EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN

Research Activities 2011

Z MBP

**ZENTRUM FÜR
MolekularBiologie
der Pflanzen**

Contents

ZMBP: Deciphering green life

Plants play a key role in life on earth and form the basis for our human existence: They assure our everyday life by providing energy, nourishment, shelter, clothing, medicine, and of course, the oxygen that we breathe. A growing world population and a concomitant shortage of natural resources make it clear that our survival will depend on sustainable, in fact increasing, agricultural crop yield. An important key to the harnessing and improvement of plants is, first of all, to understand them.

The Center for Plant Molecular Biology (ZMBP) in Tübingen, Germany, is dedicated to basic plant research. The aim of our research is to understand, at the molecular level, how plants establish an organized body plan and how they adapt their growth and development to their environment. The ZMBP was founded in 1999 as a research-oriented university institute and provides facilities for about 150 scientists in sixteen independent research teams. In the short time since its foundation, the ZMBP has developed into an internationally recognized center of excellence for plant molecular biology. The different teams not only share central service facilities but also their know-how, resulting in numerous fruitful collaborations. Until now, the different groups have been spread over three buildings. However, thanks to joint funding by the federal and state governments, a new ZMBP building is being constructed, which will gather all the groups under one roof in 2012. The spatial proximity of the different research teams will further promote collaborations and synergy effects. To ensure that the high scientific standard of the ZMBP is maintained, the institute is regularly evaluated by an international advisory board of seven to ten leading plant scientists.

An integral part of the philosophy at the ZMBP is to support young scientists at different levels of their careers. To provide an optimal ground for young group leaders, the traditional university chairs were disbanded in favour of a departmental structure and flat hierarchies. Young team leaders are provided with substantial core funding, giving them a head start into their scientific independence. The success of this concept can be measured by career opportunities of its junior members. In the last 10 years 12 former ZMBP group leaders were appointed to professorships at scientific institutions in Germany and abroad. As a consequence, the ZMBP has seen a substantial turnover in research groups. It is this turnover that keeps the ZMBP young and dynamic.

The success of any group at the ZMBP depends on its ability to attract competitive external funding. Considering its relatively short history, the performance of the ZMBP in this area has been impressive: The Tübingen University plant science community, of which the ZMBP constitutes a substantial part, leads the plant biology ranking based on competitive funding in the plant sciences of the „Deutsche Forschungsgemeinschaft", the main funding body in Germany. This funding allows research activities to be maintained at high level as documented by the progress reports of the individual groups.

The research activities of the ZMBP depend on the excellent contributions of highly dedicated graduate students and postdocs. Currently, there are some 54 Ph.D. students and 27 postdocs working at the ZMBP. The ZMBP dovetails its scientific and teaching commitments by offering a unique master's program. In the inspiring and research-oriented atmosphere of the ZMBP, this program trains students in modern and advanced cell and molecular plant

research. After completion of the master's program, students are eligible to enter the doctoral programs offered at the University of Tübingen.

Tübingen, November 2010

Georg Felix, Rita Groß-Hardt, Klaus Harter, Gerd Jürgens, Thorsten Nürnberger, Claudia Oecking, Fritz Schöffl

Current Research Group Leaders

Frédéric Brunner	Function of microbial effectors in plants and pathogen recognition
Georg Felix	Ligand specificity and activation mechanism of plant receptor kinases
Rita Groß-Hardt	Cell specification in the female gametophyte of *Arabidopsis*
Klaus Harter	Plant signal perception, transduction and integration
Andreas Hiltbrunner	From light perception to gene expression
Gerd Jürgens	Membrane traffic in development
Sascha Laubinger	MicroRNA processing and function
Sabine Müller	Molecular control of division site establishment
Thorsten Nürnberger	Plant innate immunity
Claudia Oecking	Function of plant 14-3-3 proteins
Peter Pimpl	Protein sorting in the secretory pathway
Gabriel Schaaf	Sec14 proteins and regulation of phosphoinositide homeostasis in *Arabidopsis*
Frank Schleifenbaum	Plant high resolution imaging/spectroscopy
Fritz Schöffl	Regulation of heat stress response and leaf senescence
Andreas Wachter	Alternative pre-mRNA splicing in plants
Stephan Wenkel	Environmental control of morpho-genesis in plants

Former Research Group Leaders

Jiri Friml	Auxin transport and action (Gent)
Wolf B. Frommer	Molecular physiology of metabolite transport and sensing (Stanford, USA)
Vera Hemleben	Genome organization and molecular evolution in higher plants (retired)
Frank Hochholdinger	Genetic dissection of root formation in maize (Bonn)
Martin Hülskamp	Cell morphogenesis (Cologne)
Thomas Laux	Shoot meristem development (Freiburg)
Volker Lipka	Molecular dissection of non-host resistance in *Arabidopsis* (Göttingen)
Uwe Ludewig	Molecular biology and electrophysiology of nitrogen transport (Hohenheim)
Doris Rentsch	Mechanisms for nitrogen distribution (Bern)
Thomas Schmülling	Hormonal control of growth and differentiation (Berlin)
Karin Schumacher	pH-homeostasis in the endomembrane system (Heidelberg)
Claus Schwechheimer	Regulated plant growth by regulated proteolysis (Munich)
Hanns-Ulrich Seitz	Plant-microbe interactions (retired)
John M. Ward	Sucrose transporters in long-distance transport (Minnesota)
Nico von Wirén	Transport of mineral nutrients (Gatersleben)

Developmental Genetics and Cell Biology

OVERVIEW

Developing organisms are growing populations of cells that exchange information about their relative positions and, in response, adopt specific fates. This is, in essence, the process of pattern formation that transforms the fertilized egg cell into a multicellular organism with its distinct spatial arrangement of cell types, tissues and organs. Animals establish their body organization during embryogenesis so that the newborn is a miniature adult. By contrast, plant embryogenesis generates a juvenile form, the seedling that bears little resemblance to the adult plant. During post-embryonic development, plants produce new structures, such as leaves and flowers, from self-maintaining stem-cell systems called meristems. Meristems themselves are part of the seedling body organization that is established during embryogenesis.

Plants and animals evolved independently from unicellular ancestors. It is, therefore, anticipated that plant development involves different mechanisms to those found in animal systems. For example, neighboring cells within a plant share regulatable cytoplasmic channels, called plasmodesmata, that can facilitate the passage of large molecules including transcription factors. Plants also make use of long-distance signaling, such as directional transport of the hormone auxin, to integrate developmental processes. Although surrounded by a cell wall, plant cells can signal to each other by local ligand-receptor interaction at the plasma membrane, just as animal cells do. The responses to signaling are also manifold and include transcriptional regulation of gene expression, mRNA processing and stability as well as rapid targeted degradation of gene-regulatory proteins. Mechanisms of cell-cell communication and cell-fate specification in the female gametophyte are analyzed in the research group of Rita Groß-Hardt.

Plant cells have their own distinct organization. For example, plant cells are encased by rigid cell walls that prevent them from leaving their neighborhood. As a consequence, temporal and spatial control of cell division rates and planes, as well as oriented cell expansion, underlie the morphogenetic processes that shape the embryo or individual organs. How the establishment of cell division sites is molecularly controlled is investigated in the research group of Sabine Müller.

Recent years have seen an increasing awareness of membrane traffic underlying signaling in development and plant-microbe interaction, as well as physiological processes such as nutrient transport. Secretory traffic mediates processing and delivery of secreted signaling peptides to the cell surface. Both secretory traffic and endocytosis, followed by recycling or traffic to the vacuole for degradation, provide important mechanisms for regulating the abundance and signaling activity of receptors as well as the availability of nutrient or ion transporters at their sites of action. The regulation of trafficking pathways during vesicle formation and membrane fusion is analyzed in the research group of Gerd Jürgens. Mechanisms underlying protein sorting in the trafficking pathway to the lytic vacuole are investigated in the research group of Peter Pimpl.

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Mechanisms of plant gamete formation
Rita Groß-Hardt

Formation and specification of gametes is of paramount importance for all sexually reproducing organisms. In contrast to animals, plant gametes develop in few-celled haploid structures, termed gametophytes. The female gametophyte of *Arabidopsis* consists of four distinct cell types (Fig. 1). The egg and the central cell get fertilized to form the main components of the seed. These gametes are flanked by accessory cells (synergids and antipodal cells) that partially aid fertilization.
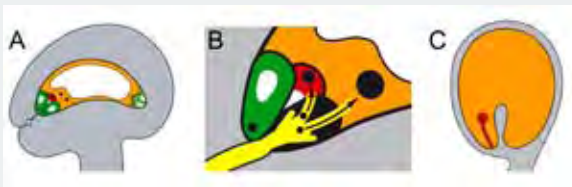

Figure 1 Female gametophyte and seed formation. (A) The female gametophyte contains seven cells, which differentiate into four distinct cell types: two synergids (dark green), one egg cell (red), one central cell containing two polar nuclei (orange) and three antipodal cells (light green). (B) The pollen tube (yellow) delivers two sperm cells, which fuse with the egg cell and the central cell. (C) The fertilization products develop into embryo (red) and endosperm (orange), respectively (Kägi et al., 2010).
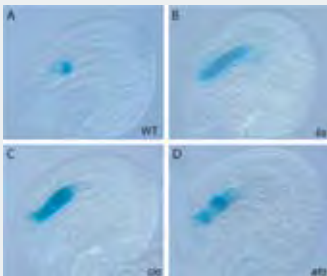

Figure 2 Expression of the egg cell marker ET1119 in wildtype and mutant gametophytes. *GUS* expression in wildtype (A) is restricted to the egg cell. By contrast, in *lis*, *clo* and *ato* gametophytes, *GUS* expression is also detected in synergids and central cell (B-D) (Moll et al., 2008)

Forward genetic screens have identified mutants with altered egg cell marker gene expression

We are interested in the intra- and intercellular mechanisms underlying the specification of the distinct cell types. To identify factors necessary for the regulation of egg cell fate, we have screened for mutants with deviating egg cell marker gene expression.

The first class of mutants identified includes the *lachesis* (*lis*), *clotho* (*clo*) and *atropos* (*ato*) mutants. All three mutants are characterized by the formation of supernumerary egg cells (Fig. 2), which differentiate at the expense of the adjacent synergids and central cell. Additionally, antipodals of mutant gametophytes can adopt a central cell fate. Our data show that all cells in the female gametophyte can differentiate into gametes, and that this gametic competence is repressed by *LIS*, *CLO* and *ATO* (Groß-Hardt et al., 2007, Moll et al., 2008).

The second class of mutants shows no, or reduced, egg cell marker expression, whereas expression of an antipodal cell marker is unchanged (Fig. 3).

Is there a link between pre-mRNA splicing and cell fate decisions in the female gametophyte?

Using a map-based cloning approach, we could show that *lis*, *clo* and *ato* are defective in the putative core spliceosomal components PRP4, Snu114 and SF3a60, respectively (Groß-Hardt et al., 2007, Moll et al., 2008). This is surprising, as the specific and late defects observed in the mutants are difficult to reconcile with general pre-mRNA splicing defects. In yeast, it was shown that defects in different core spliceosomal components result in different transcript profiles, indicating that the composition of the spliceosome critically determines transcript readout. To address whether LIS, CLO and ATO affect splicing in a substrate specific manner, we have started to establish an *in-planta* splice assay (Fig. 4) and sequenced DNA from a *clo* hypomorphic allele using paired-end Illumina sequencing (collaboration with Gunnar Rätsch, Max Planck Institute, Tübingen).
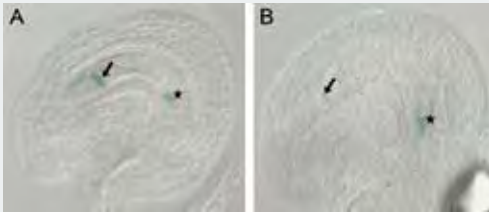

Figure 3 Ovules expressing egg- and antipodal reporters. (A) Ovule showing *GUS* expression in the egg (arrow) and antipodal cell (star). (B) Ovule with *GUS* expression in antipodals only (Kägi et al., 2010).
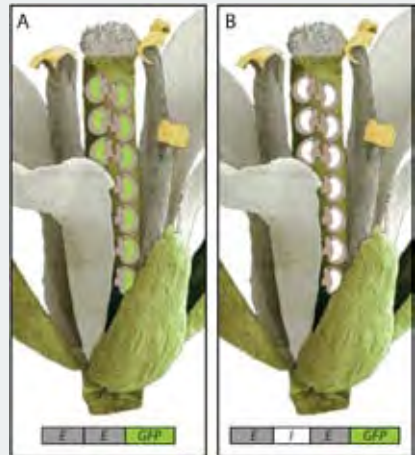
Figure 4 Principle of the *in-planta* splicing assay. For candidate genes, exon (*E*)-intron (*I*)-exon constructs have been generated and fused to a C-terminal *GFP*. As intron retention results in a nonsense transcript (B), GFP is only detected if the intron is spliced out (A).

Cell-cell communication in the female gametophyte

Promoter-reporter analysis suggests that *CLO* and *ATO* are expressed in all female gametophytic cells. By contrast, in mature female gametophytes, *pLIS::NLS_GUS* is only detected in the egg and the central cell, suggesting that the gametic competence of accessory cells is repressed by a mechanism generated in gametic cells. We are currently testing whether *LIS* is indeed only required in gametic cells by modulating *LIS* transcript amounts in a cell specific manner.

Antipodals as a model system to study the regulation of cellular lifespan

Arabidopsis contains three antipodals, which undergo programmed cell death (PCD) shortly before fertilization. As mentioned above, in *lis*, *clo* and *ato* mutants, antipodals adopt a central cell fate, which implies that the developmental program that results in antipodal PCD is repressed. It is unclear whether the ability to extend the cellular lifespan and to differentiate gametic cell fate is of adaptive significance, however it is conceivable that antipodals act as backup in case of central cell failure. Making use of mutants that show defects in antipodal PCD, we study the mechanisms that activate or bypass the cellular suicide program in these ephemeral cells.

Membrane traffic in development
Gerd Jürgens

Eukaryotic cells display functionally distinct membrane compartments such as ER, Golgi or endosomes that exchange proteins by membrane traffic involving transport vesicles (Fig. 1). In addition to maintaining the functional organization of the endomembrane system, membrane traffic regulates the concentration of signaling peptides, receptors and transporters at their site of action by delivery along the secretory pathway from the ER, by endocytosis from the plasma membrane and recycling or targeting to the vacuole for degradation.

Membrane vesicles are formed on donor membranes and fuse with specific target membranes. Vesicle formation requires the activities of conserved protein families such as ARF guanine-nucleotide exchange factors (ARF-GEFs) and their substrates, ARF GTPases, as well as specific coat proteins and GTPase-activating proteins (ARF-GAPs) (Fig. 2a). Transport
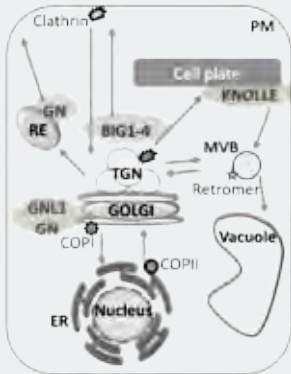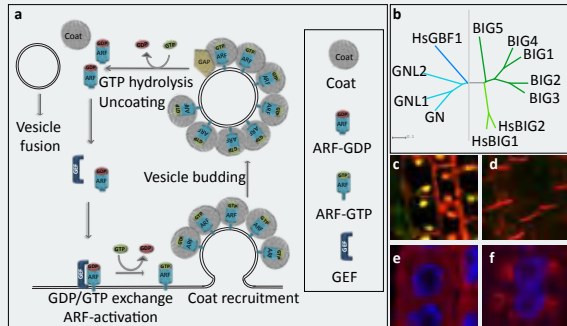

Figure 2 ARF-GEFs and vesicle formation
(a) ARF GDP/GTP cycle. Cytosolic ARF-GDP is recruited to membrane by ARF-GEF mediating GDP-GTP exchange. ARF-GAP stimulated GTP hydrolysis leads to dissociation of ARF-GDP from the membrane. (b) The *Arabidopsis* family of ARF-GEFs related to human GBF1 and BIG1. (c,d) GNOM (green) regulates polar recycling of PIN1 (red) from endosomes to the basal plasma membrane. (c) BFA-induced inhibition of GNOM activity causes accumulation of GNOM and its cargo PIN1 in BFA compartments. (d) No BFA effect in BFA-resistant GNOM plants. (e,f) GNL1 and GNOM regulate retrograde Golgi-ER traffic. BFA treatment prevents Golgi-membrane association of COPI (red) in *gnl1* mutant (e) but not in wild-type (BFA-resistant GNL1) (f). Blue, DAPI-stained nuclei.

Figure 1 Membrane trafficking pathways in *Arabidopsis*
Key regulators (ARF-GEFs GN, GNL1, BIG1-4) and coat proteins (COPI, COPII, clathrin, retromer) are indicated. ER, endoplasmic reticulum; MVB, multivesicular body; PM, plasma membrane; RE, recycling endosome; TGN, trans-Golgi network.

vesicles interact with their target membrane via RAB GTPases and tethering proteins. Subsequent membrane fusion is mediated by the formation of 4-helical trans-SNARE complexes between R-SNAREs (v-SNAREs or VAMPs) on the vesicle and Q-SNAREs or t-SNAREs (syntaxins/Qa-SNAREs and t-SNARE light chains/Qb,Qc-SNAREs or SNAP25/Qb,c-SNAREs) on the target membrane (Fig. 3). Our research addresses mechanisms of specificity during vesicle formation and vesicle fusion.

Regulation of trafficking pathways by ARF-GEFs and ARFs
The *Arabidopsis* genome encodes eight 150-220kDa large ARF-GEFs that are members of a conserved eukaryotic protein family comprising two classes (GGG, BIG): 3 (GNOM, GNL1 and GNL2) are related to the human *cis*-Golgi ARF-GEF GBF1, whereas 5 (BIG1-5) are related to the human trans-Golgi ARF-GEF BIG1 (Fig. 2b; Anders & Jürgens, 2008). GNOM and GNL1 jointly regulate the retrograde COPI-mediated traffic from the Golgi to the ER,

which is the ancient eukaryotic function of the GBF1 class (Fig. 2e,f; Richter et al., 2007). Inhibition of GNOM activity by the fungal toxin brefeldin A (BFA) in the *gnl1* mutant blocks the early secretory pathway, resulting in the retention of secretory cargo proteins in the ER. Two additional plant-specific ARF-GEF functions have evolved: GNOM mediates polar recycling of the auxin-efflux transporter PIN1 from endosomes to the basal plasma membrane, which is required for cell polarity and axis formation in embryogenesis (Fig. 2c,d; Geldner et al., 2003), whereas GNL2 plays a specific role in pollen germination and pollen-tube growth (Richter et al., unpubl.). The ARF-GEFs BIG1 to BIG4 jointly perform an essential function in the late secretory pathway, whereas the role of BIG5 is not clear (Richter et al., unpubl.).

Our research aims to analyze mechanisms underlying the pathway specificity of ARF-GEF action. To this end, we analyze how large ARF-GEFs associate with specific membrane compartments and attempt to identify their endogenous ARF substrates as well as the associated coat proteins for cargo selection.
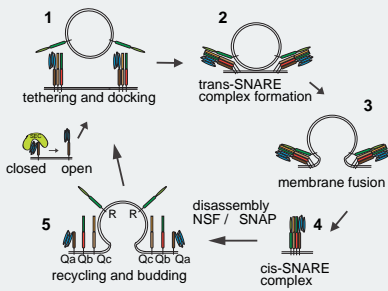

Figure 3 Membrane fusion through SNARE complex formation Disassembly of the cis-SNARE complex converts the open form of syntaxin to a closed form (5), which has to be reverted before trans-SNARE complex formation (1).
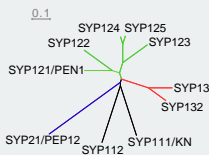

Figure 4 SYP1 family of plasma-membrane syntaxins The 3 subgroups are highlighted. Prevacuolar syntaxin PEP12 (SYP2 family) represents the outgroup.

Figure 5 Role of KNOLLE in cytokinesis
(a) KNOLLE (green) trafficking from TGN (trans-Golgi network; early mitosis) to the plane of cell division (CP, cell plate) followed by endocytosis and trafficking via the PVC (prevacuolar compartment; late) to the vacuole for degradation. Red signals represent compartment-specific markers. (b) Membrane fusion during cytokinesis is impaired in *knolle* mutants, resulting in the accumulation of unfused vesicles (top; n, nucleus).

Specificity of syntaxin action in membrane fusion

The *Arabidopsis* genome encodes 18 syntaxins, which represent 5 families (SYP1, 2, 3, 4 and 8) that are evolutionarily conserved among eukaryotes. The SYP1 family comprises 8 plasma membrane-localized members and a unique plant-specific syntaxin named KNOLLE, which is exclusively required for membrane fusion during cytokinesis (Fig. 4,5b; Müller et al., 2003).

KNOLLE is only expressed during G2/M-phase, specifically targeted from the trans-Golgi network (TGN) to the plane of cell division during cytokinesis, subsequently endocytosed from the cell plate and degraded in the vacuole (Fig. 5a; Reichardt et al., 2007).

Our research addresses mechanisms underlying the specificity of action of syntaxins in cytokinesis and secretion. To this end, we analyze how KN and other SYP1 syntaxins are targeted to their site of action, attempt to identify their endogenous interactors including SM proteins such as KEULE and other SNAREs, and analyze syntaxin dynamics and turnover.
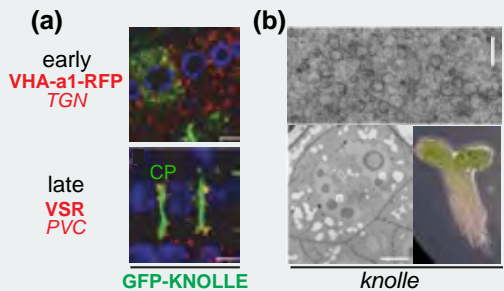
Molecular Control of Division Site Establishment

Sabine Müller

Plant cells are surrounded by rigid cell walls, which confine their shape and determine their location within tissues. Thus, the position of a new cell wall formed during cytokinesis contributes to the shape of the cell and consequently to the overall morphology of the plant.

In plants, the positional information for the future division site is specified early, at G2/M transition by the formation of the preprophase band. This transient cytoskeletal array forms at the cell cortex and demarcates the future division plane, but disassembles upon entry into mitosis. Subsequently, the division plane is identified by the absence of cytoskeletal proteins (actin depletion zone, KCA1 kinesin depletion zone) and the recruitment of specific cortical division site markers TANGLED and a component of the Ran signaling network RanGAP (Fig. 1, Müller et al., 2009).
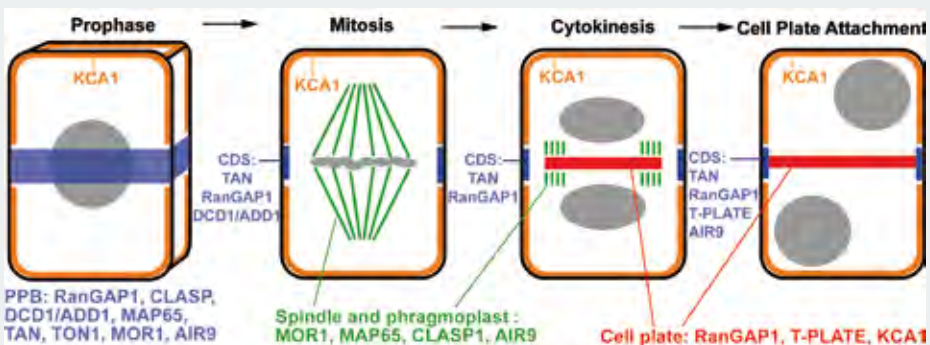

Figure 1 Schematic representation of the cortical division site throughout mitosis. Cortical division site (CDS) and associated proteins at different cell cycle stages are indicated in blue. Note that KCA1 remains absent from this site throughout mitosis.

Previously, we characterized a pair of closely related kinesins *PHRAGMOPLAST ORIENTING KINE-SIN (POK) 1* and *2*, which are implicated in the positioning of new cell walls after cell division (Müller et al., 2006). *pok1pok2* double mutants exhibit pronounced cell wall positioning defects, as a consequence of misguided phragmoplasts during cytokinesis (Fig. 2). Genetic and *in vitro* evidence suggests that POK kinesins are required for the maintenance of TANGLED and RanGAP1 at the cortical division site from metaphase onward (Walker et al., 2007, Xu et al., 2008).

In our current work we focus on the functional characterization of POK kinesins and on the identification of novel regulators of division site establishment.

Functional characterization of POKs

To investigate POK function, we generate translational fusions of full length POK and POK fragments with fluorescent protein variants to perform live cell imaging. In the context of cell cycle and cytoskeletal markers, these experiments will allow us to determine POK requirements at spatial and temporal resolution. Furthermore, we will determine the protein domains that confer sub-cellular specificity during different cell cycle stages.

Preliminary localization analysis of C-terminal POK fragments indicates that both kinesins require their C-terminal domain for localization to the cortical division site (Fig. 3). The localization pattern is reminiscent of TANGLED and RanGAP1 localization and we will perform co-localization and FRET analysis.

New regulators of the cortical division site

In yeast two hybrid screens, we have identified regulatory components of ROP signaling as interaction partners of POK-C terminal fragments. ROP signaling has recently been implicated in the regulation of actin and microtubule cytoskeleton organization (Yang, 2008). Thus, we pursue a reverse genetics approach for these novel ROP regulatory proteins to elucidate their developmental relevance and specific role in division plane establishment.
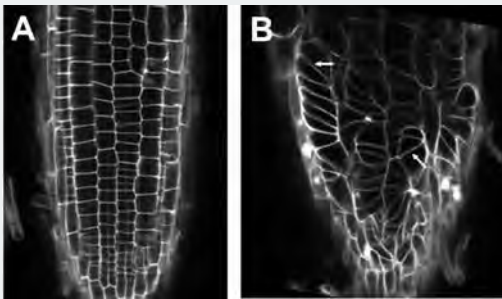

Figure 2 Aberrant cell wall positioning in *pok* double mutants. Cell walls of root meristems are visualized via Propidium Iodide staining. (A) In wild type root meristems, regular cell divisions result in an ordered pattern of cell walls. (B) In the *pok* double mutant new cell walls are inserted arbitrarily causing the irregular pattern.
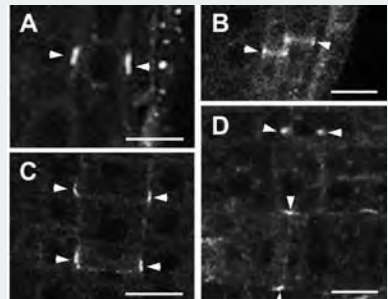
Figure 3 Localization of POK1-C terminal fragment. GFP-POK1C co-localizes with the PPB (A and B) and subsequently becomes delimited to the site of cell wall fusion during cytokinesis (C and D).

Alternatively, we use a sensitized mutant screen to identify enhancers of *pok1* single mutants which are indistinguishable from wild type plants. We have selected several mutants for further analysis. One candidate exhibits an enhanced *pok* double mutant phenotype and was confirmed to be a novel *pok1pok2* allele combination. The EMS induced mutation caused a premature Stop codon in POK2, abolishing most of the coiled coil interaction domains. We are continuing with our screen and have initiated mapping of promising candidates.

Phylogenic analysis showed that a third POK-like kinesin clusters with POK1 and POK2. It is specifically expressed during mitosis (Menges et al., 2003) and binds to RanGAP1 in a biochemical screen (D. Van Damme, personal communication). A T-DNA allele for *POK-like* is lacking transcript downstream of the T-DNA insertion and we will determine the localization pattern of POK-like.

Protein sorting in the secretory pathway

Peter Pimpl

A common feature of all eukaryotic cells is the existence of functionally distinct intracellular compartments. These compartments are dynamically interconnected via different vesicular carriers and, thus, constitute a complex network, termed the secretory pathway (Fig. 1). This pathway manufactures, distributes and degrades macro-molecules, like proteins and lipids, as a prerequisite for cellular viability and development.

Protein sorting to the lytic vacuole
Sorting of soluble proteins to the lytic vacuole requires vacuolar sorting receptors (VSRs). According to the common view, sorting is initiated by VSR-ligand interactions at the TGN, lea-
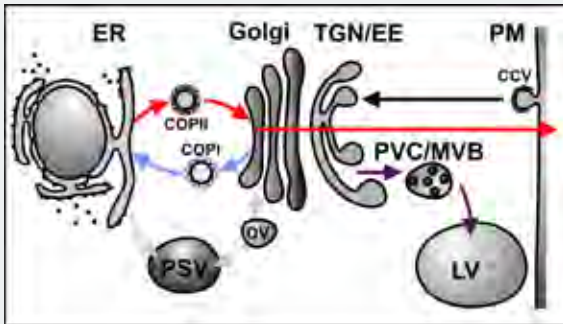

Figure 1 The plant secretory pathway. Secretion to the plasma membrane (red) leads from the ER via the Golgi and the trans-Golgi network (TGN). ER-resident proteins are retrieved back to the ER (blue). Endocytic transport (black) occurs from the PM to the TGN, the early endosome (EE). The lytic vacuole (LV) (purple) is reached via a prevacuolar compartment (PVC). Transport to the protein storage vacuole (PSV) might occur either from the ER or via dense-vesicles (DV) from the Golgi (grey).
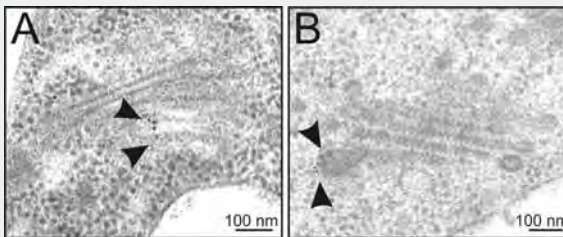

Figure 2 Retromer localizes to the TGN. IEM localization of the retromer subunits SNX2a (A) and VPS29 (B) in *Arabidopsis* roots after high-pressure freezing and *kryo*-substitution (Niemes et al., 2010a).

ding to the formation of a receptor-ligand complex. This complex is sorted into clathrin-coated vesicles, which mediate transport to the prevacuolar compartment (PVC). Upon arrival, the complex dissociates and, while the soluble cargo is delivered via fusion of the PVC with the vacuole, the receptor is recycled back to sort further ligands.

In yeast and mammalian cells, recycling of vacuolar/lysosomal sorting receptors occurs via a cytosolic protein coat, termed retromer. It consists of a heterotrimeric cargo recognition subunit (VPS35, VPS26 & VPS29) and two proteins of the sorting nexin (SNX) family, which recruit the heterotrimer and drive transport via their C-terminal coiled-coil domains.

To date, very little is known about the sorting and transport mechanisms of plant VSRs.

VSRs recycle from the TGN
In order to identify the recycling point of VSRs, we have localized retromer subunits *in situ* using immunogold electron microscopy. Surprisingly, the sorting nexin SNX2a and the VPS29

subunit of the cargo recognition complex are exclusively localized to the TGN (Fig. 2), indicating that retromer-mediated VSR recycling might already occur at the TGN.

To analyze retromer-mediated VSR recycling *in vivo*, we have manipulated retromer function by co-expressing a mutant of SNX2a, lacking the C-terminal coiled-coil domains (SNX2a-∆CC), with the fluorescent VSR-reporter GFP-BP80. The expression of SNX2a-∆CC leads to an accumulation of the VSR reporter GFP-BP80 in close proximity to the Golgi, presumably the TGN (Fig. 3). Since VSR-reporter and endogenous VSRs follow the same transport routes, it is likely that endogenous VSRs also accumulate in the presence of the SNX mutant, due to perturbed retromer function. However, the vacuolar delivery of the soluble model ligand GFP-sporamin is not affected under these conditions. This demonstrates that post-TGN transport of soluble vacuolar cargo does not require VSRs, which is in agreement with the definition of the TGN as the recycling-point of the VSRs.
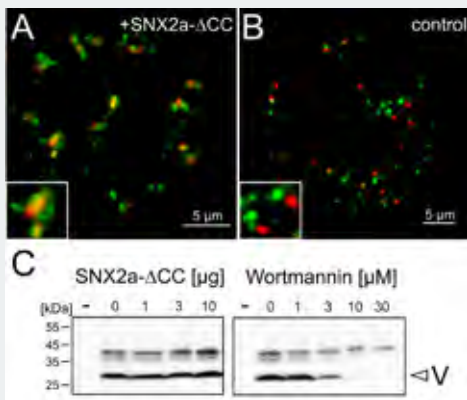

Figure 3 VSRs are not required for post-TGN sorting of soluble vacuolar cargo.
A, B) Expression of SNX2a-∆CC accumulates the VSR reporter (PVC marker) GFP-BP80 (green) in close proximity to the Golgi (red), presumably in the TGN. C) In contrast to wortmannin, SNX2a-∆ CC does not perturb vacuolar delivery of the VSR ligand GFP-sporamin as judged by the unchanged presence of the vacuolar degradation product (V) (Niemes et al., 2010a).
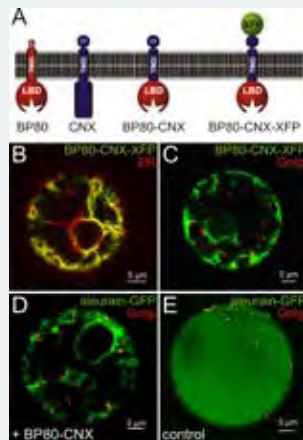

Figure 4 Receptor-ligand interaction occurs in the ER.
A) Molecular tools to analyze receptor-ligand interaction *in vivo*. B, C) BP80-CNX-XFP (green) co-localizes with the ER marker (red) and does not influence ER export of a Golgi marker (red). D, E) BP80-CNX accumulates the vacuolar reporter aleurain-GFP (green) in the ER (Niemes et al.,2010b).

VSRs sort cargo in the ER

The identification of the TGN as the recycling point of the VSRs raises the question of the location of the initial receptor-ligand interaction. VSRs and ligands are synthesized in the ER and we wanted to test whether they already interact in this compartment. We, therefore, generated ER-anchored VSR derivatives by fusing the lumenal ligand binding domain (LBD) of the VSR BP80 to the transmembrane domain (TMD) of the ER-resident chaperone calnexin (CNX) (Figure 4). Coexpression of the ER-anchored receptors with the soluble vacuolar reporter aleurain-GFP results in a strong accumulation of aleurain-GFP in the ER. This ER accumulation is specific for vacuolar cargo and does not generally perturb the ER exit.

Outlook

The future aims of the group are (a) to identify the target of the retromer-mediated route and (b) to investigate the dynamics of anterograde and retrograde VSR transport.

Genaral Genetics

OVERVIEW

The life of a plant is genetically determined but modulated by environmental cues such as light and temperature. Owing to their sessile life style, it is of the utmost importance that plants integrate environmental signals and respond by adjusting growth, differentiation and development. Thus, plants have the capacity to survive even under extreme abiotic stress conditions.

In recent years there has been a dynamic turnover of research groups and research areas in the General Genetics unit. Frank Hochholdinger, whose group is working on maize root development, has accepted a professorship at the University of Bonn and two new group leaders, Andreas Wachter (Emmy Noether fellowship) and Stephan Wenkel, joined the ZMBP.

The present research focuses on: heat shock response, leaf morphogenesis and senescence, and the mechanisms and functional roles of alternative mRNA splicing. It can be anticipated that various aspects of our research will have important implications for agriculture and plant biotechnology in the future.

The ongoing research on heat shock response is focused on the functions of the heat shock transcription factors (HSFs), which is an amazingly complex gene family in plants. The functional roles of HSFs appear to be very diverse and only a subclass seems to act as transcription factors during heat stress. HSF target genes include not only heat shock proteins but also a number of other genes involved in different cellular functions and biochemical pathways. Our long-term goals are directed towards understanding the functions of specific HSFs and HSF interactions during heat stress and the involvement of other HSFs in developmental decisions.

The regulatory network governing leaf senescence is very complex and largely unidentified. Very little is known about the developmental regulation of cell death in plants and why their protective systems against oxidative stress fail during leaf senescence. Our research is presently concentrating on the roles of WRKY transcription factors and hydrogen peroxide as a signaling molecule. As a long-term goal, a better understanding of the mechanisms integrating developmental and environmental signals at the onset of leaf senescence in annual plants is to be achieved.

The development of plant leaves is genetically determined but is also influenced by the environment. The integration of diverse signals affects physiological processes and leaf morphology. Our current work addresses the functions of different transcription factors and the role of plant hormones in leaf initiation and polarization.

The expression of many genes is regulated co- and post-transcriptionally at the mRNA level, e.g. by alternative splicing (AS) and mRNA decay. In recent years, AS has been recognized to be widespread in plants, however, many questions regarding its regulation and functional implications remain to be addressed. The current project is focused on genome-wide studies of AS and analysis of the molecular mechanisms underlying AS and nonsense-mediated decay of mRNAs in plants.

Experimental techniques include: (i) Next generation whole-genome short-read sequencing, microarray analysis, gene expression profiling and real time PCR assays, ChIP-Seq studies, proteome analysis (ii); generation/analysis of transgenic plants and gene knock out mutants; (iii) in gel staining of enzyme activities; (iv); expression of recombinant proteins in E. coli, yeast cells; (v) transient expression analyses in protoplasts and tobacco leaves; (vi) DNA-protein interaction by gel retardation, yeast-one hybrid; and ELISA-based assays; (vii) protein-protein interaction by yeast two-hybrid assays, BiFC.

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Regulation of heat stress response and leaf senescence Functions of Heat Shock Factors

Fritz Schöffl

The response to heat stress (HS) is highly conserved in organisms but owing to their sessile life style it is of the utmost importance to plants. The HS response is initiated by the activation of heat shock factors (HSF), which cause a transient expression of stress genes (e.g. heat shock proteins and others), resulting in the acquisition of higher levels of stress tolerance. HSF activation and HSP synthesis are also linked to other abiotic and biotic stresses and to developmental processes.

Class A HSF - central regulators of heat stress response

HSFs are DNA binding proteins, which recognise the conserved HSE sequences present in the promoter upstream regions of HS-genes. In *Arabidopsis,* 15 out of 21 potential HSF genes belong to subclass A, which represents transcription factors. Knock-out mutants of *HsfA1a* and *HsfA1b* revealed redundant functions required in the early phase of the HS response. In total, 112 HsfA1a/A1b target genes, including HSP, ascorbate peroxidase 2, ga-lactinolsynthase 1, were identified (Busch et al., 2005). HsfA1a and -A1b have the capacity to interact with each other in the nucleus of *Arabidopsis* protoplasts (Fig. 1) and synergistically cooperate in the expression of a number of HSF target genes, including *Hsp25.3*, *Hsp18.1-CI*, *Hsp26.5* (Li et al., 2010a, 2010b).
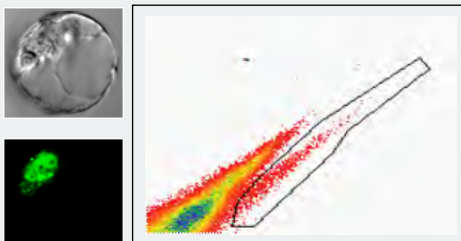

Figure 1 Interaction between class A-HSFs in the nucleus of *Arabidopsis* protoplasts. (a) bright field; (b) confocal image Bimolecular Fluorescence Complementation,; (c) quantification of fluorescent cells (boxed) by Flow cytometry (pictures by Ming Li)

Figure 2 Enhanced levels Pdf1.2 expression (a) and improved disease resistance (b) against Alternaria infections of *Arabidopsis* hsfB1/A1a knock out mutants compared to WT.
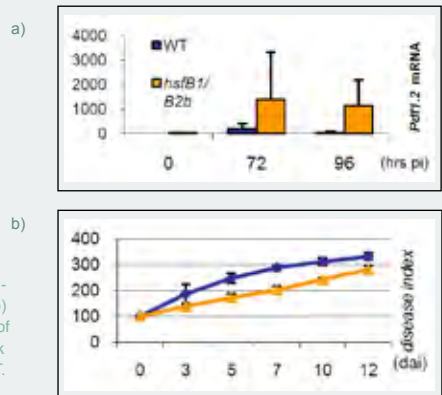
Class B HSF – regulators of biotic stress functions and development?

The five members of the *Arabidopsis* class B-HSF are devoid of a conserved activation domain and cannot act as transcription factors on their own. Microarray analysis of *hsfB1/hsfB2b* double knock-out plants revealed that *Pdf1.2* genes, which are involved in immunity against infection by necrotrophic microorganisms (Fig. 2), are the major targets (Kumar et al., 2009).

Regulation of these genes by HSF may require interaction with other proteins. Three different proteins were identified as interaction partners of both HsfB1 and HsfB2b, by yeast two-hybrid screening (Li et al., 2010a). HsfB1 and HsfB2b may be involved in regulatory networks linking abiotic and biotic stresses.

The functions of the other three *Arabidopsis* class B-HSF seem to be required under non-stress conditions in specific developmental processes.

Leaf senescence of annual plants
Ulrike Zentgraf

Natural or harvest-induced senescence is one of the major determinant factors causing agricultural crop losses, both in the field and during transport from the producer to the consumer. Besides endogenous signals, biotic and abiotic stress conditions can trigger senescence. Therefore, as a result of global climate changes, we will be forced to develop crop plants that cope better with stress conditions to avoid premature senescence. Despite the importance of the senescence processes, our knowledge on the regulation of leaf senescence is still poor. We are interested in understanding the mechanisms involved in the onset and regulation of leaf senescence of annual plants, using *Arabidopsis thaliana* as a model system.

Since many physiological changes take place during leaf senescence, a massive change in gene expression can be observed, however, not much is known about the factors regulating senescence associated gene expression. Large gene families of transcription factors, like WRKY or NAC factors, have been identified to play a regulatory role during leaf senescence. Our main interest is focused on elucidating the signaling network of the WRKY transcription factor family and its role in controlling leaf senescence. One member of the WRKY family, namely WRKY53, appears to have a central role in regulating the onset of leaf senescence. Diverse molecular analyses revealed that several different regulators are involved in the tight regulation of the WRKY53 expression, activity and degradation. Most of the WRKY53 regu-
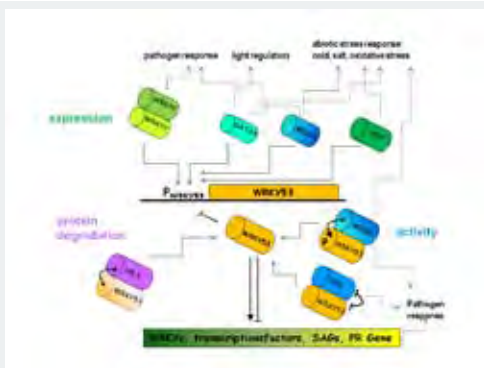

Figure 1 Model of the regulatory network of WRKY53

Figure 2 A hydrogen peroxide peak is created by the down-regulation of *CAT2* expression through GBF1 and is used as a signal to induce the expression of senescence-associated genes (SAGs).

lators have already been characterized to participate in different stress responses, indicating that WRKY53 is a node of convergence between stress response and senescence (Miao and Zentgraf 2007; 2010; for review see Zentgraf et al., 2010).

Hydrogen peroxide is thought to be one of the signaling molecules in leaf senescence. The expression of almost all WRKY53 regulators characterized so far is induced or repressed by hydrogen peroxide. The tight regulation of the H_2O_2 scavenging enzymes in development leads to an increase in H_2O_2 concentration at the bolting time. At this timepoint senescence should be turned on in all rosette leaves (Zimmermann et al., 2006). Down-regulation of CATALASE 2 expression and activity appears to be the initial step to create this peak. The bZIP transcription factor GBF1 could be identified to be responsible for the down-regulation of *CAT2* and *gbf1* mutant plant show delayed senescence (Symkowski et al., 2010)..

Alternative pre-mRNA splicing in plants

Andreas Wachter

Alternative precursor mRNA (pre-mRNA) splicing (AS) refers to the formation of multiple transcript variants from one type of pre-mRNA, by the removal of different intronic regions. With the enormous increase in the number of available transcript data in recent years, the prevalence of AS in higher eukaryotes has been revealed. For example, according to RNA sequencing data, up to 40% of all genes in *Arabidopsis thaliana* generate alternatively spliced mRNAs. AS can both increase proteome complexity and contribute to gene regulation, by the coupling of AS and mRNA turnover via nonsense-mediated decay (NMD; Fig. 1). Despite its wide distribution, many questions about the regulation and functional significance of AS events remain to be elucidated. In our work, we analyze novel regulatory mechanisms and the biological implications of AS in plants.
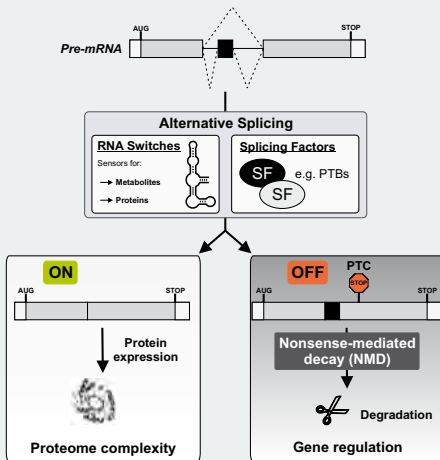

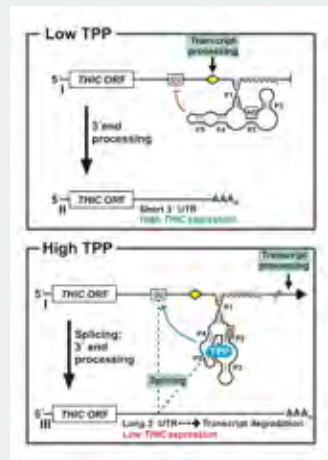**Figure 1** Regulation and functional implications of alternative splicing.

Figure 2 Plant TPP riboswitches control splicing and 3' end processing of *THIC* mRNAs.

Regulation of AS by Structured mRNA ¬Motifs

In bacteria and eukaryotes, the structural capacity and flexibility of mRNA are harnessed for gene control functions. So-called riboswitches are mRNA motifs, which can directly sense small molecules and exploit the thereby triggered structural changes for the regulation of gene expression. In eukaryotes, thiamin pyrophosphate-binding riboswitches have been shown to regulate AS (Wachter et al., 2010), affecting diverse, downstream processes, such as translation of upstream open reading frames in filamentous fungi (Cheah et al., 2007) or alternative 3' end processing in plants (Wachter et al., 2007; Fig. 2).

By combining bioinformatics with experimental strategies, novel structured mRNA motifs will be identified and characterized. This strategy has already resulted in the identification of a plant 5S rRNA mimic within the pre-mRNA of transcription factor IIIA, and a role for this element in the regulation of AS and the subsequent coordinated synthesis of ribosomal components was demonstrated (Hammond et al., 2009).

Functional Analysis of Novel Splicing ¬Regulatory Proteins in Plants

Besides the role of mRNA motifs in AS control, the functions and mechanisms of select proteinaceous splicing factors will be investigated. Our major interest is focused on *Arabidopsis* homologues of polypyrimidine tract binding proteins (PTB), a well-characterized class of mammalian splicing factors with intriguing functions in development. We have already shown that all three PTB homologues from *Arabidopsis* underlie auto- and cross-regulation via AS and NMD (Stauffer et al., 2010; Fig. 3).

In our current work, we are seeking to identify novel PTB regulation targets and, thereby, to elucidate the biological implications of plant PTB homologues. Interestingly, sub-cellular localization studies revealed the presence of PTBs in the nucleus, cytosol, and processing bodies (P-bodies; Fig. 4), pointing at functions of the plant PTB homologues in addition to splicing control.
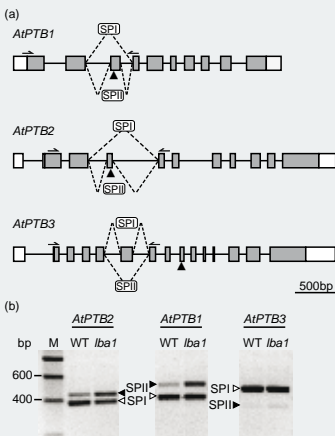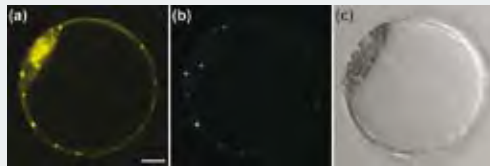

Figure 4 Sub-cellular localization of AtPTB2-YFP (a) in *Arabidopsis* protoplasts. (b) P-body marker DCP1-CFP, (c) transmission light.

Figure 3 Exon-intron structure (a) and AS patterns (b) of *Arabidopsis PTB* homologues. NMD targets are increased in the *lba1* mutant.
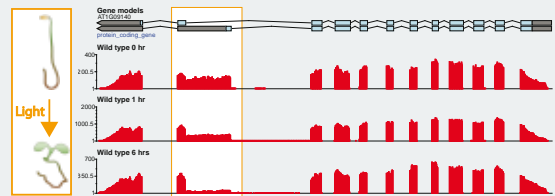

Figure 5 Alterations in AS patterns during early phase of photomorphogenesis. RNA sequencing was performed for etiolated seedlings 0, 1, and 6 h upon light exposure. Mapped reads are for gene AT1G09140.

Coupling of AS and NMD in Plant Gene Control

Recent studies have revealed an expanded scope for coupling of AS and NMD as a means of gene regulation in plants. Transcriptome-wide surveys indicate that many *Arabidopsis* transcripts possess NMD target features, such as long 3' untranslated regions (UTRs) or introns within 3' UTRs. We are interested in identifying novel natural NMD targets and revealing their functional significance in gene regulation. In this project, we apply bioinformatical approaches for the prediction of target transcripts and experimental strategies, such as next generation sequencing of wild type and NMD mutant samples for the validation of NMD turnover.

Coordinated Splicing Programs in Plant Development

AS has an enormous potential for altering the expression of large numbers of genes in a coordinated manner, as is required in many plant adaptation and developmental processes. However, it is not currently known if this potential is actually used in plants. We are employing both biased approaches starting from certain splicing regulatory proteins (e.g. PTBs) and unbiased strategies focused on defined developmental programs (e.g. light regulation, Fig. 5) to investigate a possible role of coordinated splicing programs in plants.

Environmental control of morpho-genesis in plants

Stephan Wenkel

Plants are sessile organisms that have to cope with the environment they are exposed to. Several developmental mechanisms are synchronized with the environment, like seed germination or the transition to flowering. We are interested in leaf development and how the environment can interfere with this complex process.

Plant leaves are multifunctional organs that can sense outside signals, such as temperature, day-length or pathogen attack. They integrate these cues to coordinate physiological responses, such as growth adaptation or defense responses. Plants have evolved various anatomical strategies to cope with changing environmental conditions, which is reflected in the large morphological variation in leaf shape. In addition, photosynthesis and gas-exchange take place in the leaf. Failure in the developmental program regulating leaf development often has dramatic consequences for the reproductive success of the individual. Like all seed plants, the plant body of the model plant *Arabidopsis thaliana* consists of two distinct systems, the root and the shoot. The shoot system harbors the shoot apical meristem (SAM). A SAM consists of a central zone, a peripheral zone and the developing leaf primordia (Figure 1). During the vegetative growth phase of shoot development, the population of stem cells in the SAM divides and produces the cells that will eventually form new organs. Leaves are initiated at the periphery of the SAM. In the early stages, the leaf primordium is un-polarized. No decision has been made as to which part of the primordium will adopt an adaxial (future top side) or abaxial (future bottom side) fate. Later in development, the side of the primordium that is adjacent to the SAM will become adaxialized and the side further away from the SAM will adopt an abaxial fate (Figure 1).
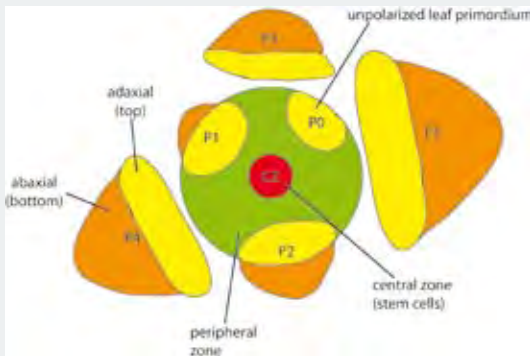
Figure 1 Schematic of the radial pattern of leaf initiation. The cells in the central zone (CZ) harbor the stem cells of the SAM that give rise to lateral organs. P0 is the youngest leaf primordium that is still unpolarized. During the course of development the primordia become more and more polarized (P1-P5).

Proper establishment of the ad/abaxial axis is of great importance, because the shape and size of the outgrowing leaf blade greatly depend on the juxtaposition of ad/abaxial tissue. If the leaf primordium is adaxialized, top side leaf cell fates dominate. This results, in extreme cases, in radial organs with top side leaf characteristics around the circumference. Conversely, radial organs with bottom side characteristics develop if the primordium is abaxialized. It was shown, in genetic mosaic plants, that (the determination of) cell fate (abaxial vs. adaxial) is not genetically determined but depends on the position of the respective cell in the developing primordium. Cells closer to the SAM will become part of the future top side, while cells in the periphery develop into the lower part of the leaf. The application of the plant hormone

auxin on the shoot apex results in the initiation of novel primordia at the place where auxin was applied, indicating that auxin plays a role in primordium initiation.

Besides hormones, several genes are known to be involved in establishing the ad/abaxial axes in Arabidopsis. We focus on the class III homeodomain leucine-zipper gene family (HD-ZIPIII genes) which is conserved throughout all land plants. *HD-ZIPIII* genes are involved in the regulation of meristem formation, leaf polarity, leaf blade outgrowth and vascular development. *HD-ZIPIII* genes encode transcription factors that have a homeodomain for DNA-binding followed by a leucine-zipper domain required for homo- and heterodimerization. They also contain a putative lipid/sterol-binding START domain and a carboxy-terminal PAS-like motif. Functions for the START-domain and the PAS-like domain have not been determined in HD-ZIPIII proteins.

Using microarray analysis, we have identified targets of the REVOLUTA (REV) transcription factor. Some of the genes regulated by REV encode hormone-biosynthetic enzymes. REV induces the expression of a gene encoding a biosynthetic enzyme involved in the synthesis of auxin (AuxE). In addition to being positively regulated by REV, the *AuxE* gene is negatively regulated by the abaxial regulator KANADI1 (KAN1; Reinhart, Wenkel and Barton, unpublished) and may, thus, be involved in establishing an auxin gradient in the incipient leaf primordium. Chromatin-immunoprecipitation experiments have further proven that the regulation by REV is direct. Current work is, therefore, focusing on the identification of the *cis* element REV interacts with.

To analyze the function of the auxin biosynthetic enzyme (AuxE) in leaf development, we have started to generate transgenic plants mis-expressing the *AuxE* gene in different leaf domains (Figure 2A).
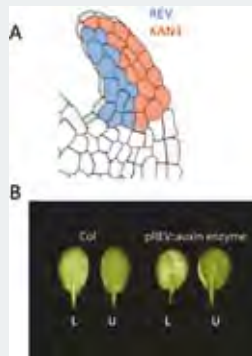
Figure 2 Mis-expression of *AuxE*.
A) Schematic of the expression pattern of REV and KAN1.
B) Mis-expression of *AuxE* using the *REV* promoter results in transgenic plants with adaxial leaves.

Transgenic plants mis-expressing AuxE in the adaxial domain using the REV promoter show an upward curling leaf phenotype reminiscent of rev gain-of-function mutants (Figure 2B). Future genetic analysis of double and triple mutants and mis-expression lines of artificial microRNAs will show the function of the REV-regulated auxin-biosynthesis in leaf development.

Besides working on endogenous signals required for leaf development, we have started to analyze how the environment (especially light) influences the initiation and development of leaves in *Arabidopsis*.

Plant Biochemistry

OVERVIEW

Microbial pattern recognition is a prerequisite for the initiation of antimicrobial defenses in all multicellular organisms, including plants. The plant immune system consists of two evolutionarily linked branches. Recognition of invariant microbial surface patterns (pathogen or microbe-associated patterns; PAMP / MAMP) through plant pattern recognition receptors is referred to as PAMP-triggered immunity (PTI) and is the basis for broad-spectrum resistance of plants against host non-adapted microbial pathogens. Suppression of PTI by microbial effectors (effector-triggered sus-ceptibility, ETS) is a prerequisite for plant infection by adapted pathogens and is likely the cause for susceptibility of many crops to virulent microbial pathogens. Co-evolution of susceptible plant hosts and host-adapted pathogens has shaped immune receptors (resistance proteins) that guard microbial effector-mediated perturbations of host cell functions and thereby trigger plant immune responses (effector-triggered immunity, ETI).

Our research activities aim at uncovering the molecular mechanisms underlying microbial sensing and activation of plant immune responses. In addition, we study the molecular mode of action of microbial effectors, in order to understand how these molecules facilitate host plant infection. In our research, we apply genetic, biochemical, cell biological and plant physiological technologies to the model plant, *Arabidopsis thaliana*. Information gained from our work harbors the potential for translational research that aims at engineering durable immunity in crop plants.
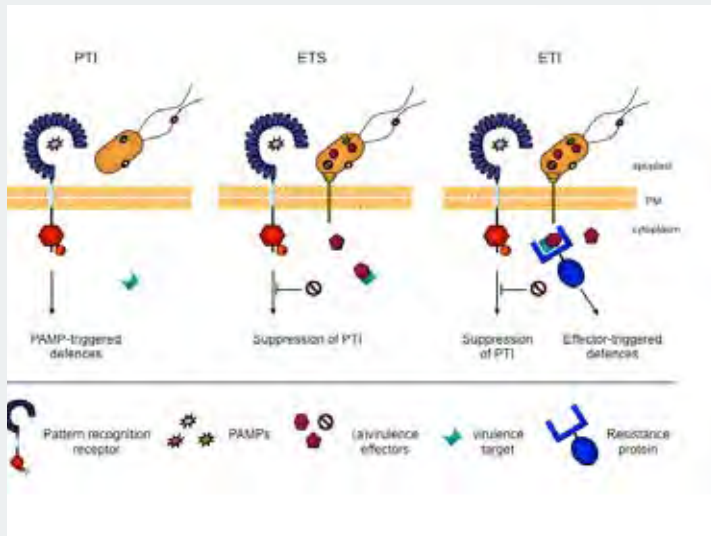

Figure 1 A schematic representation of the plant immune system
(Nürnberger and Kemmerling. Annual Plant Reviews, Wiley Blackwell. 2008, 34: 16-47)

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Function of microbial effectors in plants and pathogen recognition

Frédéric Brunner

Plants have adapted defense mechanisms to resist microbial infection. Manipulation of the immune system by pathogen-derived effectors causes plant susceptibility but the underlying molecular mechanisms remain largely elusive, especially in the case of effectors from eukaryotic pathogens like fungi or oomycetes.
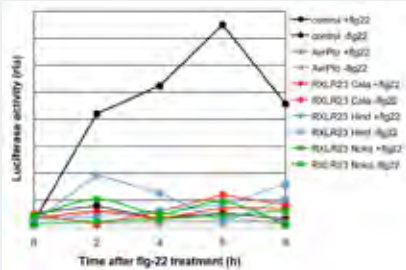

Figure 1 Suppression of PAMP-inducible promoter activity in *Arabidopsis* protoplasts by HaRXLR23.
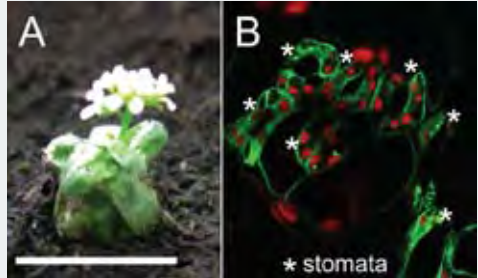

Figure 2 *Arabidopsis* plants expressing constitutively GFP-Avr2 are dwarfed (A) and display higher stomatal density (B).

The genome of several oomycetes, including the biotrophic *Hyaloperonospora arabidopsidis* (*Arabidopsis* downy mildew) or the hemibiotrophic *Phytophthora infestans* (potato and tomato late blight), encodes for several hundreds of so-called RXLR effectors. Using a cell-based assay in *Arabidopsis*, we have screened ~100 effectors from *H. arabidopsidis* and ~200 effectors from *P.infestans* (ongoing screen) for their ability to suppress the expression of a LUC-reporter genecontrolled by a PAMP-responsive promoter. Several effectors acting as suppressors of PAMP-triggered immune responses were identified. One of the most potent effectors is HaRXLR23 from *H. arabidopsidis*. *HaRXLR23* is highly polymorphic and different alleles are present in both adapted and non-adapted *H. arabidopsidis* isolates. The effector domain is under positive selection pressure, which is strong evidence for co-evolution with host proteins. Comparative transcriptome and proteome analyses in plants expressing HaRXLR23, combined with biochemical approaches (Immunoprecipitation, Yeast-two-Hybrid...), are applied to study the molecular mechanisms underlying the function of HaRXLR23.

Only a subset of effectors is assumed to impair PAMP-triggered immunity. Other effectors are supposed to target mechanisms of transport and the secretion of anti-microbial compounds and hydrolytic enzymes at the interface of the plant plasma membrane and the oomycete extrahaustorial matrix. Therefore, we are currently developing new approaches, based on biochemical and cell biology measurements, to detect novel effector functions. We have established the conditions for transient expression in *Arabidopsis* protoplasts of (defense) protein markers localized in the plasma membrane or secreted in the apoplast that are likely to be specifically targeted by RXLR effectors.

The premise for testing *P. infestans* effectors in a non-host plant species is our hypothesis that some effectors may have evolved to target universal eukaryotic factors, and high-throughput assays that exploit the huge genetic resources existing in *Arabidopsis* can guide the work with hosts. In collaboration with the research group of Paul Birch at SCRI in Dundee (UK), we are building up evidence for the virulence function of Avr2 (also an RXLR effector). *Arabidopsis* plants expressing Avr2 display a severe dwarf phenotype and an abnormal stomata phenotype. Avr2 strongly interacts with BSU1-like phosphatases – positive regulators or Brassinosteroid signaling – and current studies aim to bridge the gap between the cellular function of Avr2 and its consequence for plant development and resistance.
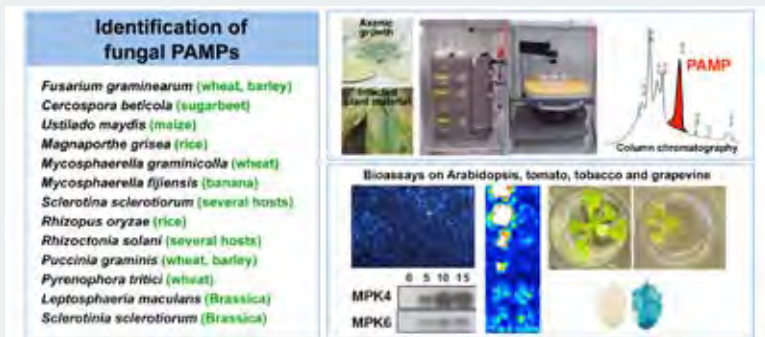

Figure 3 Experimental approach for the identification of novel PAMPs

There is only a weak colinearity of RXLR effector sequences between *H. arabidopsidis* and *P. infestans*. The difference probably reflects the fact that both oomycetes have very different host ranges and it is expected that some of these RXLR genes involved in host interactions will have rapidly diverged between the two species, as a result of strong selection for effective pathogenesis. The genome sequence of tomato is nearly complete. It will serve as a ‚blueprint‘ for Solanaceae species and allow a comparison of the *Arabidopsis-H. arabidopsidis* and Tomato-*P. infestans* model systems. This work does not only help our understanding of the molecular basis for host pathogen interactions but it will provide insights into the plasticity of the oomycete effectorome.

Fundamental discoveries about the nature and function of PAMP receptors in *Arabidopsis* offer the prospect of developing broad-spectrum disease control in crop plant species. In addition, the identification and characterization of the repertoire of recognized PAMPs will allow studies on the interplay between different perception systems and signaling pathways in defense response. We use a variety of bio-assays in order to identify novel PAMPs from oomycetes and major fungal pathogens that are recognized by *Arabidopsis* and selected crops, e.g. barley, wheat, grapevine and brassica. Collaborative projects with research groups in the UK (Cyril Zipfel, Chris Ridout), the Netherlands (Pierre de Wit) and France (Alain Pugin) address these issues.

Ligand specificity and activation mechanism of plant receptor kinases

Georg Felix

Cell surface receptors play fundamental roles in the regulation of cell behaviour by external signals. Higher plants like *Arabidopsis thaliana* have >600 genes encoding so called „receptor like kinases" (RLKs). Members of this major class of receptors are involved in regulation of growth, morphogenesis, fertilization, interaction with symbionts and detection of microbial pathogens (Fig. 1).
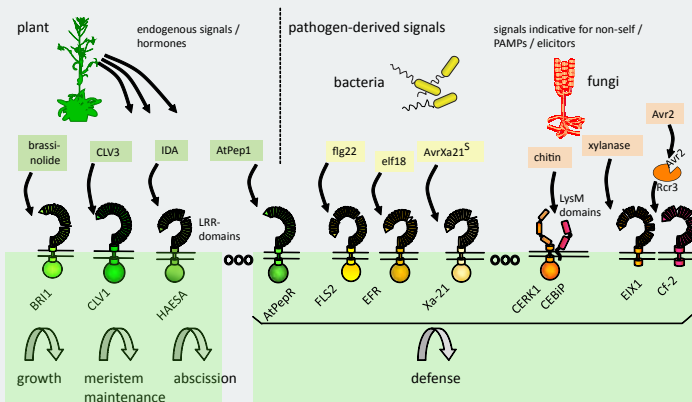

Figure 1 Examples of known ligand/receptor pairs in plants.

In previous work, we identified several microbial signals that act as ‚pathogen associated molecular patterns', so called PAMPs, and trigger basal defense responses in plants. For the two bacterial PAMPs flg22, a domain of flagellin, and elf18, the N-terminus of EF-Tu, we identified the corresponding pattern recognition receptors FLS2 and EFR, respectively. FLS2 and EFR are closely related receptor kinases belonging to the >200-membered subfamily of RLKs that contain ‚leucine rich repeats' (LRRs) in their ectodomains.

There are a number of further PAMPs and other exogenous signals that await identification of their corresponding receptors in plants. *Vice versa*, an even bigger number of RLKs still remain orphan with respect to their ligands. The identification of further ligand/receptor pairs will, thus, continue to be a challenge for future work.

Recent work has shown that ligand-dependent activation of FLS2 and EFR involves rapid complex formation with a second LRR-RLK, termed BAK1 (Fig. 2). BAK1 was originally identified as co-receptor for the receptor kinase BRI1 that regulates growth in response to the plant hormone brassinolide. Thus, surprisingly, activation of receptor kinases that regulate different types of cellular outputs seems to employ common principles and even common co-receptors.

What are the molecular structures and mechanisms that determine ligand specificity, transmembrane signaling and activation of particular output responses in these receptors? These questions build the main focus of our research efforts.

Starting with the well characterized ligand/receptor-pairs, flg22/FLS2 and elf18/EFR, we want to define the subdomains that specify ligand affinity in these receptors. For this we use bio-chemical and bio¬physical approaches that aim at direct structural elucidation. Further, we use mutational analysis and chimeric receptors, e.g. constructed as hybrids from EFR and FLS2. These receptor constructs can be rapidly tested for their binding specificity and recep-tor function, using transient expression systems (Fig. 3). With the chimeric approach we can also start to map the sites on the receptors that are responsible for complex formation with co-receptors or other steps in receptor activation.

Figure 2 Working model for receptor activation process. A) steps observed for flg22-stimulated activation of FLS2. B) brassinolide (BL)-induced activa-tion of BRI via complex formation with BAK1 as described in the literature.
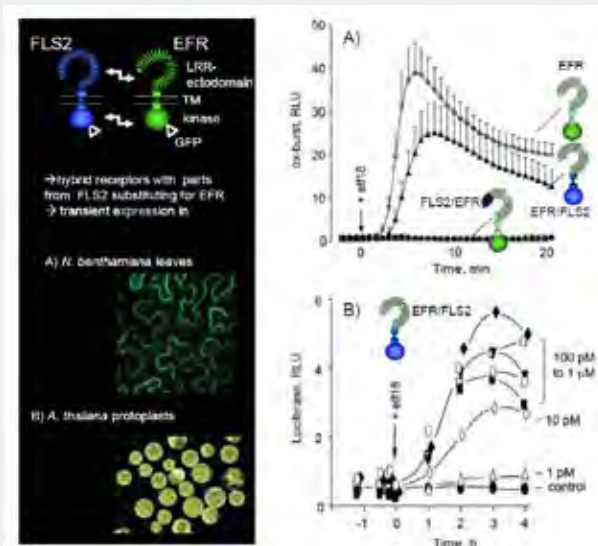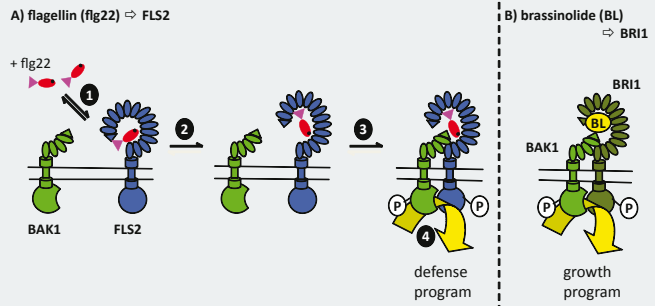
Figure 3 Two rapid and sensitive assay systems for testing functionality of chimeric receptors. A) transient expression in *Nicotiana benthamia-na* has no endogenous perception system for the PAMP elf18 and is thus highly suited for testing receptors with an EFR-type ectodomain. Results show induction of oxidative bursts, measured as relative light units (RLU) in the presence of luminol.
B) protoplasts isolated from *A. thaliana* (mutant background lacking functional EFR) can be co-transfor-med with receptor constructs and the reporter gene luciferase under a PAMP-inducible promoter. Data show luciferase activity measured as RLU in presence of luciferin.

In essence, RLKs have a modular setup with an ectodomain thought to be responsible for ligand detection, a single pass transmembrane domain and a cytoplasmic Ser/Thr prote-in kinase. A more precise definition of these modules will allow approaches with chimeric receptors consisting of domains from less related receptors. Such hybrid receptors will be useful in the search for ligand inputs for orphan receptors, for mapping the sites important for transmembrane signaling and for identifing the determinants that define the signal output programs triggered by the kinase domains.

Plant innate immunity

Thorsten Nürnberger

Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) to microbial infection constitutes an evolutionarily ancient type of immunity that is characteristic of all multicellular eukaryotic systems. Microbial patterns activating plant PTI comprise bacterial flagellin, lipopolypolysaccharides, peptidoglycans or oomycete or fungus-derived proteins, peptides or glucan and chitin oligomers. Plant pattern recognition receptors mediate microbial pattern sensing and subsequent immune activation (Fig.1).

Our research aims at a deeper molecular understanding of microbial pattern recognition in plant immunity. In particular, we work on the identification of novel microbial patterns and their corresponding plant pattern recognition receptors. We further study the molecular basis of damage-associated immune activation in plants.

We have been able to identify bacteria-derived patterns that trigger immunity-associated responses in *Arabidopsis*. These comprise, for example, protein fragments of *Ralstonia solanacearum* cell extracts. Upon purification to homogeneity, such a pattern will be used to identify the corresponding plant pattern recognition receptor.
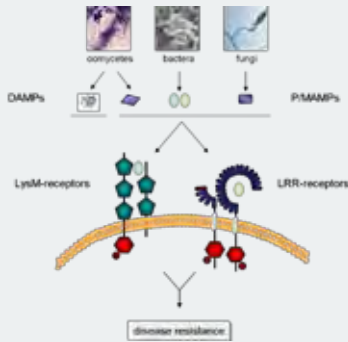
Figure 1 Identification of new D/P/MAMPs and their receptors.
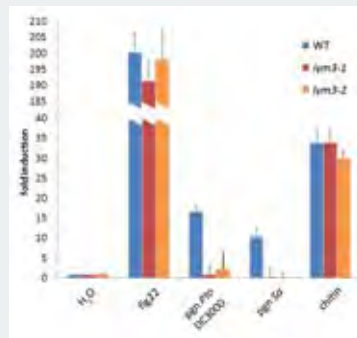
Figure 2 PGN-induced FRK1 marker gene expression is abolished in *lym3* mutants

Likewise, bacterial peptidoglycan preparations (PGN) from different bacterial sources (*S. aureus, E. coli, B. subtilis, P. syringae* pv. tomato, *Xanthomonas campestris* pv. *campestris*) trigger immunity-associated defenses in *Arabidopsis*. The *Arabidopsis* PGN perception system is mechanistically different from that for bacterial flagellin or fungal chitin. An Arabidopsis plasma membrane protein carrying two ectopic LysM domains (LYM3) has been implicated in PGN binding, PGN sensitivity (Fig.2) and plant immunity to bacterial infection. As this PGN binding protein lacks a cytoplasmic signaling domain, we now aim at understanding how the PGN signal is transduced across the plant plasma membrane.

Plants do not only respond to non self-representing microbial patterns, but also have the ability to sense endogenous, self-representing stress-associated 'danger' signals ('damage-associated molecular patterns', DAMPs). Such plant signals comprise breakdown products of cell wall pectins or cellulose, that are released during microbial infection by microbial hydrolytic enzymes or that are generated through the activities of microbial toxins. Necrosis and ethylene inducing protein 1-like proteins (NLPs) constitute a protein superfamily of which members are produced by various phytopathogenic microorganisms. NLPs trigger leaf ne-

crosis, and stimulate immunity-associated defenses in dicotyledonous plants, including *Arabidopsis*. NLPs were proposed to have dual functions in plant pathogen interactions acting both as triggers of immune responses and as toxin-like virulence factors. The crystal structure of an NLP from the phytopathogenic oomycete, *Pythium aphanidermatum*, revealed significant fold conservation between NLPs and pore-forming, cytolytic toxins produced by marine organisms (actinoporins) (Fig.3).

Expression of oomycete NLPs in an *nlp*-deficient *P. carotovorum* strain restored bacterial virulence, suggesting that NLPs of prokaryotic and eukaryotic origins are orthologous proteins. NLP mutant protein analyses revealed that identical structural features of the protein were required for cytotoxicity and for bacterial virulence, indicating that NLPs are virulence factors that contribute to host infection by plasma membrane destruction and cytolysis. In addition, NLP-mediated phytotoxicity and plant defense activation share identical fold requirements, suggesting that toxin-mediated interference with host integrity causes plant immune activation. Phytotoxin-induced cellular damage-associated activation of plant defenses is reminiscent of microbial toxin-induced inflammasome activation in vertebrates and, thus, constitutes a conserved element in eukaryote innate immunity.

Figure 3 A common toxin fold mediates microbial phytotoxicity
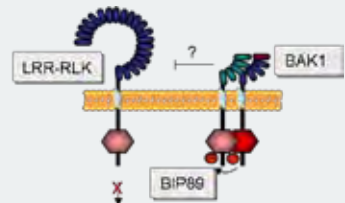
Figure 4 FRET-FLIM analyses confirm interaction of BAK1-CFP and BIP89-YFP at the plasma membrane of *Arabidopsis* protoplasts.
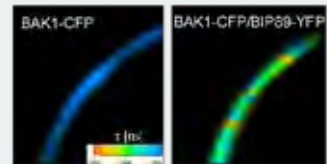
AG Kemmerling

Leucine-rich repeat receptor-like kinases (LRR-RLKs) have diverse roles in plant growth and development or serve as pattern recognition receptors in plant immunity. To identify additional plant immunity-associated LRR-RLKs, we have conducted gene expression profiling experiments with infected *Arabidopsis* plants. One of the identified LRR-RLKs is the Brassinosteroid (BL) receptor BRI1-Associated receptor Kinase 1 BAK1. We have proposed a novel, BL-independent function of BAK1 in plant cell death control. Likewise, BAK1 function in flagellin signaling has been shown to be independent of BL. Our data suggest that BAK1 interacts with multiple RLKs and acts as a central component of RLK-initiated signaling. Our research, therefore, aims at identifying additional BAK1-related processes. Several screens were performed to identify *in vitro* and *in vivo* interaction partners of BAK1. One of the identified proteins is the previously described DAMP-receptor PEPR1, which perceives the endogenous wound and pathogen inducible peptide AtPep1. Additional RLKs, as e.g. BIP89, were identified as BAK1 interaction partners (Fig.4). With functional (reverse genetics) and biochemical (phosphorylation studies) analyses, we study the BAK1 interacting proteins to elucidate their molecular mechanism and function.

Plant Physiology

Overview

Physiology studies the function of an organism, encompassing the dynamic processes of growth, differentiation, metabolism, reproduction and interaction with the abiotic and biotic environment. Modern physiology combines a wide spectrum of molecular biological, biochemical, biophysical, cell biological, genetic and functional genomics techniques and tools to obtain insight into how a plant organism functions.

Plants, as sessile organisms, face the problem that they are confined to their habitat and have to endure and adapt to a broad array of environmental conditions. To optimize growth and development in a highly competitive environment, plants depend on a multitude of molecular signal perception, processing and integration systems. Although several specific and more-or-less linear signaling pathways have been described, far less is known about the dynamics of the molecular mechanisms underlying signal processing and integration in the cells of higher plants.

The focus of our present research – conducted by six independent research groups – is predominantly on the specific features of plant signal perception, transduction, processing and integration. Furthermore, we are interested in intracellular protein trafficking, miRNA function and RNA processing. We increasingly apply quantitative physical techniques and tools, such as quantitative life time imaging and spectroscopy, for the intracellular analysis of these processes.

FG Harter: Function of plant two-component signaling systems; target gene identification, post-translational regulation and functional analysis of bZIP, WRKY and BPC transcriptional regulators; identification of regulatory gene networks, using functional genomic approaches and bioinformatics.

FG Hiltbrunner: Characterization of phytochrome interacting proteins; identification of proteins regulating phytochrome nuclear transport; evolution of phytochrome signaling; analysis of crosstalk between phytochromes and other signaling pathways.

FG Laubinger: Plant microRNA processing and function; regulation of splicing by the nuclear cap-binding complex and associated proteins; elucidation of the functions of non-coding RNAs in abiotic stress responses.

FG Oecking: Function of regulatory 14-3-3 proteins in plants; regulation of the plasma membrane H^+-ATPase; identification of 14-3-3 target proteins; elucidation of the molecular and physiological function of 14-3-3 proteins.

FG Schaaf: Regulation of phosphoinositide homeostasis and signaling by Sec14 lipid transfer proteins; molecular mechanisms by which Sec14 like proteins control metal tolerance, membrane trafficking and root hair development.

FG Schleifenbaum: High resolution spectro-microscopy; development of novel fluorescence based cell biological methods; analysis of phy signaling pathways; identification of protein-protein interactions by FRET and BiFC; development of novel functional protein fluorescence labels.

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Plant signal perception, transduction and integration

Klaus Harter

The research activities of our group focus on plant perception of environmental and hormonal stimuli and the molecular mechanisms of signal processing in the plant cell.

Two-component signaling

During evolution, higher plants adapted and developed a unique molecular signal processing mechanism, which is perfectly suited to signal perception, transduction and integration: the
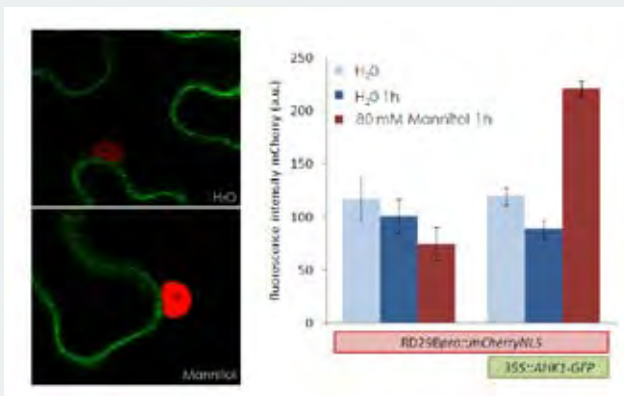

Figure 1 Reconstitution of the AHK1 response pathway in tobacco leaf cells.

two-component system (TCS). Plant TCSs are mostly composed of hybrid histidine (His) kinases (HKs), His phosphotransfer proteins (HPs) and response regulators (RRs). The general mode of TCS action is to transfer phosphoryl residues from the activated HKs via HPs to the RRs. The phosphorylation of RRs induces their interaction with other proteins or the induction of their target genes. The promiscuous interaction capability of TCS elements enables cross-talk between different signaling pathways. TCSs contribute to the perception and signal transduction, among others, of hormones and environmental cues.

The focus of our present TCS research is on the signal-dependent intracellular localization and dynamics, as well as the in vivo interaction pattern, of selected HKs, HPs and RRs involved in light, osmotic and biotic stress, ROS and cytokinin signaling. In addition to the studies on whole plant level, we aim to reconstitute complete TCS response pathways in plant cells for quantitative studies to determine the limiting factor(s), velocity and molecular determinants of TCS signaling at single cell level (Fig. 1). These quantitative data will be used for the generation of mathematical models. In collaboration with specialists at the Tübingen campus, we intend to crystallise selected HKs and HK domains and to determine their structure.
The studies on the TCS will be complemented by the quantitative cell physiological and functional analysis of other plant hormone (co-)receptors such as BRI1 and BAK1.

Transcription factor function and regulation

Transcriptional regulators play crucial roles in almost all biological processes and are classified by their DNA-binding domain. We focus on the structural, molecular and cell biological properties (DNA-binding, homo- and heterodimerisation, dynamics of intracellular and intranuclear distribution) of selected members of three distinct transcription factor families, namely bZIPs, WRKYs and BPCs (Fig. 2). In addition, we study their post-translational regulation (e.g. phosphorylation) and aim at identifying their in planta target genes. By this combinatorial approach we try to understand how the different regulatory levels contribute to their function in the regulation of plant growth and development and plant adaption to the abiotic and biotic environment.
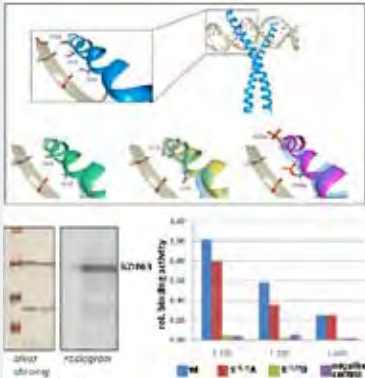

Figure 2 Influence of phosphorylation on the DNA-binding activity of bZIP63 to its cognate promoter sequence.

Figure 3 Network de-convolution.

Bioinformatics and computational biology

We are interested in unraveling gene functions by observing gene relationships as networks. Networks can be composed of multiple levels of information, for example protein-protein interaction, gene expression correlation, and gene properties. Network approaches can be used to probe signal transcription cascades and extrapolate novel gene relationships.

One aspect of transcriptional networks is the direct binding of transcription factors to their cognate DNA cis-regulatory element, which are needed to alter the activity of target genes. We apply computational biology to identify cis-elements or gather new information about cis-elements and their relationships within signal transduction cascades.

In order to solve particular biological questions, we implement cutting-edge algorithms, produced in computational biology, and develop our own algorithms and meta-approaches as needed. Meta-analysis will be more and more important in the future as more "-omics" data become available and will contribute to our understanding of how complex plant systems work.

Technology developments

In collaboration with the Schleifenbaum group, we develop and adapt highly sophisticated lifetime imaging and spectroscopy technologies, such as FIDSAM, one-chromophore FLIM, dual-focal FCS and mathematical tools, such as CILA, for the quantitative in vivo analysis of subcellular processes in living plant cells in their tissue environment. In addition, in vitro high-throughput tools are being developed for the identification and quantitative characterization of novel DNA-protein interactions.

From Light Perception to Gene Expression
Andreas Hiltbrunner

Photoreceptors in plants

Light is an abiotic factor, which is particularly important for plants. It is used as a source of energy but also provides information about the environment. To monitor the intensity, quality and direction of incident light, plants employ different types of photoreceptors, such as phototropins, cryptochromes and phytochromes. In higher plants, cryptochromes and phytochromes are the main light receptors involved in regulation of gene expression and they are important for de-etiolation, transition from vegetative growth to flowering and many other responses.
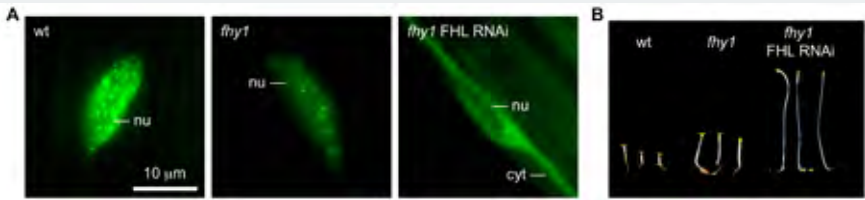
Figure 1
A. 3 day old dark grown wild type (wt), *fhy1* and *fhy1* FHL RNAi knock down seedlings expressing phyA-GFP were irradiated for 6 hours with far-red light and analyzed by fluorescence microscopy. nu, nucleus; cyt, cytosol.
B. Wild type (wt), *fhy1* and *fhy1* FHL RNAi knock down seedlings were grown for 5 days in far-red light.
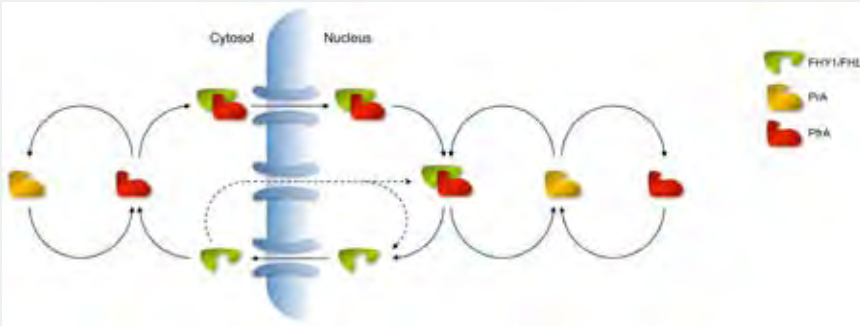
Figure 2
Current model for phyA nuclear transport. See text for details.

Phytochromes

Phytochromes are the only photoreceptors absorbing in the red and far-red light range of the spectrum. They can exist in two spectrally different forms, the inactive Pr form with maximal absorption in red light and the biologically active Pfr form, which has an absorption peak in far-red light. By absorption of light, phytochromes can reversibly interconvert between these two forms.

In *Arabidopsis*, there are five phytochromes (phyA-phyE), among which phyA and phyB are the most important. phyB is the dominating phytochrome species in adult plants and is important for the detection of the red:far-red light (R:FR) ratio. In plain sunlight, the R:FR ratio is high, whereas it is low in light reflected from or transmitted through leaves. Thus, measuring the R:FR ratio in their environment allows plants to detect potential competitors for sunlight at an early stage.

Although phytochromes are most efficiently converted to the active form by absorption of red light, phyA works as a far-red light receptor *in planta* and has been shown to be essential for survival in far-red light enriched environments, such as deep canopy shade. phyA is unique to higher plants and may have provided an adaptive advantage to early angiosperms during colonization of habitats dominated by ferns and gymnosperms. Currently, the molecular mechanism that allows phyA to function as a far-red light receptor is unknown.

Phytochrome signaling

In higher plants, phytochromes localize to the cytosol in the dark but translocate into the nucleus when activated by light. Several transcription factors essential for light perception have been shown to interact with phytochromes, suggesting that nuclear transport of the photoreceptor itself is an essential step in phytochrome signaling. In contrast, in mosses and ferns phytochromes are believed to localize to the plasma membrane and there is currently no evidence that they have a function in the nucleus.

In the past few years, we could demonstrate that the two functional homologs FHY1 and FHL are specifically required for light-regulated nuclear transport of phyA (Figure 1A), whereas phyB employs a different mechanism for translocation into the nucleus. Seedlings, which lack both FHY1 and FHL, are blind to far-red light, confirming that nuclear transport of phyA is essential for far-red light perception (Figure 1B). FHY1/FHL contain NLS and NES (nuclear import and export signal) motifs and they interact with phyA in a light dependent manner. In our current model, FHY1/FHL work as shuttle proteins, which bind to phyA in the cytosol and transport it into the nucleus (Figure 2). Interestingly, phyA nuclear transport is most efficient in far-red light, which may explain why phyA works as a far-red light receptor *in planta*.

Research projects

Our current research projects address three main questions:

1. **How did phytochrome signaling and nuclear transport evolve?**
   Cryptogams contain typical plant phytochromes, as well as homologs of components essential for phytochrome nuclear transport and signaling in higher plants. Nevertheless, cryptogam phytochromes are believed to localize to the plasma membrane and not to have a function in the nucleus. Using transgenic *Physcomitrella* lines, we investigate the molecular basis for phytochrome function in cryptogams, which is prerequisite to understand how phytochrome signaling evolved.

2. **What mechanism(s) turn(s) phyA into a far-red light receptor?**
   Red light is much more efficient than far-red light in converting phytochromes to the active form. Yet, phyA works as a far-red light receptor *in planta*. Using a systems biological approach, we investigate which step(s) in phyA signaling is/are responsible for shifting the action peak from red to far-red light.

3. **How does light signaling interact with other signaling pathways?**
   Plants have to integrate a multitude of stimuli in order to respond to their environment in an adequate way. In only a few cases the molecular mechanisms underlying signal integration are known. Using life cell imaging of double/triple transgenic plants expressing fluorescence protein tagged versions of components from different signaling pathways, we want to identify the protein complexes essential for signal integration. In addition, we are also using, or plan to use, forward and reverse genetic approaches to find components important for signal integration.

MicroRNA Processing and Function
Sascha Laubinger

Arabidopsis microRNAs (miRNAs) regulate many different aspects of plant development including root, leaf and flower development, the transition from juvenile to adult phases and adaptive developmental processes, such as the photoperiodic induction of flowering and stress adaptation. Plant miRNAs are derived from longer primary-miRNA (pri-miRNA) transcripts, which are transcribed by polymerase II and contain an imperfect fold-back structure. Pri-miRNAs are first trimmed by DCL1 to precursor-miRNAs (pre-miRNAs), from which DCL1 further excises the miRNA/miRNA* duplexes (Fig. 1). DCL1 interacts with the dsRNA-binding protein HYPONASTIC LEAVES1 (HYL1) and the zinc-finger protein SERRATE (SE), that ensure proper processing of the pri-miRNA (Fig.1). Processed miRNAs subsequently associate with one of the ten *Arabidopsis* ARGONAUTE (AGO) proteins. Most plant miRNAs exhibit a 5'-uracil, which serves as a sign for association with AGO1. AGO1/miRNA ribonucleo-protein complexes undergo base pairing with complementary regions of the target messenger RNAs (mRNAs), resulting in mRNA cleavage or translational inhibition (Fig.1).
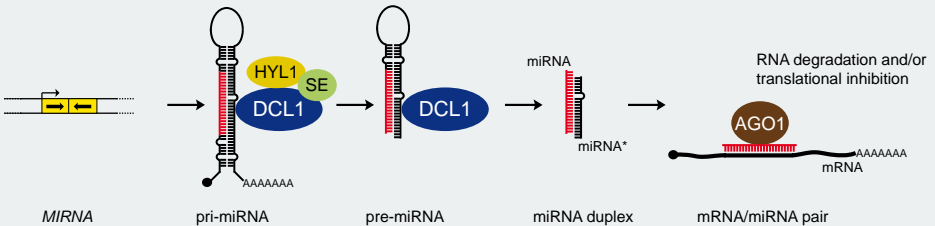

Figure 1 Overview of miRNA biogenesis in plants.

The function of the nuclear cap-binding complex (CBC) and SERRATE in mRNA splicing and miRNA processing

It has already been noted that the morphological appearance of weak *se* mutants is reminiscent of plants with mutations in ABH1/CBP80 and CBP20, which encode the two subunits of the CBC (Fig. 2A). We could show that the CBC, like SE, is necessary for the proper processing of pri-miRNAs (Laubinger et al., 2008a). Transcriptome profiling revealed that *se*, *abh1/cbp80* and *cbp20* mutants also share similar splicing defects, leading to the accumulation of many partially spliced transcripts. Introns retained in *se*, *abh1/cbp80*, and *cbp20* mutants are not affected by mutations in the other genes required for miRNA processing or function. This indicates that SE and the CBC are involved in more general RNA metabolism than the specialized miRNA processing factors DCL1 and HYL1.

Furthermore, we could show that SE directly interacts with the CBC, likely forming a higher-order nuclear cap-binding complex guiding different RNA processing factors (DCL1 or the spliceosome) to the nascent RNA molecules (Fig. 2 B, C).

The overall aim of our future research is to understand the mechanisms by which SE and the CBC regulate different RNA processing steps and how SE and the CBC can discriminate between mRNAs and pri-miRNAs, which undergo distinct maturation steps.

The function of non-coding RNAs in abiotic stress responses

In the past, genome-wide analyses of stress transcriptomes focused mainly on protein-coding RNAs. This is because standard gene-expression arrays typically represent protein-coding genes. More recent studies, however, have revealed the diverse functions of so-called non-coding RNAs in plant stress adaptation. The most prominent examples of non-coding RNAs that have important regulatory functions are miRNAs. But other classes of small and long non-coding RNAs are also implicated in regulating various plant stress responses.

We could show that some transposons and pseudogenes, which do not contain longer open-reading frames, are constitutively expressed or become transcriptionally activated after stress exposure (Laubinger et al., 2008b; Zeller et al., 2009). However, very little is known about the function of transcriptionally reactivated pseudo-genes and transposons. As pseudogenes and transposons often give rise to short interfering RNAs (siRNAs), one could speculate that these siRNAs can act *in trans* to post-transcriptionally regulate the expression of target genes.
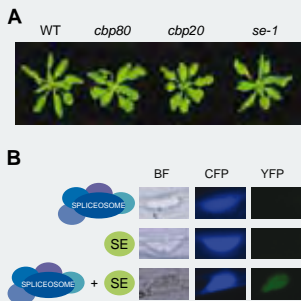

Figure 2
(A) Morphology of WT, *cbp80*, *cbp20* and *se* mutant plants.
(B) Bimolecular fluorescence complementation (BiFC) reveals interaction between SE and a component of the spliceosome, which was identified in a yeast-2-hybrid screen with SE as a bait.
(C) Proposed model for the function of SE and the CBC in pri-miRNA processing and pre-mRNA splicing.
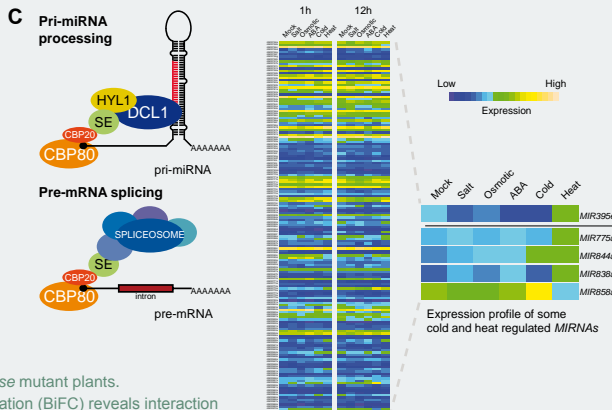
Figure 3 Heatmap of pri-miRNA expression in a *dcl1* mutant exposed to various abiotic stresses.

Several miRNAs have already been shown to be involved in plant stress responses. In order to monitor changes in the expression of pri-miRNAs under stress conditions, we conducted a whole-genome tiling-array experiment (Laubinger et al., 2010). We treated a *dcl1* mutant with salt, osmotic, cold or heat stress or directly applied the stress hormone abscisic acid (ABA). A comprehensive analysis of nearly 200 *MIRNA* genes showed that up to 10 miRNAs are differentially expressed under the respective stress conditions (Fig. 3). Heat and cold stress had a much stronger impact on the expression of the *MIRNA* gene than salt stress, osmotic stress or ABA treatment. These *MIRNA* genes will be subjected to in-depth analyses in the future.

The overall aim of our future research is to understand the function of stress regulated non-coding RNAs and miRNAs during plant stress adaptation.

Function of plant 14-3-3 proteins

Claudia Oecking

Reversible phosphorylation of proteins is a crucial signaling mechanism regulating almost all aspects of cellular life in eukaryotes. Sometimes, however, phosphorylation is not sufficient to modify the activity of the respective target protein and, in many such cases, 14-3-3 dimers come into play. They bind phosphorylated consensus motifs of diverse target proteins within the typical groove formed by each monomer (Fig 1) and operate by enforcing conformational changes, by acting as an intermolecular bridge or by modifying the subcellular localization of their clients.

Using a combination of biochemical, molecular and genetic studies, my laboratory is interested in unraveling the biological function of plant 14-3-3 proteins.

We identified 14-3-3 proteins as positive regulators of the plant plasma membrane H^+-ATPase that provides the driving force for nutrient uptake and maintenance of cell turgor. The enzyme is kept at a low activity level by its C-terminal domain, the autoinhibitory action of which is released upon phosphorylation of the penultimate residue and subsequent 14-3-3 association. The X-ray structure of 14-3-3 in complex with the entire binding motif (the C-terminal 52 residues of the H^+-pump) (Fig 1) revealed that a 14-3-3 dimer is simultaneously occupied by two H^+-ATPases, assembled in an antiparallel configuration, and forming a loop within the binding groove, thus allowing the C-terminal regions to exist together from the center of a 14-3-3 dimer. As suggested by this unusual structure, 14-3-3 association induces the assembly of an active H^+-ATPase oligomer. Furthermore, we could show that a network of kinases/phosphatases is involved in H^+-ATPase regulation, allowing gradual as well as fine-tuned adjustment of its activity. Intriguingly, phosphorylation of most if not all residues finally impacts on 14-3-3 association/dissociation.
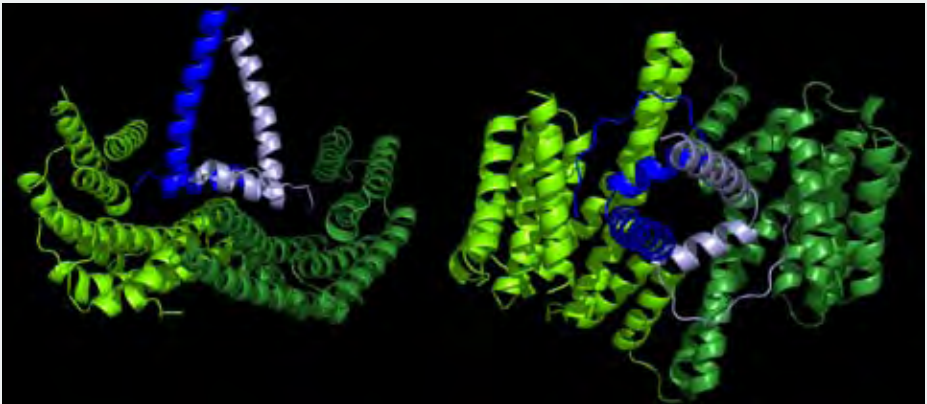

Figure 1 Coordination of the H^+-ATPase C-terminal 52 residues by a 14-3-3 dimer. Ribbon plot of 14-3-3 (individual monomers given in dark and light green, respectively) in complex with two H^+-ATPase peptides (dark and light blue, respectively). The right panel shows the view rotated by 90° about the horizontal axis.

In order to address the question of 14-3-3 function at the level of the whole organism, we analyzed the gene expression pattern of individual *Arabidopsis* 14-3-3 isoforms and performed a yeast two hybrid screen. Approximately 30% of the putative interactors could be assigned a function in signaling. In this regard, several members of both the group-A bZIP transcription factors (among those FLOWERING LOCUS D (FD)) and the NPH3 family (among those NON PHOTOTROPIC HYPOCOTYL (NPH3) and ENHANCER OF PINOID (ENP)) were captured in the screen. We confirmed these interactions by bimolecular fluorescence complementation. FD is known to act in concert with the „florigen" FT to activate floral identity genes in the shoot apex and thus, to promote flowering, while NPH3 and ENP cooperate with distinct AGC kinases to regulate polar auxin transport. In each case, the 14-3-3 binding site has been identified and the future challenge will be to determine the functional consequence of 14-3-3 association and to analyze its physiological significance *in vivo*. Preliminary experiments suggest that 14-3-3s enable FD to interact with FT, thus functioning as an intermolecular bridge.

There is an ongoing discussion concerning the pressing question of functional diversity among particular 14-3-3 isoforms (13 in *Arabidopsis*). With respect to one major phylogenetic group, the ancient epsilon group, ethanol-inducible RNA-interference was used to suppress the function of three isoforms that are ubiquitiously expressed. The ethanol-dependent reduction in the expression of the targeted isoforms is efficient and furthermore, seems to be specific (Fig 2). Remarkably, ethanol treatment revealed a dramatic growth retardation and severe development defects (Fig 2). Epinastic cotyledons, agravitropic growth and pronounced defects in root hair elongation, as well as apical hook formation, are phenotypes that are frequently observed in auxin mutants. A number of experiments indicated the 14-3-3 epsilon group members to be essential for auxin transport processes, and future experiments will aim at identifying the molecular basis of 14-3-3 dependent regulation of auxin transport.

Figure 2 The 14-3-3 epsilon group members are essential for plant growth and development. Primary root length as well as semiquantitative RT-PCR analysis of seedlings grown in the absence or presence of ethanol. Shown are the results for Col-0 and three independent transgenic lines expressing ethanol-inducible RNAi-constructs targeted against the isoforms epsilon, mu and omicron (EMO-lines).

Sec14 proteins and regulation of phosphoinositide homeostasis in Arabidopsis

Gabriel Schaaf

Phosphoinositides – phosphorylated species of phosphatidylinositol (PtdIns) - are important signaling molecules required for membrane trafficking in eukaryotic cells (Ile et al., 2006; Mueller-Roeber and Pical 2002; Bankaitis et al., 2010). Their biosynthesis is regulated by PtdIns/ phosphoinositide kinases and phosphatases.

We, and others, have found that PtdIns kinases are surprisingly inefficient enzymes on liposomal substrates (e.g. Schaaf *et al.*, 2008), raising the question of how phosphoinositide biosynthesis is regulated in living cells. Possible clues come from studies on Sec14 in yeast. Sec14 is a PtdIns/phosphatidylcholine (PtdCho) transfer protein (PITP) and the prototype of
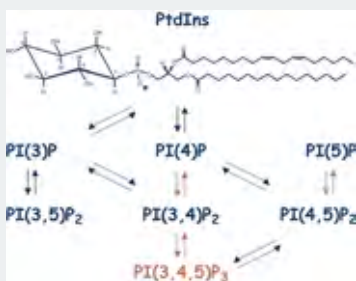

Figure 1 Phosphoinositides. Compounds and pathways depicted in orange have only been identified in animal cells so far.

Figure 2 Cartoon illustrating the Sec14 mediated *in vitro* transfer of phospholipid molecules between two distinct membrane bilayers.
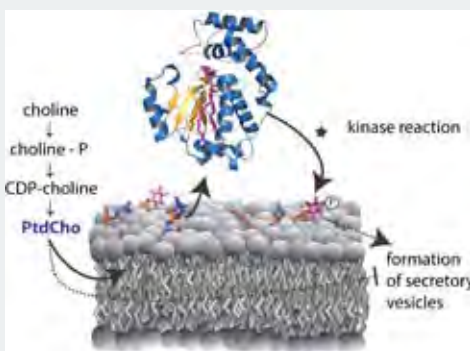
Figure 3 Sec14 presentation model. PtdCho (in blue) or its synthesis via the CDP-choline pathway (left side) is toxic to vesicle formation. Sec14 mediates a heterotypic exchange reaction of PtdIns (purple) versus PtdCho. During this exchange reaction PtdIns becomes a better substrate for 4-OH PtdIns kinases (star) thereby promoting the biosynthesis of PtdIns(4)P which stimulates the formation of secretory vesicles.

a superfamily present in all eukaryotes. Members of this family have been characterized by their ability to transfer PtdIns and PtdCho between membrane bilayers *in vitro.*

Yeast Sec14 is essential for the efficient biosynthesis of phosphoinositides and the generation of secretory vesicles from the *trans*-Golgi (Bankaitis et al., 2010). Defects in Sec14 proteins have been associated with human disease (reviewed in Phillips et al., 2005) and with a deranged membrane trafficking and lipid metabolism in a variety of different organisms, including *Arabidopsis* (Phillips et al., 2005). Our recent success in solving crystal structures of yeast Sec14 homolog Sfh1 in complex with PtdIns and PtdCho led to the following discoveries (Schaaf et al., 2006; 2008):

1. The hydrophobic pocket of Sfh1 (and Sec14) is large enough to completely accommodate a single phospholipid molecule.
2. PtdIns and PtdCho bind at remarkably different but overlapping sites.

3. Binding of both PtdIns and PtdCho are essential activities *in vivo*; both are required for phosphoinositide biosynthesis and have to reside on the same protein molecule (*cis*-configuration) to generate a biologically functional Sec14.

These findings are most consistent with an interfacial presentation model in which Sec14-bound PtdIns becomes accessible to PtdIns 4-OH kinase attack during an exchange reaction with PtdCho (Bankaitis et al., 2010; Fig. 3). PtdIns(4)P in turn activates Rab GTPase signaling and components of a functional ARF cycle, resulting in cargo recruitment, coat assembly and, finally, vesicle formation.

Plants are rich in Sec14-like PITPs. The *Arabidopsis* genome encodes 31 Sec14 homologs (Sfh), of which 11 are expressed as multidomain proteins with a C-terminal nodulin domain that shares homology with NLj16p, a late nodulin protein in *Lotus japonicus* (Ile et al., 2006). Loss of Sec14 homolog AtSFH1 results in strongly impaired root hair development (Figure 5).
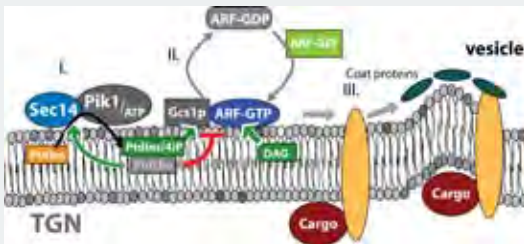

Figure 4 Sec14 dependent formation of secretory vesicles at the yeast *trans*-Golgi.
Sec14 stimulates the production of PtdIns(4)P in a PtdCho dependent manner. As a consequence the antagonistic effects of both phospholipids are kept in balance. PtdIns(4)P activates the Rab GTPase YPT32 by Rab-GEF Sec2 (not shown) and components of a functional ARF cycle. This allows recruitment of cargo and coat and finally vesicle formation.
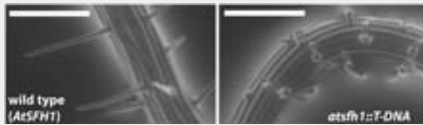
Figure 5 Environmental scanning EM of wild type roots and *atsfh1* roots (from Vincent et al., 2005, J Cell Biol 168, 801-812).
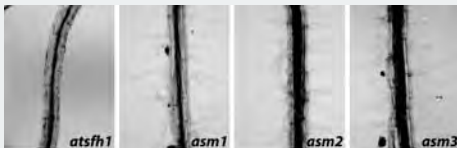
Figure 6 Identification and characterization of *atsfh1* suppressor mutants.
T-DNA insertion lines (*atsfh1*) were randomly mutagenized with EMS and 3 non-allelic, recessive mutants (*ams1-3*) were recovered. Mapping and gene identification will be performed by "next generation" deep sequencing.

We are interested in the following questions:
1. What does the reaction intermediate look like when PtdIns becomes accessible to a PtdIns kinase? Does the topology of how PtdIns exits the Sec14 molecule matter?
2. Does the exchange reaction mediated by Sec14 represent a general mechanism of lipid presentation to lipid modifying enzymes?
3. What are the lipid ligands of Sec14 homologs in *Arabidopsis* and what are their roles in membrane trafficking and lipid homeostasis?
4. What are the functional properties of the C-terminal SFH-nodulin domains in *Arabidopsis*?
5. What can we learn from recently obtained *atsfh1* suppressor mutants regarding phospholipid signaling in *Arabidopsis*?

We try to answer these questions by a combination of biochemical, structural, cell biological and genetic studies.

Plant high resolution imaging/spectroscopy

Frank Schleifenbaum

The quantification of plant subcellular processes is one key step towards a deeper understanding of molecular activity of higher plants. One promising approach is based on confocal fluorescence techniques at subcellular resolution, which offer both a highly sensitive and additionally specific tool for live cell imaging. Using autofluorescent proteins, such as the green fluorescent protein (GFP) family, distinct compartments of living plant cells can be fluorescently labeled individually. Thus, the cellular response to internal and abiotic and biotic environmental factors is accessible at a molecular level. Besides fluorescence intensity images, spectroscopic methods, such as frequency- or time-domain studies, can be used to monitor cellular processes and to study molecular interactions *in vivo.* To this end, we are active in the development of novel fluorescence spectro-microscopy techniques, which extend the spatial information obtained from conventional fluorescence microscopy by further spectroscopic parameters and thus allow for quantitative investigations.
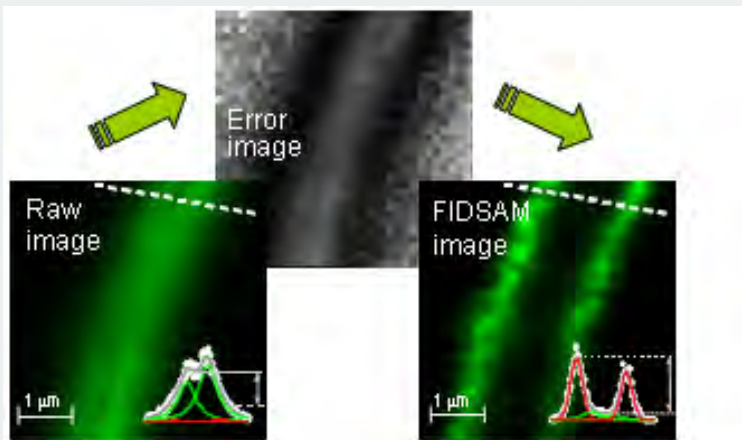
Figure 1 FIDSAM applied to plant cells expressing eGFP-LTI6b located at the plasma membrane. While in the original image (left) the eGFP-marked membranes can not be resolved due to cell wall autofluorescence, after the application of FIDSAM the two membranes become clearly visible..

FIDSAM

One major drawback in plant fluorescence microscopy is the interference of autofluorescent background. This unspecific signal typically exhibits a strong spectral and temporal overlap with common fluorescence markers, such as fluorescent proteins (GFP, YFP etc.), and, thus, cannot be filtered out by standard methods. Fluorescence intensity decay shape analysis microscopy (FIDSAM) is a robust technique to suppress and quantitatively discriminate autofluorescence from any fluorescence label-specific signal (Schleifenbaum et al., 2009). Using differences in the excited state decay statistics in autofluorescence and label-signal, FIDSAM identifies background signal without any presumptions and is, thus, applicable to most fluorescence markers regardless of their individual spectral properties.

Besides providing a decided improvement of image quality, FIDSAM is a valuable tool to avoid the misinterpretation of artefacts. Especially the monitoring of cellular dynamics, such as

vesicle formation, suffers from unspecific cellular responses, as autofluorescent vesicles that do not contain the fluorescently marked target protein can also be formed. These unspecific signals can be robustly suppressed by FIDSAM.

ocFLIM

In addition to spectral properties, the temporal evolution of the fluorescence emission is an appropriate parameter to gain insight into the local physico-chemical environment of a fluorescent dye. Applying a FLIM (fluorescence lifetime imaging microscopy) protocol, we use the local fluorescence lifetime to probe physiological parameters in living cells. The fluorescence lifetime of the brassinosteroid receptor kinase BRI1-eGFP fusion exhibits a dependency on the electrical field strength and, hence, is a read-out for the local membrane potential (Elgass et al, 2010). This way it is possible to monitor protein activity with high spatial resolution and to deduce the signaling pathways of the receptor.
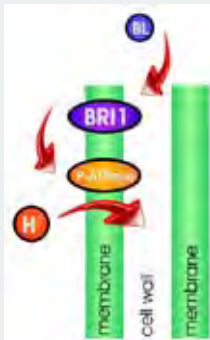
Figure 2 Model of BRI1 signalling pathway controlling early steps in cell elongation growth. After Brassinolide (BL) sensing, the P-ATPase is activated and protons are pumped into the apoplastic space. This acidification and the hyperpolarisation of the plasma membrane cause cell wall expansion. The activation of the P-ATPase can quantitatively be recorded by fluorescence lifetime measurement of BRI1-eGFP..
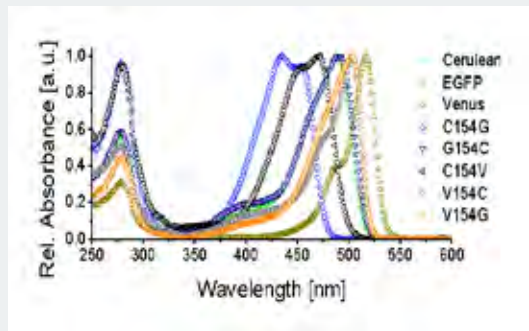
Figure 3 Fluorescence excitation spectra of different multicolour BiFC constructs..

Protein-Protein Interaction

We use confocal fluorescence microscopy to monitor specific protein-protein interactions. Here, we apply FRET (fluorescence resonance energy transfer) to carry out quantitative interaction studies, which allow the precise determination of the composition of distinct components in cellular compartments.

We further use the BiFC (Bimolecular fluorescence complementation) approach to read out close-distance protein-protein interactions, which become visible due to the recombination of two non-fluorescent YFP fragments, resulting in the formation of a functional chromophore. Here, we are active in the development of novel multicolour BiFC constructs, which are carefully characterized *in vitro*. Thus, photophysical and kinetic parameters are accessible as a prerequisite for quantitative interaction studies *in vivo*.

Central Facilities

Overview

The central facilities consist of permanent staff, providing the ZMBP and its external partners with highly specialized services in the fields of (bio)analytics, microscopy, FACS, plant transformation, plant cultivation, information technology, engineering and administration. Continuous development of methodologies and technologies provided by the different units ensure state of the art technological support for all ZMBP research groups.

Structure and staff

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ZMBP Administration
Silvia Röcker

The ZMBP Administration is responsible for the appropriate use of the human and financial resources available to the ZMBP. In detail this covers the following points:
- Implementation of the decisions of the research group leaders with regard to the human and financial resources of the ZMBP
- Financial planning to ensure that the the central tasks of the ZMBP are carried out
- Opening and management of budget accounts for the different research groups of the ZMBP
- Management of payment transactions in cooperation with the university's financial department

Analytics
Mark Stahl

The (Bio)analytics Unit focuses on the qualitative and quantitative analysis of low molecular weight analytes. Quantification of inorganic ions, primary and secondary plant metabolites, like organic acids, amino acids, carbohydrates, plant hormones or anthocyanins, is provided, as well as metabolomic approaches. Additionally, the identification and structural characterization of unknown compounds are conducted. In cooperation with the Proteom Center Tübingen (PCT) protein analytical services can also be offered.
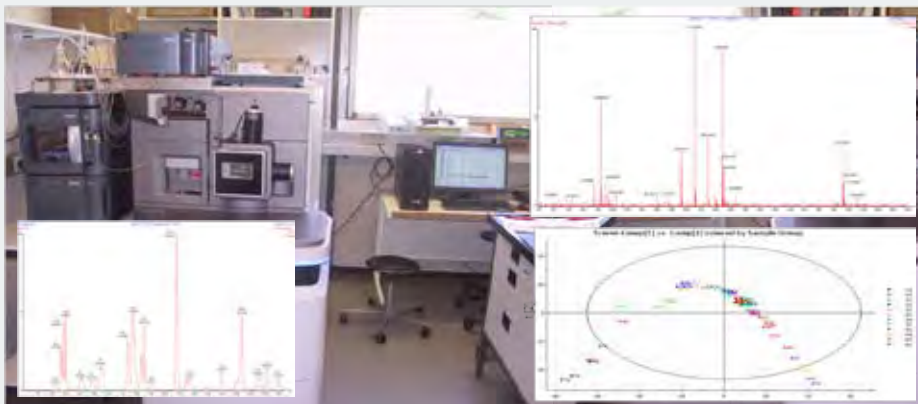
To address the large diversity of chemical and physical properties of metabolites, the following analytical techniques are available:
- High resolution mass spectrometry online coupled to HPLC (LC/MS)
- Maldi mass spectrometry
- Gas chromatography online coupled to mass spectrometry (GC/MS)
- Different HPLC systems equipped with DAD, fluorescence, radioactivity, pulsed amperometric and/or conductivity detection
- Ion chromatography equipped with conductivity detection

Microscopy

York-Dieter Stierhof

The Microscopy Unit supports research by using transmission and scanning electron microscopy, as well as conventional and confocal light microscopy, on biological samples ranging from molecules to complex tissues.

The Light- and Electron Microscopy Unit provides preparative techniques for morphological studies as well as for the immunolocalization of gene products. The focus of our methodological research is on cryoimmobilisation-based specimen preparation procedures for the improved preservation of structural integrity and antigenicity and on methods for the detection of low copy number antigens. This allows a detailed analysis of cellular processes on a molecular level in the context of complex biological systems. The current cell biological focus is on vesicle formation, sorting and trafficking (Figs. 1-3).

Techniques provided include

Negative staining, glycerol spraying, metal shadowing, freeze-drying, freeze-fracture, critical-point drying, cryoimmobilisation (high pressure freezing), freeze-substitution, (low temperature) embedding, ultrathin resin sectioning, ultrathin cryosectioning, immunogold labelling.
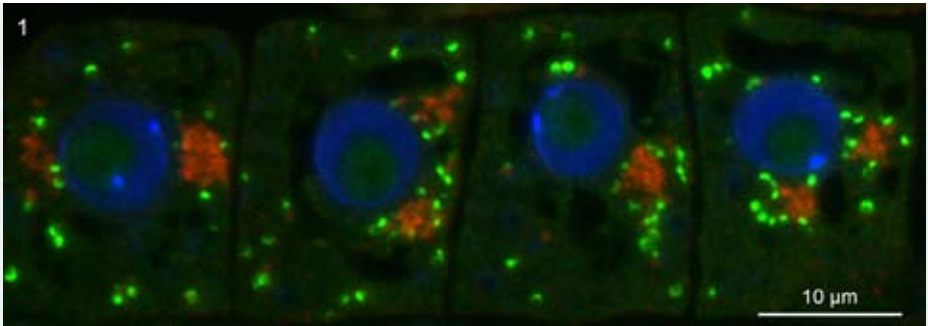
Figure 1 High resolution light microscopy on cryofixed *Arabidopsis* root tip (4 cortex cells, thin cryosection) showing immunofluorescence-labeled Arf1 containing TGN derived vesicles (red), YFP fluorescence of tagged N-Sialyl-transferase (Golgi stacks, green) and Dapi-stained DNA (blue). The root was treated with brefeldin A, resulting in vesicle aggregation (red patches).
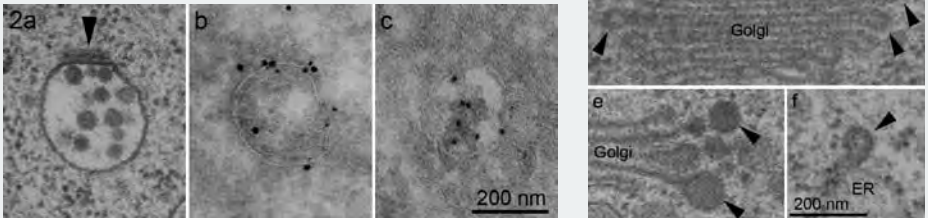
Figure 2 Immunogold localization of RabF2b/ARA7(-GFP) and the syntaxin (GFP-)KNOLLE on multivesicular bodies (MVBs) in cryofixed *Arabidopsis* root tip cells. (a) MVB with ESCRT complex, (b) gold-labeled ARA7 on MVB mem¬brane, (c) gold-labeled KNOLLE in internal vesicles of an MVB.

Figure 3 Transport vesicles in *Arabidopsis* cells (TEM). (a,b) clathrin coated vesicles budding from the plasma membrane and in the cytoplasm (c), (d) COPI vesicles and (e) secretory vesicles budding from Golgi cisternae, (f) a COPII vesicle budding from the ER.

Flow Cytometry (FACS)

Kenneth Berendzen

The Flow Cytometric Unit (FCU) provides both analytical and sorting services for protoplasts, nuclei, cells (bacteria, yeast, etc.) and cytometric suitable particles. In addition, the unit primarily strives to develop and expand novel cytometric methods applicable to plant research.

The ZMBP FCU currently possesses a MoFlo (Beckman-Coulter) with a blue (488 nm) and green (532 nm) laser. One can detect in-vivo labels GFP, YFP, RFP and mCherry as well as some stains, like propidum iodide (PI). Our machine can make 4 different sorts from the same sample and can sort into almost any collection receptacle, slides, 24, 96, 384 well plates as well as custom designs. The FCU is open to and encourages users to develop new protocols and assays.

Application examples:

- Sorting of protoplasts for the isolation of DNA, RNA or protein
- Sorting of nuclei for the isolation of DNA, RNA or protein
- Analysis of protein-protein interaction using bi-molecular fluorescence complementation (BiFC)
- Analysis of promoter transactivation assays
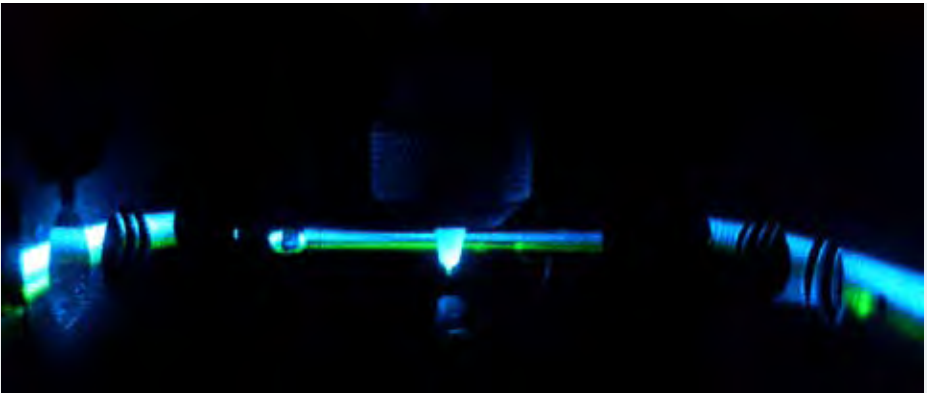
Figure 1 The refraction ring from the 488 nm and 532 nm lasers aligned to the stream.
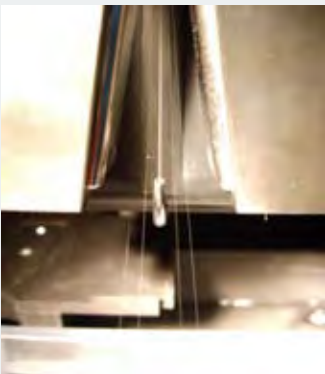
Figure 2 Generation of four sorting streams for calibration; central stream is waste. Streams are generated by polarized charging of the stream and deflection using the two deflection plates (seen to the left and right of the central stream).
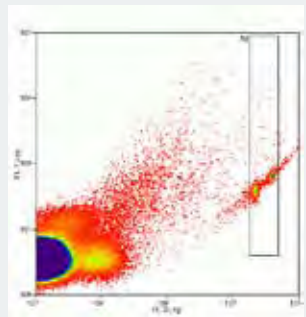
Figure 3 Detection Nicotiana tabacum ploidy level with propidium iodide.

Plant transformation

Kenneth Berendzen

The Transformation Unit offers:

Agrobacterium mediated-transformation of:
- Potato (*Solanum tuberosum* L vc. Désireé)
- Tobacco (*Nicotiana tabacum* cv. SNN)
- Tomato (*Lycopersicon esculetum* cv. Moneymaker)

PEG-mediated transformation of protoplasts from:
- Thale cress (*Arabidopsis thaliana*)
- Rice (*Oryza sativa*)

Main goals of this service unit are:
- Maintenance and distribution of expertise for plant transformation methods
- Generation of transgenic plants from crop species
- Development and standardization of new transformation procedures that could benefit the entire ZMBP

The service includes:
- Collection and maintenance of different lines and genotypes
- Cultivation of sterile plant material
- Preparation of media (for plant cultivation)
- Transformation of plant tissue(s) and protoplasts
- Regeneration and cultivation of transformants

Information technology
Dieter Steinmetz

The Information Technology Unit is responsible for all ZMBP computer systems. This covers ZMBP network and standalone PC administration, helpdesk and personal support, training and administration of the ZMBP websites.

The service includes:

- Coordination of computer systems and networks
- Computer support to staff
- Maintenance and repair of hardware
- Acquisition and installation of software
- Server Systems for science and administration
- Computer and software training
- User-specific analysis of science data
- Administration ZMBP Website

Plant cultivation
Gert Huber and Ernst Schwärzli

The Plant Cultivation Facility consists of controlled growth chambers (300 m²), computer controlled glasshouses (500 m²), standard greenhouses (250 m²) and a two-hectare field trial area. The growth chambers and computer-controlled glasshouses allow strict control of environmental conditions like temperature, humidity and light. The differently controlled cultivation areas ensure cultivation of wild type and transgenic plants as well as a wide variety of different plant species.

Workshop and engineering
Edgar Raidt and Karl Michalik

The ZMBP Workshop Unit is responsible for designing and engineering new equipment or experimental set ups. Additionally, their mechanical and electrical support guarantees the long-term operational reliability of all different scientific equipment.

Selected Publications

Developmental Genetics and Cell Biology

Research Group Groß-Hardt
Mechanisms of plant gamete formation

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Research Group Felix
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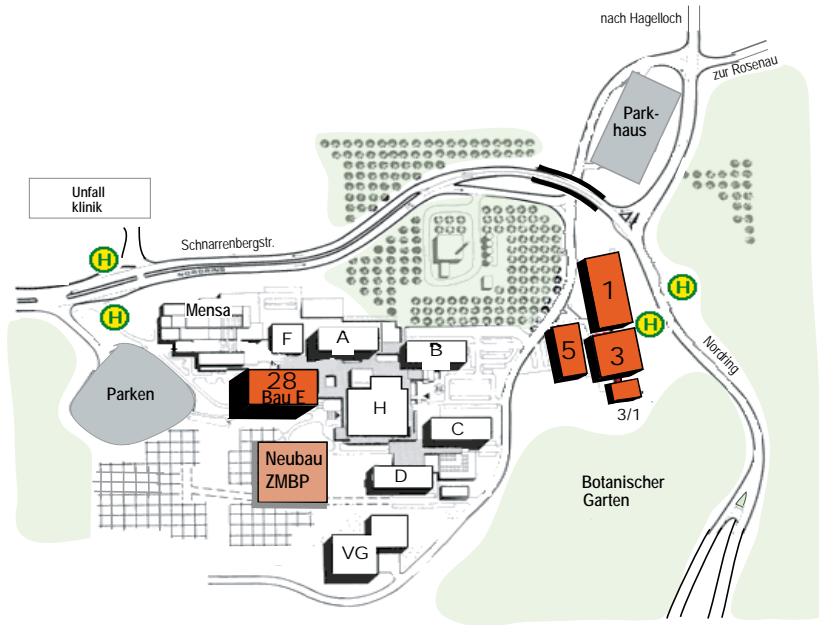
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