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Plenary lecture

PL2.1

Glutathione-dependent regulation of cell proliferation and root meristem development

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Intracellular redox homeostasis is central to the regulation of cellular functions that underpin the control of plant growth and development. Causal links between cellular redox state, cell proliferation and shoot and root meristem activity have been described but in most cases the underlying mechanisms remain to be characterized. The tripetide thiol reduced glutathione (GSH) is required for cell proliferation and root meristem formation. However, the enzymes and processes that respond to changes in GSH and the redox state of the glutathione pool are poorly characterised. Molecular genetics and pharmacological approaches, *in vivo* cell staining and redox-sensitive green fluorescent protein (roGFP) techniques were used to explore the redox state of the nucleus and cytoplasm and to identify GSH-responsive genes. Data will be presented concerning the glutathione redox potential of the nucleus and cytoplasm in the root meristem of *Arabidopsis thaliana* seedlings and how this impacts on the expression of GSH-responsive genes, particularly transcription factors and genes that are involved in the cell proliferation, redox regulation and auxin-mediated processes.

Lectures

L2.1

A membrane bound NAC transcription factor is a regulator of mitochondrial retrograde regulation of the oxidative stress

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Upon disturbance of their function by stress, mitochondria can signal to the nucleus to steer the expression of responsive genes. This mitochondria-to-nucleus communication is often referred to as mitochondrial retrograde regulation (MRR). Although reactive oxygen species and calcium are likely candidates for MRR, the protein signalling components in plants remain largely unknown. Through meta-analysis of transcriptome data, we detected a set of genes that are common and robust targets of MRR and used them as a bait to identify transcriptional regulators of MRR. In the upstream regions of these mitochondrial dysfunction regulon (MDR) genes, a *cis*-regulatory element, the mitochondrial dysfunction motif (MDM) was found that is necessary and sufficient for gene expression under various mitochondrial perturbation conditions. We demonstrated that specific NAC transcription factors mediate MRR-induced expression of the MDR genes by direct interaction with the MDM *cis*-regulatory element and trigger an increased oxidative stress tolerance.

Oral presentations

O2.1

Calcium dependent regulation of the *Arabidopsis* NADPH oxidase RbohF by CBL-CIPK complexes

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Many abiotic and biotic stimuli trigger responses that involve ROS and calcium accumulation in plants. However, the nodes of interaction between these two important second messengers are largely unknown. Rbohs are plant NADPH oxidases that are membrane localized and produce ROS. So far no ROS sensor proteins have been identified in plants. In contrast, different groups of calcium sensor proteins have been identified and characterized. Each member of the calcium sensor family designated as CBLs interacts with a defined subset of the kinase family of CIPKs. Together, the CBL-CIPK complexes translate a calcium signal into phosphorylation of a target protein. Here we report that the *Arabidopsis* kinase CIPK26 interacts with RbohF *in vivo* and phosphorylates RbohF *in vitro*. In addition, CIPK26 interacts with CBL1 and CBL9. To investigate the ROS-producing activity of RbohF *in vivo*, it was expressed heterologously in human HEK293T cells. Coexpression of CIPK26-CBL1 or CIPK26-CBL9 complexes with RbohF dramatically increased a calcium induced ROS production, while expression of RbohF and CBL1, CBL9 or CIPK26 alone did not. These results establish a direct functional interconnection between calcium and ROS signalling.

O2.2

Interplay of two PP2A B subunits in high light acclimation

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Serine/threonine protein phosphatase 2A (PP2A) family members carry out crucial functions in the regulation of signalling through phosphorelay cascades in animals and plants. The predominant form of PP2A is heterotrimer, consisting of a catalytic subunit C, a scaffold subunit A, and a highly variable regulatory subunit B, which is thought to determine the target specificity of subunit C in the PP2A holoenzyme. B'gamma subunit of PP2A was identified as a component in the crosstalk between light acclimation, disease resistance and ageing in the model plant *Arabidopsis thaliana*. Pharmacological approaches suggested that B'gamma modulates cellular ROS homeostasis through the mitochondrial ALTERNATIVE OXIDASE (AOX) pathway. Accordingly, proteomic analysis revealed that the level of AOX1D was increased in mutant plants deficient in B'gamma. Tracking structure-function relationships between B'gamma and a highly similar B'zeta further revealed that these two regulatory subunits cooperate on common signalling pathways. This was reflected in increased resistance to combined high light and drought stress in *pp2a* mutant plants.

O2.3

Dissecting the action of H_2O_2 after its metabolic formation in chloroplasts

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Reactive oxygen species (ROS) are toxic cellular metabolites that also act as signalling molecules. In previous work, we developed a novel tool to functionally dissect the action of plastidic generated-H_2O_2, using plants overexpressing glycolate oxidase (GO) in the plastids of *Arabidopsis thaliana*. Under photorespiratory conditions GO uses the glycolate derived from the oxygenase activity of RubisCO and produces H_2O_2 inside the chloroplasts. In this work GO plants were used to assess the expressional behaviour of ROS-responsive genes and transcription factors (TFs) after metabolic induction of H_2O_2 formation in chloroplasts. By using quantitative real-time PCR (qRT-PCR) platforms, we test the expression of 187 ROS-responsive genes and 1880 TFs after transferring high CO_2-grown plants to ambient CO_2 concentration. Our data revealed coordinate expression of functional related genes, including genes involved in camalexin and indole glucosinolate biosynthesis within 0,5h after induction of H_2O_2 formation in the chloroplasts. Comparative analysis using available microarray data suggests that signals for the induction of these genes through H_2O_2 may originate in chloroplast.

O2.4

Singlet oxygen emerges as a common theme in the plant response to multiple stresses

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Singlet oxygen is widely assumed to be a harmful product of excess light energy during photosynthesis. An additional source of singlet oxygen is a mutant dubbed *flu*. Through bioinformatics analysis, we found evidence for singlet oxygen production, during multiple stresses, exemplified via high correlation to the *flu* mutant. We first defined a core genes set (CGS) of 119 genes that are induced by singlet oxygen, biotic and abiotic stress and validated their expression under representative stresses and photodynamic production of singlet oxygen. Strikingly, the stress-related transcripts were induced independent of light. To examine in-vivo for the possibility of singlet oxygen production we used the fluorescent dye, Singlet Oxygen Sensor Green (SOSG). We observed induction of SOSG fluorescence under various biotic and abiotic stresses in root tips, a tissue devoid of chlorophyll, in the dark. SOSG subcellular localization studies point to its accumulation in mitochondria, peroxisomes and the nucleus suggesting multiple compartments as possible origin or target for singlet oxygen. Collectively, the results suggest that singlet oxygen can emanate from compartments other than the chloroplast in a light independent manner.

Posters

P2.1

Whole-plant acclimation to photooxidative stress: from photoprotection to leaf growth and root architecture

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Being sessile, plants respond to changing environments by adjusting their phenotypes through processes called acclimation. Recently we have demonstrated in *Arabidopsis thaliana* that fluctuating light conditions mainly induce acclimatory responses against photooxidative stress without allowing enhancement of carbon utilization. On a leaf level, acclimation entailed upregulation of thermal energy dissipation and superoxide dismutase activity as well as increased accumulation of xanthophylls. Furthermore, reduction in chlorophyll contents together with changes in leaf morphology (from cup-shaped lamina under low light to flat lamina with a shorter petiole under fluctuating light) contributed to less light absorption. In parallel with these responses in the aboveground, primary root growth decelerated in the belowground, with no significant effect on lateral root formation. However, mutants having shade-type carotenoid compositions responded to fluctuating light by decreasing the number of lateral roots while maintaining primary root growth, resulting in altered root architecture. Our results point to shoot-root signalling in whole-plant acclimation to photooxidative stress and a role of carotenoids therein.

P2.2

Real-time monitoring of the extracellular redox potential of tobacco suspensions during plant/bacterial interactions

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In both plants and animals, there has been a strong focus on reactive oxygen species and antioxidants in regard to stress responses. This has led to an awareness of the importance of "redox potential" as a prime regulatory determinant of cellular function and responses to stimuli. It has been difficult to study and monitor redox potential during many interactions, since most techniques involve metabolite extraction and quantification. We tested a method monitoring the open-circuit potential of a platinum electrode placed in plant cell suspensions treated with various bacterial strains. In most interactions with avirulent and saprophytic bacteria, a rapid oxidative increase in potential occurred several hours after inoculation. The timing and extent of the increase was unique for different bacteria. The redox responses are coincident with phenolic changes in the extracellular fluid of the suspensions. Preliminary results suggest that the increase in the open-circuit potential in some interactions may be due to oxidized phenolic intermediates. The technique offers insight into using electrodes to measure redox potential in planta.

P2.3

Involvement of reactive carbonyl species in oxidative stress-induced programmed cell death in tobacco BY-2 cells

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Reactive oxygen species (ROS) are involved in oxidative signalling in plant cells, but the mechanisms of ROS action to start signalling events have been largely unclear. Lipid-peroxide derived reactive carbonyl species (RCS), formed downstream of ROS, are recently suggested to mediate oxidative signalling. In this study, we tested the signalling role of RCS in the induction of programmed cell death (PCD) in tobacco BY-2 cells. The cells were incubated with hydrogen peroxide (H_2O_2) and we observed DNA fragmentation and TUNEL positive nuclei as the hallmarks of PCD. HPLC analysis showed that H_2O_2 treated cells accumulated significantly higher amounts of RCS such as acrolein, 4-hydroxy-(E)-2-hexenal and 4-hydroxy-(E)-2-nonenal. The RCS scavengers carnosine and hydralazine added to the H_2O_2 treated cells decreased these aldehydes and suppressed PCD. Moreover, we confirmed that acrolein induced PCD and RCS scavengers suppressed the acrolein-induced PCD. HPLC analysis also showed that several RCS were increased by the acrolein treatment and the RCS scavengers significantly lowered their levels. Thus, RCS are involved in the H_2O_2-induced PCD. These results demonstrate a signalling role of RCS in the ROS induced events in plants.

P2.4

H_2O_2 production in chloroplasts, its outward diffusion through aquaporins, and the effects of light intensity

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Production of both superoxide radical and H_2O_2 was measured in thylakoids, using ESR detectors. The increased production of these ROS at increased light intensity was shown to result from their production inside the membrane. H_2O_2 is produced there in the reaction of superoxide with PQH2, providing signalling about redox state of the PQ-pool. The appearance of H_2O_2 outside intact chloroplasts also increased in stronger light. It was observed, using H2DCF-DA producing the fluorescent DCF in the reaction with H_2O_2 that in illuminated protoplasts a cessation of cytoplasmic streaming coincided with slow-up of DCF fluorescence rise in chloroplasts and with simultaneous speed-up of such rise in cytoplasm. It was found that H_2O_2 left intact chloroplasts through aquaporins. The effect of light intensity on expression of carbonic anhydrase (CA) genes in *Arabidopsis* was studied, taking into account the link between aquaporin and CA. An increase in light intensity stimulated the expression of genes coding certain chloroplast CAs. This implies that the chloroplast aquaporins linked with these CAs have different capacities at various light intensities and thus regulate the H_2O_2 diffusion from chloroplasts.

P2.5

The impact of hydrogen peroxide and hydroxyl radical on ion channels activation in the liverwort *Conocephalum conicum*

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Bioelectric signals transmission is one of the most common features of living cells. This electric phenomenon occurs due to the fluxes of ions passing through the ion channels, located in the cell membrane. Reactive oxygen species (ROS) change the activity of ion channels. The aim of the research was to determine the influence of hydrogen peroxide (H_2O_2) in the concentrations of 10-100 mM as well as copper chloride ($CuCl_2$) and ascorbic acid (asc) – causing the generation of hydroxyl radical (OH*) in the concentrations of 1-10 mM – on activation of ion channels present in the cell membrane of the liverwort *Conocephalum conicum*. The research was conducted using the method of membrane potential measurement by intracellular microelectrodes. Application of hydroxyl radical in concentration higher than 1 mM resulted in vanishing series of action potentials (AP), leading to permanent depolarization of the membrane potential. Hydrogen peroxide caused similar response as hydroxyl radical in concentration lower than 20 mM; sustained depolarization could be observed in higher concentrations. Potassium channel inhibitor (TEA, 10 mM) and anion channel inhibitor (A9C, 2 mM) caused the suppression of AP series triggered by ROS.

P2.6

Impact of priming process on ROS concentration and the activity of enzymatic antioxidant system in sugar beet (*Beta vulgaris*) seeds

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Priming is a seed enhancement method that may improve seed performance under different environmental conditions. This process brings seed to a controlled level of humidity, which guarantees the initial course of metabolic processes associated with germination. In this particular study is characterized a new developed method for a sugar beet priming technology called SMP (Solid Matrix Priming), which involves usage of a solid substances as a water carrier. It is demonstrated that the optimal time of sugar beet seeds treatment is a period of 24 hours. Priming increases germination speed, ability and uniformity in both laboratory and field conditions. It is postulated that the antioxidant enzymatic system may be a useful marker of the effect of priming process progress. Therefore the changes in the level of reactive oxygen species (ROS) and activities of some enzymes of this system in primed sugar beet seeds are investigated. Increase in concentration of ROS (i.e. H_2O_2 and $O_2^{\bullet -}$) level at the early stage of germination of primed seeds is observed, followed then by significant decrease at the end of these process. The results indicate that alteration in ROS content is associated with modification of activity of substrate non-specific peroxidase (POX) and catalase (CAT), with the tendency to be significantly higher in primed seeds. As superoxide dismutase (SOD) activity was not affected, the POX and CAT seem to play a major role in enhancement of seed germination ability due to priming.

P2.7

Limits in the use of cPTIO as nitric oxide scavenger and EPR probe in plant cells and seedlings

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The importance of nitric oxide (NO) in plant signalling has emerged in the last decades. Despite this recognized biological role, the sensitivity and the effectiveness of the methods used for measuring NO concentration in plants are still under discussion. In the present work we report the constraints of using 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) as spin trap for quantitative measurement of NO in biological samples. The EPR analyses on *Arabidopsis* cell cultures and seedlings show that cPTIO(NNO) is degraded in tens of minutes while the (INO) compound, produced by cPTIO and NO reaction, has not even been detected. The limitations of using this spin trap for quantitative measurements of NO in plant systems are discussed. Furthermore cPTIO is widely used as NO scavenger in plant research in combination with DAF fluorescent dye. However the dependence of DAF fluorescence on cPTIO and NO concentrations have not been fully investigated so far, and may be accountable for the variability of NO-scavenging efficacy of cPTIO. In this light a systematic study on cPTIO NO-scavenging properties has been performed, since this was still lacking for plant system applications.

P2.8

Membrane-bound NAC transcription factors mediate mitochondrial retrograde regulation of the oxidative stress response

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Upon disturbance of their function by stress, mitochondria can signal to the nucleus to steer the expression of responsive genes. This mitochondria-to-nucleus communication is often referred to as mitochondrial retrograde regulation (MRR). Although reactive oxygen species and calcium are likely candidates for MRR, the protein signalling components remain largely unknown. Through meta-analysis of transcriptome data, we detected a set of genes that are common and robust targets of MRR and used them as a bait to identify transcriptional regulators of MRR. In the upstream regions of these mitochondrial dysfunction regulon (MDR) genes, a *cis*-regulatory element, the mitochondrial dysfunction motif (MDM) was found that is necessary and sufficient for gene expression under various mitochondrial perturbation conditions. Yeast one-hybrid analysis and electrophoretic mobility shift assays revealed that five transmembrane domain containing NAC transcription factors bound to the MDM *cis*-regulatory element. We further demonstrate that specific NAC transcription factors mediate MRR-induced expression of the MDR genes by direct interaction with the MDM *cis* - regulatory element and trigger an increased oxidative stress tolerance.

P2.9

High light exposure of leaves elicits rapid changes in hydrogen peroxide level: new insights and limitations using HyPer

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Exposure of leaves to high light (HL) as been shown to elicit hydrogen peroxide (H_2O_2) in cells of various tissues. H_2O_2 can elicit oxidative stress and irreversible photoinhibition. Under moderate increases in light intensity, typically <10-fold above growth intensities, H_2O_2 has been proposed to be a signalling molecule. However, we still have no accurate determinations of the amount, timing or subcellular distribution of H_2O_2. The recent development of two genetically encoded green fluorescent protein (GFP)-based sensors, HyPer and pHRed has made it possible to develop powerful methods for monitoring non-invasively the changes of H_2O_2 and pH *in vivo*.We show a family of 8 codon-optimised HyPer-based binary Ti plasmids that drive the expression of the reporter in 8 subcellular locations, using *Agrobacterium*-based transient expression in *Nicotiana benthamiana* leaves. Using transiently expressed HyPer in epidermal cells of HL-exposed leaves, changes in HyPer fluorescence will be described in the chloroplast stroma surface the chloroplast, cytosol and nucleus. This will be related to changes in H_2O_2 concentrations, but also of pH observed using the pHRed sensor.

P2.10

Cysteine-rich receptor-like kinases (CRK) in ROS signalling in *Arabidopsis thaliana*

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The receptor-like kinases (RLKs) constitute a major gene family in land plants with more than 610 members in *Arabidopsis*. RLKs have been implicated in many aspects of plant life from development to responses to the environment. The Cysteine-rich RLK (CRK) sub-family with 44 members is one of the largest RLK sub-families in *Arabidopsis*. In their extracellular region the CRKs possess two copies of the DUF (domain of unknown function) 26 domain. The DUF26 domain is characterized by a C-X8-C-X2-C motif, which might have a function in redox regulation and/or protein-protein interaction. Several CRKs have been shown to be transcriptionally induced by different stresses (Wrzaczek et al., 2010). In order to address the role of the CRKs in reactive oxygen species (ROS) signalling, sensitivity to apoplastic and chloroplastic ROS production were tested using a *crk* T-DNA insertion mutant collection. Several *crk* mutants displayed increased sensitivity towards O_3 compared to wild type. Selected *crk* knockouts also showed enhanced sensitivity to extracellular ROS production by Xanthine/Xanthine Oxidase and high light stress treatments compared to Col-0. Our data suggest that the CRKs are important elements in ROS signalling.

2.11

Bundle sheath-specific expression of the ascorbate peroxidase 2 affects photosynthesis in whole plant

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Ascorbate peroxidases (APX) are enzymes that scavenge hydrogen peroxide (H_2O_2), which despite its oxidative features, plays also an important role in signalling. APX2 is one of the cytosolic peroxidases, but is expressed exclusively in the bundle sheath cells upon stress conditions. Plants lacking the APX2 do not display altered growth under normal conditions, when compared to wild type plants (WT). However photosynthetic parameters in non-stressed plants are changed. Our results of fluorescence measurements suggest that *apx2* plants possess less reactive centres (RC), thus show impaired electron transfer through components of electron transport chain. It suggests that there may be some aberrations in the photosystems architecture in *apx2*. qPCR analyses revealed that expression of genes encoding light harvesting proteins is higher in *apx2* than in WT, what may compensate the lowered number of RCs in *apx2*. Genes involved in water splitting seem to be not affected, what stays in accordance with the measured water splitting efficiency. Our results indicate that bundle sheath-specific expression of *APX2* could influence the whole plant photosynthesis.

P2.12

Differential dynamic proteome analysis of the oxidative stress response in *Arabidopsis* wild type and catalase mutants

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A comparative study of protein expression levels in wild type plants and plants with enhanced H_2O_2 concentrations, the so called catalase mutants, was conducted in time to capture the dynamics of the H_2O_2 response. Because CAT2 is a major H_2O_2 scavenger the RNAi knockdowns of this gene can be used as an experimental model system for non-invasive H_2O_2 modulation. Both wild type plants and catalase mutants were exposed to photorespiratory inducing conditions and via shotgun proteomics it was possible to discover on a proteome-wide scale the H_2O_2 induced differences. Additionally at these time points the methionine oxidation status was monitored using the COFRADIC technology. This gel-free technique is based on the concept of diagonal chromatography and allows the isolation of specific peptides in highly complex mixtures. The method is more sensitive than two-dimensional gel-based methods, directly identifies the site of modification and avoids the problem of non-specific binding of antibodies. Assessing both changing protein abundancies and a specific posttranslational modification will create new insights in the complex dynamic proteome of plants during oxidative stress.

P2.13

Testing the role of reactive oxygen and nitrogen species in stomatal signalling

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Several lines of evidence support a role for reactive oxygen species (ROS) in stomatal signalling: a short pulse of ozone leads to transient stomatal closure, the *rbohD×rbohF* double mutant deficient in superoxide production has impaired stomatal response to ABA and mutants leading to increased ROS production in the chloroplast have defects in stomatal function. Many unanswered questions still remain – which ROS species (superoxide or hydrogen peroxide) and which site of production (apoplast, cytosol or chloroplast) are important for signalling. To determine the site of ROS perception we use a transgenic approach where specific ROS scavengers such as catalase, ascorbate peroxidase and flavodoxin are expressed with a strong guard cell specific promoter and targeted to different subcellular compartments including the cytosol, apoplast and chloroplast. Another small reactive molecule with an enigmatic function in guard cell signalling is nitric oxide (NO). NO scavengers S-NITROSOGLUTATHIONE REDUCTASE and *Vitreoscilla* hemoglobin are expressed in the guard cell cytosol to interfere with a possible NO signal pathway. We are also generating new lines with the H_2O_2 detector Hyper expressed in various guard cell compartments.

P2.14

Cysteine-rich CuZn-superoxide dismutase in the moss *Pogonatum inflexum*

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Sulfhydryl groups in proteins are target sites of redox regulation by ROS and RNS through S-S bond formation, S-gultathionylation, S-cysteinylation, S-nirosylation and oxidation to its derivatives. Superoxide dismutase (SOD) is also involved in the redox regulation by removing ROS, although it is not know that SOD activity is reversibly modulated by ROS. Most plant CuZn-SODs are devoid of Cys except two essential residues that form S-S bond, indicating the evolutional evidence that SOD protein avoided the reaction with ROS. Interestingly, however, we found that the cytosolic CuZn-SOD in the moss *Pogonatum inflexum* is a Cys-rich SOD in which C57 and C146 form a S-S bond, but C7, C22, C102 and C109 exist as free SH. To elucidate a possible function of each free SH, the activities of the site-directed mutant CuZn-SODs were examined. We produced recombinant proteins (Cys to Ala): One 0Cys-4Ala (AAAA), four 1Cys-3Ala (CAAA, ACAA, AACA, AAAC) and a 4Cys-0Ala (CCCC) as a control were generated by pGEX and BL21. Specific activities of the recombinant SODs were as follows: AAAA = ACAA >> CAAA = AACA = AAAC >> CCCC. CCCC had almost no activity. We will discuss the present results on the basis of 3D structure of the SOD.

P2.15

Signalling and multiple regulatory mechanisms for NADPH oxidase-mediated deliberate ROS production in plant cells

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We have been elucidating molecular mechanisms for stress signaling triggered by various elicitors and an osmotic shock. Common initial signaling events within minutes include fluxes of ions such as Ca^{2+} and anions as well as enzymatic ROS production predominantly mediated by respiratory burst oxidase homolog (Rboh) proteins. Hypo-osmotic shock-induced events involve the MCA family putative mechanosensitive Ca^{2+} channel component (Kurusu et al., Trends Plant Sci. 2013). We identified ion channels that positively regulate both enzymatic activation and transcription of Rbohs. By employing a heterologous expression system, we have comparatively characterized the mechanisms for activation of all 10 Rboh proteins from *Arabidopsis* by binding of Ca^{2+} to the EF-hand motifs and phosphorylation. We have also identified several proteins that interact with the N-terminal cytosolic region AtRbohs *in vivo* and regulate their ROS-producing activity. Novel chemical screening led us to identify potential compounds that affect elicitor-triggered Rboh-mediated ROS production and plant defense responses. Multiple regulatory mechanisms and physiological significance of Rboh-mediated deliberate ROS production will be discussed.

P2.16

A comparison of antioxidant systems between *Pisum sativum* and *Brassica juncea* indicates high disproportion in H_2O_2 level

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Antioxidative defense systems present in plants activate after excessive accumulation of trace elements, which leads to generation of Reactive Oxygen Species (ROS). Plants ability to withstand stress caused by trace elements differs throughout various families, species and even specimens. A study had been undertaken to assess differences in response to trace metals in two plants exhibiting dissimilar tolerance towards oxidative stress: a hyperaccumulator *Brassica juncea* and pea plant *Pisum sativum*. Hyperaccumulating plants are characterized by high tolerance to trace metals and ability to accumulate significant concentrations of metals in their aboveground tissues. Our study was aimed to determine the foundations of hyperaccumulators' advantage in dealing with oxidative stress caused by presence of lead, zinc, copper and cadmium ions. A comparison between Indian mustard and pea plant on activity of enzymes (SOD, APOX, CAT), generation of ROS, quantity of antioxidants and gene expression was conducted. It revealed high disproportion in hydrogen peroxide level, with *B. juncea* maintaining naturally high and latterly induced H_2O_2 level around one order of magnitude higher than *P. sativum.*

P2.17

Expression of UDP-dependent glycosyltransferase genes in correlation to steviol glycosides presence in *Stevia rebaudiana*

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Stevia rebaudiana accumulates a wide variety of diterpene glycosides. Due to their sweet taste, they are utilized as reduced calorie sweeteners in food products and beverages. In recent years, steviol glycosides (SGs) have been officially permitted as food additives in Europe. The aim of this research was to determine differences in the content of SGs in *Stevia rebaudiana* plants originating from *in vivo* and *in vitro* propagation. SGs content was quantified by HPLC. The gene expression of UDP-dependent glycosyltransferases (UGTs) involved in SGs synthesis was studied with gPCR. High concentration of stevioside and rebaudioside A was noted in old and young leaves of both *in vivo* and *in vitro* propagated plants. The content of SGs in nodes and internodes was low, however it was higher in *in vitro* than *in vivo* propagated plants. A significant correlation between SGs content and the transcript level of UGT 76G1 and UGT 74G1 responsible for the biosynthesis of the investigated SGs was found. It might be concluded that *in vitro* culture conditions do not influence the ability to synthesise and accumulate SGs. *In vitro* propagated *Stevia rebaudiana* plants could be used as an alternative source for SGs production.

P2.18

Molecular mechanism of chloroplastic H_2O_2-mediated stress response in *Arabidopsis*

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To clarify the function of chloroplastic H_2O_2 as signal, we generated a system for estrogen-inducible silencing of thylakoid membrane-bound ascorbate peroxidase in *Arabidopsis* plants. Using this system, we demonstrated that chloroplastic H_2O_2 is involved in the response to stress and hormones (Maruta et al. (2012) Biol. Chem. 287: 11717-11729). Here, to investigate the molecular mechanism of the signalling pathway, we isolated paraquat-induced photooxidative stress-sensitive and -insensitive mutants (*pss* and *psi*, respectively) from mutant lines of chloroplastic H_2O_2-responsive genes, and characterized functions of PSS and PSI genes. Among the mutant lines of chloroplastic H_2O_2-responsive genes, 11 *pss* and 9 *psi* mutants were isolated. Interestingly, a large number of *PSS/PSI* genes including *PSS7*, *PSS4* and *PSS9* encoded transcription factors. Functional analysis of *PSS/PSI* genes indicated that PSS7 is involved in the response to oxidative stress and salicylic acid (SA), while PSS4 is associated with the response to biotic stress in a SA-independent manner. Furthermore, PSS9, a regulator of iron homeostasis, appeared to be essential for oxidative stress tolerance.

P2.19

Transcriptomic profiling of linolenic acid-responsive genes in ROS signalling from RNA-seq data in *Arabidopsis*

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Linolenic acid (Ln) is a polyunsaturated fatty acid (18:3). It is the precursor of Jasmonic Acid (JA) phytohormone which is implicated in several abiotic and biotic stress situations generating ROS (Reactive Oxygen Species) like hydrogen peroxide (H_2O_2) (Suza W.P. et al., Plant Physiol. Biochem. 2010; 48: 337-501, 2; Hu X. et al., Plant Signal Behav. 2009; 4: 696-697). To analyze the involvement of Ln in the mechanism of gene expression regulation, *A. thaliana* cell cultures treated with Ln were analysed by Illumina RNA-seq technology. A total of 3,275 Ln-responsive genes were identified. Most of these genes were related to JA biosynthesis pathway such as Lipoxygenase (LOX) or Allene oxide cyclase (AOC) (Wasternack C., Hause B., 2013); but, an important part of them, were involved in the mechanism of response to oxidative stress caused by biotic or abiotic stress situations, like several peroxidases, detoxification of reactive carbonyls enzymes and different dehydrogenases. In summary, this new RNA-seq approach allows to extend our knowledge about the interplay between ROS and the JA signal pathway. Supported by ERDF-confinanced grant BIO2012-33904, Ministry of Science and Innovation, Spain.

P2.20

A novel proteomic approach to analyse methionine oxidation reveals protein oxidation patterns in *Arabidopsis* seeds

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Reactive oxygen species are known to oxidize various types of cellular components such as nucleic acids and proteins, thus causing various cellular damages. Oxidation of methionine, an oxidation-susceptible residue, first leads to Met sulphoxide (MetSO) and a more severe attack can result in the irreversible formation of sulphone. Oxidation of proteins is not necessarily a deleterious phenomenon and MetSO can be reversed *in vivo* by the action of methionine sulphoxide reductases (MSR). The MSR system is present in most organisms from bacteria to human; however, little information is available on MSR substrates due to the difficulty to isolate these oxidized targets. We present a novel and handy proteomic method for studying oxidized methionine in proteins based on an approach coupling 2D diagonal SDS-PAGE with specific cyanogen bromide attack after the first dimension. This approach permitted the identification by mass spectrometry of 28 *in vivo* oxidized methionine sites in 20 different proteins extracted from *Arabidopsis* seeds. The biological significance of methionine oxidation will be discussed with regards to the putative role of the identified proteins in seed germination.

P2.21

Class III peroxidases in maize plasma membranes

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Class III peroxidases in maize plasma membranes in plants the superfamily of haem peroxidases (EC 1.11.1.7) has essential functions in metabolic pathways, cell wall-related reactions and stress response. Out of this superfamily class III peroxidases are isoenzymes of the secretory pathway. In vascular plants the number of these peroxidases is extremely high. In maize (*Zea mays* L.) at least 143 distinct class III peroxidases are known (Lüthje S. et al., Phytochem. 2011; 72: 1124-1135). Modifications, either posttranscriptional or posttranslational, can generate even more isoenzymes. Class III peroxidases can be further divided into soluble isoenzymes and membrane-bound isoenzymes. Membrane-bound peroxidases have been demonstrated for the thylakoid, tonoplast and plasma membrane. A function of plasma membrane-bound peroxidases in oxidative stress has been suggested by the data of our team. In the present study localization of ZmPrx1, ZmPrx66, and ZmPrx70 has been observed via GFP-fusion proteins both in heterologous and homologous systems and immunogold labelling of native proteins in maize roots.

P2.22

Modulation of rutin-mediated alleviation of heavy metal-induced oxidative stress in *Zygophyllum fabago*

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We investigated the interaction among rutin (RTN), reactive oxygen species (ROS) and antioxidant defense system in *Zygophyllum fabago* exposed to cadmium-induced oxidative stress. *Z. fabago* were applied exogenously with 0.25 and 1 mM RTN and were exposed to oxidative stress induced by 100 and 200 mM cadmium (Cd) for 7 and 14 d (days) and total/isozymes activities of several antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR), ROS (hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) content were determined. Activities of SOD, POX, APX and GR increased in Cd-treated seedlings within the first 7 d. Moreover, *Z. fabago* affected at 14 d of stress in terms of increasing MDA and H_2O_2. A significant increase in the activities of POX, APX and GR in *Z. fabago* and remain at MDA content observed in the RTN-treated groups after 14 d of 100 mM Cd stress. However, at 200 mM Cd, induced stimulation of the CAT, POX and APX activities by RTN was not sufficient to cope with the enhanced MDA and H_2O_2. Unlike its effect at 14 d, 0.25 and 1 mM RTN could protect *Z. fabago* from harmful effects of 100 mM Cd-induced oxidative stress at 7 and 14 d.

P2.23

The effects of induced ER stress on ROS regulation and antioxidant defense in *Arabidopsis thaliana* under osmotic stress

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Accumulation of unfolded proteins in endoplasmic reticulum (ER) due to osmotic stress is called ER stress. Exogenous application of antibiotic tunicamycin inhibits protein folding and can also induce ER stress. Under ER stress, a response called "Unfolded Protein Response" occurs, which increases protein folding capacity and induces some responses that alleviate ER stress. Formation of disulfide bonds is needed for correct protein folding. During this process, reactive oxygen species (ROS) such as H_2O_2 are produced in ER, which might cause oxidative damage in cells. Plants have enzymatic and non-enzymatic antioxidant systems to prevent oxidative damage. It is important to understand how ROS affects cellular compartments and their redox state during ER stress. To explain this, *tin1* mutant (*tunicamycin induced 1*), which is insensitive to tunicamycin treatment and wild type of *Arabidopsis thaliana* were used. Level of ROS production, content of non-enzymatic antioxidants and their redox state, the activities of ROS scavenging antioxidants were determined. In addition to this, response of ER was also investigated against oxidative stress induced in chloroplasts and mitochondria by using methyl viologen and rotenone.

P2.24

Towards understanding vacuolar antioxidant mechanisms

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Plants suffering from abiotic stress are commonly facing an enhanced accumulation of reactive oxygen species (ROS) with damaging as well as signalling effects. The outcome of an environmental challenge highly depends on the delicate balance between ROS production and scavenging by both enzymatic and metabolic antioxidants. Recent *in vitro*, *in vivo* and theoretical experiments strongly suggest that sugar-(like) molecules counteract oxidative stress by acting as genuine ROS scavengers. Moreover, a concept was proposed to include the vacuole, in which numerous sugars and secondary metabolites may accumulate to a great extent, as a part of the cellular antioxidant network. Hydroxyl radicals (·OH) are the most reactive and dangerous ROS since there are no enzymatic systems known to neutralize them. The investigation of the interaction between different vacuolar compounds and ·OH reviles a correlation between the structure and ·OH–scavenging capacity of sugars and phenolic compounds. Importantly, the splitting of disaccharides and oligosaccharides emerged as a predominant outcome of the ·OH-carbohydrate interaction. Moreover, non-enzymatic synthesis of new fructan oligosaccharides was found starting from 1-kestose.

P2.25

PAD4 regulates reactive oxygen species metabolism, cell wall and wood properties in *Populus tremula* L. x *tremuloides*

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The role of *PHYTOALEXIN DEFICIENT 4* (PAD4) in woody plants is not known, therefore we characterise its function in hybrid aspen and its role in the regulation of reactive oxygen species (ROS) metabolism and wood development. Three independent transgenic lines of *P. tremula* x *tremuloides* with different suppression levels of poplar *PAD* expression were obtained. All of these lines displayed deregulated ROS metabolism, which was manifested by increased foliar ROS (H_2O_2) level, higher activities of manganese superoxide dismutase (MnSOD) and catalase (CAT) in comparison to the wild type plants. Changes in the ROS metabolism were positively correlated with significantly decreased tracheid average size and numbers, increased cell wall thickness, and increased non-photochemical quenching (NPQ). In contrast, we did not observe any significant changes in transpiration rate. The presented results suggest that the *P. tremula* x *tremuloides PAD* gene is involved in the regulation of the cellular ROS homeostasis and in cell death that is associated with wood development processes, therefore it optimises tree growth and development. This work was supported by grants: Welcome 2008/1 and PBS1/A8/16/2013.

P2.26

Functional analysis of peroxiredoxin isoforms in *Euglena gracilis*

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Euglena gracilis lacks catalase and contains ascorbate peroxidase solely in the cytosol (Ishikawa et al., 2010). Recent comprehensive gene expression analysis indicated the presence of thiol-peroxidase families including peroxiredoxin (Prx) in this alga. In this study, we focused on Prx isozymes to clarify the ROS metabolism in *Euglena*. Four full-length cDNAs encoding Prx (Prx1-4) have amplified and cloned by PCR. Homology analysis showed that Prx1-3 belong to 2-CysPrxs, and Prx4 to type II Prxs. As for subcellular localization, it has predicted that Prx1 and Prx4 localize in cytosol, while Prx2 and Prx3 in plastids and mitochondria, respectively. Each recombinant Prx protein was expressed in *E.coli* and purified to homogeneity as judged by SDS-PAGE. The Km value and catalytic efficiency (kcat/Km) of the recombinant enzymes for H_2O_2 were approx. 40 μM and 0.03 $\mu M^{-1}s^{-1}$, respectively, suggesting that Prxs are contributed to cellular compartment-specific ROS metabolism. Silencing of Prx genes by double-stranded RNA is in progress.

P2.27

PP2A controls methionine metabolism elicited by intracellular H_2O_2

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Intra-cellular signalling in plant cells is mediated by different molecules such as reactive oxygen species (ROS) and/or by protein post-translational modifications such as phosphorylation. We have identified a PP2A regulatory subunit B, PP2A-B'γ, as a cytoplasmic component in the cross-talk between light acclimation and disease resistance in *Arabidopsis thaliana*. Under moderate light intensity, PP2A-B'γ acts as a negative regulator for salicylic and jasmonic acid-dependent defense responses. In pp2a-b'γ leaves the appearance of cell death patches overlaps both with the activity of PP2A-B'γ promoter and with accumulation of H_2O_2. Moreover, the constitutive defense response affects methionine (met) metabolism, leading to changes in the methyl-activated cycle, increased methylation index and accumulation of free met and isoleucine. To identify the nature of H_2O_2 signalling and its interaction with PP2A-B'γ activity, we analyzed the soluble phosphoproteome in leaves of a double mutant, *pp2a-b'γ catalase2*. We conclude that changes in met metabolism are triggered by intracellular ROS signals and controlled by PP2A-B'γ in a daylength-dependent manner. Studies are underway to specify the target(s) of PP2A in met metabolism.

P2.28

Functional characterization of *A. thaliana* chloroplastic Prx II E

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Chloroplasts are the major subcellular location of reactive oxygen species (ROS) production in plant cells. Thus a precise regulation of ROS concentration is essential to ensure accurate function of molecular processes. Peroxiredoxins (Prx-s) constitute a family of thiol-dependent heme-free peroxidases. Prx-s detoxify a broad range of hydroperoxide substrates. The ten members of the peroxiredoxin family are abundant peroxidases located in different cell compartments in *A. thaliana* and are divided in four distinct groups. Multiple functions have been assigned to this protein family: peroxidase, chaperone, thiol oxidase, enzyme activator and redox sensor. Here, we present novel data on the type II peroxiredoxin E (Prx II E; At3g52960). The subcellular distribution, the peroxidase activity and the electron donors in the chloroplast are investigated in detail. Furthermore, posttranslational modifications of the protein are shown by mass spectrometry and Western Blot analysis. In addition, the role of Prx II E is analysed in planta under different stress conditions using plants with a reduced level of Prx II E. Thus, the study aims at elucidating the function of the yet poorly explored chloroplast type II Prx.

P2.29

WRKY transcription factors in response to ROS

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Treatment of *Arabidopsis* plants with gaseous ROS ozone (O_3) results in large transcriptional re-programming. The W-box (C/TTGACT/C), a response element considered consensus binding site for sequence-specific WRKY transcription factors, is enriched in the promoters of genes whose transcript levels increase in response to O_3. Several other genes inducible by ROS and pathogen treatments have the W-box enriched in their promoters. We are dissecting the roles of individual ROS-responsive members of the WRKY family by comparing protein-protein and protein-DNA interactions between them. Methods used include systematic evolution of ligands by exponential enrichment (SELEX) for finding in vitro DNA-binding preferences, LC-MS/MS to find proteins interacting with WRKYs, and protoplast transactivation assay for testing *in vivo* function of WRKYs on short response elements or native promoters.

P2.30

Molecular insight into LSD1-dependent cell death regulation

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Lesion Simulating Disease1 (LSD1) is a negative regulator of cell death in response to diverse stresses, both biotic and abiotic. Under nonpermissive conditions the *Arabidopsis thaliana lsd1* mutant initiates a ROS-dependent uncontrolled spread of cell death. Therefore, LSD1 was suggested to act as ROS rheostat preventing the pro-death pathway below certain oxidative stress level. Despite its evident importance in cell death regulation and plant acclimation to different stresses, still little is known about the molecular pathways involving LSD1. Our recent results demonstrated that LSD1 is a dimeric nucleo-cytoplasmic protein that is able to form BiFC complexes with Enhanced Disease Susceptibility1 (EDS1), thereby controlling EDS1-dependent cell death. To discover other LSD1-interacting proteins, we performed Tandem Affinity Purification (TAP) experiments, which indicated that the LSD1 interactome is condition-dependent. In total, we have identified 23 and 11 LSD1-interactors under control and oxidative stress conditions, respectively. Interestingly, only 2 proteins were common in both of these lists, indicating that the role of LSD1 may differ in non-stress and stress conditions.