



## The secret of stromules

### Background

#### What are stromules?

Stromules are defined as stroma-filled tubules found in angiosperm species. They extend from the surface of all kinds of plastids and can be observed in a wide range of plants. Stromules have been observed to form by inducing a various range of conditions, like for example salinity, light or drought stress. Although discovered already by Schimper in the end of the 19<sup>th</sup> century, they were forgotten and “re-discovered” using GFP technology (for a review on stromules refer to Schattat et al. 2015). Since they seemed to interconnect, they were considered in the 90ies to form a network, through which large molecules such as DNA or proteins can be exchanged. This was, however, shown to be wrong using photoconvertible fluorescent proteins (Schattat et al., 2012). This leads to the question, what these mysterious structures are actually doing. This is one of the main objectives in the PhD thesis by Toranj Rahpeyma.

#### What have we done so far?

So far, stromules have been observed in green tissue, i.e., as outgrowths of chloroplasts, in leaves. Due to the multiple cell layers and the autofluorescence of chlorophyll, this is not trivial in leaves. So, we asked ourselves, whether we can address stromules in tobacco BY-2 cells where chlorophyll is absent. Toranj Rahpeyma generated a transgenic cell line expressing a stroma-localised marker fused to mEOS, a photoconvertible fluorescent protein. This allows to see plastids. During his Bachelor thesis, Fabio Mühlberg was investigating conditions that allow to induce stromules. He could show that application of Methyl Jasmonate (MeJA) allows to induce stromules rapidly (within an hour after application). He was then investigating the mechanism underlying stromule formation and showed that stromules align with microtubules, and that elimination of microtubules by oryzalin disrupted stromule formation. The Bachelor thesis is relevant for this project, so it is made available as pdf and you should read it carefully. Still, the question, what stromules are actually doing in plant stress, remains open. Our current working hypothesis is that they might divert stress-related metabolic pathways by changing the interaction of plastids (which are the main chemical factories of plant cells) with other organelles. A major candidate is the jasmonic acid pathway.

#### Specific information on the project

Jasmonic acid is the central stress hormone of plants that regulates a lot of different adaptive responses. It is produced by the so-called oxylipin pathway initiating from peroxidation of unsaturated fatty acids ( $\alpha$ -linolenic acid) by lipoxygenase, probably in response to reactive oxygen species (ROS) as they are formed in response to stress-related perturbations of electron transport in mitochondria and plastids, but also as product of “deliberate” ROS formation by the plasma membrane located NADPH oxidase Respiratory burst oxidase Homologue, a major input for stress signalling. The oxylipins formed by the lipoxygenase activity are converted further to 2-oxophytodienoic acid (OPDA), which is then exported from the plastid into peroxisomes, where it is converted further by  $\beta$ -oxidation into jasmonic acid that is exported to the cytoplasm, where it can either be methylated into MeJA (a volatile

compound that allows for systemic signalling) or conjugated to isoleucine to yield JA-Ile the actual signal. While OPDA was first thought to be just the inactive precursor it has emerged in the meantime as signal of own virtue, able to regulate redox homeostasis in the plastid itself. For an overview on jasmonate biosynthesis and signalling you should refer to my chapter on phytohormones in the new edition of the Strasburger (Nick 2021).

Our working hypothesis on a potential stromule function can now be specified: if stromule formation is changing the interaction between plastids and peroxisomes, it should modify the conversion of OPDA into JA, which depends on this interaction.

Now, we know, how to induce stromules, we know how to prevent their formation, and we know a potential pathway that might be modulated by the formation of stromules. Still, we do not know, what the functional consequences are. This is exactly the question you will address in your project.

We have two tools that can help here: 1. A cell line expressing the mEOS plastid marker (tpFNR) that allows to visualise stromules. 2. A cell line expressing a GFP fusion of OPDA-Reductase, the first peroxisomal enzyme that converts OPDA (a manuscript describing this line is made available as pdf). The problem is that the effects we want to observe depend on jasmonate, i.e., the same compound that we use to induce the stromules. However, it is also possible to induce stromules by a factor that activates jasmonate synthesis: salt stress. How fast the salt-induced stromulation proceeds, is not totally clear, but using 50 mM of NaCl, stromules were observed if viewed the next day, so it might initiate almost as early as for MeJA.

### References

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](https://link.springer.com/chapter/10.1007/978-3-662-61943-8_12)

Schattat MH, Griffiths S, Mathur N, Barton K, Wozny MR, Dunn N, Greenwood JS, Mathur J (2012) Differential coloring reveals that plastids do not form networks for exchanging macromolecules. *Plant Cell* 24, 1465–1477

Schattat MH, Barton KA, Mathur J (2015) The myth of interconnected plastids and related phenomena. *Protoplasma* 252, 359-371. doi: 10.1007/s00709-014-0666-4.

### Scope of the study

#### Objectives of the project and approaches

- How fast is stromule formation in response to salt as compared to stromule formation in response to MeJA?
- Stromule formation can be blocked by the microtubule-eliminating drug oryzalin. What is the effect upon salt induced cellular responses?
- Microtubules can be stabilised by taxol. Will this result in the formation of stromules or does this suppress the formation of stromules?

#### WP1: Mapping the time course of stromule formation

To find out, how fast the stromule formation runs, the tpFNR-mEOS line is used. One flask is induced with MeJA, the other with 100 mM NaCl, and aliquots of cells are sampled at different time points (30 min, 1 h, 2 h,

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3 h...), imaging can be done at the Apotome. To get a certain statistical validity, at least 30-50 cells should be sampled per time point.

### **WP2: Effect of blocking stromulation by oryzalin**

Pretreatment with oryzalin can block stromule formation. How does this affect the physiology of the cells in controls and under salt stress, how in response to MeJA. Think careful about the necessary controls. The physiological parameters are viability, development of fresh weight, and cellular morphology.

### **WP3: Effect of microtubule stabilisation by taxol**

Taxol can stabilise microtubules. Does this support stromule formation (because stable microtubules are a better support), or does it suppress stromulation (because microtubules need to reorganise first). As trigger, both MeJA, or salt can be tried, the relevant time points are known from WP1.

### **Organisation of the course**

supervision: M. Sci. Toranj Rahpeyma ([Toranj.Rahpeyma@kit.edu](mailto:Toranj.Rahpeyma@kit.edu)), 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. Spinning disc microscopy
3. 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·L<sup>-1</sup> Murashige and Skoog salts (Duchefa, <http://www.duchefa.com>), 30 g·L<sup>-1</sup> sucrose, 200 mg·L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 100 mg·L<sup>-1</sup> inositol, 1 mg·L<sup>-1</sup> thiamine, and 0.2 mg·L<sup>-1</sup> (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.

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## 2. 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!

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## 3. 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)