Microtubules as Targets for New Herbicides

Background

Why are microtubules relevant for plant shape?

Microtubules constitute, together with actin filaments, the central building block for the plant cytoskeleton (plants lack intermediate filaments). The cytoskeleton is usually known for its architectural role, for instance, the shape of mammalian cells is brought about by cytoskeletal tensegrity. Plant cells, though, have a cell wall that has adopted this function. Nevertheless, the cytoskeleton is prominent here, especially in the cell cortex, adjacent to the cell wall. Why should there be a cytoskeleton, if the cell wall carries the architectural function? The general answer is that cortical microtubules serve as tracks to guide the movement of cellulose synthetising complexes moving in the cell membrane and thus define the orientation of cellulose microfibrils (Nick 2012). By control of microtubule orientation, plant cells can define the axis of cell expansion. Transverse microtubules will lead to a transverse orientation of cellulose and an elongation of the cell, while a loss of transverse orientation will lead to lateral expansion. In other words, the question, how plant shape is controlled during phase, can be broken down to the question, how plant cells control the orientation of their microtubules.

However, there is a second point, where microtubules control shape: cell division. This goes beyond the well-known role of microtubules for the mitotic spindle that is also known from animal or fungal cells. In plant cells, microtubules control the axis and symmetry of division by a structure that is found only in land plants, the preprophase band (PPB). It is formed before entry into mitosis and predicts, where and in which orientation the future cross wall is laid down. Then it mysteriously disappears, but at the site, where it was, the phragmoplast will form and control the new cell plate in telophase. Details of this enigma are discussed in the lecture on the cytoskeleton.

[26] Nick P (2012) Microtubules and the Tax Payer. Protoplasma special issue Applied Plant Cell Biology, Protoplasma 249 (Suppl 2), S81-S94

Why microtubules are an interesting target for herbicides

However, there is a second point, where microtubules control shape: cell division. This goes beyond the well-known role of microtubules for the mitotic spindle that is also known from animal or fungal cells. In plant cells, microtubules Herbicides are supposed to be specific, leaving other life forms, such as fungi, animals, or humans untouched. Therefore, the admission of new herbicides is subject to strict regulation. Due to such side-effects that had been discovered often after introduction of the herbicide, along with the development of resistance in weeds, the repertory of lead structures available for the development of herbicides has been shrinking dramatically. Over years, the development of new herbicides has also been hampered by the fact that the use of glyphosate and glufosinate along with transgenic plants engineered to be resistant to these total herbicides has sweeped the market to an extent that the development of new herbicides was not economically feasible. In the meantime, new weeds that have acquired resistance to total herbicides calls for new modes of actions and the agrochemical companies have re-launched the hunt for lead structures. Microtubules are composed of the building blocks α- and β-tubulin that are fairly conserved with exception of the C-terminus. However, the

associated proteins (MAPs) are quite different between different life forms and, thus, provide interesting targets that are specific and should allow for a new generation of herbicides with reduced risk for collateral damage. In cooperation with an industrial partner, we are currently exploring a couple of compounds that act on plant microtubules using our fluorescently tagged microtubule marker lines. The mode of action seems to follow two different patterns (**Fig. 1**) – one type of compounds leads to elimination of microtubules along with an increase of soluble tubulin dimers. These might be a mode of action comparable to known antimicrotubular herbicides such as oryzalin and trifluralin, where dimers are sequestered from integration into a microtubule, such that microtubules disappear depending on their innate turnover. The second group of compounds differs. Here, numerous small microtubule asters are produced, indicating a mechanism, where microtubule-nucleation centres are involved. The different modes of action lead to the question, whether the activity of the compounds depends on microtubular turnover, and whether the compounds act directly on tubulin, or on associated proteins. There are different associated proteins that modulate microtubular turnover. Among them, enzymes that confer post-translational modifications are of special interest, because they define different subpopulations of microtubules differing in turnover. We have been working on one of these post-translational modifications, tubulin detyrosination.

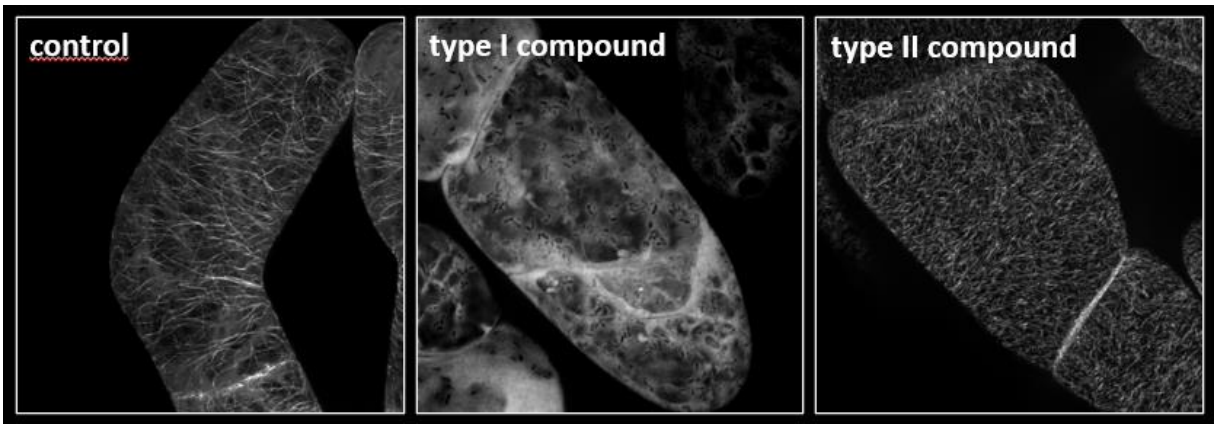

Figure 1: mode of actions of the antimicrotubular compounds in the tobacco BY-2 TuA3-GFP line after 1 h of treatment with 10 µM. Since this is an industrial project, the chemical structures of the compounds cannot be disclosed.

What is tubulin detyrosination?

Microtubules are composed of α- and β-tubulin, which are encoded in small gene families. For instance, in rice, there are three α-tubulins differing in their C-terminus, whereas most domains are fairly identical in sequence. Early attempts to explain the different populations of microtubules on the base of different tubulin isotypes turned out to be wrong, microtubules are assembled in mixtures. However, different populations can differ with respect to posttranslational modifications that are quite elaborate in the tubulins. For instance, all eukaryotic α-tubulins harbour a C-terminal tyrosine. This can be cleaved off by a tubulinyl tyrosine carboxylase (TTC), and it is religated by a tubulinyl tyrosine ligase (TTL). Since the TTC prefers microtubules over soluble dimers, whereas the TTL acts preferentially on soluble dimers, there is a correlation: stable microtubules are mostly detyrosinated, whereas dynamic microtubules are mostly tyrosinated. While the TTL has been identified from porcine brain and TTL homologues are present also in plants, the TTC has remained enigmatic – only the enzymatic activity has

been measured, but the responsible protein has not been found. Early this year, the putative TTC has been claimed to be identified in animal systems, but plant homologues seem to be absent, such that the TTC mystery remains at least for plant cells.

Why are these posttranslational modifications important? Because this provides a mechanism, by which microtubules become different in molecular terms depending on their turnover. This allows interacting proteins (such as microtubule motors) to recognise, how dynamic a microtubule is. If they bind depending on presence or absence of dynamics, we come to a functional diversity of different microtubule populations.

The difference of just one amino acid may seem quite irrelevant. However, there exist antibodies that can distinguish tyrosinated and detyrosinated tubulin, and we have developed even a one-step protocol based on affinity to ethyl-N-phenylcarbamate (a traditional blocker of potato sprouting acting on microtubules) to separate the two versions of plant α-tubulins (Wiesler et al. 2002). To find out, what the "meaning" of detyrosination might be, we have used pharmacological manipulation as approach: by feeding nitrotyrosine (which can be ligated by TTL, but not cleaved off by TTC), we created a situation, where tyrosinated microtubules were more abundant (overrunning the difference in dynamics). This caused phenotypical changes including reduced mitosis, and disorientation of cell plates (Jovanovic et al. 2010). By using a specific TTC inhibitor from Feverfew, Parthenolide, we later could show that the binding of a plant specific "wrong-way" kinesin, KCH, to cortical microtubules was altered (Schneider et al. 2015). When reorientation of microtubules was induced by auxin depletion, we could show that microtubules of different direction differ also in the degree of their tyrosination (Wiesler et al. 2002): transverse microtubules were tyrosinated, longitudinal microtubules were detyrosinated. The tyrosination of tubulin might, therefore, be one of the molecular factors, by which plants control orientation (and thus, cell and plant shape).

41. Wiesler B, Wang QY, Nick P (2002) The stability of cortical microtubules depends on their orientation. Plant J 32, 1023-1032
77. Jovanovic A, Durst S, Nick P (2010) Plant cell division is specifically affected by nitrotyrosine. J Exp Bot 61, 901-909
111. Schneider N, Ludwig H, Nick P (2015) Suppression of tubulin detyrosination by parthenolide recruits the plant specific kinesin KCH to cortical microtubules. J Exp Bot 66, 2001-2011

Our tool: a TTL from rice

The TTC is still elusive, although the enzyme activity had been discovered more than 30 years ago in microtubules from porcine brain. The TTL has been identified, however, the plant versions of TTL are quite different from their animal counterparts in harbouring long N-terminal extensions. However, they have preserved the active centers. We have identified and cloned two TTLs from rice. For one of them, TTL1, in cooperation with researchers in Korea, transgenic rice plants could be generated, where TTL1 is overexpressed in fusion with RFP. In previous work, we could show that tobacco BY-2 cells that overexpress this fusion show specific phenotypes such as disoriented cell plates (i.e. the same phenotype, we had seen with nitrotyrosine). The localisation *in vivo* appeared to be more or less cytoplasmic, which was a bit disappointing. However, we thought that this might be an overexpression artifact, because TTL-RFP was overexpressed under control of a strong promoter (CaMV-35S), such that the microtubular signal might be covered by excessive unbound TTL-RFP. We therefore stained microtubules by immunofluorescence, because, during the staining procedure, the cells are fixed and permeabilised, such that unbound protein would be washed out. In fact, we found that under these conditions, the RFP-TTL signal showed a good colocalisation with different microtubule structures, most evident for the phragmoplast (the microtubular array orienting the cell plate). This result indicates that TTL is in fact binding

microtubules in a specific way (**Fig. 1**) - the binding to the phragmoplast also would explain, why the overexpressor shows disoriented cell plates.

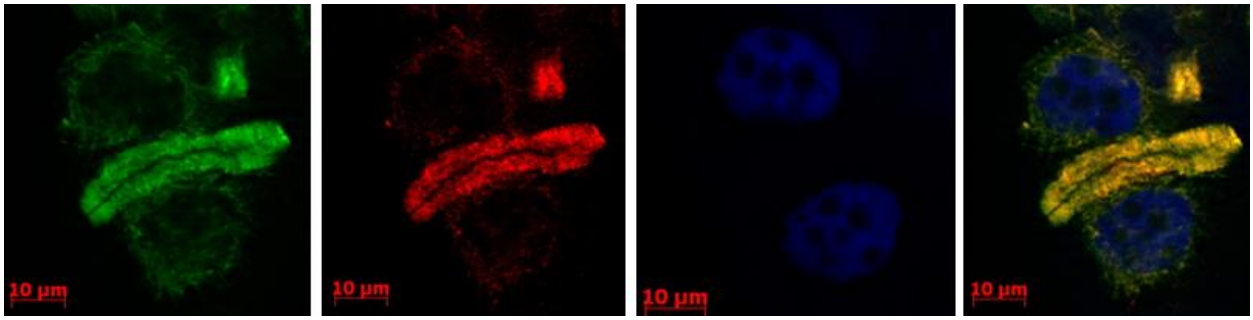

Figure 1: triple staining of a telophasic tobacco BY-2 cell, where microtubules are stained in green (using FITC-based immunostaining), TTL1 in red (using RFP as marker), and DNA in blue (using Höchst 33258). The image at the right gives a merge of all three channels, the yellow colour reports colocalisation of the green and the red signal (Steffen Durst, unpublished).

What was unexpected: the microtubules turned out to be more detyrosinated in the overexpressor, both seen for the heterologous system tobacco BY-2 as for the homologous system rice (Zhang et al. 2020). This was, as to be expected, by a reduced turnover of microtubules. In addition to the TTL overexpressor, we have in the meantime obtained a double-labelled line, where TTL as RFP fusion is visible in the background of a GFP-tagged tubulin marker line (Zhang et al. 2022).

163. Zhang K, Durst S, Zhu X, Hohenberger P, Han MJ, An GH, Sahi V, Riemann M, Nick P (2021) A rice tubulin tyrosine ligase-like 12 protein affects the dynamic and orientation of microtubules. J Int Plant Biol 63, 848-864

175. Zhang KX, Shi WJ, Zheng X, Liu X, Wang LX, Riemann M, Heintz D, Nick P (2022) A rice tubulin tyrosine ligase like 12 regulates phospholipase D activity and tubulin synthesis. Plant Science 316, 111155

Objectives of the project

- When the turnover of microtubules is reduced upon overexpression of rice TTL, this should bear on the effect of compounds that act depending on turnover (such as tubulin dimer sequestering compounds). How do microtubules respond to the compounds in the rice TTLox-RFP / TuA3-GFP line behave compared to the line where TuA3-GFP is expressed alone?

- Overexpression of TTL results in a higher level of tubulin detyrosination. A compound that binds to the C-terminus, should therefore display a different sensitivity to the compounds leading to a shift in the dose-response curve between the TTL-RFP line compared to the WT line, and of the TTL-RFP / TuA3 GFP compared to the TuA3 line. This can be scored by measuring sugar consumption or the development of fresh weight of the culture in response to different concentrations of the compounds.

- Compounds that act directly on tubulin should be more sensitive to the resulting detyrosination of microtubules compared to compounds that act on associated proteins. This should lead to a different behaviour for class-I and class-II compounds.

Approaches and workpackages

WP1. Following the microtubule response in the rice TTL-RFP / TuA3-GFP versus the TuA3-GFP line. As representative of class-I compounds, a compound is used that is called 4 (please do not forget that these

compounds come from our industrial partner and, therefore, are coded). As representative of class-II compounds, compound 7 is used. Both are administered in the same concentration (10 µM) for one hour. Then, microtubules (labelled by GFP) and TTL (labelled by RFP) are inspected by spinning-disc confocal microscopy compared to the control. This is done comparatively for the two cell lines, where TTL-RFP is present or not. The experiment is conducted with cortical microtubules. Therefore, a mature culture is used.

WP2. Dose-response curve for the compounds in dependence of TTL-RFP. To test sensitivity to a compound, the cells are cultivated in different concentration of a compound to see, whether the dose-response curve of the effect is shifted on the x-axis. Is it shifted to the right (more compound needed to get a certain effect), this means a reduced sensitivity, is it shifted to the left (less compound needed to get a certain effect), this means an increased sensitivity. If you want to learn more about this approach coming from formal physiology read the first section of the new Strasburger (page 379-381) about hormonal sensitivity versus hormonal responsiveness (the book can be obtained as e-book free of charge from the KIT-BIB, when you are a KIT-student). Practically, there are limitations when you have to handle four cell lines with several concentrations. So, we will confine the concentrations on three: 0 µM, 1 µM, 10 µM, which will allow to see, whether the slope of the line is changing and tell to what extent 10 µM was already to the saturation. There are different ways to score the response – to extract the maximum of data points from one experiment, the measurement should be non-destructive. Since this is a quantitative experiment, standardisation is crucial. Since the behaviour of a cell line is strongly dependent on the amount of inoculum, we use the same amount of fresh weight (0.5 g) for inoculation.

Here, we have found three approaches:

- Sugar consumption. The cells get 3% sucrose as carbon source in the beginning of the cycle. This sugar is consumed during the cycle. This can be measured in a non-destructive way: a small volume of supernatant can be sampled, and the remaining sucrose determined by refractometry (using a so-called Brix-meter, as it is used in food industry). To get insight into the timing, the sampling is done at several time points (start of the cycle, after ¼, ½, ¾ of the cycle, end of the cycle).

- Fresh weight. The culture grows by cell division and subsequent cell expansion. These processes differ in their sucrose demand. Therefore, to get a full picture of growth, the increase in cell fresh weight is scored using an aliquot of 1 ml. The starting point does not need to be measured, because the inoculum is selected such that it corresponds to 0.5 g. Since the fresh weight in the beginning is very small, a measurement at ¼ of the cycle does not make much sense, because the variability of the readout is too high, Thus, for the fresh-weight experiment, it is sufficient to score at ½, ¾ and at the end of the cycle.

- Since the elimination of microtubules will eventually kill the cell, a readout for mortality is useful. There exist two approaches – one is a vitality stain by fluorescein diacetate (FDA), the other is a dye exclusion assay by Evans Blue, a dye that is not membrane permeable and, thus, can only stain cells that have lost membrane integrity (these are the dead cells). The FDA approach is not feasible in cells that express a fluorescent marker. Therefore, the Evans Blue approach is used. This can be done with the cells that had been used for the fresh weight measurement to minimise the volume that has to be taken from a running culture.

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).

Cell lines used in the experiment

- Non-transformed Bright Yellow 2 (BY-2) Wild type
- TuA3-GFP: this line is expressing tobacco tubulin α3 (NtTuA3) as GFP fusion (GFP fused to the N-terminus to let the C-terminus, where the MAPs bind unmasked) under control of the strong and constitutive Cauliflower Mosaic Virus 35S promoter (CaMV 35S). This line allows to see microtubules. Compared to the wild type, microtubules show mildly reduced turnover, leading to perturbations of cell axis. Note: These can be rescued by adding 5 µM of the natural auxin indole acetic acid at the time of subcultivation. For the context of the current experiment, we will not do this, because we would otherwise need to test the effect of auxin in all the lines, which is beyond the time frame of the experiment. For the subsequent Bachelor thesis, this should be considered, though.
- TTL-RFP: this line is expressing a tubulin tyrosine ligase from rice in fusion with RFP under control of a CaMV 35S promoter. It shows a higher detyrosination level of α-tubulin and a reduced turnover of microtubules leading to a higher resistance to compounds that act by sequestering tubulin dimers from integration into the microtubules (such as the herbicide oryzalin). Details are given in Zhang et al. 2021 (this paper is essential for your work. You should read it carefully).
- TTL-RFP / TuA3-GFP: this line is combining the two markers, such that the behaviour of microtubules can be followed in a situation, where TTL is overexpressed. This allowed to address the role of microtubule dynamics for cold sensing and to discover interesting regulatory feedbacks of microtubule integrity upon tubulin transcripts. Details are given in Zhang et al. 2022 (also this paper is essential for your work).

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

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. 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 built

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.