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Title: A Root Hair Assay to Expedite Cell Death Research

Running Head: A Root Hair Assay

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Summary:

Programmed cell death can be defined as an organized cellular destruction and can be activated throughout plant development, as a defence response against invading pathogens or during environmental stress. The root hair assay presented herein enables *in vivo* quantitative measurements of programmed cell death based on the morphological changes of dying root hairs. Application of this novel, simple technique eliminates the need for establishing cell suspension cultures, resulting in a significant reduction in time, cost and labour input. Here, we present a detailed root hair assay protocol for the dicotyledonous model plant *Arabidopsis thaliana*, where results from germination to scoring of cell death can be obtained within 7 days. We also suggest and present a panel of cell death inducing treatments which can be used to study abiotic stress- and mycotoxin-induced programmed cell death in the root hair system in Arabidopsis. A root hair assay protocol for the monocotyledonous model species *Brachypodium distachyon* is also included.

Keywords: programmed cell death – root hair assay – Arabidopsis – apoptosis-like

1. Introduction

Programmed cell death (PCD) describes a number of processes that result in a highly controlled and organised form of cellular destruction, activated in every part of the plant, throughout its entire life cycle *(1).* It is an indispensable facet of plant development, senescence, defence responses and architecture (*2-4).* In the last twenty years research has increased our understanding of several facets relating to the induction and destruction of cells undergoing PCD, however its central execution mechanisms are still relatively poorly understood compared to animal cell death programmes. Plant PCD can often be characterized by a specific morphology: the condensation of cell contents and retraction of plasma

membrane from the cell wall, resulting in a visible gap, and this type of death has been named apoptosis-like PCD (AL-PCD) *(5, 6).* This morphological hallmark feature has been commonly observed following PCD events occurring in response to abiotic stress and pathogen attack *(7)*, during developmental PCD, such as during leaf morphogenesis in *Monstera (8)* and lace plant leaves *(9)*, the culmination of senescence *(10, 11)* and in embryogenic associated suspensor cells *(4)*.

PCD research in plants has been hindered to some extent by a lack of convenient and reliable methods which facilitate quantitative measurement of rates of *in vivo* cell death as cells undergoing PCD are often buried within living tissue, which impedes large-scale visual scoring of living and dead cells. Several methods have recently been used to monitor *in vivo* rates of PCD, for example, the lace plant (*Aponogeton madagascariensis)* facilitates studies of developmental PCD in real time *in vivo (12).* Another method is the root hair assay, which allows quantitative measurements of stress-induced AL-PCD *in vivo (13)*. Root hairs, already considered a classic system for studying plant cell growth *(14)*, also provide a robust tool for determination of PCD rates in whole plants. In order to expand the scope of potential applications of the root hair assay, the technique has subsequently been optimized for high throughput performance and a panel of cell death inducing treatments has been developed for use with the assay. To date, the root hair assay has proved useful in several research contexts. For example the assay has been used to demonstrate a reduction in stress-induced PCD levels in Arabidopsis plants overexpressing the Defender Against Apoptotic Death-1 (DAD1) protein *(13)*. This result was in accordance with earlier *in vitro* results obtained using a protoplast system *(15).* Obviously the root hair assay is technically simpler and often quicker than protoplast isolation and this is one of major advantages of using root hairs, indeed the root hair assay can also be used as an alternative to establishing cell suspension cultures and therefore is a convenient tool for rapid determination of changes in stress-induced PCD in

transgenic or mutant lines. Root hairs can also be used in to study the role of genes and gene products in cell death, for example, Blanvillain et al., *(16)* used this technique to characterize two mutant alleles of the Kiss of Death peptide coding gene to demonstrate there was a reduced PCD phenotype after a 55°C heat treatment. The root hair assay is therefore a promising tool for the rapid screening of mutant populations in order to identify novel genes involved in AL-PCD regulation. Additionally the root hair system is also a convenient tool to investigate potential modulators of AL-PCD for example caspase inhibitors *(13)*. Root hairs are easily accessible for chemical manipulations, as the cells are exposed on the root surface. Their natural function is absorption of water and nutrients and therefore the uptake of chemicals that are potential cell death modulators is facilitated. A combination of pharmacological and genetic approaches also means the root hair assay is a suitable technique for dissecting more complex cell signalling processes. For example it has been recently used to identify components of salicylic acid induced programmed cell death and autophagy (*17)*.

2. Materials

2.1. Growth of plant material

2.1.1. Growth of Arabidopsis seedlings

1. 1.5 ml microfuge tubes
2. Growth medium: MS half-strength basal salt mixture (Duchefa), 1% (w/v) sucrose, adjust pH to 5.8 with KOH, add 1.5 % agar (w/v) and autoclave. Pour into 12x12 cm square sterile Petri dishes (approximately 25 ml of growth medium per plate).
3. Sterilizing solution: 20% (v/v) commercial bleach (final concentration of sodium hypochlorite ~1% v/v)
4. Sterile deionised water

5. 1000 µl pipettes and pipette tips
6. 200 µl pipettes and pipette tips with ends cut-off

2.1.2. Growth of *Brachypodium distachyon* seedlings

1. Forceps, sterilized in autoclave
2. Sterile deionised water
3. Sterilizing solution: 20 % (v/v) commercial bleach (final concentration of sodium hypochlorite ~1% v/v)
4. Filter paper (90 mm Ø discs), sterilized in autoclave
5. Sterile Petri dishes (90 mm Ø)

2.2. Induction of PCD in root hairs of Arabidopsis

1. Forceps
2. 24-well plates (e.g. Cellstar multiwell culture plates, Greiner Bio-One GmbH)
3. Sterile deionised water
4. For heat treatment: waterbath (not shaking) (Grant OLS200), Leucopore tape
5. For NaCl treatment: 100 mM NaCl solution in deionised water, prepared beforehand and stored at 4^{o}C
6. For hydrogen peroxide treatment: 30% w/v H_2O_2 stock solution (Sigma-Aldrich)
7. For SA treatment: 0.1 M SA stock solution in ethanol, prepare directly prior to use
8. For fumonisin B1 (FB1) treatment: 1 mM stock solution in deionised water, prepare beforehand and store at -20^{o}C

2.3. Induction of PCD in root hairs of *Brachypodium distachyon* by heat treatment

1. Forceps
2. 24-well plates (e.g. Cellstar multiwell culture plates, Greiner Bio-One GmbH)

3. Sterile deionised water
4. Waterbath with no stirring (Grant OLS200), Leucopore tape

2.4. Root hair assay

1. Forceps
2. Razors (optional)
3. Fluorescein diacetate (FDA) stock solution in acetone (0.1%, w/v). Store at -20^{o}C and dilute to 0.001% w/v in deionised water directly prior to use.
4. Standard microscope slides and cover-slips
5. White and fluorescent light microscope (e.g. Olympus BX60)
6. Three mechanical counters

3. Methods

3.1. Growth of plant material

The root hair assay is a technique based on the examination of the morphology of individual cells. Relatively short root hairs and moderately dense root hair distribution facilitates observation of single cells and also reduces mechanical damage during handling of the plant material. The preliminary optimization of species specific growth protocols that result in these short dense root hairs will be of significant benefit in facilitating subsequent root hair scoring assays in different species (*see* **Note 1**). Herein, we present growth protocols that work well for us for two model species, *Arabidopsis thaliana* and *Brachypodium distachyon*,

3.1.1. Growth of Arabidopsis seedlings

1. This method is adapted from **Ref. 13**
2. Place Arabidopsis seeds in the labelled 1.5 ml microfuge tube. All subsequent steps should be performed using sterile techniques in a laminar flow hood.

3. Add 1 ml of sterilizing solution and mix by inverting the tube a few times. Sterilize for 20 min with mixing every 5 min.
4. Remove the sterilizing solution by pipetting and wash seeds with 1 ml of sterile deionised water 4 times.
5. Sow seeds in 2 to 3 lines on solid growth medium using 200 µl cut-off pipette tips. Preferably, individual seeds should be separated by 0.5 cm. Seal Petri dishes with Parafilm and vernalize in the dark, at 4^{o}C for 1 day.
6. Place Petri dishes at 22^{o}C, in constant light, in a vertical position, so that the seeds are germinating in horizontal lines, to allow the roots to grow down the surface. Seedlings should be used for experiments when 5-6 days old.

3.1.2. Growth of *Brachypodium distachyon* seedlings.

1. Soak mature *Brachypodium distachyon* seeds in sterile deionised water at room temperature for 3 hr in a Petri dish.
2. Using fingers, carefully remove lemma from the seeds. Collect the seeds in deionised water in a Petri dish.
3. In the sterile flow cabinet, drain seeds from water and add sterilizing solution. Gently shake for 4 min. Rinse 4 times with sterile deionised water. Place seeds in a sterile Petri dish on two layers of filter paper soaked with sterile deionised water. Seal the Petri dish with Parafilm.
4. Vernalize for 4 days in the dark at 4ºC to synchronise germination and transfer to 25ºC with a 16-hr photoperiod.
5. Seedlings can be used as soon as the emerging radicle is approximately 1 cm long (typically after 1 to 2 days).

3.2. Induction of PCD in root hairs of Arabidopsis.

24-well culture plates (**Fig. 1**) provide a convenient system for simultaneous treatment of up to 24 seedlings, making the root hair assay a promising tool for high-throughput screening of mutant or transgenic lines (*see* **Note 2**). A panel of cell death inducing treatments, including temperature, NaCl, hydrogen peroxide, salicylic acid and mycotoxin fumonisin B1 is presented and can be expanded to suit the purpose of planned experiments (*see* **Note 3**).

1. Heat treatment: Fill each well of a 24-well culture plate with 1 ml of sterile deionised water. Using forceps, transfer Arabidopsis seedlings to individual wells. Seal the lid of multiwell plate with Leucopore tape. Set the waterbath (with shaking switched off) to the desired temperature. Perform the heat treatment by allowing the culture plate to float on the surface of water. Following the heat shock incubate seedlings in the light at 22^{o}C until scoring for PCD rates. Typically, a 10 min heat shock at temperatures between 49 to 51^{o}C results in induction of 30 to 60% AL-PCD within 24 hr.
2. NaCl treatment: warm 100 mM NaCl solution to room temperature. Fill wells of multiwell plates with 1 ml of NaCl solution or 1 ml of deionised water. Perform the stress treatment by incubating seedlings in NaCl for 5 min followed by transfer to deionised water. Following the treatment incubate seedlings in the light at 22^{o}C until scoring for PCD rates. Typically, treatment results in induction of 50 – 60% AL-PCD within 24 hr.
3. H_2O_2 treatment: Dilute 30% w/v H_2O_2 stock in deionised water. Fill wells of multiwell plates with 1 ml of H_2O_2 solution or 1 ml of deionised water. Perform the stress treatment by incubating seedlings in H_2O_2 for 5 min followed by transfer to deionised water. Following the treatment incubate seedlings in the light at 22^{o}C until scoring for PCD rates. Typically, treatment with 15 mM H_2O_2 results in induction of 30 - 40% of AL-PCD within 24 hr.

4. SA treatment: Dilute SA stock in deionised water. Fill wells of multiwell plates with 1 ml of SA solution. Perform the stress treatment by incubating seedlings in SA solution in the light at 22^{o}C until scoring for PCD rates. Typically, treatment with 60 to 75 µM SA results in induction of 30 - 70% AL-PCD within 24 hr.
5. FB1 treatment: Dilute mycotoxin stock solution in deionised water. Fill wells of multiwell plates with 1 ml of FB1 solution. Perform the stress treatment by incubating seedlings in FB1 solution in the light at 22^{o}C until scoring for PCD rates. Typically, treatment with 50 µM FB1 results in induction of 30 - 40% of AL-PCD within 24 hr.

3.3. Induction of PCD in root hairs of Brachypodium by heat treatment

Perform the heat treatment according to protocol recommended for Arabidopsis seedlings. Typically, 10 min heat shock at temperature between 49 to 51^{o}C results in induction of 40 -60 % AL-PCD within 24 hr.

3.4. Root hair assay

1. This method is adapted from **Ref. 13.**
2. Select the time point after cell death induction when the root hair assay will be performed (*see* **Note 4**).
3. Prepare 0.001% w/v FDA solution directly prior to use. Whole Arabidopsis seedlings can be stained directly on the microscope slide and immediately examined under white and fluorescent light. For Brachypodium seedlings, carefully cut off the radicle with a razor blade before FDA staining and placing the cover-slip on (*see* **Note 5**).
4. Examine root hairs under fluorescent and white light, starting from the root tip (*see* **Note 6**). The root hair assay is based on observation of root hairs morphology and result of FDA staining (**Fig. 2**). Score root hairs positive for FDA staining (exhibiting

green fluorescence) as alive. Examine the morphology of FDA-negative root hairs and score them as AL-PCD, if they exhibit cytoplasm condensation and retraction of protoplast away from the cell wall (*see* **Note 7**). If the root hair is FDA negative and does not exhibit AL-PCD morphology, score it as necrotic (*see* **Note 8**). Record your results using mechanical counters until you score at least 100 root hairs.

4. Notes

1. Root hair length and density can be varied and optimised for scoring by changing factors such as light regime, growth system type (e.g. solid medium or hydroponic culture), growth medium type, composition (concentration of salts) and hardness (concentration of gelling agent). Testing different growth conditions can facilitate identification of conditions producing seedlings with root hair characteristics for optimized cell death type scoring.
2. To prevent high background cell death rates due to mechanical damage, seedlings should be handled very carefully with forceps during transfer from the growth medium to the cell death inducing treatment solution.
3. While establishing a protocol for cell death induction in the root hair system with a novel chemical agent, it may be useful to test a wide range of concentrations. From our experience, concentrations of chemical agents which induce AL-PCD in Arabidopsis root hairs are often significantly lower than in cell suspension culture, possibly due to absorptive function of root hairs, favouring uptake of chemicals.
4. AL-PCD morphology takes several hours/days to develop, depending on the type and intensity of the cell death inducing stimuli. Typically, 24 hr after the cell death induction, AL-PCD in root hairs is fully developed and significant condensation of the cell content can be clearly recognized. However, we have used the root hair assay

to investigate AL-PCD at earlier time points in *Arabidopsis thaliana,* characterized by less advanced, although still recognizable, levels of cytoplasm condensation and a smaller gap between the cell wall and retracted protoplast.

5. If the background FDA staining is excessive, seedlings can be washed and mounted on the microscope slide in deionised water.
6. In order to correctly identify root hairs as viable it is often useful to adjust the focus on the individual cell under fluorescent light with the white light switched-off.
7. AL-PCD morphology is characterized by a gap between the protoplast and cell wall, usually being readily recognizable at the tip of the root hair. However, occasionally, cytoplasm condensation leaves a clearly identifiable gap between the cell wall and retracted protoplast only in the middle part of the root hair or close to its base (**Fig. 3a, b**). In certain root hairs, the cytoplasm does not retract as a complete unit and splitting of the cell content into two or more parts can be observed (**Fig. 3c, d**). Occasionally, especially in case of the relatively short root hairs the cytoplasm retracts almost completely to the root hair base cell (**Fig. 3d, e**).
8. Necrosis is a rapid cell death that usually takes place in case of overwhelming stress on injury and is not associated with cytoplasm retraction. For example, in the case of heat treatment, the percentage of AL-PCD in root hairs increases with temperature increases up to 65°C, whereas further increases of temperature cause root hairs to die by necrosis rather than AL-PCD *(13).*

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Figures legends:

Fig. 1. 24-well culture plate used for AL-PCD cell death inducing treatments with Arabidopsis seedlings.

Fig. 2. AL-PCD morphology in root hairs of *Arabidopsis thaliana* (A-C) and *Brachypodium distachyon* (D-F). **(A, D)** Living root hair stained with FDA and viewed under both white and fluorescent light. **(B, E)** Root hair 24 hr after a 10 min heat shock at 51°C. The root hair shows condensation of the cytoplasm and no FDA staining, indicating it has undergone AL-PCD. **(C, F)** Root hair 24 hr after a 10 min heat shock at 75°C. No FDA staining and no retraction of the cytoplasm can be observed. Scalebar: 10 μm.

Fig. 3. Different appearances of AL-PCD morphology in the root hairs of Arabidopsis thaliana. Cytoplasm condensation leaving a clearly identifiable gap between the cell wall and retracted protoplast in the middle part of the root hair or close to its base (A,B). Condensed cell content split into two parts (C,D). Condensed cell content almost completely retracted to the base cell (D,E).