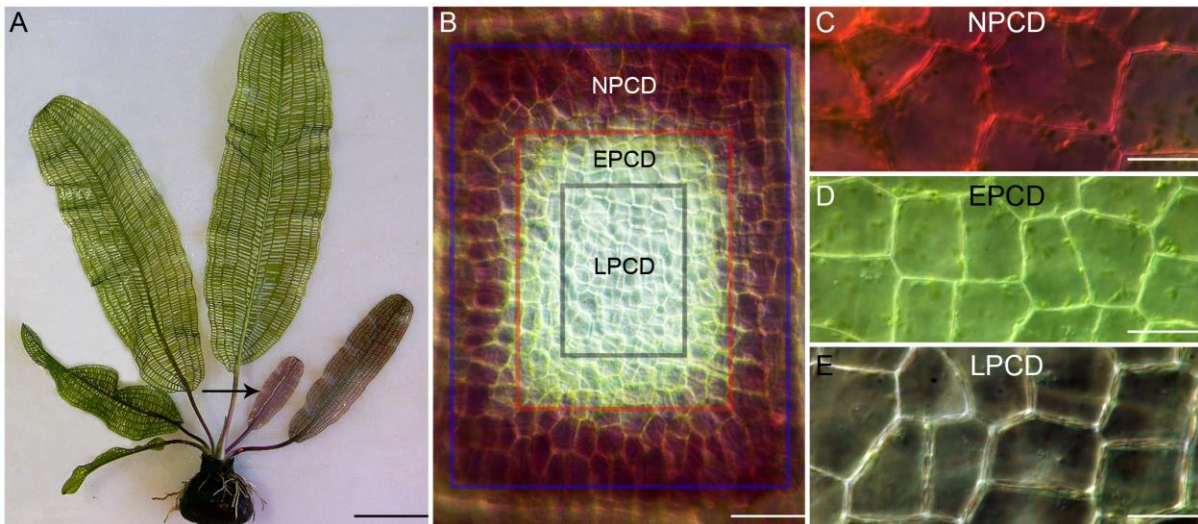
491 Figure 1



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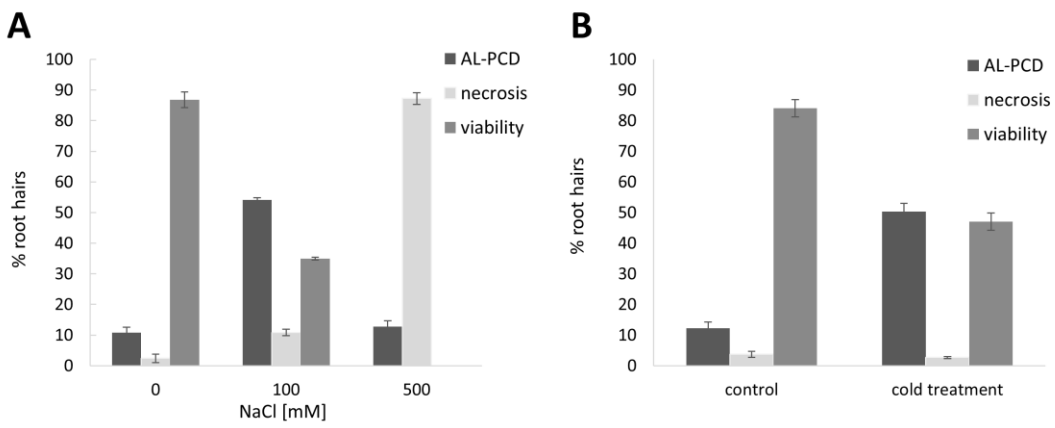
493 Figure 2

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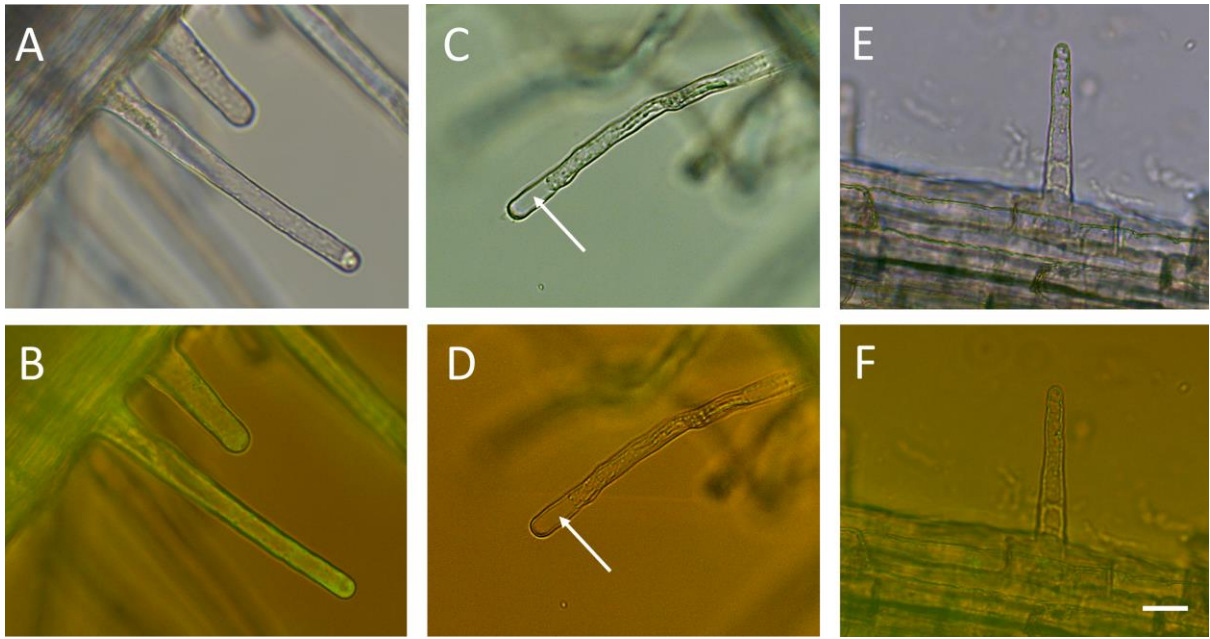
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496 Figure 3



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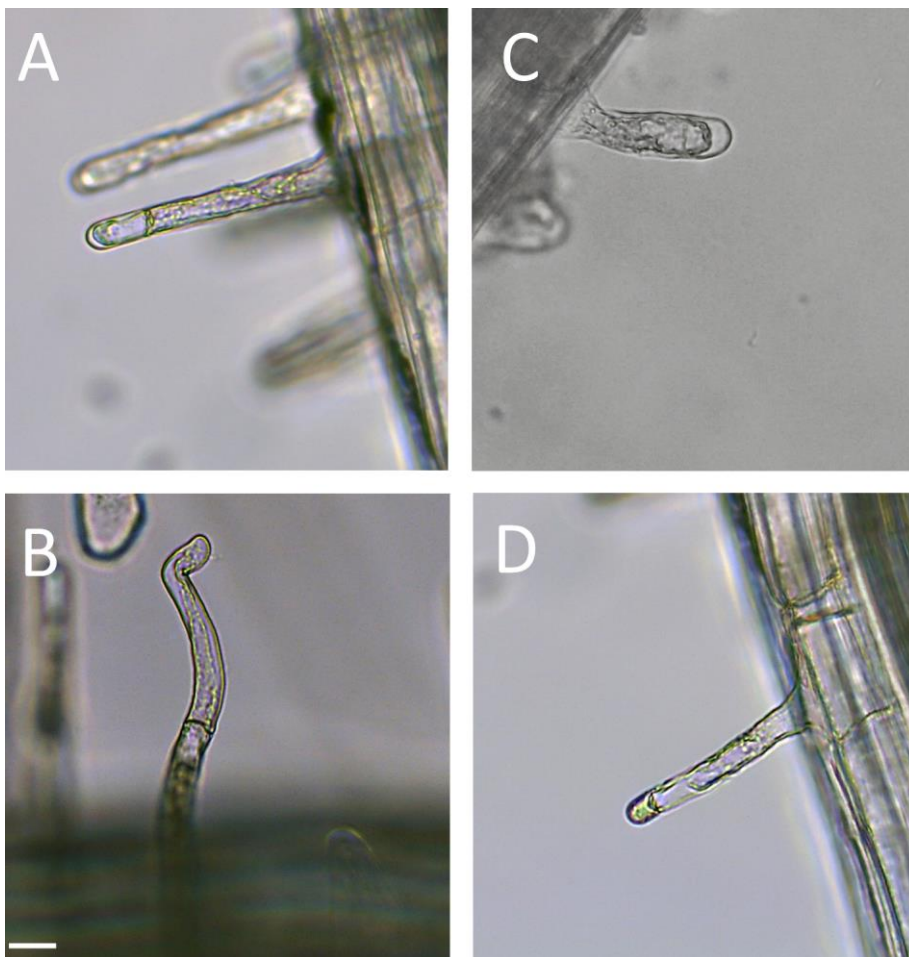
498 Figure 4



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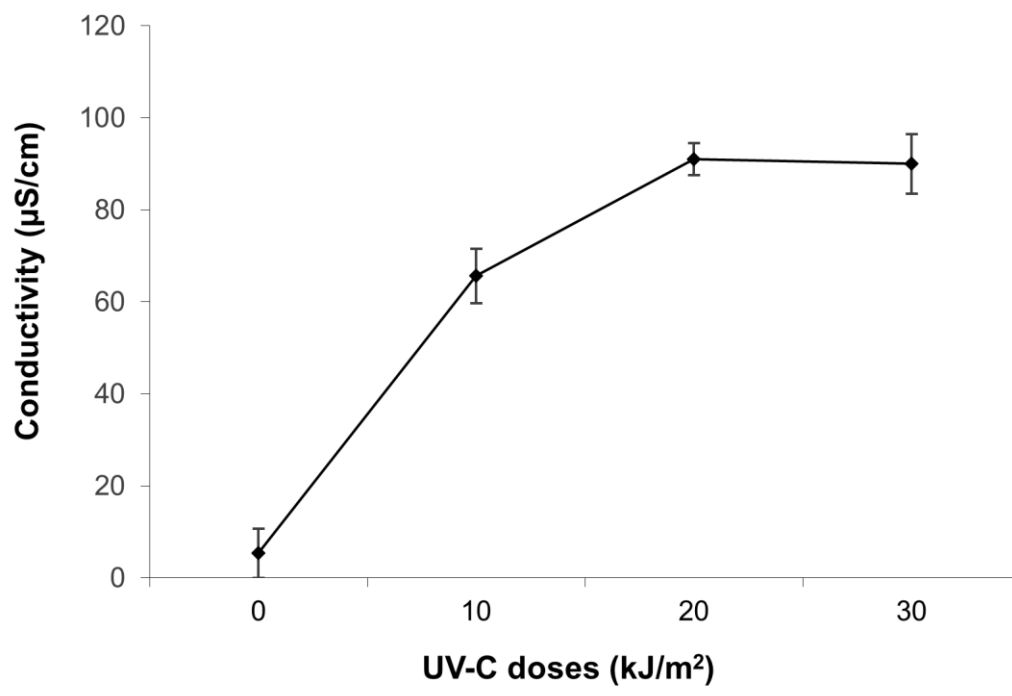
Figure 5



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Figure 6



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504 Figure 7

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