Protocols

1. Determination of secretive structures
2. Cultivation of BY-2 cells
3. Phenotyping of the cells

1. Determination of secretive structures

To determine secretive structures of the three species, leaves will be inspected from both sides using a Keyence three-dimensional microscope. The microscope allows to see surface structures without the need for sectioning. The three species will be analysed on a quantitative level by determining the number of glandular scales. It is also important to know, how these structures develop, therefore a developmental series from the apex (young leaf) to the base (older leaves) will be investigated. Is the differentiation into glandular scales happening earlier than leaf expansion or later? So, the area of the leaves that are counted has to be determined as well, such that it is possible to calculate the density of glands per area. Leaf area can be measured from digital images using the ImageJ freeware (https://imagej.nih.gov/ij/)

Here, how it looks like for *Mentha x piperita* (image Tizian Ehrlich) for a magnification of 200 x (which is recommended for your experiments). The glands are clearly visible as round structures with a whitish centre, in the abaxial side, there are also stomata, but they are much smaller and hardly visible here. In addition, there are sometimes other trichomes that should also be counted. Reason: the meristemoids that differentiate can do this into different structures, therefore there might be a relationship between glandular scales and other trichomes – we do not know, but it would be interesting, because this would indicate differences in the activity of certain transcription factors.

What you should determine:

1. Density of glandular scales on both sides (the dimension is given by the size bar)
2. Density of other trichome structures
3. Area of the entire leaf, such that the total number of glands per leaf can be calculated
4. Do this along the axis of the plant starting from the apical (smallest) to the basal (largest) leaves
5. To get a statistical base, collect the data for three individuals per species

2. 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. This line is kept in 50 $\mu g \cdot mL^{-1}$ Kanamycin, if there is enough time, also actin filaments can be addressed in GF11 (30 $\mu g \cdot mL^{-1}$ Hygromycin), a line, where actin filaments are visible.

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.

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

To test the allelopathic effect of the Mentha oils or its compounds a cell viability assay is performed with a non-permeating cell dye called Evans blue. This dye could only enter the cell if its membrane gets disrupted before [Baker and Mock 1994]. As a cause, it stains dead cells blue. For this, 200 µl of a three day old cell culture is transferred to a tube and incubated with 1 µl of the oil for 30 min. The suspension is put on a cell filter tube and stained for 5 min in 2.5 % Evans Blue. Afterwards the cells are washed in BY-2 medium three times for five minutes each. Then 20 µl of the suspension is transferred onto a slide and observed under the Axio Imager.Z1 ApoTome. The percentage of observed cells that are unstained is calculated and indicates the cell viability in percentage. 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 3000 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.

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)