Kinetin-induced Programmed Cell Death

Background

Why should a cell commit suicide?

When cells generate an organism, their mutual relationship changes dramatically: the competition of inidividual cells for resources and survival has to change into cooperation of all cells. The clearest hallmark of completed self organisation is the sacrifice of the invidual cell for the sake of the entire population. This so-called programmed cell death is observed in all multicellular organisms, precursors even in colonies of unicellular organisms. When a cell recognises that it is damaged, it will arrest proliferation and instead initiate programmed cell death. This ensures genetic integrity of the organism and thus its survival. However, for the individual cell this is disadvantageous, of course - the dead cell will not get profit from its own sacrifice. When cells return to the egoism of individual life and proliferate, although they are damaged, this will lead to cancer. Cancer in plants seems to be non-existing, but plant cells often undergo programmed cell death, when they are attacked by microbes. This allows to contain the intruder and to seal the damaged site. But how can plant cells actually sense their integrity? A different form of cell death is not linked with stress and damage, but with developmental processes. For instance, a root that has to grow through compacted soil needs to form aerenchymatic tissue to allow for oxygen access. This aerenchyma is produced by cells that die to give way for air. Here, suicide is a normal part of development. A similar example is the formation of lignified xylem in vascular bundles.

What have we done so far?

To study aerenchyma formation in a root, is possible, but to address the cellular events leading to programmed cell death is technically difficult. We have therefore asked ourselves, whether we can mimick this using tobacco BY-2 cells as model. These cells derive from parenchymatic tissue and are able of developmental PCD, for instance during vascular bundle formation and, in the root during aerenchyma formation. During a cooperative project with Prof. Dr. Andrzej Kaźmierczak from Łódź University (Poland), we could show that kinetin can indeed induce programmed cell death in these cells, correlating with an arrest of the cell cycle, a deregulation of DNA replication, a loss of plasma membrane integrity, a subsequent permeabilisation of the nuclear envelope, an increase of cytosolic calcium, a suppression of callose deposition, and a complete loss of microtubule integrity, while actin microfilaments persist. This work is laid down in detail in a manuscript that is currently under revision (Kaźmierczak et al., Kinetin Induces Microtubular Breakdown, Cell Cycle Arrest and Programmed Cell Death in Tobacco BY-2 Cells). We discuss these findings in the context of a working model, where kinetin, mediated by calcium, causes the breakdown of the microtubule network, which, either by release of executing proteins, or by mitotic catastrophe, will result in PCD.

Question: The role of ethylene

Kinetin is usually a phytohormone that is inducing cell proliferation and regeneration, i.e., in processes that are just the opposite of PCD (for review see Schaller et al., 2015). Among the phytohormones that are discussed as regulator of PCD, ethylene is the most prominent. Its role in senescence of flowers, fruits and leaf abscission are well established (for review see my chapter on phytohormones, Nick 2021). As gaseous hormone, ethylene is well suited to act as signal for soil compaction. In fact, it can cause a reorientation of cortical microtubules, leading to thicker shoots and roots, such that the plant has a higher pushing force to get its way through compacted soil. The ethylene-induced microtubule response was, by the way, the first example for a signal-dependent change of morphogenesis that depends on microtubules (Lang et al. 1982).

These considerations lead to the research questions to be addressed during this project: Is ethylene mediating kinetin-induced PCD in tobacco BY-2? Is ethylene involved in the microtubule response triggered by cytokinin? Is the microtubule response a parallel phenomenon with PCD, or is the microtubule response required for kinetin-induced PCD?

Specific information on the project

The time course and concentrations for the kinetin treatment were analysed previously (Kaźmierczak et al., under revision). A concentration of 50 µM, added at the time of subcultivation, is sufficient to induce a clear response. The response seems to be slow, however, changes in proliferation and cell death can be observed from day 3 onwards.

To measure ethylene, as volatile hormone, is not trivial, but since this hormone is very relevant for fruit ripening, there exist devices that allow to measure ethylene in the gas phase through changes of conductivity. This device is available for the project through Prof. Dr. Kaźmierczak, who is currently working as visiting scholar in the department.

To follow the microtubule responses, we make use of a transgenic tobacco BY-2 cell line that is expressing a tobacco tubulin α3 in fusion with GFP. Details on this line are available through https://www.botanik.kit.edu/botzell/intranet/2729.php (access with username: theseus, password: Ariadne). Since the GFP-fusion with tubulin is driven by a CaMV 35S promoter, there is a phenotype with the cell axis, which was criticised by the reviewers in the manuscript mentioned above. However, it is possible to rescue this phenotype by feeding external auxin (indole acetic acid, 5 µM) at time of subcultivation.

To find out, whether ethylene is needed for PCD and/or microtubule reorganization, it would also be important to disrupt either ethylene synthesis or ethylene signalling. A very good review on inhibitors is given in Schaller and Binder (2017), which can be accessed through Researchgate under the link
https://www.researchgate.net/publication/315065967_Inhibitors_of_Ethylene_Biosynthesis_and_Signaling

As inhibitor for ethylene synthesis, 2-Aminoethyoxyvinyl glycine (AVG) represents the state of the art. Due to problems in international delivery (the compound is shipped from the US), this will not work out in time. As classical alternative, we can use a low concentration (10 µM) of $AgNO_3$ or of $CoCl_2$ compounds that are able to bind ethylene and remove it.

References

- Lang, J.M., Eisinger, W.R. & Green, P.B. (1982) Effects of ethylene on the orientation of microtubules and cellulose microfibrils of pea epicotyl cells with polylamellate cell walls. Protoplasma 110, 5–14 (1982). https://doi.org/10.1007/BF01314675
- Nick P (2021) Kontrolle der Entwicklung durch Phytohormone in Strasburger − Lehrbuch der Pflanzenwissenschaften, pp 377-422. https://link.springer.com/chapter/10.1007/978-3-662-61943-8_12
- Schaller GE, Street IH, Kieber JJ (2014) Cytokinin and the cell cycle. Curr Opin Plant Biol 21, 7-15. doi: 10.1016/j.pbi.2014.05.015.

Scope of the study

Objectives of the project and approaches

- Does kinetin lead to a release of ethylene? At what time course?
- How fast is the effect of kinetin on microtubules? Does this precede or follow ethylene release?
- Can we block cell death and/or the microtubule response by silver or cobalt ions?

Note: the course is this year overbooked because there are far more Master students than usual. Therefore, we have bottlenecks with the use of the spinning disc microscope and the use of the clean benches. This requires adjustments with the other teams. We use an online calendar for this.

WP1: Time course of ethylene release

Ethylene is sampled using a mobile device on a daily base, once in control cells, once in cells that grow in the presence of 50 µM kinetin. This project is already ongoing, but to get reliable data, several replications are needed that can be conducted in the subsequent weeks. In parallel, physiological parameters should be measured. These include non-invasive assays in small aliquots, such as measuring sugar consumption as readout for cell expansion or determining fresh weight as readout for culture growth, but also the number of cells per cell file as readout for the degree of normal differentiation.

WP2: Microtubule responses

So far, the time course of the microtubule has not been addressed – does it precede PCD and ethylene release? Therefore, the tubulin marker line will be treated with kinetin, and microtubules are assessed on a daily basis. Depending on the outcome, a zoom-in to the relevant time course is conceivable. To be able to detect an effect on cell axiality, these cells are cultivated in presence of 5 µM of indole acetic acid.

WP3: inhibitor treatments

The ethylene binders $AgNO_3$ or of $CoCl_2$ (both at 10 µM) are administered either in combination with 50 µM or in the absence of kinetin (as control to see potential side effects of these inhibitors) and the parameters described in WP1 and WP2 are assessed.

Organisation of the course

supervision: Prof. Dr. Andrzej Kaźmierczak (andrzej.kazmierczak@biol.uni.lodz.pl), participation in our usual Cell Biology Group meetings, Thu 10-12, seminar room 506-507 is expected (refer to the plan at the blackboard opposite of the escalator).

The course starts with the lecture block. In parallel, experiments can already be launched.

Protocols

1. Cultivation of BY-2 cells
2. Measuring ethylene
3. Spinning disc microscopy
4. Phenotyping of the cells

1. Cultivation of tobacco BY-2 cells

Cultivation tobacco cells. BY-2 (*Nicotiana tabacum* L. cv Bright Yellow 2) suspension cells (Nagata et al., 1992) are cultivated in liquid medium containing 4.3 $g{\cdot}L^{-1}$ Murashige and Skoog salts (Duchefa, http://www.duchefa.com), 30 $g{\cdot}L^{-1}$ sucrose, 200 m $g{\cdot}L^{-1}$ KH_2PO_4, 100 $mg{\cdot}L^{-1}$ inositol, 1 m $g{\cdot}L^{-1}$ thiamine, and 0.2 $mg{\cdot}L^{-1}$ (0.9 µM) 2,4-D, pH 5.8. The cells are subcultivated weekly, inoculating a defined quantity of stationary cells* into fresh medium (30 mL) in 100-mL Erlenmeyer flasks. The cells are incubated at 25°C under constant shaking on a KS260 basic orbital shaker (IKA Labortechnik, http://www.ika.de) at 150 rpm. Every three weeks the stock BY-2 calli are subcultured on media solidified with 0.8% (w/v) agar (Roth, http://www.carlroth.com). NOTE: only use sterile cut tips, when handling cells, make sure that cells are well suspended (resuspend properly before pipetting).

Note: the medium is not buffered, the pH is fluctuating easily. Take enough time to adjust the pH of 5.8

Note: to get standardisation, the inoculum has to be standardised – usually, people take around 1-1.5 ml of mature suspension depending on the density. This is done by intuition. If a culture is growing more slowly, and the inoculum is too low, the time course will be delayed, although at the end the culture may reach saturation (just later). A more standardised way is to use a constant amount of fresh weight. To simplify the procedure, you can transfer 1 ml of source culture into a tipped Eppendorf tube and spin down at 10000 g for 5 min (in a table centrifuge, lab 508.1). Then take off the supernatant with a pipette, without stirring up the sediment, drain off excess liquid and way the tube against an empty tube. Now, you know, how many cells are in 1 ml. Then you calculate, what volume of suspension you need to get a defined fresh weight. A good value is 0.5 g. This fresh weight should be used throughout. The same procedure can be used with aliquots during the culture cycle to assess differences in growth

Note: for cultivation the TuA3-GFP cells, supplement with 5 µM of indole acetic acid at the time of subcultivation to rescue the cell-axis phenotype of that line.

Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 cell line as the "Hela" cell in the cell biology of higher plants. Int Rev Cytol 132, 1–30.

2. Measuring ethylene

Ethylene is measured from the gas phase of the flask which requires that the cells are cultivated with a tube allowing to exctract the gas phase. The measurement is done with a portable device based on changes in surface charge of the sensor.

3. Spinning disc microscopy

Signals are observed using the AxioObserver Z1 (Zeiss, Jena, Germany) inverted microscope equipped with a laser dual spinning disk scan head from Yokogawa (Yokogawa CSU-X1 Spinning Disk Unit, Yokogawa Electric Corporation, Tokyo, Japan), a cooled digital CCD camera (AxioCamMRm; Zeiss), and the 488 nm laser line attached to the spinning disk confocal scan head. Images are recorded using a

Plan-Apochromat 63x/1.44 DIC oil objective operated via the Zen 2012 (Blue edition) software platform. ONLY AFTER INSTRUCTION!

4. Phenotyping

1. **To measure cell death, there are two approaches available**

1.A. Viability assay with FDA

Fluorescein Diacetate (FDA) is a non-fluorescent derivative of fluorescein, it is cleaved by esterases in the cytoplasm of plant cells, such that the green fluorescent fluorescein is released. A green signal, thus, indicates the presence of esterase that should not be present, if the cell is dead (the persistence of esterase is a limitation of this assay). The stock solution (please note the respective concentration for the protocol!) of this light-sensitive compound is kept in the dark in 96% at -20°C. was kept in the fridge at -8 °C due to its high light sensitivity and because it was dissolved in 96 % Ethanol. For staining, 0.5 µl FDA solution are directly diluted into the 500 µl sample and analysed by MosaiX imaging to avoid sampling bias: After mixing, 50 µl of the suspension are viewed under an AxioImager.Z1 ApoTome Microscope (Zeiss, Jena, Germany) equipped with the AxioVision Software Rel. 4.8 (Zeiss). MosaiX images of the size 5x5 are recorded through an EC Plan-Neofluor objective (10x, N.A. 0.3) through filter set 38 HE (Zeiss, excitation at 470 nm, beam splitter at 495 nm, emission at 525 nm). Around 500 cells are scored.

1.B. Mortality assay with Evans Blue

After treatment for 24 h, 500 µl of each sample are transferred into custom-made staining chambers (Nick et al., 2000) to remove the medium and to determine mortality by the Evans Blue exclusion test (Gaff and Okong'O-Ogola, 1971): The cells are incubated for 3-5 min in 2.5% (w/v) Evans Blue (Sigma-Aldrich) dissolved in Millipore water, and then washed with MS medium three times. The membrane-impermeable dye can penetrate only into dead cells, such that the frequency of blue cells can be scored under AxioImager Z.1 microscope (Zeiss, Jena, Germany) using differential interference contrast. Mortality is calculated as the ratio of dead cells over the total number of cells. Results are represented as mean values from three independent experimental series (biological replications) with 500 cells per individual data point. Differences between treatment and control are tested for significance using Student's t-test.

2. **To measure culture growth, there are two approaches available**

2.A. Fresh weight

Aliquots of 1 ml are removed under the clean bench and transferred into tipped Eppendorf reaction tubes. Spin down in the table centrifuge at 10000 g for 5 min. This gives a compact sediment, such that you can remove the supernatant carefully with a pipet. Make sure that the sediment does not come off (start at the side where the sediment is lower), after removal carefully tilt the tube and drain excess liquid. Weigh against an empty tube. The fresh weight can then be calculated for the volume of the flask (30 ml).

2.B. Sugar consumption

When the cells grow, they consume the sugar from the medium. This can be used to monitor growth. Especially during cell expansion, when the cells have to build a lot of new cell wall (cellulose is build from sugar!), the sugar content drops rapidly. Sugar concentrations can be measured either by reflectometry using test strips, or, even more simply, by using a commercial Brix-meter as it is used for wine making. Here, a small aliquot of medium is measured for its rotation using polarised light, since sugars as chiral molecules turn the plane of the polarised light, which can be used to quantify the concentration.

3. **To quantify cellular differentiation**

Tobacco BY-2 cells undergo a developmental cycle during the cultivation, beginning with a lag phase, where the nucleus is moving into the cell centre, a proliferation phase, where the cells pass through 3-4 cycles of cell division and form a pluricellular file, an expansion phase, where cell volume increases strongly, and a fragmentation phase, where the cell files split up into smaller units and eventually into single cells. The formation of the pluricellular file is controlled by a polar flow of auxin from the tip cell towards the basal cell of the file. This flow is synchronising the cell cycles. This polarity is a very sensitive readout for normal development and can be used to monitor, whether cells decide to leave the path of normal development in response to kinetin. The synchrony can be quantified, by counting the number of files with 1, 2, 3, 4, … cells and calculating the frequency in %. If auxin transport is functional, the even-numbered files are frequent, the odd-numbered are rare. The best time point is at the transition between proliferation and expansion (day 3-4).

Details on quantitative aspects of BY-2 development can be found here

133. Huang X, Maisch J, Nick P (2017) Sensory role of actin in auxin-dependent responses of tobacco BY-2. J Plant Physiol 218, 6-15

(available through our website do research – publications)