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Triggering event for cell dedifferentiation in Physcomitrella patens

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Stem cell research is important to many people because of its applications in medicine and disease therapy. However, understanding how to use stem cells in medical treatments is just one side of stem cell research. Another equally important facet to study is how stem cells are maintained and controlled in other living systems. Bryophytes—or mosses—are a unique group of plants that are able to turn any cell in their bodies into an entirely new plant. This ability is not found in any other organism, and how they are able to do this is not entirely known yet. Thus, in this study, I plan to investigate what events trigger cell dedifferentiation that leads to the regrowth of entire organisms from single leaf cells. Specifically, I will be testing whether cells innately contain molecules that prevent each other from degenerating back into stem cells. I will do this by removing cell contents from in-tact leaf cells and observing whether I can induce new plants to grow from the remaining leaf cells. Obtaining a holistic view of stem cell control and maintenance is vital in stem cell research, and it is my hope that understanding the stem-cell like qualities of bryophytes will lead to better understanding of stem cells in human models as well.

Triggering Event for Cell Dedifferentiation in *Physcomitrella patens*

Introduction

Bryophytes (or mosses) are one of the few groups of organisms that can fully dedifferentiate any cell in their body. Everything—including the leafy gametophyte (haploid), rhizoids (haploid), and even the sporophyte (diploid)—has demonstrated the ability to dedifferentiate into completely totipotent protonematal cells (Giles 1971, Westerdijk 1907, Von Wettstein 1924, von Maltzahn 1959). In these organisms, cell dedifferentiation appears to be triggered by excising structures from the parental plant, making it one of the few organisms whose regenerative capabilities are limited by neither previous cell determination nor pleuripotency. The process for cell dedifferentiation in bryophytes, which is mostly studied in leaves, is triggered by excision from the main plant and results in the formation of 'chloronema'. Chloronema is the totipotent stage directly following spore germination and is characterized by its numerous chloroplasts (Giles 1971, Ishikawa et al 2011, Sakakibara et al 2014, Kofuji & Hasebe 2014). Newly formed chloronema will then give rise to an entirely new organism via the usual developmental processes.

Unfortunately, the processes and mechanisms surrounding dedifferentiation in bryophytes is complicated and fairly inconsistent across species. Some species like *Funaria* will produce numerous apical chloronema cells across entire excised leaves, whereas species like *Physcomitrella patens* will produce protonema from just cells bordering the detached boundary of the leaf (Giles 1971, Westerdijk 1907). Others yet do not differentiate at all: the genus *Dawsonia* will increase chloroplast number and cell size but ultimately never form functional protonema (Von Wettstein 1924). Not all cells are created equal either. Often, there is a gradient of most differentiable tissue to least. For instance, sporophytic tissue will dedifferentiate more readily near the apex whereas protonema will dedifferentiate more often near the basal area than the tip (Von Wettstein 1924). Indeed, the dedifferentiation process of bryophytes is complex and seemingly difficult to understand.

There are a handful of papers investigating the cellular changes associated with dedifferentiation in mosses (Ishikawa et al 2011, Sakakibara et al 2014). Ishikawa et al (2011) identified two essential proteins involved in triggering the dedifferentiation event in *P. patens*: CDKA and CDKD (cyclin-dependent kinases). CDKA was found to be an activator for CDKD and is present in all cells at all times, whereas CDKD is only transcribed in cells at detachment sites. However, although current studies offer insight into dedifferentiation pathways, the actual triggering mechanism for dedifferentiation in mosses still remains a mystery.

There are some theories as to how dedifferentiation is triggered in excised moss fragments. Von Maltzahn (1959) suggested that leaves (and other tissue)

receive signals from the apex that specify *not* to dedifferentiate. Therefore, when leaves are removed from the main plant the lack of this signal results in spontaneous activation of CDKA/CDKD and other pathways. This system—where a deficiency of certain molecules causes 'instability' in cellular state— is not unprecedented. Similar systems are seen in angiosperms, which use auxin as a concentration-dependent signal for injury response. Normally, there is a constant auxin flow from the apical tip to the peripheral tissues, but when tissues are damaged along this flow it slows or blocks the flow of auxin. This results in a build up of auxin on the basal side and a deficiency of auxin on the apical side, which initiates transcription to begin healing and regeneration (Asahina et al 2011, Read and Ross 2011). One can imagine how bryophytes may use a similar system to signal to leaves when dedifferentiation is necessary.

The objective of my project is to identify a possible trigger for cell dedifferentiation in bryophytes. I hypothesize that there is the presence of some 'constant' signal in each cell that stabilizes neighbouring cells. This hypothesis is supported by the presence of 'persistent' factors such as CDKA that are involved in activation of dedifferentiation factors, and is based on auxin-induced wound repair models in angiosperms. I plan to address my hypothesis by removing the cytoplasm of neighbouring cells, in hopes to induce cell dedifferentiation in select cells due to the removal of particular 'stabilizing' signals.

Significance

Stem cell research is important for a variety of reasons. Understanding how stem cells function will contribute to our overall understanding of stem cell functioning. This may be indirectly applicable to stem cell therapy research in the medical field. Additionally, learning about how other systems handle stem cells may lead to analogous pathways within our own bodies. Other applications of cell totipotency can be used in farming techniques. Being able to master cell dedifferentiation to create cloned strains of certain foods may be beneficial to many farmers and save time and money. Bryophytes give a unique perspective into stem cell maintenance because they are able to dedifferentiate every single cell in their body to become totipotent. Thus, understanding the triggering events and pathways involved in cell dedifferentiation may lead to better understanding of how to reverse or halt cell differentiation in other systems.

Experimental Approach:

For my experiment, I plan to remove the cytoplasm of leaf cells from in-tact gametophytes. I chose the species *Physcomitrella patens* because of its historical use in bryophyte research (Reski 1999). It is commonly used in gene studies because homologous recombination in this system is fairly simple, and reproducing clones is easy due to its ability to regenerate by fragmentation. Additionally, since

Physcomitrella patens is part of the class Bryophyta, it generally does not possess any 'special' features associated with some other classes of bryophytes (Van der Poorten & GOffinet 2010). Thus, *P. patens* can provide a simple, effective model to broadly represent bryophytic characteristics.

All protocols, except cytoplasmic extraction, have been successfully used in other bryophyte experiments, whereas removal of cell contents has been demonstrated on other cell models such as neurons.

Culturing of *P. patens*

First, I will obtain a line of *P. patens* similar to the ones found in Ishikawa et al. (2011). I would use the ProCYCD;1:NLS-GFP- GUS #263 *P. patens* line described in their paper, which used the polyethylene glycol–mediated transformation system created by Nishiyama et al (2000) and Shaefer (1994) to introduce a GFP signal to the CDKD protein. Cultures will be kept on BCDAT media, which is used for cultivation of protonema and gametophores (Ishikawa 2011, Hiwatashi and Hasebe 2004).

Ten stock plates of *P. patens* will be grown and incubated at 25^0 C in continuous white light using BCDAT media (Nishiyama et al 2000) and transplanted every 8 weeks to ensure optimal growth. Experimental gametophytes will be cultivated for 4 weeks at 25^0 C on cellophane-lined media to produce gametophytic shoots (Ishikawa et al 2011). The cellophane prevents protonema and rhizoids from growing into the agar to make gametophyte isolation easier. Gametophytes will then be removed with tweezers and placed horizontally on fresh BCDAT plates for experimentation.

Treatments

There will be 14 plates total with a single gametophyte on each. Ten of these gametophytes will be subject to all four treatments on different leaves to account for differences in individuals or media. The remaining four gametophytes will have 3 replicates of each type of treatment to ensure treatments are not affecting the results of each other. Treatments will be randomized in position along a single gametophyte so as to prevent any leaf-age effects. Leaves for treatment will be chosen based on how flat against the media they are: leaves that lie flat on the cellophane will be preferentially chosen to ensure any budding protonema will have sufficient nutrients.

1. No-cytoplasm treatment

Cytoplasm from an entire row of cells across the broadest part of the leaf will be removed by glass micropipettes stretched out to have a tip with a 0.2um diameter (Mackler 1992). Originally, the extraction of cell contents was done on hippocampal neuron cells. The cells of bryophytes are very delicate so the integrity of the cell wall

should not pose a problem. Visual examination of the cells will confirm the absence of cytoplasmic contents.

2. Puncture-control (Negative control 1)

Using the same micropipettes and procedures as in treatment (1), cells will be punctured but not aspirated.

3. Excise-control (Positive control)

Using a razor blade, single leaves will be cut at the broadest part and moved 1cm away from the main shoot using sterilized tweezers. I will be careful not to damage the cells as I am moving the leaf fragment.

4. In-tact control (Negative control 2)

Leaves will remain in-tact and un-punctured on the main stem.

Observation

Using a light microscope, I will make and record observations at 1h, 12h, 24h, 48h, and 2 days. The following traits will be noted:

- chloroplast count, with counts from surrounding cells for comparison—this is associated with cell dedifferentiation (Giles 1971, Ishikawa et al. 2011, Sakakibara et al 2014)
- Budding or abnormal growth
- Cell division (the formation of the apical chloronemal cell)

Additionally, I will also look at GFP fluorescence under UV light to evaluate CDKD expression.

Finally, I will look at total protonemal growth after 2 days (Kofuji & Hasebe 2014)

Possible Results:

No-cytoplasm treatments:

If my hypothesis is correct, I expect to see:

- CDKD-GFP expression and protonemal growth in 'neighbouring' cells on the 'No-cytoplasm' treatments within 24 hours of treatment (Ishikawa et al 2011)
- CDKD-GFP expression and protonemal growth in cells adjacent to the cut site on the 'Excise-control' treatments within 24 hours of treatment
- No CDKD-GFP expression and no protonemal growth in 'Puncture-control' treatments and 'In-tact control' treatments.

No cytoplasm treatments:

The cells bordering the row of no-cytoplasm cells are expected to give rise to chloronema. If cells on both sides grow chloronema, it would suggest that each cell has the same stabilizing signal, and that there is no polarity regarding this signal. However, if there were some kind of polarity (for example, if the signal is being excreted by the apical growth cell), then one would expect only the cells apical of the

no-cytoplasm divide to dedifferentiate into chloronema. An unexpected result would be if CDKD-GFP expression and chloronema formation were not displayed together: this would suggest that either CDKD activation is not exclusive to dedifferentiation, or that it may not be necessary for dedifferentiation.

Puncture-control treatments:

Assuming the puncture-control cells did not lose too much cytoplasm and that they retained the ability to repair themselves, I would expect to see no chloronemal growth. However, it is possible that the puncture would result in enough loss of cytoplasm to initiate cell dedifferentiation on neighbouring cells. I would use visual examination to observe the extent of cell damage due to puncturing. If it were evident that the puncture control had lost a substantial amount of cytoplasm, then I would have to find a new way to remove the cytoplasm while minimizing cell damage.

Excise-control treatments

Ishikawa et al (2011) tested this treatment under nearly identical conditions; so any deviations from this result would be unexpected. The only difference between Ishikawa et al (2011)'s treatment and my experiment is the presence of the whole-gametophyte 1cm away from the excised leaf. In the Ishikawa et al (2011) experiment, they transferred the excised leaf onto a separate plate, whereas I plan to keep the excised leaf on the same plate. The 1cm distance is relatively arbitrary, and chosen based on space restriction on the petri plate. Any differences in results would require further testing to examine why.

In-tact treatments

It is expected that these leaves will not show any dedifferentiation because they were not wounded in any way. Most literature states that attached leaves will not grow protonema, but there have been exceptions. It is unclear why this is the case, but I suspect it is because of damage done to cells when leaves are excised from the whole gametophyte.

An alternative result that would contradict my hypothesis is as follows:

- No CDKD-GFP expression and protonemal growth in 'neighbouring' cells on the 'No-cytoplasm' treatments within 24 hours of treatment
- CDKD-GFP expression and protonemal growth in cells that were punctured in the 'Puncture-control' treatments
- CDKD-GFP expression and protonemal growth in cells adjacent to the cut site on the 'Excise-control' treatments within 24 hours of treatment
- No CDKD-GFP expression and no protonemal growth in 'In-tact control' treatments.

These results would suggest that dedifferentiation is likely triggered by differential gene expression in response to cell damage, rather than the loss of a 'stabilizing' factor. With no cytoplasm (and thus no transcriptional machinery) in the no-

cytoplasm treatments, cells would not be able to express wounding signals. Puncture-controls treatments, on the other hand, would show CDKD-GFP expression and protonema formation because injury to the cell wall directly causes transcriptional changes within the cell (including expression of CDKD). It is possible that exposure of cell cytoplasm to differential osmotic conditions causes differential gene expression, leading to cell dedifferentiation. This would also explain why some species of bryophytes show ubiquitous cell dedifferentiation while some only show it on cells bordering cut sites: the leaves removed from certain species may be more delicate than others, and are thus more prone to damage than others.

By examining the results from all four treatments, I hope to narrow down the possible triggering events for cell dedifferentiation. By establishing the mechanism by which cells dedifferentiate in bryophytes, future research can focus further on what specific molecules or signals are involved in this process. Elucidating the process of cell dedifferentiation in bryophytes, as mentioned above, will lead to improved understanding of stem cells in general and will potentially be applicable in both health- and agricultural-related matters