The Plant Vascular System: A Macromolecular Information Superhighway

William J. Lucas

Section of Plant Biology, College of Biological Sciences, University of California-Davis, USA

A new paradigm is emerging in which plants utilize proteins and RNA as non-cell-autonomously acting signaling macromolecules to mediate local and long-distance regulation over physiological and developmental processes. The cell-to-cell pathway for the trafficking of these non-cell-autonomous proteins (NCAPs) and RNA, in the form of ribonucleoprotein (RNP) complexes, is established by plasmodesmata (PD), the intercellular organelles unique to plants. The interconnection of local tissues to the phloem sieve tube system, through PD, establishes an integrated supracellular organism. Regulation of these local and long-distance macromolecular trafficking networks is likely essential for the coordinated exchange of information between distantly located plant organs to orchestrate events at the whole plant level. In angiosperms, the sieve tube system is comprised of two main cell types, the sieve elements (SEs) and their associated companion cells (CCs). At maturity, the enucleate SEs are highly modified to create a low-resistance pathway, the sieve tube, for the translocation of photoassimilates; CCs function in the maintenance of the associated SEs. Specialized, branched PD interconnect these two cell types, thereby forming the CC-SE complex. Analysis of the phloem sap collected from a number of species has demonstrated that the translocation stream contains a complex set of proteins (~ 1000 in number). Detailed analyses of many of these phloem proteins have demonstrated their capacity for cell-to-cell movement through PD: thus, the entry and exit of these phloem proteins appears to be regulated by the CC-SE PD. Interestingly, a number of these phloem proteins can bind to RNA and one, CmPP16, has been shown to mediate the cell-to-cell and long-distance translocation of RNA, in a non-sequence-specific manner.

These studies provide support for the concept that the CC-SE complex has the machinery necessary to mediate long-distance delivery of NCAPs and RNPs. This notion is consistent with the discovery that the phloem translocation stream contains a specific population of RNA molecules (>1,500 mRNA and many 1000s of si/miRNA species). Grafting experiments have proven that many of these RNA molecules are translocated within the phloem and, in some situations, delivery of these RNA have

been correlated with development of specific phenotypes within the shoot apex. Systemic spread of RNA interference (RNAi), a sequence-specific RNA degradation process, is also consistent with the concept that RNA can be delivered to distant organs, through the phloem. Thus, PD and the sieve tube system act, in concert, to establish an information superhighway in plants.

To advance our understanding of the roles played by PD and the phloem, in the trafficking of NCAPs and RNPs, we are currently developing proteomic-based strategies to (a) elucidate the supramolecular structure of PD, (b) establish the function of the phloem-mobile proteins, and (c) develop a phloem transcriptome for the RNA molecules (both large and small) that function within the context of the enucleate SEs. The impact of these findings on studies in plant biology will be discussed in terms of RNA as a long-distance information macromolecule within the plant kingdom.

Plant Neurobiology: Why Now, and What For?

František Baluška[1,4], Dieter Volkmann[1], Peter Barlow[2], Stefano Mancuso[3,4]

[1] *University of Bonn, Germany*
[2] *University of Bristol, UK*
[3] *University of Florence, Italy*
[4] *Plant Neurobiology Laboratory (Florence & Bonn)*

Recent advances in plant cell biology, molecular biology, and ecology have accumulated a critical mass of data which are not 'digestible' within the framework of these, now classical, disciplines of plant sciences. New approaches are required, and these should be characterized by system-like analysis of information acquisition, storage, processing, and the making of decisions. Plants retrieve from the abiotic environment information critical for their survival, especially relating to light and gravity, two physical forces pervading the universe. Intriguingly, the translation of these physical forces into plant activities – typically differential growth responses – is based on the transcellular transport of auxin, which help to bring about the final shape of the plant body. Thus, this information-bearing molecule is central to our call for plant neurobiology.

Although the history of auxin can be traced back to the Darwin's early experiments with phototropism of coleoptiles, we still know almost nothing about its peculiar features. Let us examine the mystery of this unique molecule. Although auxin can be synthesized probably in each plant cell, it is tediously transported from cell to cell throughout the plant body. Similarly puzzling is the well-known phenomenon that, although the auxin molecule is sufficiently small to pass easily through plasmodesmatal channels, plants cells somehow manage to prevent this direct cell-to-cell means of auxin transport. Rather, plants maintain an energetically costly system based on vesicle trafficking, closely resembling neuronal and immunological cell-cell communication, in order to drive transcellular auxin transport. The next peculiarity is that when extracellular auxin hits the plasma membrane, it induces electric responses based on the ABP1 auxin-binding protein. All this suggests that auxin, besides hormone- and morphogen-like properties, also possesses neurotransmitter-like properties. As the cell-to-cell transport of auxin is also involved in plant response the light and gravity, as well as to vesicular trafficking, plant neurobiology is needed to explain this great mystery of plant nature.

In a recent Nature article, DeWeese and Zador (2006) loosely defined neurobiology as having three basic characteristics: 1) all biological systems (organisms)

are embedded within a physical environment that shapes their organization and behaviour; 2) in order to survive, all biological systems need to effectively retrieve of information from their physical environment; 3) neuronal activity is essential to translate information sensed concerning the environment into electrical impulses which are then capable of rapid transformation into biological signals that induce motoric responses. Importantly, also communication from the biotic environment is physically mediated through the senses: hearing, seeing, feeling, or smelling; all this is based on the laws of physics. In short, the neurobiological apparatus translates sensory information first into electrical impulses and only then into biological information inducing organismal actions. Human perception of the outside world relies on so-called 'neural code' which links together sensory signals and neural responses.

Similarly in plants, numerous parameters of the physical environment, especially light and gravity, are monitored. Specialized cells (e.g., root cap statocytes and root transition zone cells) are evolutionarily optimised to translate sensory information obtained from this environment into motoric responses (e.g. gravibending of root apices). Moreover, physical forces, influences, and insults, all induce immediate electrical responses in plants. Obviously, one task is to make a connection betwee all these events with the molecules and cellular processes which are known from neurobiology, and for which there is firm or emerging evidence from plants. Finally, we need to understand those processes which transform physical information (e.g. light, gravity, temperature, mechanical and osmotic forces etc.) into biological information. Particularly, we need to know if it is possible to convert physical information directly into biological information without inducing any bioelectrical responses, or if physical information needs to be first transformed into bioelectrical information before it can be translated into purely biological information. For this, we need a merging of classical electrophysiology with cell biology and molecular biology. Obviously, plant neurobiology as a new branch of plant sciences is not only justified but also is very competent to solve the new and urgent questions of contemporary plant biology.

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How and Why do Plants Support Electrical Long-distance Signals?

Rainer Stahlberg, Robert E Cleland, and Elizabeth Van Volkenburgh

University of Washington, POB 355325, Seattle WA 98195, USA

L. Hermann, W. Nernst and J. Bernstein determined our modern understanding of transmission of action potentials (APs) by demonstrating that excitation travels not as a direct electrical current but in the form of an area of altered ion currents along a frequently myelin-coated plasma membrane [1]. After characterizing APs in animal nerves, finding similar propagating electrical signals in nerveless plants was unexpected [2]. When neurobiologists later discovered that maintenance of large amplitudes of APs over long distances was achieved by the intermittent renewal of the signal by electrogenic (mostly depolarizing) chemicals (neurotransmitters) in synapses, the degree of astonishment and curiosity about plant signals should have increased even more [1,2]. After all, (i) plant cells are separated by thick cell walls that leave no space for synaptic structures and (ii) their sessile life style leaves most plants in no need for fast signals. In spite of such consideration we know today that plants have not only one but two types of propagating, electrical long-distance signals; (i) action potentials (APs), which they share with animals and (ii) slow wave potentials (SWP, also called "variation" potentials), which are unique to vascular plants [2, 3]. The mechanisms of how plants can support APs and SWPs over long distances of hundreds of cells is one of the great challenges in the emerging field of plant neurobiology together with the exploration of their roles in signaling internal and external situations from one plant part to another.

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Gravity as Physical Force Shaping Plants

Dieter Volkamnn

IZMB, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany

Gravity is the most constant environmental factor which influences both ontogeny as well as phylogeny, and in particular the diversity, of land living organisms. As prominent mechanical signal, gravity controls a large number of plant genes belonging to different categories, including signalling, metabolism, cytoskeleton, vesicle trafficking, and cell wall. Several signal transduction cascades have been proposed for the gravity controlled processes like gravitropism and gravimorphogenesis. Experiments under temporarily reduced gravity conditions (parabolic flights) suggest that extremely fast electrical signals, like action potenials (APs), as well as very rapid changes in oxygen and nitric oxide (NO) concentrations, play a crucial role in these plant morphogenesis processes. The cell wall-plasma membrane-cytoskeleton continuum, known as the Ingber's tensegrity concept, in connection with vesicle trafficking and cytoskeleton dynamics, will be discussed as structural basis of a common mechanism enabling gravity to shape the plants.

Potential Calcium Sensor Proteins Function in Perception and/or Response to Environmental Stimuli

Yu-Chang Tsai, Nikkí A. Delk, Elizabeth McCormack, Naweed I. Chowdhury, Roque Sanchez and Janet Braam

Biochemistry and Cell Biology, Rice University, Houston TX 77005-1892 USA

Plants are highly responsive to diverse stimuli enabling them to acclimate and thrive under diverse environmental conditions. Fluctuations in free cytosolic calcium (Ca^{2+}) serve as second messengers in transducing perceived stimuli into cellular responses. The quintessential Ca^{2+} sensor is calmodulin (CaM), which upon binding Ca^{2+} undergoes a profound conformational change that affects the activity of interacting proteins. In this way, Ca^{2+} signals are converted to changes in target protein activity and consequent physiological responses. Arabidopsis has a large gene family encoding CaM and CaM-like (CML) proteins (McCormack and Braam, 2003; McCormack et al., 2005). To reveal functions of Arabidopsis CaMs and CMLs, we are characterizing phenotypes of plants harboring mutations in a subset of the gene family members. CML11 is an unusual CaM isoform that has a polymorphic glutamine-rich domain at the amino terminus. *CML11* insertional mutants show defects in root hydrotropism. *CML23* and *CML24*, closely related paralogs, regulate flowering time through the photoperiod and autonomous pathways, affecting both *CONSTANS* and *FLOWERING LOCUS C* expression. The altered transition to flowering is a consequence, at least in part, of altered nitric oxide accumulation in the mutants. Thus, these potential Ca^{2+} sensors have specialized and specific roles in plant physiology enabling diverse behaviors, including response to water stress and seasonal regulation of the transition to flowering.

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Cellular Signals Governing Vascular Tissue Organization in Plants

Hiroo Fukuda

Department of Biological Sciences, Graduate School of Science, the University of Tokyo, Hongo, Tokyo 113-0033, Japan

The body plan of plants is governed by a combination of clonal fate and positional information that is provided by local signals, as is commonly seen in multicellular organisms. Recent advances in molecular genetics and cell biology have uncovered unique mechanisms of plant body formation. We have focused on the vascular system, because the vascular system becomes a good model system for understanding molecular mechanism of plant body formation and also becomes a good target for improving crops and trees. The vascular system is composed of various types of vascular cells, which usually differentiate at predicted position and at predicted timing according to genetic programs and cell-cell communications [(1)]. Here I report our recent findings about genetic program and cell-cell communication in association with vascular organization.

1）Vesicle transport plays a role in continuous formation of vascular strands

We isolated Arabidopsis *van1-van7* mutants with discontinuous vascular strands [(2)]. Of them, the *van3* mutant has been analyzed in detailed, revealing that the *VAN3* gene encodes an ARF-GAP, which regulates vesicle transport from the *trans* Golgi network [(3)]. This protein seems to be involved in auxin perception but not closely in auxin transport which is regulated by GNOM ARF-GEF, resulting in fragmented vascular formation. We also found that a dynamin protein is bound to the VAN3 protein specifically and may function in continuous formation of the vascular system together with the VAN3 protein [(4)].

2）Various extracellular factors regulate vascular cell differentiation

We have searched an extracellular factor(s) promoting tracheary element differentiation from medium of *Zinnia* xylogenic culture, in which isolated mesophyll cells transdifferentiate into xylem cells [(5)]. Isolation of the factor, called xylogen and its gene indicated that xylogen is a non-classical arabinogalactan protein with non-specific lipid transfer protein-like sequence. Two similar genes are found in Arabidopsis genome sequence. Introduction of the genes into tobacco BY-2 culture cells induced a xylogen activity in culture medium, demonstrating that the genes encode xylogen. Double mutants of these genes exhibited a distinctive phenotype defective in vascular continuity. Because the xylogen gene is expressed in

procambium or xylem precursor cells, xylogen may function as an apoplastic signal molecule directing xylem cell specification continuously [5]. We also have found other factors promoting tracheary element differentiation, brassinosteroids [7,8] and phytosulfokine in *Zinnia* xylogenic culture. Recently we identified a novel peptide that inhibits tracheary element differentiation from medium of *Zinnia* non-xylogenic culture. These findings clearly indicated that spatial and temporal expression of these intercellular factors.

3）Systematic analysis of gene expression with DNA arrays for *Zinnia* and *Arabidopsis* xylogenic cultures revealed master genes directing xylem cell differentiation

Using a *Zinnia* xylogenic culture we have revealed comprehensive gene expression profiles [9]. Recently we newly established Arabidopsis cell culture in which cultured cells differentiate into xylem cells at high frequency. Using the culture system, we also performed a comprehensive analysis of gene expression during xylem cell differentiation. The two comprehensive analyses revealed that similar gene sets are expressed in association with xylem cell differentiation. Detailed analysis of gene function revealed a new class of transcription factors, VND6 and VND7, which can induce xylem cell differentiation [10]. Interestingly, VND6 and VND7 induce different types of xylem cells, that is, VND6 induces metaxylem vessel cells and VND7 does protoxylem vessel cells. On the other hand. the suppression of function of *VND6* and *VND7*, results in the inhibition of metaxylem and protoxylem formation, respectively. These results indicate that *VND6* and *VND7* are master genes for inducing xylem cell differentiation.

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Rice ARF-GAP Mediates Auxin Influx and Auxin-dependent Root Growth

Xiaolei Zhuang[1], Jiafu Jiang[1], Junhua Li[1], Qibin Ma[1], Yunyuan Xu[1], Yongbiao Xue[2], Zhihong Xu[1], Kang Chong[1]

[1]*Research Center for Molecular Developmental Biology, Key Laboratory of Photosynthesis and Molecular Environmental Physiology, Institute of Botany; Chinese Academy of Sciences, Beijing 100093, China;*
[2]*Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100086, China*

Development and organogenesis in plants are highly dependent on polar auxin transport, which requires the proper asymmetric localization of both auxin influx and efflux carriers. Auxin efflux carriers such as PIN1 are regulated by GNOM ADP ribosylation factor (ARF)-GEF in Arabidopsis, a GDP/GTP exchange factor for a small G protein of ARF-GTPase. However, little is known about the regulation of auxin influx carriers. Here we identified *OsAGAP* gene in rice which encoded a protein with predicted structure similar to ARFGAP. It was able to stimulate the GTPase activity of rice ARF. Furthermore, OsAGAP can rescue the defect of vesicular transport in the yeast *gcs1* Δ *glo3* Δ double-mutant cells. Transgenic *Arabidopsis* with *OsAGAP* constitutively expression showed reduced apical dominance, shorter primary roots, increasing number of longer adventitious roots. Overexpression of OsAGAP in rice, an activating protein for ARF-GTPase, impaired polar auxin transport and reduced lateral root development and growth. OsAGAP was expressed mainly in the cortex, vascular tissues and meristematic cells under the root cap. The transgenic plant phenotype of reduced number of lateral roots was rescued by treatment with 1-naphthyl acetic acid (NAA), which can enter the cells via diffusion independent of auxin influx carriers, but not by indole 3-acetic acid (IAA), which requires influx carriers to enter the cells. Total [^{3}H]IAA transported in roots of transgenic plants was significantly reduced as compared with that in the wild type. The auxin influx carrier AUX1 is asymmetrically localized in the plasma membrane in wild-type plants but in the cytoplasm in transgenic Arabidopsis plants overexpressing OsAGAP. OsAGAP-overexpressed rice plants also showed altered vesicle trafficking. Our data and other evidences support a previously undescribed model of PAT regulation: a loop mechanism mediated by ARF-GAP and GEF is involved in regulating polar auxin transport at influx and efflux carriers, which controls root development in plants.

Gamma Aminobutyric Acid (GABA) Metabolism in Plants: Analysis of *knock-out* Mutants

Anke Hüser[1], Rainer Waadt[2], Dennis Fink[1], Iris Schmitz[1], Ulf-Ingo Flügge[1], and Frank Ludewig[1]

[1]Botanical Institute II, University of Cologne, Gyrhofstr. 15, 50931 Cologne, Germany
[2]Botanical Institute and Botanical Garden, University of Münster, Schlossplatz 4, 48149 Münster, Germany

GABA metabolism is compartmentalized. Anabolism takes place in the cytosol and catabolism occurs in mitochondria. The GABA catabolic *ssadh* mutant is strongly impaired in growth, most likely due to the accumulation of a toxic compound. Two candidate metabolic intermediates (SSA and GHB) were analyzed for their responsibility to cause the phenotypic aberrances of mutant plants.
The *ssadh* phenotype can be rescued by simultaneously knocking out the *gaba-t* gene, the gene upstream in GABA catabolism. This phenotype suppression can be explained by preventing the intermediate to accumulate in the double *knock-out* plants.
Based on this finding and seeking for unknown genes being involved in GABA metabolism an *ssadh* suppressor screen has been performed, where *ssadh* mutants have been mutagenized using EMS. Suppressor mutants have been collected and analyzed from the M2 generation.

Ultimately, EMS mutagenized genes should be mapped and cloned to assign a function for them in GABA metabolism or regulation of the pathway.

Functional Characterization of Plant Steroid Binging Protein

Hong-Wei Xue

National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Chinese Academy of Sciences;
Partner Group of Max-Planck-Institute of Molecular Plant Physiology (MPI-MP) on "plant Molecular Physiology and Signal Transduction"

Steroid-binding proteins (SBPs) are essential for growth and development of both animals and higher plants. Most known animal SBPs not only regulate the concentrations of general available steroids but also function in steroid signal transduction through interacting with specific receptors on the plasma membrane of target cells, thus directly involve in regulation of cell growth and differentiation (Rosner *et al*., 1999; Breuner and Orchinik, 2002). Although some SBP coding genes have been annotated in higher plants, less studies on the isolation and functional characterization of plant SBP to date. Based on the homologous analysis, we identify one putative arabiopsis MSBP1. Full-length cDNA was isolated and recombinant expressed MSBP1 was used for biochemical characteristics analysis. MSBP1 could bind progesterone, 5-dihydrotestosterone, 24-epi-brassinolide (24-eBL), and stigmasterol with different affinities in vitro, demonstrating the presence of a steroid-binding protein in higher plants.

Transgenic plants overexpressing MSBP1 showed short hypocotyl phenotype and increased steroid binding capacity in membrane fractions, indicating that MSBP1 negatively regulates hypocotyl elongation and as a negative regulator of cell elongation in *Arabidopsis thaliana*. Altered expressions of genes involved in cell elongation, such as expansins and extensins, were detected, indicating that enhanced MSBP1 affected a regulatory pathway for cell elongation. Suppression or overexpression of MSBP1 resulted in enhanced or reduced sensitivities, respectively, to exogenous progesterone and 24-eBL, suggesting a negative role of MSBP1 in steroid signaling. Further studies indicate that MSBP1 involve in BR signaling through interaction with BAK1.

Evidence that Leaf Attachment is Required for Auxin-Induced Inhibition of Leaf Expansion in *Arabidopsis*

Christopher P. Keller and Morgan L. Grundstad

Department of Biology, Minot State University, Minot, ND 58707

Previous work suggests auxin (indole-3-acetic acid) may function in leaf expansion. Increasing the auxin content of intact expanding leaves of *Arabidopsis* and *Phaseolus*, either through exogenous application or through trapping the endogenous hormone in leaves, results in inhibition of leaf expansion.[1] Paradoxically, other work has clearly shown that treatment of excised leaf strips from tobacco (*Nicotiana*) with auxin stimulates rather than inhibits growth.[2] Auxin treatment, whether of intact or of excised leaf tissue results in epinastic curvature due to relatively greater growth by the adaxial side of the tissue. The current, not yet complete, project reexamines the auxin growth sensitivity of attached leaves and excised leaves and leaf tissues of *Arabidopsis* attempting to determine if the reversed growth response to auxin of excised tissues is a wound response or a result of detachment from the plant.

For our experiments, 10-14 day old soil-grown *Arabidopsis* with both the first two true leaves 2.7-3.3 mm in diameter were used - one randomly selected leaf to be the treatment leaf and the other a control. Depending on the experiment, digital images used to determination initial leaf or leaf strip area of intact attached leaves, detached leaves, excised leaf strips (0.7 mm wide cut transversely at midleaf), or wounded attached leaves (sliced transversely from leaf edge to near midvein in three places). Treatment solutions included: full strength Murashige and Skoog media, 10 mM KCl, 0.1 mM Mes/Btp (pH 6.0), +/- indole-3-acetic acid at various concentrations (between 1 μM and 1 mM). Attached leaves and wounded attached leaves received either a 5μl drop of the treatment or control solution. Detached leaves and leaf strips were incubated in 3 mL of the same solutions. After 24 hours, the area of the variously treated leaves and strips was re-determined.

The growth of intact attached leaves was found to be relatively insensitive to auxin. While lower concentrations of IAA were ineffective, 300 μM and 1 mM were inhibitory. For example, at 300 μM the area of IAA treated leaves increased 51.7 +/- 10.1(95% C.L.)% compared to 77.2 +/- 6.3 % for the controls (n=12). The growth of detached leaves floated on IAA was sensitive to the same high concentrations but here growth was increased. For example, at 300 μM the area of auxin treated leaves increased 52.3 +/- 3.1 % compared to 43.7 +/- 4.5 % for the controls (n=12). Leaf

strips incubated in auxin grew significantly more than control leaves across a range of concentrations 10 μM and higher. In limited tests, wounded attached leaves were significantly inhibited by auxin. Applied at 50 μM, the area of auxin treated leaves increased 14.9 +/- 6.9 % compared to 35.2 +/- 8.8 % for the controls (n=20). In the data so far collected the growth of attached leaves, whether intact or wounded, is inhibited by auxin treatment while detached leaves and strips grow more. Possible explanations will be discussed.

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Viroid to Probe Intracellular and Systemic RNA Trafficking in Plants

Biao Ding

Department of Plant Cell and Molecular Biology and Plant Biotechnology Center, 1060 Carmack Rd., Ohio State University, Columbus, OH 43210 USA

RNA trafficking plays important roles in plant growth and development and plant-pathogen interactions. How an RNA traffics within a cell and across specific cellular boundaries is a central mechanistic issue that is poorly understood. Viroid infection provides an excellent model system to address this issue. Viroids are small, noncoding and autonomously replicating RNAs that infect plants. Without encoding proteins, the viroid RNA genome must have evolved structural motifs to exploit an endogenous system for trafficking. We have developed methods that combine single cell replication and whole plant infection assays to investigate how Potato spindle tuber viroid (PSTVd) traffics within a cell and systemically throughout a plant. Our studies show that the strand polarity of PSTVd RNAs can dictate subnuclear trafficking and localization patterns. We have also obtained evidence that trafficking of the PSTVd RNA across different cellular boundaries is mediated by distinct RNA motifs. These findings have important implications in investigating the general regulatory mechanisms of RNA trafficking in plants, including characterizing cell-specific protein factors that recognize an RNA for trafficking between different cells and in particular directions.

Genetic and Proteomic Analysis of Gravity Signal Transduction in *Arabidopsis* Roots

Patrick H. Masson, Li-Sen Young, Narayana Murthy UM, Gregory Sabat[2], Benjamin R. Harrison, John Stanga, Carolyn Neal, Laura Vaughn

Laboratory of Genetics and UW Mass-Spec Facility[2], University of Wisconsin-Madison, 425G Henry Mall, Madison, WI 53706, USA

Arabidopsis roots respond to gravistimulation by developing a curvature that is modulated by a lateral gradient of auxin. This gradient originates in the columella statocytes, and is associated with a lateral repositioning of the PIN3 auxin efflux facilitator in these cells. We used genetics to identify proteins that contribute to gravity signal transduction in the statocytes. ARG1 and ARL2 are needed for lateral auxin transport across the cap. ARG1 is associated with the vesicular trafficking pathway, suggesting it regulates PIN3 function or trafficking. Accordingly, immunolocalization studies confirm a lack of PIN3 relocalization in gravistimulated statocytes of *arg1-2* and *arl2-1* mutant root caps. Genetic modifiers of *arg1-2* were obtained and shown to enhance the gravitropic defect of *arg1-2*. The corresponding proteins function in interpretation of the gravity signal. Furthermore, a proteomic approach allowed identification of root-tip proteins that are differentially represented early in response to gravistimulation. Subsequent reverse genetic studies demonstrated a role for adenosine kinase and the AdoMet pathway in gravity signal transduction (Support from NASA and NSF).

Polarity Control of Auxin Transport

Rujin Chen, Ashverya Laxmi and Jianwei Pan

Plant Biology Division, Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401 USA

Polar auxin transport plays an important role in many plant developmental processes and responses to the environment. It is mediated by plasma-membrane localized auxin influx and efflux carrier proteins of AUX1 and PIN families of proteins, respectively. In the Arabidopsis genome, there are 8 closely related and 7 distantly-related PIN sequences. Loss-of-function analysis and over-expression in heterologous systems of PINs suggested that PIN proteins are the facilitator/regulator/component of the auxin efflux carriers. PIN proteins are undergoing constitutive cycling between plasma membrane and endocytic compartments called endosomes. Experimental evidence suggests that this cycling is important for PIN function as the auxin efflux facilitator and is subject to feedback regulation by auxin. We are interested in understanding how the polarity of auxin transport is established at the molecular and cellular levels. Our inhibitor studies suggest that protein phosphorylation/dephosphorylation plays a role in the control of auxin transport.

Signaling in Chloroplast Movement

Halina Gabrys, Anna Anielska-Mazur, Agnieszka Katarzyna Banas, Weronika Krzeszowiec

Department of Plant Physiology and Biochemistry, Faculty of Biotechnology, Jagiellonian University, Kraków, Poland

Chloroplasts change position in plant cells to optimize light conditions for photosynthesis. In leaves of higher plants these movements are activated by blue light absorbed by phototropins. Phototropin2 controls avoidance responses of chloroplasts in strong light whereas phototropin1 mediates both, accumulation responses in weak light and avoidance responses. Chloroplasts move along actin tracks using myosins as motor molecules. The signal transduction pathway downstream the photoreceptor remains largely unknown. Our study focused on the chloroplast end of the pathway. In particular, we asked the question whether information on the spectral range, intensity and direction of the actinic light is transmitted to the actomyosin system, and if so, in what way it is reflected in the operation of this system. Using transgenic tobacco plants we showed that wavelength differences are not sensed by actin filaments. The actin cytoskeleton was visualised due to expression of a human plastin (actin bundling protein) gene fused with GFP. Images of blue and red-irradiated actin network were similar. The filaments responded however to fluence rate: strong light caused their widening and diffusion, reversible in weak light. No blue specific effect was detected. A potential role of Ca^{2+} ions in light signal transduction was addressed in experiments with several compounds disturbing calcium homeostasis. The results emphasize the necessity of calcium for proper organization and dynamics of the actin network. Its role as a secondary messenger remains elusive. Immunofluorescence studies using antimyosin antibodies provide evidence that myosins residing on chloroplast surface may be a target of strong blue light signal. Only in this light were these myosins detached from the chloroplast surface. The strong blue light-activated separation of myosin did not occur in the *phot2* mutant of *Arabidopsis thaliana* deficient in phototropin2. This finding sheds a new light on the mechanism operating at the chloroplast-myosin interface and on the mechanism of chloroplast movements in general. Intracellular responses of the actin cytoskeleton and chloroplasts to blue light signals are modulated by the presence of sugars in the leaf apoplast. This modulation occurs at the level of gene expression and is pretty complex, depending on the sugar type, concentration and length of exposure. A hexokinase-dependent signaling pathway is partly involved in this effect. This has been shown in experiments using a non-metabolisable glucose analog, 3-O-methylglucose that did not affect the movement apparatus. On the other hand, Hexokinase1, the unique known *Arabidopsis* hexose sensor, effected only a reduction in the avoidance response amplitude.

A ROP GTPase Signaling Network in the Control of Plant Cell Morphogenesis

Ying Fu, Shundai Li, Tongda Xu, Ying Gu and Zhenbiao Yang

Department of Botany and Plant Sciences, Center of Plant Cell Biology, University of California at Riverside

Rho-family small GTPases are ubiquitous signaling switches in eukaryotic signal transduction. They are considered master regulators that control many important cellular, developmental, and physiological processes through their ability to coordinate multiple pathways and feedback loops. Plants possess a unique subfamily of RHO GTPases, known as ROP, that regulate a diverse array of processes ranging from polar cell growth through hormone responses to defense reactions. However, the molecular mechanisms of Rho GTPase signaling in plants remain poorly understood. We are investigating ROP GTPase signaling networks using two model systems: tip growth in pollen tubes and intercalary cell growth in pavement cells that form jigsaw puzzle appearance. In this talk, I will focus on our recent understanding of how the ROP2 GTPase signaling network coordinates the organization of actin microfilaments (MFs) and microtubules (MTs) to achieve the intercalary cell growth in pavement cells. Both MFs and MTs play an important role in cell morphogenesis, and earlier drug experiments suggest that these two cytoskeletal elements interact and coordinate to modulate cell shape formation. The intercalary growth in pavement cells of *Arabidopsis* leaves provide an exciting system to investigate how these cytoskeletal elements coordinate with each other at the molecular level to regulate cell growth and cell shape formation. Earlier studies suggest well-ordered cortical MTs, which are found in the indenting zone, promoting indentation, while more recent studies suggest that lobe formation and outgrowth requires cortical fine MFs that are localized to the tip of outgrowing lobes. We have shown that ROP2 GTPase controls intercellular growth to from interlocking lobes and indentations by coordinating these two types of cytoskeleton. ROP2 regulates these two cellular targets using two molecular targets, RIC4 and RIC1. ROP2 is activated at the site of lobe formation and growth and then activates RIC4, leading to the formation of localized fine cortical MFs. In the meantime, active ROP2 inhibits RIC1, which are associated with the indenting zone and promotes ordering of cortical MTs. Therefore, in lobe-forming zone, ROP2 promotes outgrowth by activating actin assembly and suppressing MT ordering. Interestingly, in the indenting zone, RIC1-dependent well-ordered MTs suppresses ROP2 activation, assuring that no

ROP2 activity is present in this region. Our ongoing research is aimed at understanding how ROP2 is activated in the lobe-forming zone, how RIC1 is activated in the indenting zone, how cortical MTs regulate ROP2 activity, and whether cell-cell communication is important for the coordination of lobe growth and indentation formation between neighboring cells. Progress in answering these questions will be discussed.

Roles of Syntaxins in Disease Resistance

Hans Thordal-Christensen, Ziguo Zhang, Angela Feechan, Carsten Pedersen

Dept. of Agricultural Sciences, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Copenhagen, Denmark

Plant disease resistance is the result of the collective activity of several separate defence mechanisms. In genetic analyses, we have previously discovered that syntaxin SYP121 in *Arabidopsis* is required for penetration resistance (Collins et al., 2003; Assaad et al., 2004). SYP121 is probably necessary for vesicle trafficking leading to formation of papillae, which are local cell wall appositions functioning as barriers against fungal penetration. The closely related SYP122 is not required for penetration resistance.

Other defence mechanisms are controlled by different signalling pathways, which are activated upon pathogen attack. The signalling compounds salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) each define such pathways. In addition, plant cells can undergo the "hypersensitive response" (HR), a program cell death (PCD) reaction, that confer resistance to biotrophic pathogens. In mutant studies, we have examined the involvement of SYP121 and SYP122 in these four signalling pathways, and found that the syntaxins act as negative regulators of all four. While SYP121 is the primary regulatory protein, SYP122 is partially able to take over it role. The release of this negative regulation in the *syp121 syp122* double mutant results in strong PCD-mediated resistance to an otherwise virulent powdery mildew fungus.

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Regulation of pH Homeostasis in *Arabidopsis* by a Protein Kinase

Yan Guo

National Institute of Biological Sciences, Beijing 100093 China

Regulation of cellular pH is an important part of plant responses to several hormonal and environmental cues such auxin, blue light and fungal elicitors. However, little is known about the signaling components that mediate cellular pH homeostasis in plants. Here we report that an Arabidopsis serine/threonine protein kinase, PKS5, is a critical regulator of cellular pH homeostasis and plant responses to alkaline conditions. Loss-of-function pks5 mutant plants are more tolerant of high external pH. PKS5 negatively regulates the activity of plasma membrane H^+-ATPase, and it can phosphorylate the H^+-ATPase AHA2 at a novel site, Ser-931, in the C-terminal regulatory domain. We show that PKS5 interacts with an EF-hand calcium-binding protein SCaBP1, and that high external pH can trigger an increase in the concentration of cytosolic free Ca^{2+}. Yeast reconstitution experiments show that the activity of AHA2 in vivo is repressed by SCaBP1 and PKS5, and this repression requires Ser-931. These results suggest that PKS5 is part of a calcium-signaling pathway mediating H^+-ATPase regulation.

Involvement of Vesicular Trafficking in Abiotic Stress Tolerance

Miguel A. Botella, Arnaldo Schapire, David Posé, Abel Rosado, and Victoriano Valpuesta

Laboratory of Plant Biochemistry and Biotechnology. Department of Biochemistry and Molecular Biology, Facultad de Ciencias, Universidad de Málaga, Málaga, Spain

We have identified a number of Arabidopsis mutants that show hypersensitivity to several abiotic stresses. Two of these mutants suggest that membrane trafficking is important for abiotic stress tolerance. The *746* mutant was identified in a screening of plants that showed hypersensitivity to NaCl stress. Identification of the gene altered in this mutant encodes for a protein with homology to synaptotagmins. Synaptotagmins are proteins that share a common structure: an N-terminal transmembrane domain, a linker segment and two C2 domains. The *dry2* mutant is hypersensitive to oxidative stress and the gene altered encodes a protein involved in sterol biosynthesis. Previous analysis of Arabidopsis mutants affected in sterol composition demonstrated the importance of sterol biosynthesis in cell polarity and auxin efflux. The importance of vesicular trafficking relative to abiotic stress tolerance will be discussed.

Lipid-Mediated Signaling in Plant Stress Responses

Xuemin Wang

Department of Biology, University of Missouri, St. Louis, MO 63121; Danforth Plant Science Center, St. Louis, MO 63121, USA

Cell membranes are the initial and focal points of stimulus perception and signaling messenger production. Phosphatidic acid (PA) is the simplest membrane phospholipid and also a central intermediate for the synthesis of membrane lipids and storage lipids. The metabolism of PA occupies a key position in glycerolipid metabolism and membrane biogenesis. It is the regulatory function of PA, however, that has attracted increasing attention in recent years. PA is involved in various cellular processes, such as signal transduction, membrane trafficking, secretion, and cytoskeletal rearrangement. The effects of PA have been linked to the survival, proliferation, reproduction, and response to abiotic and biotic stresses. Signaling PA can be produced by multiple enzymes, and the activation of specific enzymes regulates the timing, location, and molecular species of PA. The modes of PA action are multifaceted and include membrane tethering, direct modulation of enzymatic activity, and effects on membrane structures and metabolism. Phospholipase D (PLD) is one super-family of enzymes that produce PA. Recent results have provided insights into the molecular mechanism by which the PLD family and PA molecular species mediate stress signaling. The functions of specific PLDs and PA have been linked to programmed cell death and plant tolerance to water, temperature, and nutrient stresses. This talk will discuss the multifaceted roles and the mechanisms of action of PLDs and PA in mediating different plant stress responses. It will also describe lipidomics approaches used to systematically understanding membrane lipid functions.

Plant Molecular Response to Al Toxicity in Acid Soil: Regulation of Organic Acid Exudation

Hideaki Matsumoto[1,*], Hong Shen[1,2], Takayuki Sasaki[1], Yoko Yamamoto[1], Mineo Yamaguchi[1,3], Hiroki Osawa[1,4], Peter R. Ryan[5], and Emmanuel Delhaize[5]

[1]*Research Institute for Bioresources, Okayama University, Kurashiki 710-0046, Japan*
[2]*College of Natural Resources and Environment, South China Agricultural University, Guangzhou 510642, China*
[3]*Div0ision of Plant Science, 1-31 Agriculture Building University of Missouri Columbia, MO 65211 USA*
[4]*Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, 113-8657 Japan*
[5]*CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia*

Acid soils occupy 30% or 3950 billion ha of the world's ice free land area and are the largest problem soils for the crop production. Thus attention has been paid to improving the crop production in acid soils. The adverse effect of acid soil on plant growth is strongly related to the toxicity of Al^{3+}. Some plants acquires Al tolerance simply by exclusion of toxic Al^{3+} by chelation with exuded organic acids from root by Al. *Malate exudation from wheat root*: Activation of malate efflux occurred in 5 min after exposure of root apex of Al tolerant wheat to Al^{3+}. The channel highly permeable to malate was demonstrated in Al-tolerant wheat by the whole patch clamp method but the gene encoding the malate transporter had not been discovered until recently. We cloned *ALMT1* gene (aluminum-activated malate transporter) expressed dominantly in the root apices of the Al-tolerant wheat line. *ALMT1* localized in the plasma membrane (PM) and constitutively expressed in the root apices of the Al-tolerant line at greater levels than in the Al-sensitive line (1). Transgenic barley expressing *ALMT1* demonstrated that *ALMT1* encoding Al-activated malate transporter is capable of conferring Al tolerance to plant cells (2).

Citrate exudation from soybean root: Al initiates citrate efflux from the soybean root apices 30min after the addition of Al. PM H^+-ATPase regulated the efflux of citrate by Al. Vanadate and fusicoccin extended inhibitory and stimulatory effects on the Al-induced efflux of citrate. Higher activity of PM H^+-ATPase coincided with more citrate efflux in Al-resistant than –sensitive soybean cultivars. The increase of PM H^+-ATPase activity by Al was caused by transcriptional and translational regulation. PM H^+-ATPase activity and expression were higher in an Al-resistant cultivar than in Al-sensitive cultivar. Al activated the

threonine-oriented phosphorylation of PM H^+-ATPase. The up-regulation of PM H^+-ATPase activity was associated with the secretion of citrate from soybean roots [(3)].

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The E3 Ubiquitin Ligase Activity of *Arabidopsis* PLANT U-BOX17 and Its Functional Tobacco Homolog ACRE276 Are Required for Cell Death and Defense

Cheng-Wei Yang[a], Rocio Gonza´ lez-Lamothe[b], Richard A. Ewan[a], Owen Rowland[b], Hirofumi Yoshioka[b], Matt Shenton[a], Heng Ye[a], Elizabeth O'Donnell[a], Jonathan D.G. Jones[b], and Ari Sadanandom[a]

[a] *Plant Science Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom*
[b] *Sainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, United Kingdom*

Previous analysis of transcriptional changes after elicitation of Cf-9 transgenic tobacco (*Nicotiana tabacum*) by Avr9 peptide revealed a rapidly upregulated gene, ACRE276. We show that ACRE276 is transiently induced in wounded leaves within 15 min, but upon Avr9 elicitor treatment, this upregulation is enhanced and maintained until cell death onset in Cf-9 tobacco. ACRE276 RNA interference (RNAi) silencing in tobacco results in loss of hypersensitive response (HR) specified by Cf resistance genes. CRE276 RNAi plants are also compromised for HR mediated by the tobacco mosaic virus defense elicitor p50. Silencing tomato (*Lycopersicon esculentum*) ACRE276 leads to breakdown of Cf-9–specified resistance against Cladosporium fulvum leaf mold. We confirmed that tobacco ACRE276 is an E3 ubiquitin ligase requiring an intact U-box domain. Bioinformatic analyses revealed *Arabidopsis thaliana* PLANT U-BOX17 (PUB17) and Brassica napus ARC1 as the closest homologs of tobacco ACRE276. Transiently expressing PUB17 in Cf-9 tobacco silenced for ACRE276 restores HR, while mutant PUB17 lacking E3 ligase activity fails to do so, demonstrating that PUB17 ligase activity is crucial for defense signaling. Arabidopsis PUB17 knockout plants are compromised in RPM1- and RPS4-mediated resistance against Pseudomonas syringae pv tomato containing avirulence genes AvrB and AvrRPS4, respectively. We identify a conserved class of U-box ARMADILLO repeat E3 ligases that are positive regulators of cell death and defense across the Solanaceae and Brassicaceae.

Cheng-Wei Yang, Rocio Gonzalez-Lamothe, Richard A. Ewan, Owen Rowland, Hirofumi Yoshioka, Heng Ye, Elizabeth O' donnell, Jonathan DG Jones, Ari Sadanandom(2006) The E3 Ubiquitin Ligase Activity of *Arabidopsis* PLANT U-BOX17 and Its Functional Tobacco Homolog ACRE276 Is Required for Cell Death and Defence. Plant Cell, 10.1105/tpc.105.039198

Plant Immunophilins Regulate ABC Transporter Activity and Cell Morphogenesis

Burkhard Schulz[1], Markus Geisler[2], Enrico Martinoia[2], Wendy Peer[1] and Angus Murphy[1]

[1] *Department of Horticulture, Purdue University, West Lafayette, IN 47907, USA*
[2] *University of Zurich, Institute of Plant Biology, CH-8008 Zurich, Switzerland*

E-mail: bschulz@purdue.edu

Polar transport of the phytohormone auxin is required for the plant polarity and coordinated development. Plant homologs of human multiple drug resistance/P-glycoproteins (MDR/PGPs) have been implicated in auxin transport, as defects in AtPGP1 and AtPGP19 result in reductions of growth and auxin transport in Arabidopsis (atpgp1, atpgp19), maize (brachytic2) and sorghum (dwarf3). AtPGP1 exhibits non-polar plasma membrane localization at the shoot and root apices, as well as polar localization above the root apex. Protoplasts from *atpgp1* leaf mesophyll cells exhibit reduced efflux of natural and synthetic auxins with reduced sensitivity to auxin efflux inhibitors such as NPA and Quercetin.

Expression of AtPGP1 in yeast and in mammalian cell expression systems results in enhanced efflux of indole-3-acetic acid (IAA) and the synthetic auxin 1-naphthalene acetic acid (1-NAA), but not the inactive auxin 2-NAA. AtPGP1-mediated efflux is again sensitive to auxin efflux and ABC transporter inhibitors.

The plasma membrane bound FKBP-like immunophilin protein TWD1 (TWISTED DWARF1/FKBP42) from Arabidopsis physically interacts with ABC transporters AtPGP1 and AtPGP19. Disruption of the TWD1 gene in Arabidopsis results in dwarfed plants exhibiting a reduction in cell elongation as well as desorientation of cell growth. This leads to strong epinasty of leaves and size reduction of organs. Biochemical analysis of resembling dwarf phenotypes of the double mutants atpgp1/atpgp19 and of the single mutant twd1 suggested a positive regulatory role of TWD1 on AtPGP-mediated auxin export activities. We verified the regulatory effect of TWD1 on PGP-mediated auxin efflux by employing plant specific as well as heterologous auxin transport systems. Using an IAA-specific microelectrode we demonstrate that IAA influx in the root elongation zone is reduced and shifted apically in *atpgp* and *twd1* mutant roots. As a consequence, *atpgp1*/*atpgp19* and *twd1* mutant roots reveal elevated levels of free IAA in the elongation zone and above. Yeast and plant transport data suggest that TWD1 defines not only transport activities but also substrate specificities.

Emerging Roles of Endocytosis in Plant Development

Akihiko Nakano[1,2], Tatsuaki Go[1,2], Wakana Uchida[2], Satoko Arakawa-Kobatashi[2] and Takashi Ueda[1]

[1]*Dept. of Biol. Sciences, Grad, School of Science, University of Tokyo, Bunkyo-ku, Tokyo 113-0033,*
[2]*RIKEN Discovery Research Institute, Wako, Saitama 351-0198, Japan*

Endocytosis plays important roles in various functions of plants, such as polar transport of auxin, establishment of cell polarity, cell plate formation during cytokinesis, and cell wall morphogenesis. To understand molecular mechanisms of endocytosis in plants, we are focusing our attention on Rab5 GTPases. In mammalian cells, Rab5 is known to organize many events relating endocytosis, such as homotypic fusion between early endosomes, alteration of lipid composition of the endosomal membrane, and signal transduction through endosomes via specific interactions with effector proteins. *Arabidopsis thaliana* contains three Rab5-related GTPases (conventional Ara7 and Rha1, and plant-unique Ara6) in the genome. These three Rab5 GTPases are localized on differentiated plant endosomes and regulate endosomal membrane fusion. We have recently identified an *Arabidopsis* Rab5-specific guanine nucleotide exchange factor (GEF), AtVps9a, and shown that this is the sole activator of the all three Rab5 members. Two tagged lines are available for the AtVPS9a locus. In the *atvps9a-1* mutant whose GEF activity is completely lost, embryogenesis is arrested at the torpedo stage. In the *atvps9a-2*, a leaky allele lacking the C-terminal regulatory domain, shoot appears to develop normally but elongation of the primary root was severely affected. These results indicate that endocytosis plays very basic roles in plant development.

Cloning and Functional Characterization of Arsenate Reductase from Rice

Gui-Lan Duan[1], Barry P Rosen[2], Yi-Ping Tong[3], and Yong-Guan Zhu[1]*

[1] *Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences;*
[2] *Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, MI 48201, USA;*
[3] *Institute of Genetics and Developmental Biology, Chinese Academy of Sciences*
**Corresponding author*

Rice (*Oryza sativa*) is the staple food for more than half the world's population. Arsenic ingestion through by rice consumption attracts more and more attention in the last few years. The genetic engineering techniques can be hopeful in improving the yield and quality of rice grown in arsenic-impacted soils.

The rice genome contains two genes with significant homology to the CDC25 from *Arabidopsis thaliana* and *ACR2* from *S. cerevisiae: OsACR2.1* (137 aa, 14,963 Da; GenBankTM accession number AY860059) and *OsACR2.2* (130 aa, 14,330 Da; GenBankTM accession number AY860058). The predicted proteins contain the $HC(X)_5R$ catalytic motif, and exhibit high sequence identity with *ScAcr2p* (21.25% and 26.24%, respectively) and AtCDC25 (50.98% and 55.48%, respectively). Both genes complemented the *E. coli* mutant strains with *ArsC* deletion and yeast mutant strains with a disrupted *ACR2* gene. Detailed molecular evidence will be presented.

Sec14p-like Phosphatidylinositol Transfer Proteins in *Arabidopsis*

Patrick Vincent and Vytas A. Bankaitis

Department of Cell & Developmental Biology, University of North Carolina, Chapel Hill, NC 27599-7090 USA

Phosphatidylinositol transfer proteins (PITPs) represent novel regulators of specific signaling interfaces between lipid metabolism and membrane trafficking. The Sec14p-like PITPs major comprise a eukaryotic protein superfamily that is highly represented in plants. A particularly interesting group of plant Sec14p-like proteins is the large and uncharacterized *Arabidopsis* Sec14p-nodulin domain family. AtSfh1p, one such Sec14p-nodulin protein, is a focus of our work. AtSfh1p is a PITP that regulates a specific stage in root hair development by integrating phosphoinositide signaling with polarized membrane trafficking in developing *Arabidopsis* root hairs. Compromise of this signaling node results in deranged polarized root hair expansion in a manner that coincides with loss of tip-directed PtdIns(4,5)P_2, dispersal of secretory vesicles from the tip cytoplasm, loss of the tip *f*-actin network, precocious Ca^{2+} entry into root hairs, and disorganization of the root hair MT cytoskeleton. We propose that Sec14p-nodulin domain proteins represent a family of regulators of polarized membrane growth in plants.

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The *Arabidosis thaliana* SNARE Protein PEN1 Localizes to Endosomes and at Sites of Polarized Endosomal Secretion in Root Cells

Boris Voigt[1,2,3], Hans Thordal-Christensen[4], Stefano Mancuso[2,3], Diedrik Menzel[1], František Baluška[1,3]

[1] *IZMB, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany*
[2] *Dept. Horticulture, University of Firenze, Viale delle idee 30, 50019 Sesto F.no (FI), Italy*
[3] *Plant Neurobiology Laboratory, Viale delle idee 30, Sesto F.no (FI), Italy; Kirschallee 1, Bonn, Germany*
[4] *Plant and Soil Science Laboratory, Dept. of Agricultural Sciences, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40,1871 Frederiksberg C, Copenhagen, Denmark*

The syntaxin SYP121/PEN1 was first isolated in a non-host penetration resistance screen in *Arabidosis thaliana*. SYP121/PEN1 shows a localization at the plasma membrane and endomembrane compartments in leaves. It is accumulating at papillae upon pathogen infection. In knock-out mutants, the papilla formation is delayed and the penetration resistance is decreased (Collins et al. 2003, Assaad et al. 2004). The syntaxin SYP121/PEN1 is well expressed in many root cells and has there probably different functions than penetration resistance. We show that it is localized, besides to the plasma membrane, also to numerous vesicular compartments of root cells. These are of endosomal nature and obviously participate in the polarized endosomal secretion in tip growing root hairs as well as in cytokinetic root cells. Both the root hair apices as well as cytokinetic cell plates are sites of the polarized endosomal secretion (Voigt et al. 2005, Ovecka et al. 2005, Dhonukshe et al. 2006, Dettmer et al. 2006). Importantly, PEN1 labelled endosomes exhibit a Ca^{2+} dependent behavior. All this suggests that the SYP121/PEN1 function in calcium triggered polarized secretion in root cells. As papilla also represents an example of polarized secretion, (Schmelzer 2002), our data suggest for the first time that the syntaxin SYP121/PEN1 is driving calcium-regulated polarized secretion based on secretory endosomes.

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Hydrogen Peroxide-Based Signals Co-Ordinated the Response to Sodium Stress in Plants

Guoyong An, Chun-Peng Song

Lab of Plant Stress Biology, College of Life Sciences, Henan University, Kaifeng 475001, China

It is now clear that hydrogen peroxide (H_2O_2) acted as the second messenger involved in abscisic acid (ABA) signaling in the regulation of stomatal behavior. H_2O_2 generation is induced in plants following exposure to a wide variety of abiotic and biotic stimuli, including salt, drought, UV irradiation and ABA treatment, etc. Roots are exposed in soil and always challenged by salt stress. It is not clear how plant root cells sense and transduce the stress signal to aerial parts of plants, e.g. stomata, to adjust stress adaptation. Here we report that H_2O_2 generation in guard cells mediates stomatal behavior to control the water loss resulting in development of salt tolerance under salt stress. We used infrared camera, laser scanning confocal microscope and patch clamp to identify the long-distance signal from root cells as component of guard cell signaling. The stomata of *Vicia faba* leaves closed when plants were treated by 300 mM NaCl in the soil for 3 hours, thereby accompanying the increase of temperature of leaf surface. Monitoring Na^+ changes in guard cells by using fluorescent probe Sodium-Green AM indicate that the concentration of Na^+ of guard cells from the plant challenged with NaCl was higher than that of control. Exogenous NaCl could induce both closure of stomata and enhancement of H_2O_2 production in guard cell. CAT and DPI partly reversed the stomatal closure induced by NaCl. The pattern of stomatal behavior in response to H_2O_2 and NaCl is similar. Whole-cell recording showed that NaCl could inhibit inward K^+ current of guard cell. These results suggested that stomata could probably act as sensor after NaCl treatment to co-ordinate whole plant to adapt to salt stress through transduction of long-distance signal.

Extracellular ATP and signalling in Arabidopsis

A. R. Slabas[1], W.J.Simon[1], K.Lindsey[1], S.Chivasa[2, 1]

1. *The Integrative Cell Biology Laboratory, School of Biological and Biomedical Sciences, University of Durham, South Rd, Durham DH1 3LE UK.*
2. *Creative Gene Technology, School of Biological and Biomedical Sciences, University of Durham, South Rd, Durham DH1 3LE UK.*

Following a proteomic investigation of the extracellular matrix of Arabidopsis, we observed that a number of proteins contained potential phosphorylation domains [1]. This prompted us to investigate if extracellular ATP existed in plants and if so, what the function of the ATP was. Using ^{32}P-phosphate labelling of cell cultures, we have demonstrated that extracellular ATP exists. We have used ATP depletion systems to observe the physiological effects of extracellular ATP removal. Removal of ATP results in cell death – this is a phenomenon which is not only observed in suspension cultures, but also in whole plants. Addition of non-hydrolysable ATP also results in cell death –indicating that extracellular ATP is essential for cell viability.

Fumonisin B1 [FB1] triggers depletion of extracellular ATP, which precedes cell death. Addition of exogenous ATP rescues the cells from FB1-mediated cell death, indicating that extracellular ATP may be an important component of sensing pathogen attack [2]. We are currently using both transcriptomic and proteomic approaches to try and identify the candidate genes in this cell death pathway.

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Apoplastic Calmodulin: Existence, Functions, and Transmembrane Mechanism

Daye Sun, Ying Sun

Institute of Molecular Cell Biology, Hebei Normal University, Shijiazhuang 050016

Calmodulin (CaM), traditionally a well known intracellular calcium sensor that mediates many signaling pathway, has been found in the apoplast of many species of plants, and functions of apoplastic CaM in accelerating cell proliferation, protoplast cell wall regeneration, and in promoting pollen germination and tube growth, have also been described physiologically in our previous studies.

To provide further genetic evidence for the existence and functions of apoplastic CaM to support our hypothesis that apoplastic CaM might be used as a polypeptide signal in plant development, we first verified cell wall localization of CaM in living cells by visualizing fluorescence of GFP fusion protein with soybean CaM(SCAM), and interestingly found that highly conserved isoform SCaM-1, -2, -3, but not divergent isoform SCaM-4,-5, all of which are stably expressed in tobacco cells are preferably secreted. Next we confirmed the promotion effect of apoplastic CaM on pollen germination and tube growth by phenotypic analyzing *Arabidopsis* wild type pollen, transgenic pollen over-expressing apoplastic AtCaM-2, a conserved isoform in *Arabidopsis*, and transgenic pollen with apoplastic CaM attenuated by over-expressing apoplastic CaM-binding peptide.

To understanding transmembrane mechanism of apoplastic CaM function, we did radio-ligand binding assay with 35S-labeled AtCaM-2, and disclosed the specific, reversible, and saturable calmodulin binding sites on the surface of both A. thaliana suspension-cultured cells and its protoplasts. Chemical crosslinking of 35S-labeled AtCaM-2 further revealed 117- and 41-kDa plasma membrane proteins, which might be promising candidates for receptor-like protein specifically bound to apoplastic CaM. Besides, phospholipase C activity, cytosolic Ca2+ concentration were also found to be increased specifically in response to exogenous CaM when applied outside of pollen protoplast. Furthermore, patch clump experiment showed that heterotrimeric Gα protein regulated pollen plasma membrane Ca2+ channel is involved in mediating apoplastic CaM signal. All our findings suggest that apoplastic CaM may be a polypeptide signal in plants development, and its signaling pathway is different from that of intracellular CaM.

Redox-Responsive Calcium Signaling in Plants

Tomonori Kawano[1], Takuya Furuichi[2], and Takashi Kadono[1]

[1] *Graduate School of Environmental Engineering, The University of Kitakyushu, Kitakyushu 808-0135, Japan*
[2] *Graduate School of Medicine, Nagoya University, 65 Tsurumai, Nagoya 466-8550, Japan*

Calcium ion (Ca^{2+}), one of the major necessary elements in living cells, plays an essential role as an intracellular secondary messenger in plant cells. When plant cells are exposed to environmental stresses or perceive signaling molecules, calcium channels can be transiently activated to convert these stimuli into intracellular events. In the present talk, our recent studies on the redox-responsive calcium signaling mechanism in tobacco (*Nicotiana tabacum* L.) cells will be described.

Three cell lines of tobacco cell suspension cultures expressing aequorin genes were used to study the changes in cytosolic calcium concentration ($[Ca^{2+}]_c$) in responses to extracellular redox changes. The $[Ca^{2+}]_c$ elevation-stimulating oxidative stimuli related to reactive oxygen species (ROS) studied here include hydrogen peroxide, salicylic acid, aluminum ion and ozone. Salicylic acid and aluminum added to the cells rapidly stimulate the generation of superoxide anion via peroxidase- and NADPH oxidase-dependent mechanisms, respectively, and in turn superoxide induces the influx of Ca^{2+} into the cells. In tobacco cell suspension cultures, exposure to ozonized air can result in generation of a variety of ROS such as singlet oxygen, hydrogen peroxide and hydroxyl radicals and these ROS members eventually stimulate the influx of Ca^{2+} into the cells. When the entry of Ca^{2+} into the cells were prevented by Ca^{2+} chelators such as BAPTA and EGTA, the ozone-induced cell death was significantly prevented, supporting our view that Ca^{2+} plays a key role in oxidative cell death. Our data suggested that the recently characterized TPC1-type Ca^{2+}-permeable channels behave as the key oxidative stress-responsive Ca^{2+}-permeable channels involved in transient increase in $[Ca^{2+}]_c$.

In addition to oxidative stresses, effect of reducing environments in the extracellular space on $[Ca^{2+}]_c$ was tested by adding some low molecule thiols such as cysteine, *N*-acetylcysteine, glutathione, dithiothreitol and 2-mercaptoethanol. Similarly to the responses to oxidative stresses, tobacco cells responded to extracellularly added thiols by rapidly elevating $[Ca^{2+}]_c$. Such thiol-induced increases in $[Ca^{2+}]_c$ were shown to be sensitive to Ca^{2+} chelators and Ca^{2+} channel blockers thus suggesting the involvement of Ca^{2+} channels as the targets of extracellular thiols. We are presently trying to propose a conceivable model that explains the plant calcium responses to both oxidative and reductive environments surrounding the plant cells.

Histone Modification: A Link Between Signals and Programs

Shunong Bai

College of Life Sciences, Peking University, Beijing, 100871

It is widely accepted that plant development is heavily affected by environmental signals. During previous two decades, through analyzing Arabidopsis mutants, people were enabled to dissect the developmental events those are responded to environmental signals. It is interesting that the investigations were concentrated at the two ends of an integrated process: the dissection of the signaling system at the one end, centered with the signaling receptors; and the identification of the components involved in the developmental program at the other. Taking the cellular patterning of root epidermis as an example, genes such as CPC, GL2 and WER have been isolated and identified to play roles in determining the patterning process. The highly positional dependence to the cortical cells and the identification of SCM gene suggested a "positional cue" is involved in directing the patterning. But little is known how the signals linked with the patterning genes. Such a situation is not a solo case. How to link signals and programs in developmental events is an obvious challenge to our understanding of regulatory mechanism in plant development. Our work on histone acetylation affecting the cellular patterning of root epidermis suggested that it is possible to approach the link not from the both ends inward, but from the center outward by deciphering the mechanism of the chromatin regulation of gene expression.

Signalling to the Actin Cytoskeleton

Patrick. J. Hussey

The Integrative Cell Biology Laboratory, School of Biological and Biomedical Sciences, University of Durham, South Rd., Durham DH1 3LE, UK

One of the most intriguing issues in plant cytoskeleton biology is that whereas the fundamental components of the cytoskeleton appear similar to their animal counterparts (actin and microtubules) the actin regulatory proteins in particular are utilised (e.g. SCAR/WAVE **)** and regulated (e.g ADF) differently. This is perhaps not surprising because the modes of development in plants and in animals are different. For example embryogenesis in animals is dependent on cell migrations whereas plant cells cannot move. In plants the simple body plan is established during embryo development but most organ development is established after embryogenesis and after seed germination. Plant cells have to respond to different environmental cues in order to maximise energy production, to take up nutrients from the soil, to reproduce and to protect from pathogen invasion. In all these cases the cytoskeleton has to respond to signals and reorganise to generate organelle movement and cell expansion, polarise cell growth and thicken the cell wall. My lab is involved in elucidating the plant signalling pathways that control actin reorganisations that govern plant cell morphogenesis and this presentation will give an overview of the key stimulus responsive molecules of the actin cytoskeleton.

How to Stop a Pollen Tube? Signaling to the Targets of Self-incompatibility in *Papaver* Pollen

Steven G. Thomas, Shutian Li, Shanjin Huang[1], Christopher J. Staiger[1] and Vernonica Franklin-Tong

School of Biosciences, University of Birmingham, Edgbaston, Birmingham. B15 2TT, UK [1]Dept. Biological Sciences & The Bindley Bioscience Center, Purdue University, West Lafayette IN 47907-1392, USA

Sexual reproduction in higher plants involves interactions between pollen and pistil. Self incompatibility (SI) prevents self-fertilization and is an important mechanism for promoting outbreeding. SI is controlled by the *S*-locus; discrimination occurs between incompatible ("self") pollen, which is rejected, while compatible ("non-self") pollen can achieve fertilization. In *Papaver rhoeas,* S proteins encoded by the pistil part of the *S*-locus interact with incompatible pollen. The "self" SI interaction triggers a Ca^{2+}-dependent signalling cascade, resulting in rapid depolymerization of the actin cytoskeleton and inhibition tip growth in incompatible pollen. A MAP kinase, p56, is also activated specifically in incompatible pollen. We recently showed that programmed cell death (PCD) involving a caspase-3-like activity is triggered by SI. This provides a precise mechanism for the specific destruction of "self" pollen.

We are currently investigating a possible role for actin depolymerization in signaling to PCD and also whether the p56-MAPK might signal to PCD. Recent data providing evidence for their involvement in SI-mediated PCD will be discussed.

As the SI-induced F-actin depolymerization was far in excess of that required to inhibit growth, this suggested an additional function. We have used drugs that affect actin dynamics, latrunculin B and jasplakinolide, to explore a possible role for actin depolymerization in signaling to PCD. Our data show that alterations to actin dynamics play a functional role in the early stages of the PCD signaling cascade in *Papaver* pollen. Furthermore, a significant alleviation of SI-induced PCD in incompatible pollen was achieved by adding jasplakinolide, showing that actin depolymerization plays a functional role in SI-induced PCD. We believe this represents the first demonstration of signaling between the actin cytoskeleton and PCD in plants.

Because MAPKs are known to play a role in PCD in both animal and plant cells, we have investigated a possible link between the p56-MAPK and PCD triggered by SI. Using the MAPK inhibitor, U0126, we have obtained evidence that this blocks both p56-MAPK activation and DNA fragmentation induced by SI. This provides the first evidence that the p56-MAPK is involved in signaling to PCD in the SI response.

The Cytoskeleton and Intercellular Communication

Christine Faulkner[1,2], Leila Blackman[3], David Collings[3], Robyn Overall[1]

[1]*School of Biological Sciences, University of Sydney, NSW, Australia*
[2]*Present address: Institute of Molecular Plant Sciences, University of Edinburgh, Scotland*
[3]*Plant Cell Biology Group, Research School of Biological Sciences, Australian National University, ACT, Australia*

As conduits for electrical signals, developmental messages, water and metabolites between cells, plasmodesmata are central in the function and development of plants as well as the integration of their responses to environmental signals. In plant cells, the cytoskeleton has a myriad of functions from orchestrating cytokinesis and directing cellulose deposition through to driving cytoplasmic streaming and vesicle and organelle motility. The identification of the cytoskeletal components actin (White et al, 1994, Blackman and Overall, 1998), myosin (e.g. Blackman and Overall, 1998, Reichelt et al, 1999), centrin (Blackman et al, 1998) and Arp3 (Van Gestel et al, 2003) in plasmodesmata suggests that the cytoskeleton also plays a role in the trafficking to and through plasmodesmata. Tropomyosin is an actin-binding protein thought to be involved in a range of functions associated with the actin cytoskeleton, including the regulation of myosin binding to actin filaments, but to date no tropomyosin-like proteins have been conclusively identified in plant genomes. Anti-tropomyosin antibodies localised to plasmodesmata in the green alga *Chara corallina*. These antibodies also localised to other structures including actin cables. Anti-tropomyosin antibodies labelled plasmodesmata of *Arabidopsis thaliana* and leek tissue. Western blot analysis identified a 75kDa and a 55kDa protein in *Chara* protein extracts, a single protein at 42.5 kDa in *A. thaliana* extracts and two proteins at 58.5 and 54 kDa in leek extracts. The 75 kDa protein from *Chara* was present in protein extracts from cell walls containing plasmodesmata (nodal complex walls) and was absent from the external cell walls of internodes that do not contain plasmodesmata, again suggesting that a tropomyosin-like protein is associated with plasmodesmata**.**

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Cloning and Characterization of a Ca^{2+}-dependent Actin-binding Protein from Lily Pollen

Yun Xiang and Haiyun Ren

Key Laboratory of Cell Proliferation and Regulation Biology of Ministry of Education, and College of Life Science, Beijing Normal University, Beijing 100875, People's Republic of China

As the only known Ca^{2+}-dependent actin binding proteins, villin/gelsolin/fragmin superfamily proteins play important roles in pollen germination and the tube growth. However, bioinformatic analysis shows that separate genes for gelsolin/severin do not exist in *Arabidopsis* and rice (*Oryza sativa* L.), and it is generally accepted that these proteins might be encoded by mRNA splicing variants of villins. Here we succeeded in cloning an identical full-length cDNA of 989 bp from two different cDNA libraries of lily pollen by PCR, respectively. It encoded a predicted protein of 263 amino acids that shared 100% identity to N-terminus of P-135-ABP (a lily villin) except for the 6 amino acids in C-terminus. In addition, the entire 3'UTR of the cDNA was totally different from that of cDNA encoding P-135-ABP. The deduced LdABP29 contained G1, G2 and part of G3 domains. Biochemical analysis showed that the purified recombinant LdABP29 could accelerate actin nucleation, block barbed ends and sever actin filaments in a Ca^{2+}-dependent manner *in vitro*. Over expression of LdABP29 in tobacco BY-2 cells resulted in fragmentation of actin filaments. These results suggest that there exists a separate gene that encodes the small molecular mass protein of villin/gelsolin/fragmin superfamily in plants and LdABP29 is a new member of the superfamily, which may participate in regulating the organization of actin cytoskeleton in living plant cells.

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Plant Prevacuolar Compartments: Secretory Trafficking and Endocytosis

Sheung Kwan Lam, Yu Chung Tse, Louse Lo, Junqi Wang, Hong-Ye Li, Yansong Miao and Liwen Jiang

Department of Biology and Molecular Biotechnology Program, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

Prevacuolar compartments (PVCs) are membrane-bound organelles that mediate protein trafficking in the secretory and endocytic pathways. We have previously demonstrated that vacuolar sorting receptor (VSR) proteins concentrated on PVCs [1] and VSR-marked PVCs in tobacco BY-2 cells were multivesicular bodies (MVBs) [2], which might also involve in receptor-mediated endocytosis because MVBs colocalized with internalized endosomal marker FM4-64 [2-4]. Using transgenic BY-2 cells expressing a GFP fusion involving endocytosis, we have also identified early endosomal compartment in BY-2 cells [5]. To further study PVC biogenesis and PVC-mediated protein traffic, we have purified PVCs from both tobacco BY-2 cells and *Arabidopsis* cells for proteomics analysis via both 2D gel MS/MS and 1D gel LC-MS/MS [6]. Current studies focus on characterization of newly identified PVC proteins for their subcellular localization and functional roles. Supported by grants from the Research Grants Council of Hong Kong to L Jiang.

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An Important Pool of Sucrose Linked to Starch Biosynthesis is Taken up by Endocytosis in Heterotrophic Cells

Edurne Baroja-Fernandez[1], Ed Etxeberria[2], Francisco José Muñoz[1], María Teresa Morán-Zorzano[1], Nora Alonso-Casajús[1], Pedro Gonzalez[2] and Javier Pozueta-Romero[1]

[1]*Agrobioteknologia Instituta, Nafarroako Unibertsitate Publikoa, Gobierno de Navarra and Consejo Superior de Investigaciones Científicas, Mutiloako etorbidea zenbaki gabe, 31192 Mutiloabeti, Nafarroa, Spain;*
[2]*University of Florida, IFAS, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL, 33850, USA.*

We have recently shown the occurrence of endocytic sucrose uptake in heterotrophic cells (Etxeberria et al. Plant Cell Physiol. 46, 474-481). Whether this mechanism is involved in the sucrose-starch conversion process was investigated by comparing the rates of starch accumulation in sycamore cells cultured in the presence or absence of the endocytic inhibitors wortmannin and LY294002. These analyses revealed a two-phase process involving an initial 120 min wortmannin- and LY294002- insensitive starch accumulation period, followed by a prolonged phase that was arrested by the endocytic inhibitors. Both wortmannin and LY294002 led to a strong reduction of the intracellular levels of both sucrose and the starch precursor molecule, the ADPglucose. No changes in maximum catalytic activities of enzymes closely linked to starch and sucrose metabolism occurred in cells cultured with endocytic inhibitors. In addition, starch accumulation was unaffected by endocytic inhibitors when cells were cultured with glucose. These results provide a first indication that an important pool of sucrose incorporated into the cell is taken up by endocytosis prior to its subsequent conversion into starch in heterotrophic cells. This conclusion was further substantiated by experiments showing that sucrose-starch conversion was strongly prevented by both wortmannin and LY294002 in both potato tuber discs and developing barley endosperms.

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Multivesicular Compartments "in Action" in Response to Infection by the Barley Powdery Mildew Fungus

Qianli An[1*], Ralph Hückelhoven[2], Katrin Ehlers[1], Karl-Heinz Kogel[2], and Aart J. E. van Bel[1]

[1]*Institute of General Botany, Justus-Liebig-University Giessen, Senckenbergstrasse 17, D-35390 Giessen, Germany*
[2]*Institute of Phytopathology and Applied Zoology, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany*

The recent finding that *Arabidopsis* and barley syntaxins known to mediate membrane fusion are required for the papilla-associated penetration resistance to powdery mildew fungi highlights the function of vesicle trafficking in plant–pathogen interactions (Assaad et al., 2004; Collins et al., 2003). Plant resistance to powdery mildew fungi is performed by arrest of fungal penetration attempts through polarized secretion of defense materials and deposition of papillae, by restriction of fungal infection through resistance-gene-mediated hypersensitive cell death response, or a combination of both depending on the host-fungus combination.

In barley leaves, light-microscopically visible vesicle-like bodies intensively stained by H_2O_2-reactive dyes frequently accumulate around papillae, in which the penetration attempt of barley powdery mildew fungi is halted. By using transmission electron microscopy in combination with cytochemical localization of H_2O_2, we demonstrated that the conspicuous H_2O_2-containing vesicle-like bodies were actually small papillae instead of cytoplasmic vesicles and that large multivesicular compartments including multivesicular bodies and paramural bodies, some of which contained H_2O_2, proliferated near papillae. Moreover, we observed intravacuolar multivesicular bodies with double limiting membranes, of which the outer one was seemingly derived from the tonoplast, and intravacuolar vesicle aggregates, which might result from a degradation of the limiting membranes of the intravacuolar multivesicular bodies (An et al., 2006). These vesicular structures also proliferated at the periphery of the intact cells neighboring the hypersensitive dying cells in the resistant *MLA12*-barley. All plasmodesmata between intact cells and hypersensitive cells were constricted or blocked by cell wall appositions frequently associated with paramural bodies.

Together, multivesicular bodies seemingly followed two distinct pathways: either they fused with the plasma membrane to release their internal vesicles into the paramural space or they were engulfed by the tonoplast for degradation in the vacuole. They appeared to secret building blocks for deposition of cell wall appositions to

prevent the fungal penetration and to contain the hypersensitive cell death. They may be also involved in internalization of deleterious materials, damaged membranes, elicitors, and elicitor receptors.

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Signal and Assimilate Phloem Translocation: Understand the Controlling Mechanisms of Nutrient Supply and Seed Development in the Economically Important Crops

Hong Li Wang

Department of Biology, University of Arkansas, Little Rock, Arkansas 72204, USA

It would greatly benefit our economics and society if we can develop crops that have higher biomass production which can be used for fuels and fibers; and high yield of grains with better nutritional quality for human and animal feeds. Biomass production of crop is mainly determined by plant's ability to produce high levels of assimilates over a wide range of environmental conditions, while the crop yield is mainly depending on its capacity to efficiently transport and accumulate a high proportional assimilates into the harvesting organs such as seeds and grains. Phloem translocation of signals and assimilates plays significant roles on controlling of nutrient supply and seed development of economically important crops such as wheat. This work presents our long-term research on the elucidation of the cellular pathways of assimilate transport in the developing wheat grain, and our understanding on the physiological, cellular and molecular bases of phloem translocation in crop plants, especially their underlying controlling and regulatory mechanisms. Our work revealed that the transfer cells which are differentiated from the nucellar projection and the modified aleurone cells in the wheat grains enhance the preferential accumulation of assimilates into the endosperm tissues. Moreover, this mechanism can also be applied to improve nutrient assimilation (mineral nutrient acquisition and biomass production) in the source tissues. Our findings provide novel insights into the manipulation of the genes involved in the controlling points, for the breeding of ideal crops, the optimization of cropping systems and the improvement of agricultural management.

Keywords: Signals, assimilates, phloem translocation, controlling mechanisms, seed development; crop yield, nutritional quality, transfer cells, wheat.

Electrophysiological Studies on Mechano-perception and Wounding Response in Green Axon, Characean Cells

Teruo Shimmen

Department of Life Science, Graduate School of Life Science, University of Hyogo, Harima Science Park City, Hyogo 678-1297, Japan

Mechanical and wounding stresses are serious problem for plants. It is suggested that the plasma membrane first perceives such stresses and the electrical signal plays pivotal roles. In higher plants composed of complex tissues, it is hard to monitor signals from target cell. On the other hand, it is easy in Characeae because of its simple morphology. In addition, large cell size makes the electrical measurement easy and simple.

Mechano-sensing: By dropping a glass rod on a internodal cell of *Chara corallina*, receptor potential was induced. The intensity of the stimuli can be simply controlled by either the weight of the glass rod or height from which the glass rod is dropped. Systematic analysis indicated that activation of chloride channel is involved in generation of the receptor potential. In addition, involvement of calcium channel was also indicated.

Wounding response: When plants were suffered from wounding, the initial signal should be received by a cell neighboring to the killed cell. In higher plants, it is very hard to selectively record the electrical response of these cells. I prepared a specimen comprising two adjoining internodal cells. One internodal cell (victim cell) was killed by cutting and change of the membrane potential in another internodal cell (receptor cell) was analyzed. Upon cutting a victim cell, a receptor cell generated four kinds of depolarizing response; (1) rapid depolarization, (2) long-lasting depolarization, (3) action potentials and (4) small spikes. Both rapid and long-lasting depolarizations are generated at the distal end and action potential was at the flank region of the cell. At the peak of the long-lasting depolarization, an action potential was induced and transmitted along the internodal cell. Thus, the wounding signal is perceived at the nodal end and the long-lasting depolarization is a kind of receptor potential. At the peak of the long-lasting depolarization, action potentials were induced and transmitted along the cell. The role of K^+ released from the victim cell in induction of long-lasting depolarization was indicated. Characeae can be an ideal material for analysis of electrical signals in stress response.

Brassinosteroids and ABA Regulate Plasma Membrane Anion Channels in Addition to Proton Pumps in *Arabidopsis Thaliana* Cells

Jean-Pierre Rona, Mathias Brault, AM Pennarun, Zahia Amiar, Michèle Monestiez, Bernadette Biligui, Karine Madiona and Zongshen Zhang

Electrophysiologie des Membranes, EA 3514, Université Paris VII, 2 place Jussieu, 75005 Paris, France

The plant growth regulators 28-homobrassinolide (HBL) and abscisic acid (ABA) play key roles in the control of plant development and cell volume by regulating ion channel activities and water exchanges across the plasma membrane (PM).

In *Arabidopsis thaliana* suspension cells, our results clearly show that both HBL and ABA had opposite effect on the modulation of the proton pump and anion channel activity. These modulations were associated with the control of the PM electrical gradient magnitude involved in phytohormones signaling pathways.

Using experiments employing combined voltage clamping and continuous measurement of extracellular pH during PM phytohormone signalings on cells where physiological wall functions are maintained, we demonstrate that HBL induced both medium acidification (ΔpH $\approx$ 0.45 units in less than 10 min) and PM hyperpolarization (ΔEm $\approx$ -12 mV), whereas ABA simultaneously induced rapid alkalinization of the medium (ΔpH $\approx$ 0.06 units) and PM depolarization (ΔEm $\approx$ 6 mV). These data revealed that the PM H^+-ATPase is activated by HBL (Zhang et al. 2005), but inhibited by ABA (Brault et al. 2004) in *A. thaliana* suspension cells. Upon ABA treatment, we observed an increase in the anion current (anion efflux) in suspension cells (ΔI $\approx$ 62%). This increase is abolished by a subsequent addition of the anion channel inhibitor 9-AC (ΔI $\approx$ 17%) or strongly reduced when ABA was added in the presence of 9-AC. In opposite manner, we observed HBL treatment decrease anion current in suspension cells (ΔI $\approx$ 70%) during the PM hyperpolarization. Therefore, anion channels may also be good candidates, in addition to proton pumps for the controls of PM potential during the responses to phytohormones.

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On the Track of Ca^{2+} Permeable Channels

Karina Schulz, Bernd Mueller-Roeber, Barbara Koehler

University of Potsdam, Institute for Biochemistry and Biology, Molecular Biology, Karl-Liebknecht-Str. 25, H. 20, 14476 Golm, Germany

After the complete sequencing of the *Arabidopsis* genome the functional characterisation of unknown membrane proteins and to find out their physiological roles in the plant is one of the major challenges to date. We focus on the identification of putative plant ion channels involved in Ca^{2+}-based signalling processes. Beyond dispute, Ca^{2+} is an important, ubiquitous second messenger during development and in responses to various stresses. Although candidate genes like cyclic-nucleotide activated channels and glutamate receptors are possible pathways for Ca^{2+} entry (Demidchick *et al.*, 2002), the direct link to plant Ca^{2+} channels like they have been identified on the electrophysiological level (e.g. Hamilton *et al.*, 2000; Köhler and Blatt, 2002; Dreyer *et al.*, 2004) is still lacking. Based on sequence similarity to the conserved pore region of animal calcium channels and on the presence of at least six transmembrane spanning domains candidate genes from several multigene families were cloned. All of the candidate genes are of unknown function and do not belong to the cyclic-nucleotide activated channels or glutamate receptors. Candidate genes were investigated with respect to permeability for Ca^{2+} and monovalent cations (K^+, Na^+) in heterologous expression systems. *Xenopus* oocytes and yeast stably expressing reconstituted aequorin were used. Five of the candidate genes representing five multigene families with 2 to 13 members affected the intracellular Ca^{2+} homeostasis of yeast. These were investigated further. The knowledge of the subcellular distribution and the expression pattern within the plant is crucial for developing approaches to assign functions. Therefore, GFP-fusion proteins were transiently expressed in tobacco BY2 protoplasts to determine the intracellular localisation. Two of the candidate genes were located in the plasma membrane, two were located in endomembranes, and one was located in mitochondria. Promoter-GUS studies of the candidate genes were done end partly extended to the whole gene family. Some of the genes were expressed throughout the plant. Others showed a very specific expression pattern. Available information from databases was compiled. A portrait comprising current knowledge of the selected gene families will be presented.

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Mechano-perception: Thermodynamics of Mechanosensitive Ion Channels

V. S. Markin, M.I.Volkova-Gugeshashvili, A.G.Volkov.

Department of Chemistry, Oakwood College, 7000 Adventist Blvd. Huntsville, Alabama 35896 USA

Plants respond to external stimuli. This includes mechanical response of plants to mechanical stimuli that occurs via mechanosensory ion channels (MSC). These channels are activated by mechanical stress and then transduce this information into electrical signals. MS channels are involved in the growth, development and response to environmental stress in higher plants. Detailed analyses of the electrophysiology in higher plants are difficult because such plants are composed of complex tissues. The large cells of the charophytes facilitate electrophysiological measurements and allow studying MS ion channels at the level of single cells. This showed that their functioning is based on the same principles as much better studied MS channel in bacterial and animal cells, which are immersed into lipid matrix and are transducing mechanical stress in the membrane.

Mechanosensitivity of ion channels are conventionally interpreted as being driven by a change of in-plane area A_{msc} of mechanosensitive comples. This, however, does not include any factors relating to membrane thickness, spontaneous curvature or changes in channel shape, length or stiffness. Since the open probability of a channel is sensitive to all these factors, we constructed a general thermodynamic formalism. Corresponding equations comprise the basis for the analysis of the behavior of mechanosensitive channels in lipids of different geometric and chemical properties ─ properties such as the hydrophobic mismatch at the boundary between the protein and lipid, and effects of changes in the bilayer intrinsic curvature caused by the adsorption of amphipaths. This model predicts that the mid-point $\gamma_{1/2}$ and the $slope_{1/2}$ of the gating curve are not generally independent. Using this relationship, we predicted the line tension at the channel/lipid border of MscL as ~ 10 pN, much less than the line tension of aqueous pores in pure lipid membranes. The channel appears quite well matched to its lipid environment. Using gramicidin as a model system, we have explained its observed conversion from stretch-activated to stretch-inactivated gating as a function of bilayer thickness and composition.

We have also identified two types of shape sensitive mechanotransduction: one-sided and two-sided shape activation. These effects are particularly relevant to the activity of amphipaths and to curvature sensitive channels.

Electrophysiology of mechanosensitive responses of Venus flytrap and Mimosa are analyzed using ultra fast data acquisition PXI system from National Instruments. Touching of mechanosensitive hairs of Venus flytrap induced action potentials between lobes and midrib with duration about 1 ms and speed of propagation about 10 m/s.

Properties and Physiological Roles of ROS-activated Cation Channels in Higher Plants

Vadim Demidchik

Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, CO43SQ, United Kingdom

Reactive Oxygen Species (ROS) are involved in such important physiological phenomena in plants as stress responses, growth and development, regulation of stomatal aperture, gravitropism and others. One of mechanisms of ROS action on plant physiological processes is through their effect on plasma membrane cation channels – proteins catalyzing an exchange of cations between the cell and the environment (Demidchik et al., 2002, Annu Rev Plant Biol 53: 67). Here, I present data obtained in collaboration with several laboratories (Cambridge, Plymouth and John Innes Centre) on properties and physiological roles of Ca^{2+}- and K^+-permeable cation channels in *Arabidopsis thaliana* root cells. We showed that application of ROS (hydroxyl radicals) at the extracellular side of the plasma membrane in whole-cell patches caused an activation of inwardly rectifying Ca^{2+} conductance and outwardly rectifying K^+ conductance (Demidchik et al., 2003, J Cell Sci 116: 81). ROS-induced conductances in elongation zone and root hairs were 2-3 times larger than in mature epidermis and 10-20 times larger than in pericycle. This suggested an involvement of ROS-activated cation channels of root epidermal cells to the perception of external stimuli, growth and development. Pharmacological analyses showed that ROS-activated Ca^{2+} conductance was mediated by a new group of Ca^{2+}-permeable nonselective cation channels having different properties than nonselective cation channels involved in toxic Na^+ influx (Demidchik & Tester, 2002; Plant Physiol 128: 379). ROS-activated K^+ conductance was mediated by outwardly rectifying K^+ channels because it was blocked by TEA^+ and showed high selectivity to K^+ over other the range of monovalent cations. Single-channel studies in outside-out patches demonstrated an activation of hyperpolarisation-activated 14.5-pS channel (20 mM $CaCl_2$ in the bathing solution) in response to H_2O_2 applied at the intracellular side of the plasma membrane. Properties of this ROS-activated single channel resembled properties of constitutive hyperpolarisation-activated Ca^{2+} channels. Single-channel analyses of K^+ outward conductances are in progress. Using MIFE® technique we have found that H_2O_2 and hydroxyl radicals activate significant transient Ca^{2+} influx of and K^+ efflux in intact roots. In elongation zone ROS-activated cation fluxes were larger than in mature epidermis. Experiments with

Ca^{2+}-aequorin luminometry showed that extracellular ROS accumulation is induced by abiotic and biotic stresses and that this accumulation is accompanied by the activation Ca^{2+} influx through ROS-activated cation channels. Using K^+ photometry we have found that oxidative stress-induced K^+ release from plant tissues is mediated by ROS-activated K^+ channels. A critical role of ROS-activated Ca^{2+} channels in plant elongation growth was demonstrated in experiments with *Arabidopsis* root hair deficient mutant (*rhd2*) (Foreman et al., 2003, Nature 422: 442). We have shown that impaired HADPH oxidase activity results in decreased production of extracellular ROS that leads to decreased Ca^{2+} influx through ROS-activated cation channels and stops Ca^{2+}-dependent elongation of root cells (root hairs and cells of elongation zone). In conclusion, our data show that ROS-activated cation channels are critical systems for sensing environmental stresses and regulation of plant elongation growth.

Myotubularins: Novel Members of Phosphoinositide-Based Signalling Pathways in Plants

Andrej Hlavacka[1,2], Dieter Volkmann[1], Diedrik Menzel[1], Zoya Avramova[3], František Baluška[1]

[1] *IZMB, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany*
[2] *LINV, Dip. Horticulture, Viale delle idee 30, 50019 Sesto F.no (FI), Italy*
[3] *School of Biological Sciences, University of Nebraska, Lincoln, NE 68588-0118, USA*

Myotubularins are dual-specificity phosphatases that use PI(3,5)P_2 or PI(3)P as substrate and convert it into PI(5)P. They have been first identified in humans where the mutation of hMTM1 (human myotubularin 1) causes Charcot-Marie-Tooth syndrome that results into the muscular myopathy disease. By screening the *Arabidopsis* genome, we have identified 2 myotubularin homologues – AtF18K10 and AtT32M21. Based on bioinformatical studies, we have found, that both of these proteins exhibit similar structure and properties comparing to human ones. Despite the high similarities on the protein level and in the protein structure, these genes have evolved in the different way than the ones from animals and humans. They form a separate clade on the phylogenetical tree and they might also have a different roles. Plant myotubularins posses a consensus active site Cx_5R within the PTP (Protein Tyrosine Phosphatase) domain and a myotubularin-related domain. In the case of AtT32M21, there is also a PH-GRAM domain (domain found in glucosyltransferases, myotubularins and other putative membrane-associated proteins). We have also found a SET-interacting domain (SID) overlaping with PTP domain. This domain is interacting with proteins carrying well conserved SET (SuVar (3–9)-E(z)-trithorax) domain. Recently, we have reported (Alvarez-Venegas et al. 2006) that *Arabidopsis* trithorax homologue 1 (ATX1), belonging to the *Trithorax* gene family, contains SET domain. Moreover, we have also found that ATX1 binds specifically PI(5)P and is necessary for the proper plant development and stress response. *Arabidopsis* myotubularins could form a complex with ATX1 and provide a substrate for this protein. Therefore, through balancing the level of phosphoinositides, there might regulate the development of the plant body and stress response.

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Transition Zone in the Root Apex: Oxygen Influx and Nitric Oxide Production During Temporary Changes in Gravity Conditions

S. Mancuso[1], S. Mugnai[1], E. Azzarello[1], C.Pandolfi[1], A. Hlavacka[1,2], B.Voigt[1,2], F. Baluska[1,2], D. Volkmann[2]

[1]*LINV (Laboratorio Internazionale Neurobiologia Vegetale), Department of Horticulture, University of Florence, Italy,*
[2]*Institut für Zelluläre und Molekulare Botanik, University of Bonn, Germany*
Email: Stefano.mancuso@unifi.it

Oxygen influx changed in the transition zone after varying root position from the vertical to the horizontal on ground, showing a gravity-regulated asymmetry. Specifically, 18 ± 2 sec after changing the root position oxygen influx increased only on the upper root side of the transition zone, remaining stable on the lower one. Considering that the tilting procedure took around 15 s, the first O_2 signal can be hypothesized to appear just few seconds after gravistimulation. This rapid change in the oxygen flux into root apices is by far the fastest ever reported plant response to gravity. In order to study this phenomenon in a real microgravity condition, an experiment has been set up on a parabolic flight. Oxygen and nitric oxide flushes have been monitored during normal, hyper- and microgravity conditions in roots of *Zea mays* seedlings. During parabolic flight a clear and distinct signal in oxygen and NO fluxes has been detected only in the apex zone, starting just 2.0 ± 0.5 s after the imposition of microgravity conditions. No significant changes have been noticed neither in normal nor in hypergravity conditions. The significance of these results on the nature of the graviperception will be discussed.

Herbivore-Induced Volatiles in Plant Defense: Early and Late Events in Enemy-recognition and Response

Wilhelm Boland, Axel Mithöfer, Gen-Ichiro Arimura, Heiko Vogel, Jürgen Kroymann

Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, Germany
Email: Boland@ice.mpg.de

Herbivore feeding elicits defence responses in the infested plants, typically the emission of a blend of volatile organic compounds (VOCs) that mediates interactions with the parasites or enemies of the herbivore. To study the impact of individual factors which may contribute to the stimulation of volatile biosynthesis or control the composition of a blend, a mechanical caterpillar (MecWorm) has been engineered, which very closely resembles the herbivore-caused tissue damage in terms of a similar physical appearance and a long lasting wounding period on defined leaf areas. In many plants the mechanical treatment was sufficient to induce a blend of VOCs as known from real herbivore feeding.[1] The defence patterns could be modified by addition of salivary secretions from the feeding insect to the wounded leaf, demonstrating that the salivary secretions also have a strong impact on the composition of the blend. This was further established by microarrays comprising the whole genome of *A. thaliana*. In total about 5000 genes were either up- or down regulated after simple mechanical damage. By Principal Component analysis the different treatments of the leaves of *A. thaliana*, such as mechanical damage, feeding by a specialized insect (Diamond Back Moth), and a generalist herbivore (Beet Army Worm), could be clearly distinguished by a typical set of differently affected genes. Interestingly, the salivary secretions of the feeding insects seem to silence locally the gene expression in the damaged leaf, compared to the effect of mechanical wounding, but in distant leaves a significant reprogramming occurs that is not observed after the MecWorm treatment.[2]

Other elements of damage recognition and internal signaling comprise membrane depolarization and influx of Ca^{2+}-ions.[3] Some of the effects can be induced by low molecular *N*-acyl glutamines, which are typical and widespread components within salivary sections of Lepidopteran larvae.[4] The *N*-acyl glutamines are amphiphilic compounds that cause depolarization and membrane destabilization resulting in a simultaneous up-regulation of the octadecanoid and the salicylate signaling pathways. Depending on the extent of the activation of the signal-transduction routes (jasmonic

acid, salicylic acid, ethylene, and Ca^{2+}-influx) and depending on their syn- or antagonistic interaction, the biosynthesis of phytoalexins (volatiles) is either induced, suppressed or modulated.[5] The complexity of interactions will be presented at the molecular level; consequences and the impact on plant-insect interactions will be discussed.[6]

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Role of Auxin Signaling in Communication among Maize Plants

E. Van Volkenburgh[1,*], M. Fellner[2], E.D. Ford[1]

[1]Biology Department and College of Forest Resources, University of Washington, USA
[2]Department of Cell Biology and Genetics, Palacky University in Olomouc, Czech Republic

Plants sense each other by communicating via changes in the ratio of red to far-red light (R:FR ratio) inside the canopy. In response to crowding, and thus warnings of competition from others, shade-avoiding plants invoke a syndrome of responses including stimulation of elongation growth, reduced branching, and a redistribution of leaves to the top of canopy (shade avoidance responses). It was proposed that extension growth induced by neighbor detection and shade is the result of R:FR-regulated auxin distribution (Morelli and Ruberti 2000). Plant breeders, when selecting corn (*Zea mays* L.) plants with high yield in dense plantings, created hybrids with reduced sensitivity to neighbors. We have tested the possibility that the physiological consequence of the selection involves changes in responsiveness to light and auxin (see also Fellner et al. 2003). Etiolated seedlings of a hybrid originally released in the 1930's (307) elongated significantly more than seedlings of a modern hybrid released in the 1990's (3394). The level of endogenous auxin and activity of polar auxin transport (PAT) were similar in both genotypes. The 1990's hybrid shows resistance to auxin- and light-induced responses at the seedling, cell, and molecular levels. Intact 3394 plants exhibited less responsiveness to the inhibitory effect of R, FR, and W, auxin, antiauxin, and inhibitors of PAT. Excised mesocotyl tissue from 3394 seedlings also showed low responsiveness to NAA. Cells of 3394 were insensitive to auxin- and light-induced hyperpolarization of the plasma membrane. Expression of *ABP4* was much less in 3394 than in 307, and in contrast to 307, it was not up-regulated by NAA, R and FR. Preliminary analysis of *abp* mutants suggests that ABPs may be involved in development of leaf angle in corn.

Corn breeders inadvertently selected hybrids for reduced sensitivity to neighbors. Modern plants have reduced sensitivity to auxin, not to light. By analogy they are blind to each other, not because their eyes don't see, but because their brains don't process the image. Instead of having a signaling system responsive to changes in R:FR, these plants appear to be 'hard-wired' in a shade-avoidant posture with upright leaves. One consequence of the more vertical canopy architecture is that modern

hybrids shade themselves and each other less, allowing more light to power photosynthesis in the leaf subtending the developing ear, thus generating higher yield.

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Compatible Solutes as Regulatory Agents in Plant Adaptive Responses to Environment

Tracey Cuin, Zhonghua Chen and Sergey Shabala

School Agricultural Science, University of Tasmania, Private bag 54, Hobart 7001, Tasmania, Australia

When confronted with a hostile environment (such as salinity, drought or low temperatures), plants respond with a significant elevation in the level of compatible solutes in the cytosol, ameliorating the detrimental effects of stress on cell metabolism [1]. Mechanisms of such amelioration are not fully understood. It has been previously suggested that the functions of compatible solutes are not likely to be limited by conventional osmoprotection, but may also include a regulatory role in adjusting metabolic pathways to altered environmental conditions [2]. Physiological assessment of over 70 barley cultivars in our laboratory has suggested that the difference between salt-sensitive and salt-tolerant barley cultivars was conferred essentially by their ability to retain K^+ and minimise the magnitude of NaCl-induced K^+ efflux [3]. At the same time, salt-tolerant cultivars showed attenuated K^+ efflux responses to a hydroxyl radical ($OH^\bullet$) -generating Cu^{2+}/ascorbate (Cu/a) mixture. Therefore, it was hypothesised that one function of compatible solutes in stress-induced responses is in scavenging ROS and maintaining cytosolic K^+ homeostasis by preventing NaCl-induced K^+ leakage from the cell. This hypothesis was investigated using the non-invasive MIFE ion flux measuring technique. In both barley and Arabidopsis plants, low (0.5 to 5 mM) concentrations of exogenously supplied proline or glycine betaine significantly reduced NaCl-induced K^+ efflux from plant roots in a dose-response manner. The above mitigating effect was instantaneous, implying that large intracellular concentrations of compatible solutes are not required for an amelioratory role in salt tolerance. Exogenously supplied betaine also significantly enhanced NaCl-induced H^+ efflux, but only in preincubated roots, implying some alternative mechanism of regulation. Sap K^+ and Na^+ analysis and membrane potential measurements are also consistent with the model that one function of compatible solutes is in maintaining cytosolic K^+ homeostasis by preventing NaCl-induced K^+ leakage from the cell, possibly through the enhanced activity of H^+-ATPase, controlling voltage-dependent outward-rectifying K^+ channels and creating the electrochemical gradient necessary for secondary ion transport processes. Root preincubation in low concentrations of compatible solutes also significantly reduced the extent of the $OH^\bullet$-induced potassium efflux. Importantly, such reduction was

found not only for osmolytes for which a role is free-radical scavenging has been demonstrated *in vitro* experiments, but also for compounds thus far not shown to act as free-radical scavengers. This indicates that compatible solutes must play some other (regulatory) roles in addition to free-radical scavenging, in mitigating the damaging effects of oxidative stress. Overall, these data provide the first direct evidence for regulation of ion fluxes across the plasma membrane by physiologically relevant low concentrations of compatible solutes. This may be important for understanding the mechanisms of stress tolerance in plants and development of salt-tolerant crop species.

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Plants as Environmental Biosensors

Maya I. Volkova-Gugeshashvili[1], Alexander G. Volkov[1] and Vladislav S. Markin[2]

[1]*Department of Chemistry, Oakwood College, 7000 Adventist Blvd., Huntsville, AL 35896, USA*
[2]*Department of Anesthesiology UT Southwestern Medical Center, Dallas, TX 75390-9068*

Plants are continuously exposed to a wide variety of perturbations including variation of temperature and/or light, mechanical forces, gravity, air and soil pollution, drought, deficiency or surplus of nutrients, attacks by insects and pathogens, etc., and hence, it is essential for all plants to have survival sensory mechanisms against such perturbations. As a consequence, plants generate various types of intracellular and intercellular electrical signals mostly in the form of action potentials or variation potentials in response to these environmental changes [(1-2)]. However, over a long period, only certain plants with rapid and highly noticeable responses to environmental stresses have received much attention from plant scientists. Of particular interest to our recent studies on ultra fast action potential in green plants, we discuss in this review the possibility of utilizing green plants as fast biosensors for molecular recognition of the direction of light, monitoring the environment, and detecting the insect attacks as well as the effects of pesticides and defoliants.

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Spatially Regulated Changes in Proton Pumping, Accumulation of Wall Phenolics, Wall Extensibility and Growth Along the Elongation Zone of Water Stressed Maize Roots

Peter M Neumann and [1]Ling Fan

Plant physiology lab, Dept of water, environmental and agricultural engineering, Faculty of Civil and Environmental Engineering, Technion IIT, Haifa 32000, Israel
[1]Institute of Nuclear and Biological Technology, Xinjiang Academy of Agricultural Sciences, Urumqi 830000, China

Accelerating segmental growth rates from 0-3 mm behind the tips of intact maize (Zea mays L) seedling primary roots were well maintained under a 48 h water deficit and correlated with outward flux of protons (Fan and Neumann, 2004). However, segmental growth rates, proton flux and wall mechanical-extensibility decreased progressively 3 to 9 mm behind the tip and more so under water deficit. Since exogenous acidification did not reverse these decreases, additional growth-regulatory factors were involved. The root expression of 2 gene transcripts involved in lignin biosynthesis, cinnamoyl-CoA reductase 1 and 2, increased after only 1 h of water deficit and before the onset of decreases in wall extensibility. We therefore investigated the possibility that spatially-localized increases in deposition of wall-linked phenolic compounds such as lignins and ferulic esters might stiffen the expanding cell-walls and cause irreversible deceleration of root growth. Progressive increases in wall-linked phenolics were detected by comparing Fourier transform IR-spectra and UV-fluorescence images of isolated cell walls from control and water–stressed roots at 0 to 3, 3 to 6 and 6 to 9 mm behind the tip. The increases in UV fluorescence and lignin-staining induced under water deficit co-located to cell walls of vascular tissues in the stele. Longitudinal bisection of the elongation zone resulted in inward curvature, indicating that the inner stelar tissues were rate-limiting for growth (Fan et al., 2006). We suggest that spatially-localized wall stiffening and deceleration of growth in the basal elongation zone involves regulated increases in the deposition of wall-bound phenolics. These changes may facilitate acclimation to water deficit by diverting resources to the tip meristem. They could be mediated by gradients of growth regulatory signals or of cell responsiveness to such signals.

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Plant Electrophysiology: Effects of Ion Channel Blockers on Signal Transduction in Green Plants

Alexander G. Volkov[1,*], Vladislav S. Markin[2], Maia I. Volkova-Gugeshashvili[1]

[1]Department of Chemistry, Oakwood College, Huntsville, AL 35896
[2]Department of Anesthesiology UT Southwestern Medical Center, Dallas, TX 75390-9068

Ion transport plays a fundamental role in many biophysical processes in plant cells, including the generation of cell turgor, energy, signal transduction, and metabolite distribution. Ion channels also play an important role in the signal transduction in higher plants. Action potentials in higher plants may be the information carriers in intercellular and intracellular communication in the presence of environmental challenges [1-3]. Action potentials take an active part in the expedient character of response reactions of plants. These impulses transfer a signal about the changes of conditions in a conducting bundle of a plant. Impulses travel in either direction, from the root system to the point of growth and or from the point of growth to the root system [2]. The response reactions of plant tissues and organs can be local or transmitted from cell to cell over long distances. The transfer of excitation has a complicated character accompanied by an internal change in cells and tissues. Inhibition of Ca^{2+} channels blocks propagation of action potentials induced by electrical stimulus or phototropism. We have found that tetraethylammonium chloride, a potassium channel inhibitor, blocks the propagation of action potentials induced by phototropism, thermal or mechanical stresses in soybean plants. (This work was supported by NASA Grant NAG8-1888.)

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Can Plants Discover Neighboring Plants by Volatile Signalling?

Velemir Ninkovic, Robert Glinwood, and Jan Pettersson

Swedish University of Agricultural Sciences, Department of Entomology, Box 7044, SE-750 07 Uppsala, Sweden

We have introduced the term 'allelobiosis' to describe a type of plant-plant interaction via chemicals (Pettersson et al. 2003; Ninkovic et al. 2006). The three key aspects of our definition of allelobiosis are (1) the interaction occurs between undamaged plants, (2) the interaction may be beneficial for the receiving plant and (3) the responses of the receiving plant affect organisms at other trophic levels. Aspect (1) distinguishes allelobiosis from plant-damage/stress signalling, in which chemicals are released by infected/infested plants, while aspect (3) distinguishes allelobiosis from allelopathy.

We have studied a model system consisting of different cultivated and wild genotypes of barley (*Hordeum vulgare* spp.), aphids, and aphid natural enemies. Data on the temporal dynamics of plant responses volatile cues, in terms of aphid acceptance and leaf temperature, show major differences between allelobiosis-related volatiles and stress-related signals such as methyl salicylate and methyl jasmonate. Furthermore, allelobiosis interactions in *Hordeum* are genotype-dependent, and are manifested only in certain combinations of inducing and responding genotypes. Certain *Hordeum* genotypes that are already resistant to aphids show further significant reductions in aphid growth rate after exposure to volatiles from certain other genotypes.

Changes in the pattern of biomass allocation in responding barley plants suggest that responses may be beneficial in the context of potential competition with neighbouring plants (Ninkovic 2003). The effects of allelobiosis are apparent over three trophic levels; certain barley cultivars become more attractive to the natural enemies of aphids after exposure to volatiles from certain other barley cultivars.

We believe that allelobiosis represents a mechanism by which certain barley genotypes can detect potential competition from neighbouring plants. The changes in plant status induced by allelobiosis affect the interaction of the plant with both aphid herbivores and their natural enemies, and may therefore be ecologically important.

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External Glutamate Modifies Root Growth in *Arabidopsis*

Pia Walch-Liu, Tony Remans and Brian Forde

Biological Sciences, Lancaster University, Bailrigg, Lancaster LA1 4YQ, United Kingdom

The sessile nature of plants demands a highly sophisticated sensory system monitoring environmental changes and an extreme flexible physiological and physical plasticity in order to respond to this changes efficiently. Plants are known to forage for localised supplies of nitrate by proliferating their lateral roots within nitrate-rich patches. There is increasing evidence that amino acids can be an important source of N for many plant species. Is it possible that roots also possess sensory mechanisms that enable them to more efficiently exploit heterogenous supplies of organic N? Studies with *Arabidopsis* have shown that exogenous L-glutamate (L-Glu), one of the most abundant forms of soluble organic N in the soil, is able to elicit complex changes in root architecture. L-glutamate, at μM concentrations, is perceived at the primary root tip in a stereospecific manner and inhibits mitotic activity in the root apical meristem. Surprisingly, mitotic activity in the developing lateral root is insensitive to L-Glu, but lateral roots acquire L-Glu sensitivity later in their development. The plant's sensitivity to L-Glu is under the influence of a number of environmental factors (such as light intensity) and different ecotypes of *Arabidopsis* differ markedly in their L-Glu sensitivity. We have used populations of recombinant inbred lines (Ler x Col-0, C24 x Col-0), together with near isogenic lines (NILs), to identify major QTLs for L-Glu sensitivity to chromosomes 2, 3 and 5. A screen of a fast neutron bombardment population has yielded a number of mutants showing altered L-Glu sensitivity and the deletions responsible are being mapped. Through this combination of approaches we aim to elucidate the mechanism of L-Glu sensing in the Arabidopsis root tip.

A 14-AA Peptide Derived from CLV3 is Sufficient to Regulate the Stem Cell Population in *Arabidopsis*

Martijn Fiers* and Chun-Ming Liu[+]

**Plant Research International, Wageningen, The Netherlands; [+]Center for Signal Transduction & Metabolomics, Institute of Botany, [+]Chinese Academy of Sciences, China*

Stem cells positioned in the central zone of the plant shoot apical meristem (SAM) are the source of totipotent cells, which give rise continuously to new organs post-embryonically. These slow-dividing cells maintain simultaneously two antagonistic events, cell proliferation and cell differentiation in a similar manner to animal stem cells. The determination of the fate of the progeny cells is made by a population-based mechanism in which signals from neighboring cells play the most important role. Genetic experiments have shown that, as part of a feedback regulatory loop, the stem cell-promoting transcription factor *WUSCHEL* (*WUS)*, which is expressed in the stem cell organizing center (OC), provides a positive signal to maintain an undifferentiated state; while CLAVATA3 (CLV3) interacts with the underlying CLV1/CLV2 receptor complex to act as a negative signal to restrict the number of stem cells.

Previous work suggested that *CLV3* encodes a mobile ligand that acts in a non-cell autonomous fashion for intercellular communication. CLV3 belongs to a family of small proteins, named CLV3/ESR (CLE), found in plants and parasitic nematodes. They share an N-terminal secretion signal (SS) and a conserved 14-amino acid (AA) CLE motif at or near their C-termini. Over-expression of several *CLE* genes, such as *CLV3*, *CLE19* and *CLE40* from Arabidopsis and *SYV46* from nematode, causes a termination of the root meristem (Casamitjana-Martínez et al., 2003; Fiers et al., 2004). Recently we showed that CLE19p and CLE40p, peptides corresponding to the 14-amino acid CLV3/ESR (CLE) motif of CLV3 and CLE40, respectively, are able to mimic the over-expression phenotype of the corresponding genes, triggering the consumption of root meristem in vitro (Fiers et al., 2005). Through deletion analyses, we demonstrate further that the CLE motif of CLV3, together with its secretion signal, is sufficient to complement clv3-2 defects. A synthetic peptide, CLV3p, corresponding to this motif, is able to restrict the size of the SAM in vitro in a CLV1-dependent manner. Peptides derived from CLE40, CLE5, CLE19 and CLE22 confer a various degree of complementation, as reported before in transgenic assay. Application of CLV3p restricts the expression of the stem-cell promoting

transcription factor WUSCHEL (WUS) in *clv3-2*. We thus propose that the CLE motif is the functional cue of CLV3, and the mode of action in different CLEs may be the same.

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Sugar Signaling to Cytoskeleton

Agnieszka K. Banas, Weronika Krzeszowiec, Halina Gabrys

Department of Plant Physiology and Biochemistry, Faculty of Biotechnology, Jagiellonian University, Kraków, Poland

Besides being a source of carbon skeletons sugars may also act as signal molecules. They are involved in the regulation of gene expression, enzyme activity and numerous cellular processes. Hexokinase is the only hexose sensor identified so far in plant cells. In the presence of glucose, it has been shown to induce microfilament depolymerization *in vitro*. Abundant evidence points to interactions between sugars/hexokinase and actin *in vivo* in animal cells. In contrast, very little is known about the influence of sugars on plant cytoskeleton. Glucose, sucrose and mannose have been shown to inhibit light-induced chloroplast movements in higher plants. These intracellular movements are based on actin cytoskeleton. The aim of this study was to investigate the effect of exogenously delivered sugars on actin cytoskeleton in leaf mesophyll cells of *Arabidopsis thaliana* and *Nicotiana tabacum*. Detached leaves were incubated on sugar-enriched agar plates for 2 days. Influence of glucose, sucrose and mannose on microfilaments was tested. Neither glucose nor sucrose affected the microfilament architecture in *Arabidopsis*. In contrast, mannose caused a complete destruction of actin cytoskeleton, reducing a branched network to fluorescent foci within 24 h. Interestingly, *Nicotiana* mesophyll cells turned out to be insensitive to mannose. This insensitivity was probably connected with the presence of phosphomannose isomerase in tobacco cells. Mannose is widely used as a selection marker for transformation of plants lacking enzymes responsible for the metabolism of mannose-6-phosphate. Exposure to this hexose is linked with DNA fragmentation and release of cytochrome c from mitochondria in *Arabidospsis* roots and in the maize suspension culture. Both responses are known as features of programmed cell death. However, no DNA laddering was observed in *Arabidopsis* leaves exposed to mannose.

Regulation of Starch Composition in the ArabidopsisLeaves by Pad1 and Pad2 Proteins

Chao Chen, Wei Cheng, Xiaojin Zhou and Yingdian Wang*

Laboratory of Plant Development Physiology and Molecular Biology, College of Life Sciences, Beijing Normal University, Beijing 100875, China
**Corresponding author: Yingdian Wang (Tel: +86-10-58808195; Fax: +86-10-58809077; E-mail: ydwang@bnu.edu.cn)*

Starch is the main component of the maize kernel, which is used for hundreds of food and nonfood products. Amylose extender (ae) of rice and maize are controlled by SBEIIb deficient. The ae mutants of rice have decreased levels of amylopectin chains (DP≦17), increased levels of intermediate and long amylopectin chains. To understand the molecular basis for the variations of the amylose contents, we examined transcripts for key starch branching enzyme (SBEIIb). SBEIIb functions as a critical master regulator in starch composition show low transcription in high amylose content maize plant, but high transcription in low amylose content maize plant.

In the present study, using the yeast two-hybrid system, a protein named Zmpa4 that similar with Arabidopsis pad1 and pad2 was identified as a ZmSBEIIb-binding protein. Invitro specific interaction between SBEIIb and Zmpa4 was confirmed by *E. coli* and yeast expression products. When overexpressed in *E. coli* cells, the GST-Zmpa4 could co-precipitate with ZmSBEIIb from yeast proteins. This interaction requires the motif 1 and 2 of ZmSBEIIb and the C-terminus of Zmpa4. Plant lines carrying T-DNA insertions that disrupt the *Arabidopsis* pad1 or pad2 exhibited the altered starch composition in the leaves. The ZmSBEIIb interaction proteins identified in this screen are new targets for studies of starch metabolism in higher plants.

Key words: amylopectin, amylose, protein-protein interactions, starch branching enzyme, alph 4 subunit of 20s proteasome.

RACK1 Mediates Multiple Hormone Responsiveness and Developmental Processes in *Arabidopsis*

Jin-Gui Chen[1,2*], Hemayet Ullah[2,7], Brenda Temple[4], Jiansheng Liang[2,6], Jianjun Guo[1], José M. Alonso[5,8], Joseph R. Ecker[5], and Alan M. Jones[2,3]

[1] *Department of Botany, University of British Columbia, Vancouver, British Columbia, V6T 1Z4 Canada*

[2] *Department of Biology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA*

[3] *Department of Pharmacology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA*

[4] *Structural BioInformatics Core Facility, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA*

[5] *Plant Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037, USA*

[6] *College of Bioscience and Biotechnology, Yangzhou University, Yangzhou 225009, P.R. China*

[7] *Present address: Department of Biology, Howard University, Washington, DC 20059, USA*

[8] *Present address: Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695, USA*

E-mail: jingui@interchange.ubc.ca

The scaffold protein RACK1 (*R*eceptor for *A*ctivated *C K*inase *1*) serves as an integrative point for diverse signal transduction pathways. The Arabidopsis genome contains three RACK1 orthologs, however, little is known about their functions. We report here that one member of this gene family, *RACK1A*, previously identified as the Arabidopsis homolog of the tobacco *arcA* gene, mediates hormone responses and plays a regulatory role in multiple developmental processes. *RACK1A* expresses ubiquitously in Arabidopsis. Loss-of-function mutations in RACK1A confer defects in multiple developmental processes including seed germination, leaf production, and flowering. *rack1a* mutants displayed reduced sensitivity to gibberellin and brassinosteroid in seed germination, hypersensitivity to abscisic acid in seed germination and early seedling development, and hyposensitivity to auxin in adventitious and lateral root formation. These results provide the first genetic evidence that RACK1A may have a regulatory role in diverse signal transduction pathways.

Disruption of Actin Filaments by Latrunculin B Affects Cell Wall Construction in *Picea meyeri* Pollen Tube Via Vesicle Trafficking Disturbance

Tong Chen[1,2], Nianjun Teng[1,2], Xiaoqin Wu[1,2], Yuhua Wang[1,2], Wei Tang[3], Jozef Šamaj[4,5], František Baluška[4,6] and Jinxing Lin[1*]

[1] *Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China*
[2] *Graduate School of Chinese Academy of Sciences, Beijing 100049, China*
[3] *Department of Biology, Howell Science Complex, East Carolina University, Greenville, NC27858-4353, USA*
[4]*Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany*
[5]*Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademicka 2, SK-95007, Nitra, Slovak Republic*
[6]*Institute of Botany, Slovak Academy of Sciences, Dubravska 14, SK-84223, Bratislava, Slovak Republic*

*E-mail: linjx@ibcas.ac.cn

The involvement of actin filaments (AFs) in vesicle trafficking, cell wall construction and tip growth were investigated during pollen tube development of *Picea meyeri.* Pollen germination and tube elongation were inhibited in a dose-dependent manner by the latrunculin B treatment. The fine actin filaments (AFs) were broken down into disorganized fragments showing a tendency of aggregation. FM4-64 labelling revealed that the dynamic balance of vesicle trafficking was perturbed due to F-actin disruption and the fountain-like cytoplasmic pattern changed into disorganized Brownian movement. The configuration and/or distribution of cell wall components, such as pectins, callose, cellulose as well as arabinogalactan proteins also dramatically changed after the LATB application. FTIR analysis further established significant changes in the chemical composition of the wall material. Our results indicated that depolymerization of actin filaments affects the distribution and configuration of cell wall components in *Picea meyeri* pollen tube via vesicle trafficking disturbance.

Characterization of Differentially Expressed Pollen Tube Proteins: Towards Understanding of the Regulation of Ca^{2+}-Calmodulin in Pollen Tube Development in Conifer

Tong Chen[1], Xiaoqin Wu[1], František Baluška[2,3], Jozef Šamaj[2,4], Jinxing Lin[1*]

[1] *Key Laboratory of Photosynthesis and Molecular Environmental Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China*
[2]*Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany*
[3]*Institute of Botany, Slovak Academy of Sciences, Dubravska 14, SK-84223, Bratislava, Slovak Republic*
[4]*Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademicka 2, SK-95007, Nitra, Slovak Republic*

*E-mail: linjx@ibcas.ac.cn

We investigated the roles of Ca^{2+}-CaM signaling pathway in the development of pollen tube of *Picea meyeri* Rehd. et Wils., using calmodulin antagonist trifluoperazine (TFP) under quantitatively controlled conditions. Proteomic approach was employed to analyze protein expression profile changes during pollen germination and subsequent tube growth after inhibition of calmodulin functions. Two-dimensional electrophoresis and staining with Coomassie Brilliant Blue revealed over 800 protein spots. A total of 76 of these were reproducibly differentially displayed at different hours with varying doses of TFP, and 57 differentially expressed proteins were identified by tandem mass spectrometry. These proteins were grouped into distinct functional categories including carbohydrate and energy metabolism, signaling, cell expansion, defense and stress response, etc. Moreover, perturbation of Ca^{2+}-calmodulin signaling dissipated the tip-focused $[Ca^{2+}]_c$ gradient and dramatically increased calcium concentration in the cytoplasm. Morphology of mitochondria, Golgi stacks along with a differential expression of proteins involved in their functions was also affected. In the meantime, the patterns of endocytosis/exocytosis and cell wall construction were obviously changed after inhibitor application. These findings provide new insights into the sophisticate mechanism of calmodulin functions in pollen tube development and its interaction with energy-producing pathways, signaling and cell expansion machinery.

Cloning and Expression Analysis of the Hexokinase Family in the Developing Spikelets of Rice (*Oryza sativa* L.)

Wei Cheng, Xiaojin Zhou, Jie Li, Chao Chen, Yingdian Wang*

Laboratory of Plant Development Physiology and Molecular Biology, College of Life Sciences, Beijing Normal University, 19 XinJieKouWai Avenue, Beijing 100875, China

**Corresponding author: Yingdian Wang (Tel: +86-10-58808195; Fax: +86-10-58809077; E-mail: ydwang@bnu.edu.cn)*

The carbon metabolism and sugar signal transduction is critical in the plant growth and development processes. Plant hexokinase not only catalyzes the production of hexose 6-phosphates but also acts as a sugar sensor plays an important role in sugar sensing and signaling (Jang *et al*., 1997; Coruzzi and Bush, 2001; Moore *et al*., 2003).

To identify the hexokinase gene family in rice, we searched the databases of both the whole genome and full-length cDNAs of rice carried out with the known OsHXK I and OsHXK II sequences, and isolated eight new rice hexokinase cDNAs designated *OsHXK1*-*OsHXK8* (AY884164-AY884171). The rice hexokinase gene family is to be comprised by those ten genes. All *OsHXKs* have a highly conserved genomic structure consisting of nine exons, except for *OsHXK1* with single exons. Those genes mapped to one of three chromosomes: *OsHXK2*, *OsHXK4*, *OsHXK5* and *OsHXK8* to chromosome 1; *OsHXK3*, *OsHXK6*, *OsHXK7* and *OsHXK I* to chromosome 5; *OsHXK1 OsHXK II* to chromosome 7. The deduced proteins of obtained HXK genes were predicted to contain adenosine phosphate binding sites and substrate recognition sequences, which were highly conserved compared with known HXKs, suggesting the ten *OsHXKs* are likely to encode functional HXKs. The predicted molecular mass of those OsHXKs are much closer in size, except OsHXK4 and OsHXK6. Isoelectirc point values were calculated to be ranged from 4.81 to 6.77. Multi-sequence alignment analysis of hexokinase proteins in rice and other species suggested that all plant hexokinases are to be grouped into four classes.

The transcript levels of the HXK family were confirmed in several organs and developing spikelets in rice. Different expression patterns of *OsHXKs* were observed at the different stages of plant development. The expression results of *OsHXKs* in different tissues suggest that those ten HXKs may possess diverse physiological functions in different tissues, whereas the function of specific HXKs in metabolism and sugar sensing during rice caryopsis development still need to be further clarified.

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Fluid Phase Endocytotic Uptake of Artificial Nano-Spheres and Fluorescent Quantum Dots by Sycamore Cultured Cells: Evidence for the Distribution of Solutes to Different Intracellular Compartments

Ed Etxeberria[1,*] Pedro Gonzalez[1], Edurne Baroja-Fernandez[2] and Javier Pozueta-Romero[2]

[1] *University of Florida, Institute of Food and Agricultural Sciences, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850-2299, USA, Department of Horticultural Sciences,*

[2] *Agrobioteknologia Instituta, Consejo Superior de Investigaciones Cientificas and Nafarroako Unibertsitate Publikoa, Mutiloako etorbidea zembaki gabe, 31192 Mutiloabeti, Nafarroa, Spain;*

E-mail: eje@crec.ifas.ufl.edu; javier.pozueta@unavarra.es

Fluid phase endocytic uptake of external solutes in plant cells was further substantiated using artificial polystyrene nano-spheres (40 nm) and CdSe/ZnS quantum dots (20 nm). Both types of artificial nano-particles were taken up by sycamore cultured cells. However, whereas polystyrene nano-spheres were delivered to the central vacuole, CdSe/ZnS nano-dots were sequestered into cytoplasmic vesicular structures. Using dextran-Texas Red (mw, 3,000; d-TR) as additional marker, confocal micrographs confirmed the distinct topographic distribution of CdSe/ZnS quantum dots within the cell. Initially, d-TR and CdSe/ZnS quantum dots co-localized within cytoplasmic vesicles. After 18 h incubation, d-TR was distinctly localized in the vacuole whereas CdSe/ZnS quantum dots remained sequestered in cytoplasmic membranous compartments. The data provide a first evidence for the rapid distribution of solutes taken up by endocytosis to distinctive intracellular compartments.

Short-term Systemic Reactions of Photosynthetic Apparatus of Tobacco Plants to Local Burning in Relation to Propagating of Electrical Signal and Accumulation of Signaling Molecules in the Distant Leaves

Vladimíra Hlaváčková[1,*], Pavel Krchňák[1], Martin Kubala[1], Jan Nauš[1], Ondřej Novák[2], Miroslav Strnad[2]

1 Laboratory of Biophysics, Department of Experimental Physics, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic
2 Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany ASCR, Šlechtitelů 11, 783 71 Olomouc, Czech Republic

*E-mail: hlavacko@prfnw.upol.cz

Local burning events have been shown to induce short-term changes in photosynthetic parameters of distal leaves in various plants that appear to be mediated by electrical signals. Longer-term changes (within hours) in hormone and heat-shock proteins levels have also been reported. However, the mechanisms whereby electrical and/or chemical signals induce the short-term photosynthetic (or heat-shock protein) responses are largely unknown. We studied short-term (up to 1 h) systemic responses of tobacco (*Nicotiana tabacum* cv. Samsun) plants to local burning of an upper leaf by measuring the following variables in a distant leaf: extracellular electrical potentials (EEPs); gas exchange parameters; fast chlorophyll fluorescence induction; endogenous concentrations of three putative chemical signaling compounds – abscisic (ABA), jasmonic (JA), salicylic (SA) acids and content of heat-shock proteins (Hsp70 family). A decrease in EEPs in the distant leaves started to decline within 10-20 s of the beginning of the treatment, fell sharply for ca. 1-3 min, and then tended to recover within the following hour. The measured gasometric parameters (stomatal conductance and the rates of transpiration and CO_2 assimilation) started to decrease 5 - 7 min after local burning, suggesting that the electrical signals may induce stomatal closure. Simultaneously, systemic increases in the endogenous ABA concentration were followed by huge systemic rises in endogenous JA levels started after ca. 15 min, providing the first evidence of short-term systemic accumulation of these plant hormones in responses to local burning. Furthermore, JA appears to have an inhibitory effect on CO_2 assimilation. Also an increase in Hsp70 content was detectable both locally and systemically within 1 hour after local burning. The correlations between the kinetics of the systemic EEP, stomatal, photosynthetic, ABA, JA and Hsp70 responses suggest that (i) electrical signals (probably induced by a propagating

hydraulic signal) may trigger chemical defence-related signaling pathways in tobacco plants; (ii) both electrical and chemical signals are interactively involved in the induction of short-term systemic stomatal closure and subsequent reductions in the rate of transpiration and CO_2 assimilation; (iii) systemic accumulation of heat-shock proteins may be induced by chemical and/or electrical signals propagating after local burning events.

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Opposite Patterns of ABA and IAA in Relation to the Cambium Periodicity in *Eucommia ulmoides* Oliv

Xinqiang He, Kalima-N'Koma Mwange, Hongwei Hou, Wanfeng Li, Keming Cui

College of Life Sciences, Peking University, Beijing 100871, China

E-mail: hexq@pku.edu.cn

The seasonal change of abscisic acid (ABA) and Indole-3-acetic acid (IAA) and their relationship with the cambial activity in *Eucommia ulmoides* tree were investigated by ABA and IAA immunolocalization, quantification, and systematic monitoring of vascular cell layers production. ABA and IAA clearly displayed opposite annual distribution patterns. In the active period, both immunolocalization and HPLC detected an abrupt decrease of ABA, reaching its lowest level in summer. During dormancy, ABA started increasing in the first quiescence (Q1) (autumn), peaked in the rest (winter), and gradually decreased from the onset of the second quiescence (Q2) (end winter). IAA showed a reverse pattern to that of ABA: it sharply increased in the active period, but noticeably decreased from the commencement of the first quiescence. The concomitant IAA-ABA distribution and seasonal changes in vascular tissues greatly correlated with xylem and phloem cell production, and late wood differentiation and maturation. Meanwhile, A cDNA clone of auxin binding protein 1 (ABP1), the putative auxin receptor, was isolated. Spatio-temporal expression patterns showed that ABP1 transcript abundance in the cambium tissues was high, low and remarkably scarce respectively in active, quiescent and resting tree. Results strongly support the role of ABP1 in mediating the IAA signals, which could boost cambium reactivation during cambium quiescence, and the results could also explain why IAA could not reactivate a resting cambium. This ABP1 expression pattern correlated positively with that of IAA, but negatively with that of ABA. It also correlated closely with auxin sensitivity during cambial activity periodicity.

Keywords: ABA, ABP1, cambium periodicity, dormancy, *Eucommia ulmoides*, IAA

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Analysis of Metabolite Content in *Arabidopsis* GABA Shunt Mutants

Anke Hüser, Ulf-Ingo Flügge, Frank Ludewig

Botanical Instiute, University of Cologne, Gyrhofstr, 15, D-50931 Cologne, Germany

Email: Anke.Hueser@uni-koeln.de

GABA (gamma-amino butyric acid) is a four carbon amino acid, found in all organisms. In plants, mutants of both catabolic genes (gaba-t and ssadh) display phenotypic deviations to WT. *ssadh* knock-out plants are severely affected in growth, probably due to the accumulation of a toxic compound. Two candidate substances (GHB and SSA), that can be converted into each other, were examined for their capacity to affect plant growth.

WT and mutant plants were grown on half-strength MS-media containing different amounts of either SSA or GHB to test which of the compounds are causing the *ssadh* phenotype. Plant growth was observed and the metabolite content was determined using GC/MS techniques.

WT or gaba-t mutant plants were affected in growth by increased concentrations of SSA or GHB added to the media, but to a lesser extent than *gaba-t ssadh* double knock-out plants. By determining the GHB content in plant extracts using GC/MS, higher amounts of GHB were found in plants grown on GHB containing media. These results indicate that SSA might be the causative substance for the observed ssadh phenotype.

To verify these results, knock-out mutants in the ghbdh gene were isolated and analyzed for their response to GHB or SSA in comparison to WT plants.

Aluminium sensitivity of *Arabidopsis* roots: unique status of the transition zone in plasma membrane properties, endosomal behaviour, nitric oxide production and internalization of aluminium

Peter Illéš[1], Markus Schlicht[2], Ján Pavlovkin[1], Irene Lichtscheidl[3], František Baluška[1,2*], Miroslav Ovečka[1]

[1] *Institute of Botany, Slovak Academy of Sciences, Bratislava*
[2] *Institute of Cellular and Molecular Botany, University of Bonn, Bonn*
[3] *Institution of Cell Imaging and Ultrastructure Research, University of Vienna, Vienna*

***E-mail:** baluska@uni-bonn.de

One of the most relevant problems of recent research on aluminium phytotoxicity is to define the primary site of aluminium action on a cellular and subcellular level. In addition, data on the fate of internalized aluminium during plant recovery are completely missing. We studied the extent of aluminium internalization during the recovery from aluminium stress in living roots of *Arabidopsis thaliana* by non-invasive *in vivo* microscopy in real time. We document evidently distinct sensitivity of the cells passing through different developmental zones. Aluminium exposure caused rapid depolarization of the plasma membrane. It was much more extensive in cells of the distal portion of the transition zone than in the proximal portion of the transition zone. Also full recovery of the membrane potential after removal of external aluminium was slower in cells of the distal portion of the transition zone compared to cells of its proximal part. Apoplastic aluminium internalized extensively during the recovery phase into endosomal/vacuolar compartments in the most aluminium sensitive cells of the distal portion of the transition zone. Importantly, internalization of aluminium is spatially restricted to the pectin-recycling zone. Aluminium interfered with endosomal behaviour and inhibited the formation of BFA-induced compartments in these cells. Moreover, cells of the distal portion of the transition zone emitted large amounts of nitric oxide (NO) and this was blocked by aluminium treatment. All these data suggest that the most sensitive status of the distal portion of the transition zone towards aluminium reflects the specific mode of aluminium sensing within the root apex.

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Comparison Analysis of the ADP-ribosylation Factors Gene Family in Rice

Jie Li, Wei Cheng, Chao Chen, Xiaojin Zhou, Yingdian Wang*

Laboratory of Plant Development Physiology and Molecular Biology, College of Life Sciences, Beijing Normal University, 19 XinJieKouWai Avenue, Beijing 100875, China
**Corresponding author: Yingdian Wang*

(Tel: +86-10-58808195; Fax: +86-10-58809077; E-mail: ydwang@bnu.edu.cn)

ADP-ribosylation factors (Arf), a subfamily of the Ras superfamily of GTP-binding proteins, was originally described as a cytosolic cofactor activity required for cholera toxin to ADP-ribosylate the α-subunit of the Gs heterotrimeric G-protein (Munro, 2005). They play important roles in intracellular trafficking and regulate a wide variety of intracellular signaling in eukaryotic cells including plant cells (Memon, 2004). In rice, the development of endosperm may be closely related with the process of vesicle trafficking. Vesicle trafficking delivers proteins to intracellular and extracellular compartments, cellulose synthase to the plasma membrane, and non-cellulosic polysaccharides to the cell wall (Gebbie *et al*., 2005). Therefore, the vesicle trafficking is essential in the process of cellulization and proliferation of endosperm cells.

Few studies about ARF gene family were reported in rice now. To clarify the molecular mechanisms of endosperm development process, the members of ARF gene family were predicted according to the rice genomic database. To search ARF genes associated with the development of rice endosperm, the various analysis were made within those predicted ARF genes.

In this study, a genome-wide analysis of rice ARF genes was carried out, and eight new rice ARF genes were identified according the known the sequences of OsARF1 and OsARF2. The ten putative *OsARFs* could fall into two classes based on the comparison of the gene structures and the sequences of deduced amino acid with other known plant and animal ARFs. Six of the ten *OsARFs* are highly analogous with the class1 *ARF* subfamily members in animals and Arabidopsis, while the other *ARF* genes are only identified in plants. The analysis of the six class1 *ARF* gene sequences reveal that they are consist of six exons and five introns. Moreover their amino acid sequences are also highly conserved with similarity over 97%. The other four *ARFs* are less conserved than the class1 *ARF* subfamily members, but contain the similarity more than 80% in their amino acid sequences. RT-PCR analysis was performed in order to investigate the mRNA expression pattern of each member of the *ARF* gene

family in rice. The results show that one of the Os*ARF* genes, *OsARF3*, which belongs to the class1 *ARF* subfamily specifically expressed in young leaf, root and seed. It was demonstrated that *OsARF3* could complement yeast *arf1 arf2* mutants and its GFP-fusion is localized to the Golgi apparatus in plant cells like its animal counterpart. The physiological role of OsARF3 in rice will be further analyzed.

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Intracellular Localization of Integrin-like Protein and Its Roles in Osmotic Stress-induced ABA Biosynthesis in *Zea Mays*

Bing Lv[1], Feng Chen[1], Zhonghua Gong[1], Hong Xie[1], Jianhua Zhang[2], Jiansheng Liang[1*]

[1]*College of Bioscience and Biotechnology, Key Laboratory of Crop Genetics and Physiology of Jiangsu Province, Yangzhou University, Yangzhou, People's Republic of China, 225009*
[2]*Biology Department, Hong Kong Baptist College, Kowloon Tang, Hong Kong*

*E-mail: jsliang@mail.yzu.edu.cn

Plants have evolved many mechanisms to cope with adverse environmental stresses. Abscisic acid (ABA) accumulates significantly in plant cells in response to drought conditions, which has been considered as a major mechanism for plants to enhance drought tolerance. In this study, we explore the possible mechanisms how plant cells perceive the osmotic stress, and as a consequence, induce cells to biosynthesize ABA. Our results showed that a great difference existed between protoplasts and cells in responses to osmotic stress in induction of ABA biosynthesis, implying that cell wall and/or cell wall-plasma membrane interaction play important roles in perceiving osmotic stress. Western Blotting and immuno-fluorescence localization experiment, using polyantibody against rabbit integrin β1, showed that there existed a protein in roots of *Zea Mays*, which was similar to integrin protein of animals and it mainly localized in insoluble fraction of plant cells. Treatment with GRGDS, a synthetic pentapeptide containing RGD domain, which interacted specifically with integrin protein and thus blocked the cell wall-plasma membrane interaction, significantly inhibited osmotic stress induced ABA biosynthesis in cells, but not in protoplasts. Accordingly, we concluded that cell wall and/or cell wall-plasma membrane interaction mediated by integrin-like protein played important roles in osmotic stress- induced ABA biosynthesis in *Zea Mays*.

Cloning, Expression and Characterization of a Plastidial N-glycosylated ADP-glucose Hydrolase

Yohei Nanjo[1], Hiromasa Oka[1], Noriko Ikarashi[1], Kentaro Kaneko[1], Aya Kitajima[1], Toshiaki Mitsui[1], Francisco José Muñoz[2], Milagros Rodríguez-López[2], Edurne Baroja-Fernández[2], Javier Pozueta-Romero[2]

[1] *Laboratories of Plant and Microbial Genome Control and Department of Applied Biological Chemistry, Niigata University, 2-8050 Ikarashi, Niigata 950-2181, Japan;*

[2] *Agrobioteknologiako Instituta, Nafarroako Unibertsitate Publikoa, Gobierno de Navarra and Consejo Superior de Investigaciones Científicas, Mutiloako etorbidea zenbaki gabe, 31192 Mutiloabeti, Nafarroa, Spain*

*E-mail: t.mitsui@agr.niigata-u.ac.jp ; francisco.munoz@unavarra.es

A nucleotide pyrophosphatase/phosphodiesterase (NPP) activity has been shown to occur in the plastidial compartment of both mono- and di-cotyledonous plants that catalyzes the hydrolytic breakdown of ADPglucose (ADPG) (Rodríguez-López et al. 2000 Proc. Natl. Acad. Sci. USA 97, 8705-8710). To further our knowledge about the protein entity responsible for this activity, two NPPs from rice and barley seedlings have been purified and characterized. Both enzymes are glycosylated, since they bind to Concanavalin A, stain with periodic acid-Schiff reagent and can be digested by Endo-H. A complete rice NPP encoding cDNA, designated as *OsNPP1*, was isolated, characterized and overexpressed in transgenic plants displaying high ADPG hydrolytic activity. Computer searches of data banks revealed that OsNPP1 belongs to a functionally divergent group of plant nucleotide hydrolases. OsNPP1 contains numerous N-glycosylation sites and a cleavable hydrophobic signal sequence that does not match with the N-terminal part of the mature protein. Both immunocytochemical analyses and confocal-fluorescence microscopy of rice cells expressing OsNPP1 fused with the green fluorescent protein (GFP) revealed that OsNPP1-GFP occurs in the plastidial compartment.

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Effects of Auxin Inhibitors and Glutamate on Electrotropism of Maize Roots

Camilla Pandolfi, Elisa Azzarello, Sergio Mugnai, Stefano Mancuso*

LINV, Dip. Ortoflorofrutticoltura, University of Florence, viale delle Idee 30, 50019 Sesto Fiorentino, Italy

* Email: stefano.mancuso@unifi.it

Like all living tissues, the cells of plant roots produce an electric field due to the activities of ion transporters. This ion movement creates a flow of current through the tissue and the formation of electrical potential differences across the membrane.

As a consequence of this ion transport activities, plant roots generate long-lasting electric fields in the apoplast and rhizosphere (Weisenseel et al., 1992). These electric fields can polarize cells and tissues and can affect growth of the root. For instance, electric fields may generate a lateral asymmetry of ions and hormones in the elongating zone. Application of electric fields can modify the direction of growth of certain plant cells or organs. This phenomenon, known as electrotropism, has been reported in fungi (McGillavray and Gow NAR 1986) and algae (Brower and Giddings 1980) as well as in roots (Fondren and Moore 1987; Schrank,1959), and shoots (Schrank,1959) of higher plants. The preferred direction of growth relative to the applied electric field varies with the type of cell or organ tested and, in some cases, is species dependent.

In our experiments we examined the effect of electric field on the primary root of maize (*Zea mays L.*). At first we used different intensity of voltage, observing a root curvature towards cathode. Electric field greater than 1 V/cm caused important reduction in root elongation. Then we added other substance to the medium using an electric field of 0,2 V/cm not to interfere with root growth .

Electrotropism was partially inhibited when we used different concentration of TIBA and NPA.

Another interesting phenomenon was observed adding different concentration of glutamate to the medium. Glutamate is one of the 20 standard aminoacids but it is also the most abundant excitatory neurotransmitter in the nervous system. Numerous glutamate receptor-like (*GLR*) genes have been identified in plant genomes, and plant GLRs are predicted, on the basis of sequence homology, to retain ligand-binding and ion channel activity (Davenport 2002). Electrotropic effect on root growth was inverted in presence of glutamate: roots turn towards anode and show a corkscrew

kind of growth. BMAA, a glutamate antagonist, inhibited this glutamate effect on the root apex electrotropism.

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Effect of Secondary Metabolites Associated with Anaerobic Soil Conditions on Ion Fluxes and Electrophysiology in Barley Roots

Jiayin Pang[1], Tracey Cuin[1], Lana Shabala[1], Meixue Zhou[1], Neville Mendham[1], Jiansheng Liang[2], Sergey Shabala[1]

[1]*School of Agricultural Science, University of Tasmania, Private Bag 54, Hobart, Tasmania, 7005, Australia*
[2]*College of Bioscience and Biotechnology, Yangzhou University, Yangzhou, 225009, China*

*Email: jypang@postoffice.utas.edu.au

Waterlogging stress is traditionally associated with O_2 depletion. In addition, a significant accumulation of toxic substances from the microbial reduction processes has been widely reported in waterlogged soil. In this study, the effects of several secondary metabolites (phenolic acids, monocarboxylic acids and Mn^{2+}) on nutrient (K^+, H^+ and Ca^{2+}) acquisition of barley roots were investigated using the non-invasive MIFE® system. All three lower monocarboxylic acids (formic, acetic and propionic acids) and three phenolic acids (benzoic, 2-hydroxybenzoic, 4-hydroxybenzoic acids) caused immediate net influx of H^+ and the reduction of K^+ uptake, while Mn^{2+} treatment caused K^+ to quickly return to the initial level following the net efflux in the first few minutes and gradual increase of H^+ influx. Phenolic acids slightly increased the influx of Ca^{2+} immediate after treatment, but not in other chemicals. Plant roots showed different responses of ion fluxes and membrane potential to these chemicals in the long term (24 h). 24 h treatment with all chemicals significantly reduced the K^+ uptake, and the adverse effects of phenolic acids were smaller than with monocarboxylic acids and Mn^{2+}. Treatment with monocarboxylic acids for 24 h reversed H^+ from net efflux to net influx, while all three phenolic acids did not cause significant effects compared with the control. Phenolic acids caused significant net Ca^{2+} efflux from roots pre-treated for 24 h. The possible model explaining effects of secondary metabolites on membrane transport activity is suggested.

IBA Promotes Lateral Root Formation Via IBA-induced NO Formation and ß-oxidation-like IBA–to–IAA Conversion in Peroxisomes of Pericycle and Endodermis Cells

Markus Schlicht[1], **Diedrik Menzel**[1], **František Baluška**[1]

[1] *Institute of Cellular and Molecular Botany, University of Bonn, Bonn*

Indole butyric acid (IBA) is considered to be an inactive form of auxin, which had to be converted to indole acetic acid (IAA) to show biological activities relevant for the plant growth and development. A process similar to the ß-oxidation of fatty acids within peroxisomes is implicated in this conversion. Currently valid model suggest that ß-oxidation-like process converts IBA to IAA to cause any biological effects.

However, this model can not convincingly explain why IBA induces stronger effects, than IAA, on the formation of lateral roots. Furthermore the *lrt1*-mutant of rice is resistent against the inhibitory effect of auxins (IAA, 2,4-D and IBA) on the root elongation and only IBA, but not IAA, can induce lateral roots (Chhun et al. 2003). The same phenomenon is true also for the *lrt1*-mutant of maize (this work). Moreover, levels of free IBA are nearly equal to the levels of free IAA in young seedlings (Ludwig-Müller and Cohen 2002). Astonishingly, a polar transport of IBA in roots takes place which is independent of PIN1 and AUX1 proteins and not inhibited by the IAA efflux inhibitors like NPA (Rashotte et al. 2003). Mutants with a disturbed polar IBA transport, but normal polar IAA transport, are showing phenotypes which are typical for the polar IAA transport related mutants. For example, these seedlings show disturbed gravisensing (*rib1*, Poupart et al. 2005) or fewer lateral roots (*arm2*, Chhun et al. 2005). Altogether, IBA emerges as biologically active auxin.

Our present results, based on the IAA specific antibody (Schlicht et al. 2006), are showing a first direct proof for a possible IBA-conversion within peroxisomes of endodermis and pericycle. In addition, we show that this tissue-specific ß-oxidation-like conversion is needed for the IBA activity stimulating the lateral root induction via nitric oxide (NO) production. The IBA-induced NO production, in combination with the tissue specific IBA-to-IAA conversion, explain why the weak auxin IBA strongly promotes the lateral root formation despite showing only weak activities for other typical actions of IAA.

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Interaction of Photoreceptors: Signal Memory in *Mougeotia*

Tadeusz Walczak, Halina Gabryś*

Department of Plant Physiology and Biochemistry, Faculty of Biotechnology, Jagiellonian University, Kraków, Poland

Although most plants use blue light for activating chloroplast movements, some ferns, mosses and green algae use two spectral regions, red and blue. Among these species the filamentous alga *Mougeotia scalaris* rotates its ribbon-shaped chloroplast towards a face position in weak blue light and in red light irrespective of its fluence rate, and towards a profile position in strong blue light alone or combined with red. According to the classical hypothesis developed by Haupt in the sixties, the red-controlled rotation is mediated by a gradient of P_{fr} form of phytochrome bound in spirally ordered arrays at the plasma membrane. Evidence for the involvement of an independent blue-absorbing photoreceptor (phototropin?) in the control of weak-light activated response has been provided more than two decades ago [1]. Interaction between the two photoreceptors in the generation of the strong light (face-to-profile) response was investigated by using high energy blue and red light pulses. This technique provided a time-resolved excitation of both photoreceptor systems. The results demonstrated an indirect nature of the interaction between the photoreceptors [2,3]. First, a stable product was formed in a blue light-mediated reaction, having a lifetime of ca. 2 min at room temperature. Subsequently, this blue light signal carrier interacted with phytochrome in its physiologically active, far-red absorbing P_{fr} form. As a result of this interaction a long-lived product was formed capable of inducing the face-to-profile rotation. The induction required elimination of the P_{fr} gradient. The information about the latent face-to-profile response could be stored in the cell for at least 40 min, and displayed after application of far-red light eliminating the phytochrome gradient. Even if the classical phytochrome will be substituted by a recently discovered neochrome as a novel partner of the reaction, its interaction with a phototropin seems to be a prerequisite of the response of *Mougeotia* chloroplasts to strong light.

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The Subcellular Localization and Blue Light-induced Dynamic Relocalization of Phototropin 1 in Root and Shoot Cells of *Arabidopsis thaliana*

Yinglang Wan[1], William R Eisinger[3], Weronika Krzeszowiec[4], Halina Gabrys[4], František Baluška[1], David W. Ehrhardt[2,] Winslow R Briggs[2]

[1]*Institute of Cellular and Molecular Botany,University of Bonn, Kieschallee 1, D-53115 Bonn, Germany*
[2]*Carnegie Institution Department of Plant Biology, 260 Panama Street, CA94305 Stanford, USA*
[3] *Santa Clara University, 500 El Camino Real, CA 95053 Santa Clara, USA*
[4]*The Jan Zurzycki Institute of Molecular Biology, Jagiellonian University, Krakow, Poland*

The blue light receptor phototropin 1 (phot1) is a flavin-binding light-activated protein kinase. It serves as the major photoreceptor for phototropism in both roots and shoots. Although the activation of the C-terminal kinase domain has been characterized, the signal transduction pathway between perception of blue light by phot1 and phototropic responses remains unresolved. In continuation of our previous study with phot1-GFP-transformed Arabidopsis plants lacking phot1 (Sakamoto et al., 2002), here we have investigated patterns of subcellular localizations of phot1-GFP in different cell types in shoots and roots. Driven from the Phot1 promoter, the PHOT1:GFP fusion protein is expressed in almost all plant cells. Expression is especially strong in cells of shoot and root tips. We observed different patterns of phot1 expression and localization in cells of different tissues. For example, in cortical cells of the shoot elongation zone, PHOT1:GFP can be detected mainly at the plasma membrane (PM). GFP signal was especially strong at the apical pole of the cell formed irregular longitudinal stripes along the regions of lateral walls in closest contact with the neighboring cells. In root cortical cells, PHOT1:GFP was mainly observed at the cell poles, where it formed a semicircle pattern. The localization to lateral walls observed in the hypocotyl was not detected in root cortical cells. Following blue-light irradiation, PHOT1:GFP relocalized within approximately xx minutes, forming sharp punctae at the PM and also a marked increase in signal in the cytosol. The extent of relocalization is related to the intensity of the blue light and the exposure time. Experiments with brefeldin A demonstrated that the increased signal in the cytosol does not arrive from the ER, and suggested that blue-light activates endocytosis and recycling of membrane-bound PHOT1:GFP. In support of this notion, both cold treatment and depolymerization of all F-actin with latrunculin B inhibit PHOT1:GFP internalization. Moreover, internalized PHOT1:GFP co-localized with FM4-64 labeled compartments within the cytoplasm, suggesting that PHOT1:GFP may become associated with endosomes in this process. We propose that the BL-induced PHOT1 recycling between the plasma membrane and endiosomes may be involved in the signal-transduction pathway for the phototropism of plant organs.

Dynamic Imaging of Secretory Vesicles in Living Pollen Tubes of *Picea meyeri* using Evanescent Wave Microscopy

Xiaohua Wang[1,2], Yan Teng [3], Qinli Wang[1,2], Xiaojuan Li[1,2], Maozhong Zheng[1,2], Jozef Šamaj[4,5], František Baluška[4,6] and Jinxing Lin[1*]

[1] *Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China*
[2] *Graduate School of Chinese Academy of Sciences, Beijing 100049, China*
[3] *Department of Biology, Howell Science Complex, East Carolina University, Greenville, NC27858-4353, USA*
[4] *Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany*
[5] *Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademicka 2, SK-95007, Nitra, Slovak Republic*
[6] *Institute of Botany, Slovak Academy of Sciences, Dubravska 14, SK-84223, Bratislava, Slovak Republic*

*E-mail: linjx@ibcas.ac.cn

Evanescent wave excitation was used to visualize individual, FM4-64-labeled secretory vesicles in an optical slice proximal to the plasma membrane of *Picea meyeri* pollen tubes. A standard upright microscope was modified to accommodate the optics used to direct a laser beam at a variable angle. Under evanescent wave microscopy or total internal reflection fluorescence microscopy, fluorophores localized near the surface were excited with evanescent waves, which decay exponentially with distance from the interface. Evanescent waves with penetration depths of 60 to 400 nm were generated by varying the angle of incidence of the laser beam. Kinetic analysis of vesicle trafficking was made through a ~300-nm optical section beneath the plasma membrane using time-lapse evanescent wave imaging of individual fluorescently labeled vesicles. Two-dimensional (2-D) trajectories of individual vesicles were obtained from the resulting time-resolved image stacks and were used to characterize the vesicles in terms of their average fluorescence and mobility, expressed here as the 2-D diffusion coefficient D $^{(2)}$. The velocity and direction of vesicle motions, frame-to-frame displacement, and vesicle trajectories were also calculated. Analysis of individual vesicles revealed for the first time that two types of motion are present, and that vesicles in living pollen tubes exhibit complicated behaviors and oscillations that differ from the simple Brownian motion reported in previous investigations. Furthermore, disruption of the actin cytoskeleton had a much more pronounced effect on vesicle mobility than did disruption of the microtubules, suggesting that actin cytoskeleton plays a primary role in vesicle mobility.

Reorganization of Cytoskeleton in Rice Cells during the Early Stages of Disease Development Caused by *Magnaporthe grisea*

Min-He Yang[1], Zhong Zheng[2]

[1]*College of Life Science, Fujian Normal University, Fuzhou, Fujian 350007, China;*
[2]*Department of Plant Protection, Zhejiang University, Hangzhou, Zhejiang 430070 China*

The procedures of immunostaining of microtubules and microfilaments were examined in inner epidermal cells of rice leaf sheath. The results demonstrated that microtubules were successfully detected in detached single layer of rice leaf sheath after fixed in a mixed solution of 4% paraformaldehyde and 1% glutaraldehyde combined with enzyme digestion of rice cell wall for 1-2 min. Netted distribution of microtubules was observed in inner epidermal cells of rice leaf sheath. Microfilaments were distributed in parallel with the longitudinal axis of rice cell. The results also suggested that actin filaments were sensitive to paraformaldehyde and glutaraldehyde fixation. And pretreatment of rice tissue with 1mmol/L MBS (3-maeimidobenzoic acid N-hydroxysuccinimide ester) for 30 min was critical for the detection of microfilaments in cells of rice leaf sheath. *M. grisea* penetration resulted in rapid and striking reorganization of microtubules and microfilaments in cells of rice leaf sheath. The distribution patterns of cytoskeleton in the incompatible interaction of rice and *M. grisea* were different from those in compatible interaction. In cells undergoing resistant response, microtubules and microfilaments of rice cells radiated to fungal penetration site during the process of fungal penetration. Then they were gradually fragmented and eliminated from host cells after hypersensitive cell death occurred. However, in cells undergoing susceptible response, the cytoskeleton was severely damaged and fragmented quickly at the initial stage of fungal penetration. As the disease development, fine bundles of cytoskeleton were seldom observed in host cells although the cytoskeleton of *M. grisea* was normally detected.

Glutamic Acid Decarboxylase is Involved in the Regulation of Tobacco Pollen Tube Growth

Guanghui Yu, Mengxiang Sun*

Key Laboratory of MOE for Plant Developmental Biology, College of Life Sciences, Wuhan University, Wuhan 430072, China

E-mail: mxsun@whu.edu.cn ; yusheen@163.com

Calcium is a key regulator of pollen tube growth, but little is known concerning the downstream components of the signaling pathways involved. Glutamic acid decarboxylase (GAD)， as a down-regulatory molecule of Calmodulin, was investigated in present work. To analyze its potential roles in the pollen tubes growth, both Ca^{2+} chelator ethyleneglycol-bis (β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA) and Calmodulin antagonist trifluoperazine (TFP) were employed in the experiments. The results showed that GAD was constituently expressed in the pollen grains and pollen tubes. Moreover, it was tip localized and asymmetrically distributed in the pollen tubes. The inhibitory effect of the EGTA and TFP on the growth of pollen tubes could be partially ameliorated by the addition of 1mmol/L exogenous γ-Aminobutyric acid (GABA). This was further confirmed by the use of 3-mercaptopropinic acid (3-MPA), which is a relatively specific inhibitor of GAD. The experiments demonstrated that the exogenous GABA could also decrease the inhibitory effect of 3-MPA (at lower concentration) on pollen tube growths (statistical significance $p<0.01$). Additionally, coming along with the morphological alteration of pollen-tube tip after 3-MPA treatment at higher concentration, actin filaments disorganized, the speed of pollen tubes growth and cytoplasmic streaming were much decreased, orientation of vesicles trafficking was also misleaded. Our results suggest that GAD is a down modulatory molecular of Ca^{2+}/CaM and plays an essential role in the regulation of pollen tube growth possibly via controlling the traffic of secretory vesicles.

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Roles of the Ubiquitin/Proteasome Pathway in Pollen Tube Growth with Emphasis on MG132-Induced Alterations in Ultrastructure, Cytoskeleton and Cell Wall Components

Xianyong Sheng [1, 2] Zhenghai Hu [2], Hongfei Lü[1], Xiaohua Wang[1], František Baluška[3, 4] Jozef Šamaj[3, 5], and Jinxing Lin[1]

[1] Institute of Botany, The Chinese Academy of Sciences, Key Laboratory of Photosynthesis and Molecular Environment Physiology, Beijing 100093, China; [2] College of life Science, Northwest University, Xi'an, 710069, China; [3] Institute of Cellular and Molecular Botany, Rheinische Friedrich- Wilhelms- University Bonn, Department of Plant Cell Biology, Kirschallee 1, D-53115 Bonn, Germany; [4] Institute of Botany, Slovak Academy of Sciences, Dubravska 14, SK-84223, Bratislava, Slovak Republic; [5] Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademicka 2, SK-95007, Nitra, Slovak Republic

The ubiquitin/proteasome pathway represents one of the most important proteolytic systems in eukaryotes and has been proposed as being involved in pollen tube growth, but the mechanism of this involvement is still unclear. Here, we report that proteasome inhibitors MG132 and epoxomicin significantly prevented *Picea wilsonii* pollen tube development and markedly altered tube morphology in a dose- and time-dependent manner; while hardly similar effects were detected when Cys-protease inhibitor E-64 was used. Fluorogenic kinetic assays using fluorogenic substrate sLLVY-AMC confirmed MG132-induced inhibition of proteasome activity. The inhibitor-induced accumulation of ubiquitinated proteins was also observed using immunoblotting. TEM revealed that MG132 induces ER-derived cytoplasmic vacuolization. Immunogold-labeling analysis demonstrated a significant accumulation of ubiquitinated proteins in degraded cytosol and dilated ER in MG132-treated pollen tubes. Fluorescence labeling with FITC-phalloidin and β-tubulin antibody revealed that MG132 disrupts the organization of F-actin and microtubules, and consequently affects cytoplasmic streaming in pollen tubes. However, tip-focused Ca^{2+} gradient, albeit reduced, seemingly persists after MG132 treatment. Finally, fluorescence labeling with anti-pectin antibodies and calcofluor indicated that MG132 treatment induces a sharp decline in pectins and cellulose. This result was confirmed by FTIR analysis, thus demonstrating for the first time the inhibitor-induced weakening of tube walls. Taken together, these findings suggest that MG132 treatment promotes the accumulation of ubiquitinated proteins in pollen tubes, which induces ER-derived cytoplasmic vacuolization and depolymerization of cytoskeleton, and consequently strongly affects the deposition of cell wall components, providing a mechanistic framework for the functions of proteasome in the tip growth of pollen tubes.

Proteomic Analysis of Differentially Expressed Protein by Mannitol-induced Osmotic Stresses in Rice

Xin Zang*, Setsuko Komatsu and Tuansheng Shi***

**Bioengineering Department, Zhengzhou University, Zhengzhou 450052, China*
***National Institute of Agrobiological Sciences, Tsukuba 305-8602, Japan*

Plants are continuously exposed to biotic and abiotic stresses that endanger their survival. Among abiotic stresses, osmotic stress is one of the most severe, caused by drought, high salinity and cold stresses in nature. In this study, to investigate the response of rice to osmotic stress induced by mannitol, proteins from basal part of rice leaf sheath were screened by a proteomics approach.

Two-week-old rice seedings were treated with 400 mM mannitol for 48 h. After separation of proteins from basal part of leaf sheath by two-dimensional polyacrylamide gel electrophoresis, 327 proteins were identified. Among them, 15 proteins significantly responded to osmotic stress by up- or down regulation, as 12 proteins were increased while 3 proteins were decreased. The expression profile of unique proteins was further confirmed by dose- and time-dependent parameters. The changed protein spots were analyzed by protein sequencer and mass spectrometry. Homology searches were carried out using the FastA or Mascot search tools. N-terminal sequences were successfully obtained for eight of these proteins. They were 26S proteasome regulatory subunit, calreticulin precursor, heat shock protein, dnaK-type molecular chaperone, uroporphyrinogen decarboxylase, two proteins encoded by Oryza sativa genomic DNA and a function unknown protein. All the rest protein spots were N-terminally blocked. One of them was identified as glutathione S-transferase by internal sequencing. Among the other seven proteins, three were not identified while four were identified as endosperm lumina bnding protein, lipid transfer protein, glyoxalase I and alpha subunit of 20S proteasome.

Moreover, comparative proteomics approach was carried out among different stress treatments, different tissues and different cultivars. These findings have important implications for understanding the biochemical and molecular mechanisms of plant adaptation and response to osmotic stress.

Interactions Between Shoot Derived Auxin Transport, Root Auxin Metabolism and Phenolic Accumulation in the Regulation of Iron-deficiency Stress Responses in Red Clover

Shao Jian Zheng[1], Chong Wei Jin[2]

[1]*Key State Laboratory of Plant Physiology and Biochemistry, College of Life Science;*
[2]*College of Environmental and Resource Science, Zhejiang University, Hangzhou 310058, China*

In the present research, an Fe-efficient plant species, red clover (*Trifolium pratense* L.), was used to investigate the role of shoot-derived signals and phenolic metabolism in regulating Fe-deficiency responses. Both the application of the inhibitor of polar auxin (IAA) transport, TIBA (2, 3, 5-triiodobenoic acid) to the stem as well as decapitation of the shoot apex, significantly decreased root ferric reductase activity induced by Fe deficiency. The inclusion of exogenous IAA in the nutrient solution bathing the roots only slightly enhanced the ferric reductase activity in the Fe-sufficient plant, but remarkably enhanced this activity in Fe-deficient plants. In addition, both stem TIBA application and shoot decapitation completely inhibited the stimulated root proton extrusion and subapical root hair development. Exogenous IAA application to roots of Fe-sufficient plants also induced subapical-root hair development, further supporting the role of shoot-derived IAA in these responses. Another of the Fe deficiency stress responses, root phenolic secretion, was not affected by stem TIBA treatment, suggesting that shoot-derived IAA is not involved in regulating this Fe-deficiency stress response. While Fe deficiency enhanced root phenolic levels, it inhibited root IAA-oxidase activity and increased the endogenous root IAA levels significantly. A link between these two processes is suggested by the demonstration that, phenolics extracted from roots of Fe deficient plants inhibited IAA-oxidase activity *in vitro*, and this inhibition was greater than with phenolic extracted from roots of Fe sufficient plants. Based on these observations, we propose a model where under Fe deficiency stress in dicots, an increase in root phenolic concentrations plays a role in regulating root IAA levels through an inhibition of root IAA oxidase activity. This response, along with a stimulation of shoot to root IAA transport in response to Fe deficiency, leads to an increase in root IAA levels, which in turn help induce increased root ferric reductase activity, proton extrusion and subapical-root hair development. This is the first report to demonstrate a significant role for the commonly recognized phenomena of phenolic accumulation in dicot roots under Fe deficiency in helping regulate a suite of Fe deficiency responses through interactions with root IAA oxidase activity. These findings are helping to advance our understanding of the signaling pathways associated with the response of Strategy I plants to Fe deficiency.

Cytoskeleton Antagonistic effects on Mitochondria Motilities Revealed by Dynamic Imaging of Mitochondria in Living Pollen Tubes

Maozhong Zheng[1], Yan Teng [2], Xiaohua Wang[1], Qinli Wang[1] and Jinxing Lin[1]

[1] *Key Laboratory of Photosynthesis and Molecular Environment Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China*
[2] *Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China*

Mitochondria has been revealed to serve as fundamental elements in intracellular signaling, such as in calcium signal and participants in cell death programs, except for being envisioned to be cellular power plants. Previous investigations showed that actively mitochondrial movements were indispensable to fulfill strategic mitochondrial localization at particular subcellular sites. Up to date, mitochondrial dynamics have been studied extensively using yeast or animals, and are known to vary considerably depending on the cell type and organism studied. For plant cells, few studies have been conducted and therefore little is known about dynamics of plant mitochondria. None has been reported on pollen tubes with actively cytoplsamic streaming.

In order to determine how cytoskeleton and the relevant molecular motors affect mitochondrial motility and to study the mechanisms underlying the mitochondrial distribution, transporting and positioning in *Picea wilsonii* pollen tubes, the dynamic nature of mitochondria motion was characterized after the pollen tubes were stained with mito-tracker Red dye and treated with three cytoskeleton inhibitors and relevant molecular motor inhibitors by means of laser scanning confocal microscope and evanescent wave microscope..

The results showed that the actin filament disrupting drug latrunculin B and the myosin ATPase inhibitor 2, 3-butanedione 2-monoxime severely decreased the mitochondrial motility (decreased 82.2% and 61.0% respectively). Interestingly, microtubules disrupting agent oryzalin and dynein inhibitor vanadate slightly increased the motility, while kinesin inhibitor AMP-PNP showed no affect and taxol slightly inhibited mitochondrial motility. Further observations through evanescent microscope provided the two-dimensional (2-D) trajectories, 2-Ddiffusion coefficient, velocity and direction of mitochondria motions. It was found that mitochondria can move toward the tip, away from the tip or across the tube in different trajectories. Four classes of mitochondrial movement are detected: rapid movements at average

velocity of 7.1μm/s, slower movement at average velocities of 1.6μm/s, wiggling mitochondria at average velocities 0.74μm/s and no movement at all. Drug treatment complicatedly altered mitochondrial dynamics, including velocity and distribution. This research demonstrated that microtubules and microfilaments are both responsible for mitochondrial motility and distribution in *Picea wilsonii* pollen tubes. Actin filaments served as track for mitochondrial transport, which was primarily powered by myosin ATPase while the most of mitochondria perhaps can use microtubule polymerization dynamics for the positionging. In the other hand, microtubule also may be affected mitochondria motility by mediating, direct and indirect, microfilament organization.