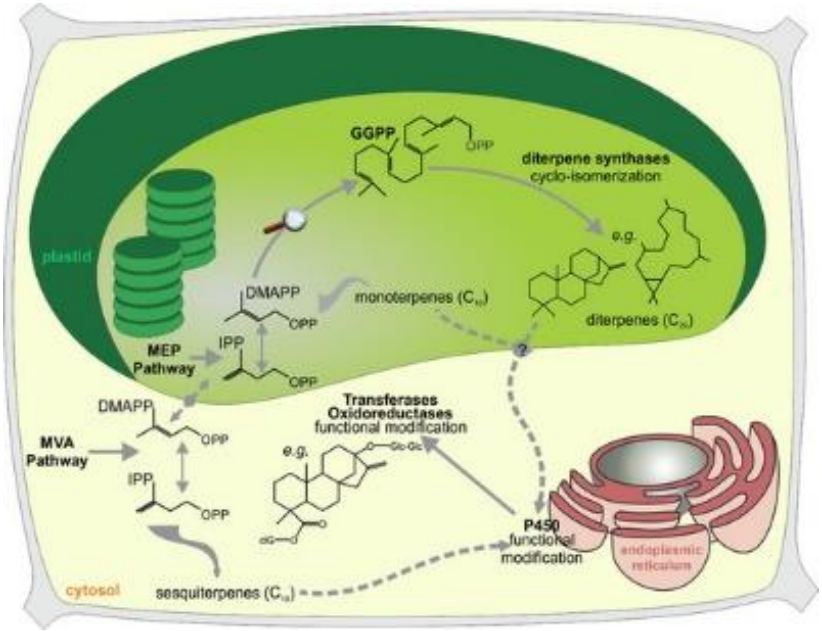
The secret of stromules

Background

What are stromules and why are they interesting?

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 19th 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. Stromules are interesting beyond the fact that morphogenetic changes of a semi-autonomous organelle like a plastid stimulate many interesting questions on the degree of autonomy and the interaction with the "host cell". However, since plastids are the main chemical factory of plant cells, there is also the question, whether they might correspond to changes in metabolic activity. The project will address this for the terpenoid pathway, which gives rise to numerous secondary compounds and is occurring in two versions – one in the cytoplasm, the other in the plastid. Both use isoprenoid building blocks, but then lead to different outputs: the cytosolic pathway produces sesquiterpenes, and the plastid pathway produces monoterpenes. There is a crosstalk, and this cosstalk might change by the formation of stromules.



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), but also of sodium stress 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 and scope of study

Both, the plastidic and the cytosolic pathways begin with an activated isoprene derivative, isopentenyl-pyrophosphate (IPP). This is, however, metabolised differently:

- In the plastid, this goes to geranyl-pyrophosphate (GPP), leading to monoterpenes
- In the cytoplasm, the IPP is converted to farnesyl-pyrophosphate (FPP), leading to sesquiterpenes

Our tool are NBD-conjugated derivatives of these two key compounds that are green fluorescent (Kim et al. 2004). This allows us to see their localisation by fluorescence microscopy. Where are they localised and how does this localisation respond to the induction of stromules?

In addition a terpene synthase from maize has been cloned and introduced into the GATEWAY vector system, which allows to see the localisation of this key enzyme. In the long term, this can be combined with the NBD-conjugates, but first, a transgenic cell has to be generated, which is beyond the time frame of a F-module, but might be achieved during the Bachelor thesis.

Objectives of the project and approaches

- How are NBD-GPP and NBD-FPP localised?
- Can we pinpoint the labelled structures, either by co-labelling with a red fluorescent marker, or by the use of specific inhibitors?
- Can we modulate the localisation by induction of stromules using MeJA?
- How is terpene synthase localised, how does this relate to the patterns seen for NBD-GPP and NBD-FPP and how does it respond to stromule formation (this objective is accessible only after completion of the respective transgenic line and, therefore, not part of the F-module, but of the subsequent Bachelor thesis.

WP1: Mapping the localisation of NBD-GPP and NBD-FPP

This will first be done in non-transformed WT BY-2 cells. Depending on the pattern, other fluorescent marker lines will be employed (for instance the tpFNR-mEOS line labelling plastids – here, mEOS could be switched to red prior to labelling with the NBD compounds. The patterns should first be secured qualitatively, later, also quantitative characterisation might be envisaged. Important is also the time course – how fast are the compounds entering and what is observed during their entrance, does the pattern change over time? By using

red-fluorescent labels such as ER-Tracker Red, or MitoTracker Red the relationship of the NBD signal with those organelles can be assessed. Alternatively, red fluorescent marker lines might be employed from our collection.

WP2: Effect of inducing stromulation by MeJA

MeJA can induce stromules in around 1 h. Does this change the pattern of NBD-GPP and NBD-FPP. The design of the experiment depends on the outcome of WP1 – is the pattern different, when the NBD-compounds are given prior to MeJA as compared to a set-up, where stromules are induced first and then the NBD-compounds added?

WP3: Effect of inihibitors

We already know that stromulation can be blocked by the microtubule blocker oryzalin. Does this change the localisation of the NBD-compounds? The ER can be modified by Brefeldin A, a fungal toxin that blocks the budding of vesicles, such that the ER is forming large lacunae. If this is seen for the NBD-compounds, it would indicate ER localisation. A third target might be actin filaments that structure ER and its interaction with organelles and can be eliminated by Latrunculin B.

WP4: generating a terpene synthase GFP expressing line

This will be not part of the module, but of the subsequent Bachelor thesis. This WP uses the usual routine by introducing the transgene (originating from maize) into the modular GATEWAY vector system and a transformation protocol via the Agrobacterium system. Depending on the progress of this process, the localisation and function of this terpene synthase can be studied, by using the repertory of approaches worked out in WP1-3.

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

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.

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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 $g \cdot L^{-1}$ 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.

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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!

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)