Mitteilungen und Nachrichten

4th Sulphyton Workshop, 5th – 8th September 2013 in Athens, Greece

In September 2013 the 4th Sulphyton workshop[1] took place in Athens, organized by the Agricultural University of Athens. The international plant sulfur research community has set up the Plant Sulfur Network[2], which organizes scientific meetings on a regular basis covering all topics of plant sulfur research. The International Plant Sulfur Workshop series are organized every 3 years. In the between years the Sulphyton Workshops take place, which provide an open platform for the presentation and discussion of ongoing plant sulfur research. The main topics of the meetings are:

- plant sulfur nutrition
- sulfur and crop quality
- post genomic technologies
- cross-talk of metabolic pathways interacting with sulfur
- managing sulfur nutrition
- diagnosing sulfur deficiency
- sulfur in plant stress response
- regulation of sulfur assimilation pathways and sulfur metabolism
- interaction between sulfur and nitrogen metabolism or other nutrients

The 4th Sulphyton workshop focused on plant sulfur metabolism and its regulation, the effects of sulfur nutrition on sulfur secondary metabolism, the role of the enzyme sulfite oxidase in plants and the effects of stress such as light stress, high salt and heavy metals on sulfur metabolism. Extended abstracts of the talks are presented here along with a brief description of the background of the workshop's scientific excursion, during which the highly contaminated heavy metals and sulfides mining sites of Lavrion were visited.

The 9th and 10th International Plant Sulfur Workshops will be held in Freiburg, Germany (April 14–17, 2014) and in Goslar, Germany (September 2–5, 2015) respectively, whilst the 5th Sulphyton Workshop will be held in Groningen, The Netherlands (September 1–4, 2016).

[1] www.aua.gr/sulphyton4
[2] www.plantsulfur.org

(Dr. Elke Bloem, Julius Kühn-Institut Braunschweig)

Abstracts:

1) Systems biology of plant sulphate metabolism: The OAS module

Hans-Michael Hubberten, Mutsumi Watanabe, Elmien Heyneke, Fayezeh Aarabi, Sarah J. Whitcomb, Rainer Hoefgen
Max Planck Institute of Molecular Plant Physiology, Science Park Potsdam-Golm, Germany
E-Mail: hoefgen@mpimp-golm.mpg.de

O-acetyl-L-serine (OAS) provides the carbon backbone for cysteine synthesis. OAS is formed through the activity of serine acetyltransferase (SERAT). Upon sulfate starvation most of the intermediate metabolites of the sulfate assimilation pathway are present in reduced amounts while OAS accumulates. Additionally, resupply of sulfate quickly reverts OAS accumulation to its normal very low level. Thus, OAS appears to be a suitable candidate for a signaling molecule for the sulfur status of a plant. Further, support for a signaling function of OAS comes from sulfur (S) assimilation by enteric bacteria. OAS accumulates under S depletion and autocatalytically converts to N-acetylserine (NAS), which is sensed and activates the sulfur assimilation operon. In plant systems OAS accumulation from sulfate starvation or OAS application induces genes of the sulfur assimilatory pathway, namely SULTR1.1 and SULTR1.2 (encoding sulfate transporters) and APR1-3 (encoding 5'-adenylylsulfate reductases). However, previous studies showed an induction of sulfate starvation-responsive SULTR family 1 members before accumulation of OAS in the respective tissues. Therefore OAS has been assumed to integrate sulfur metabolism with carbon and nitrogen metabolism. The difficulty in all prior investigations is that the concentrations of various potential signaling molecules within sulfate metabolism change upon sulfate depletion, making it difficult to assign functions to distinct metabolites. Cellular sulfate, sulfite and sulfide as well as cysteine, glutathione (GSH), and *S*-adenosylmethionine are reduced, while inversely OAS and reactive oxygen species (ROS) increase.

Database evaluations revealed two experiments in which OAS accumulation occurred independently of changes in sulfate availability to the plants. First, *Arabidopsis thaliana* plants grown under a normal day light cycle transiently accumulated OAS during the night. And second, plants transferred from constant light to darkness exhibited a short transient OAS peak within minutes after transfer, while sulfate, sulfide, sulfite, cysteine and GSH levels remained unaltered. In both experiments, a set of genes was identified which apparently respond to OAS accumulation based on their slightly time shifted induction relative to OAS. In order to experimentally test this assumption, transgenic Arabidopsis plants were generated with a SERAT gene under the control of a dexamethasone inducible promoter. Dexamethasone induction of the SERAT transgene resulted in accumulation of OAS, while within a time window of up to 6 hours after induction no other sulfur related metabolites changed in concentration. Later, cysteine and GSH accumulated, as is well known from previous experiments. When applying very stringent selection conditions in all above mentioned experimental conditions, the following genes consistently accumulated: adenosine 5'-phosphosulfate reductase 3 (APR3; At4g21990), sulfur deficiency induced 1 (sdi1, previously termed MS5-1; At5g48850), sulfur deficiency induced 2 (sdi2, previously termed MS5-2; At1g04770), low sulfur induced 1 (LSU-1; At3g49580), serine hydroxymethyltransferase 7 (SHM7; At1g36370) and ChaC-like protein (ChaC; At5g26220). These genes are known to be among the most strongly induced genes from the transcriptome studies of sulfate starvation mentioned above.

Using these genes to query about 1400 transcriptome datasets (www.attedII.jp) resulted in the identification of a stable cluster of co-expressed genes. In addition to the above mentioned six core genes, the OAS cluster contained further sulfur pathway related genes, i.e. the vacuolar sulfate transporters (SULTR4;1, At5g13550, and SULTR4;2, At3g12520), and APR1 (At4g04610) and APR3 (At1g62180). Further members of this OAS cluster are LSU2 (At5g24660), a beta glucosidase with putative myrosinase function (BGLU28; At2g44460) and an unknown protein, which responds strongly to sulfate starvation (At1g12030). Interestingly most of these genes are also induced under selenium treatment, which mimics sulfate starvation, or under conditions inducing ROS species, such as menadione treatment or cadmium stress.

In conclusion, it can be assumed that OAS plays a role as a signaling molecule in the plant's response to sulfate deprivation, though it must be clearly stated that OAS is not the only necessary signal. Further, we can assume that OAS acts as sig-

naling molecule in various other, seemingly unrelated physiological responses to environmental signals or stresses, such as dark-light shifts, diurnal rhythms, and responses to ROS inducing conditions. Speculatively the OAS cluster genes are mobilized as part of a response module to various stimuli or stresses.

2) Arabidopsis cytoplasmic serine acetyltransferase interacts with putative transcription factor SCL11

Anna Wawrzyńska, Monika Mierzwińska, Agnieszka Sirko
Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland
E-Mail: blaszczyk@ibb.waw.pl

Cysteine biosynthesis represents the final step of sulfate assimilatory reduction and the sole *entry point* of reduced sulfur into an organic form in plants. Cysteine is not only a protein constituent, but a substrate for methionine biosynthesis, and a precursor for the synthesis of essential molecules such as glutathione, thiamine, biotin and coenzyme A. Cysteine biosynthesis in higher plants involves the cysteine synthase complex (CSC), which consists of serine acetyltransferase (SAT) and O-acetylserine(thiol)lyase (OAS-TL) enzymes. SAT transfers an acetyl moiety of acetyl-CoA to serine, producing *O*-acetylserine (OAS), which in turn accepts sulfide in reaction catalyzed by OAS-TL, releasing cysteine. The Arabidopsis genome contains five SAT genes and nine OAS-TL genes, with SAT1, 3 and 5 as well as OAS-TLA1, B and C being the predominantly expressed isoforms. The formation of CSC alters the activities of both enzymes and serves SAT activation but OAS-TL deactivation. The stability of CSC is controlled by OAS and sulfide concentrations. When sulfate is limiting CSC dissociates turning SAT inactive; however, when there is surplus of sulfate CSC is stabilized by sulfide and the subsequent synthesis of cysteine is very efficient. SAT and OAS-TL isoforms are present in multiple cellular compartments; however, the relevance of CSC formation in each compartment for flux control of cysteine synthesis remains controversial.

Based on the findings that CSC formation controls cellular sulfur homeostasis, we hypothesized that activity of either SAT or OAS-TL either alone or complexed may be additionally modulated by interactions with other proteins. It is also possible that such an interaction would transfer the sulfur status message to the other proteins to alter their function. There are already several reports showing the ability of CSC proteins to interact with other proteins. It has been demonstrated that cytoplasmic isoform of OAS-TLA1 interacts with plasma-membrane sulfate transporter SULTR1;2. The binding of OAS-TLA1 inhibits the activity of the transporter and coordinates the internalization of sulfate with the energetic/metabolic state of the cell. In another report cyclophilin CYP20-3 (also known as "ROC4") of the chloroplast stroma was found to function *in vivo* in assisting the folding or assembly of SAT1 enzyme to form the CSC in chloroplast facilitating the biosynthesis of cysteine. Along with other antioxidants, the newly formed cysteine is essential for the biosynthesis of glutathione that enables the chloroplast of the buildup of cellular reduction potential mitigating detrimental effects of ROS. An alternate and more direct regulatory link between sulfur metabolism and cellular redox state is a putative interaction of thioredoxin with OAS-TL in mitochondria as indicated by proteomic studies.

Here, we report the application of a yeast two-hybrid method (Y2H) that used the entire cytoplasmic CSC of *Arabidopsis thaliana* to search for interacting proteins. The cytoplasmic CSC was chosen because it may play rather regulatory then biosynthetic function, as the mitochondrial SAT/OASTL was shown to be most important for cysteine synthesis. The region encoding SAT5 was fused with the GAL4 binding domain and introduced on the same vector with OAS-TLA1 coding region into the yeast cell. The searched cDNA library was made from 5-week old *Arabidopsis thaliana* plants starved for sulfur for two days. This approach let us to identify, among four other putative partners of the CSC, Scarecraw-like11 (SCL11) encoded by At5g59450. The protein belongs to the GRAS protein family, which is unique to plants. GRAS proteins are typically composed of 400–770 amino acid residues and exhibit considerable sequence homology to each other in their respective C-termini with the highly divergent N-termini. Although the Arabidopsis genome encodes at least 33 GRAS protein family members only a few have been characterized so far. These proteins play a crucial role in diverse plant growth and development processes, ranging from gibberelin signaling, root radial pattering, light signal transduction, and axillary shoot meristem formation. It is generally believed that GRAS proteins could be involved in transcriptional regulation; however, for many of the proteins with SCL11 among them, the exact function has yet to be determined. *SCL11* was reported to be expressed strongly in roots, whereas in flower its expression was detected mainly in sepals and upper region of carpel.

We were interested whether the formation of the CSC is a prerequisite for the interaction with SCL11. More detailed studies using Y2H revealed that SCL11 binds to SAT5 but not to OAS-TLA1. Additionally, the interaction domain is likely present in a structurally conserved region of SAT because SCL11 was also able to interact with chloroplastic SAT1 and mitochondrial SAT3. The physical interaction between SAT5 and SCL11 was next independently validated using two techniques: pull-down of the proteins overexpressed in *Escherichia coli* and Bimolecular Fluorescence Complementation (BiFC) of the proteins overexpressed in the leaves of *Nicotiana benthamiana*. BiFC revealed the cytoplasmic localization of the interaction with the clear exclusion from the nucleus. The independent studies on the cellular localization of SCL11-GFP fusion in *Nicotiana benthamiana* leaves demonstrated the protein can localize to cytoplasm and nucleus, although *in silico* studies revealed no NLS present in SCL11 sequence. The nuclear localization might suggest the function of SCL11 in transcription. Additional hint comes from the results of Y2H. When fused with the GAL4 binding domain, SCL11 was able to activate the reporter genes even without interaction with SAT5 (auto-activation), suggesting it could act as transcription activator in yeast, as it was similarly noted for another 20 GRAS family members. We did not observe any phenotypic abnormalities for the Arabidopsis knock-out mutant *scl11*. All *scl11* seedlings were indistinguishable from the wild-type when grown for ten days in different conditions (sulfur deficient media, osmotic stress, high glucose, addition of abscisic acid or 1-aminocyclopropane-1-carboxylic acid). Also the adult *scl11* plants show normal morphology, despite smaller or delayed flowering appearance. However, more careful observation of these mutant plants in comparison with the allelic mutant plants revealed that phenotypes in different alleles fell into the range of variation as observed among wild-type plants grown simultaneously.

Based on the findings discussed above, we hypothesize that the interaction between SAT5 and SCL11 might serve to transfer the sulfur status signal to the transcriptional machinery. SCL11 does not have to directly bind to DNA but could also act indirectly as coactivator, as it has transactivation abilities, or could interact with transcription factors to modulate gene expression. Another possibility, as the precise role of SCL11 is not

yet known, is that it may modulate (positively or negatively) the activity of SAT5 in certain conditions. However, the confirmation of any of these hypotheses needs further studies.

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3) Analysis of the tobacco *UP15* gene induced during sulfur and nitrogen deficiency

Katarzyna Zientara-Rytter, Anna Znój, Anna Wawrzynska, Agnieszka Sirko
Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland
E-Mail: asirko@ibb.waw.pl

Sulfur is an essential macro-element, which in many geographical regions is a limiting factor for plant biomass production. Low sulfur availability in soils resulting from decreased atmospheric pollution or usage of S-free fertilizers decreases quantity and quality of plant yield. Since the importance of sufficient supply of S on plant yield has become apparent, much greater emphasis is put on studies focused on adaptation, tolerance and changes in metabolic pathways in plants exposed to S deficiency stress, as well as on regulatory aspects of plants response to S limitation. Despite several years of intensive studies, the details of these regulatory mechanisms are still unknown and many questions concerning signaling networks and significant regulatory factors that take a part in response to S-deficit remain unanswered. Analyses of genes encoding proteins of unknown function seem to be a reasonable approach to help clarify these problems.

The *UP15* gene from tobacco (*Nicotiana tabacum* cv LABurley21) was identified in our laboratory as induced by short term S starvation. Two independent clones corresponding to *UP15* were found during screening for differentially regulated genes by suppression subtractive hybridization (SSH) method. Regulation of *UP15* gene by S deficiency in young and mature leaves of tobacco was verified by quantitative RT-PCR. Moreover, *UP15* appeared to be up-regulated even stronger than by S-deficiency in the conditions of nitrogen deficiency. *UP15* encodes a small (168 amino acids) Gly-rich protein of unknown function. Near the C-terminal part of this protein the sequence for nuclear localization signal (NLS) can be found, however, *in silico* analysis revealed that several other subcellular localizations of the UP15 protein should be considered, including chloroplasts. To determine function of UP15 protein and to identify protein partners that would be able to interact with this protein *in vivo* under S-deficit, the yeast-2-hybrid (Y2H) system was applied with cDNA library prepared from two-month-old tobacco plants maintained for 2 days in S deficient conditions. One of the candidate clones identified in this experiment encoded part of amidophosphoribosyltransferase (ATase) called also glutamine phosphoribosylpyrophosphate amidotransferase (GPAT). This enzyme is localized in stroma of chloroplast and is responsible for the first step of purine biosynthesis by transforming glutamate into glutamine. Interaction between UP15 and full length ATase was confirmed by Y2H and *in vitro* by pull-down assay of the proteins expressed in bacterial cells. However, it still remains to be demonstrated if both proteins interact in plant cells. The, preliminary results indicated that the full length ATase fused to fluorescent protein (YFP or CFP) could be detected, as expected, in close proximity or inside the chloroplasts. However, the UP15 protein seems to be unstable and several independent trials failed to demonstrate presence of the UP15-YFP fusion proteins *in planta*. Thus, it is tempting to speculate that UP15 is a regulatory unit quickly degraded in plant cell.

The detected interaction might suggest that UP15 plays a role in adaptation of plant metabolism to the imbalanced nitrogen-sulfur homeostasis due to reduced availability of sulfur or nitrogen source. However, the hypothetical role of UP15 in this regulatory network remains to be determined.

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4) Using metabolic engineering to improve the nutritive quality of rice

Sarah J Whitcomb, Nguyen Huu Cuong, Franziska Brueckner, Holger Hesse, Rainer Hoefgen
Max Planck Institute of Molecular Plant Physiology, Science Park Potsdam-Golm, Germany
E-Mail: hoefgen@mpimp-golm.mpg.de

Rice is a staple food for 3 billion people and can account for a significant proportion of their dietary protein, especially in the developing nations of Asia. However, rice protein is deficient in several amino acids essential in diets of non-ruminant animals, including lysine, tryptophan, and methionine. Unless the diet is supplemented with other protein sources, these amino acid deficiencies can result in significant health consequences for humans and significantly stifled growth in animals. Traditional rice breeding programs have developed high yielding varieties with higher seed protein levels (e.g. IR64), but these varieties are still deficient in several essential amino acids. A more targeted approach to improve the amino acid balance in rice is seed-specific expression of exogenous storage proteins to 'pull' essential amino acids into the seed. Sunflower seed albumin (SSA), with its very high methionine and cysteine contents (16% and 8% respectively), is one of the most sulfur-rich storage proteins known. In addition, SSA is rumen stable, meaning that the improved amino acid profile of SSA rice would be bioavailable, even to ruminant animals like sheep, goats, and cattle. Despite achieving high SSA expression in transgenic rice seed, overall levels of essential amino acids remained nearly unchanged. This suggests that free amino acid pools, in particular methionine and cysteine, limit protein expression in this context, and that limiting amino acids are redirected into SSA expression from endogenous seed proteins. An alternative targeted approach to improve the nutritive quality of rice seeds is to increase the biosynthesis of cysteine and methionine. Extensive work in model organisms such as Arabidopsis and tobacco and crops such as potato suggests that overexpression of serine acetyltransferase and feedback-insensitive cystathionine-gamma-synthase would have the potential to increase the synthesis of cysteine and methionine to the point where these free amino acids are no longer limiting to storage protein expression. These 'push' approaches proved to be somewhat successful in increasing seed protein incorporated methionine, but the gains fell short of producing greatly improved nutritive quality. Since neither manipulation of sink nor source strength proved to be entirely sufficient on its own, we are combining these two approaches in one rice line. We anticipate that this "Push plus Pull" approach will result in rice with greatly improved nutritive quality for human and animal consumption.

5) Alterations of seed yield and quality in sulphur-limited *Brassica napus* L.

Philippe D'Hooghe[1,2,3], Lucie Dubousset[1,2,3], Karine Gallardo[4], Stanislav Kopriva[5], Jean-Christophe Avice[1,2,3], Jacques Trouverie[1,2,3]
[1] Normandie Univ, France
[2] UNICAEN, UMR 950 Ecophysiologie Végétale, Agronomie & nutritions, Caen, France
[3] INRA, UMR 950 Ecophysiologie Végétale, Agronomie & nutritions, Caen, France
[4] INRA, UMR1347 Agroécologie, Dijon, France
[5] John Innes Centre, Norwich Research Park, Norwich, UK
E-Mail: jacques.trouverie@unicaen.fr

Sulphur (S) limitation reduces seed yield and quality of various crops such as cereals, mainly used for producing flour, or oilseed rape, a high S demanding plant cultivated for its nutritional value for animal and human feeding. In this work, we describe the impacts of S restriction applied at the bolting (LS32), early flowering (LS53) or start of pod filling (LS70) stages on *Brassica napus* seed composition. For this purpose, lipids were analysed by Near-infrared spectroscopy and two-dimensional electrophoresis were performed on total proteins extracted from mature seeds. The major S compounds of mature seeds were also determined. The reduction of protein quality observed for all LS seeds was related to a reduction of S-rich seed storage protein (SSP) accumulation (as Cruciferin Cru4) at benefit of S-poor SSP (as Cruciferin BnC1). Through this adaptive response, the protein contents of LS70 and LS53 seeds were not affected, but it was reduced for LS32 seeds. The reduction of lipid content in LS53 and LS32 seeds was primarily associated with a reduction of C18 derivates. The ω6/ω3 ratio was increased in LS53 and LS32 seeds. Modulations of proteins associated with lipid storage and carbohydrate metabolism (reduction of caleosines, glyoxysomal malate synthase, thiazole biosynthetic enzyme THI1; accumulation of citrate synthase) could be involved in the alteration of lipid composition of LS53 and LS32 seeds. The accumulation of proteins associated with stress response and a lower level of glutathione in LS53 and LS32 seeds may decrease seed resistance to biotic/abiotic stresses during conservation and germination.

6) How abiotic stress affects glucosinolate biosynthesis in plants

Elke Bloem[1], Kathrin Gualato[1], Silvia Haneklaus[1], Maik Kleinwächter[2], Jana Paulsen[2], Ewald Schnug[1], Dirk Selmar[2]
[1] Julius Kühn-Institut, Institute for Crop and Soil Science, Braunschweig, Germany
[2] Institute for Plant Biology, TU, Braunschweig, Germany
E-Mail: elke.bloem@jki.bund.de

The glucosinolate (GSL) content is an important quality parameter in plants such as mustard and nasturtium. Both crops are used as spices and in phytopharmaceutical products. Agrotechnical measures such as sulfur fertilization are well known to increase the concentration of sulfur-containing secondary compounds in plants and contribute to the compliance with minimum quality demands. The worldwide scientific interest in using GSLs and their degradation products as anti-carcinogenic agents and reported beneficial health effects of GSL-containing vegetables give reason to maximize the GSL content in harvest products. Yet, another politically promoted objective is to increase the acreage of medicinal plants that are grown in Germany. A beneficial side effect is that biodiversity on agricultural soils will improve. It was the aim of the presented experiments to increase the GSL content in different plants by triggering stress response in the plants.

The impact of different stress parameters on the growth and GSL content of *Tropaeolum majus*, *Sinapis alba* and *Brassica juncea* was investigated in greenhouse experiments. Drought stress (soil water content of 4–8 volume % in comparison to 12–18 volume % in the control), salt application (2 mg NaCl per day with irrigation water) and methyljasmonate (MeJA) spray application (4 ml of a 200 µMol l^{-1} MeJA) were investigated. Different plant features were recorded to evaluate the impact of the treatments on plant performance and stress response. For this purpose evapo-transpiration, biomass development, specific leaf weight, plant pigments, plant thiols, GSL content and GSL yield were determined. Plants were harvested three times during the vegetation period.

Stress response was triggered by marginal doses of stress factors in order to increase the GSL content without negatively affecting crop and GSL yield. Drought and MeJA application reduced biomass production in all three crops by 25–40% while moderate salt applications slightly increased yield.

In all three plant species marginal drought stress and/or the application of MeJA increased the concentration of the prevailing GSLs on a dry weight basis. In *Tropaeolum majus* the glucotropaeolin concentration increased on an average by 21% with drought and by 31.5% after MeJA application at all sampling dates. But only MeJA application increased also the glucotropaeolin yield by 10% while drought and salt application reduced the glucotropaeolin yield. Similar results were found for sinalbin and sinigrin in *Sinapis alba* and *Brassica juncea*, respectively. Drought and MeJA increased the sinalbin concentration in *Sinapis alba* but MeJA was the only elicitor that yielded a 1.7-fold increase of the sinalbin yield in comparison to the control. In *Brassica juncea* MeJA increased the sinigrin concentration and resulted in a 2.4-fold higher sinigrin yield. Salt applications did not affect the GSL concentration significantly.

It can be concluded that MeJA application proved to be a suitable measures to increase the GSL content and yield of *Tropaeolum majus*, *Sinapis alba* and *Brassica juncea*. Though slight drought stress increased the GSL concentration, it reduced biomass production and GSL yield. Marginal salt application showed no effect on the GSL content, while crop yield was slightly increased.

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7) The impact of sulfate, hydrogen sulfide and sulfur dioxide on glucosinolate metabolism in Brassica species

Tahereh Aghajanzadeh[1], Malcolm J. Hawkesford[2], Anna Koprivova[3], Stanislav Kopriva[3] and Luit J. De Kok[1]
[1] Laboratory of Plant Physiology, University of Groningen, The Netherlands
[2] Plant Science Department, Rothamsted Research, Harpenden, Hertfordshire, UK[3]Department of Metabolic Biology, John Innes Centre, Norwich Research Park, Norwich, UK
E-Mail: l.j.de.kok@rug.nl

Glucosinolates are secondary sulfur compounds, especially found in *Brassicaceae*, which may function in plant defense against insects, herbivory and pathogens and have anti-carcinogenic properties. The content of the glucosinolates varies strongly between species, cultivars, developmental stage and may be

affected by the plant sulfur supply/status. In addition to sulfate taken up by the roots, *Brassica* species are able to utilize atmospheric sulfur gasses, *viz*. H_2S or SO_2 taken up by the leaves as sulfur source for growth. In the current study the impact of H_2S or SO_2 on glucosinolate metabolism was studied in two *Brassica* species characterized by a high (*B. juncea*) and low (*B. rapa*) glucosinolate content. 10-day-old seedlings were grown on a 25% Hoagland nutrient solution containing 0.5 mM sulfate for 3 days and subsequently transferred to fresh 25% Hoagland solution at 0 mM sulfate (−S) or 0.5 mM sulfate (+S) and exposed to 0.25 µl l^{-1} H_2S or SO_2 for 7 days. At an ample sulfate supply, exposure of both species to H_2S or SO_2 hardly affected content and composition of glucosinolate. H_2S or SO_2 exposure resulted in a slight decrease in expression of APS reductase expression whereas that of APS kinase and ATP sulfurylase remained unaffected. Sulfate-deprivation of plants resulted in a decreased biomass production and glucosinolate content. Expression of APS reductase was strongly enhanced in sulfate-deprived plants but expression of both APS kinase and ATP sulfurylase hardly changed. When sulfate-deprived plants were exposed to H_2S or SO_2, plant growth was restored, however, the glucosinolate content remained lower than that of sulfate-sufficient plants. Moreover, the expression of APS reductase was partially down-regulated again, whereas expression of APS kinase and ATP sulfurylase remained unaffected.

8) Effect of S-limitation on osmotic potential components in oilseed rape leaves: towards the development of early indicators?

Elise Sorin[1,2,3,4], Anne Maillard[1,2,3], José Maria Garciamina[5], Angel Mari Zamarreno[5], Mustapha Arkoun[4], Florence Cruz[4], Jean-Claude Yvin[4], Philippe Etienne[1,2,3], Alain Ourry[1,2,3]
[1] Normandie Univ, France
[2] UNICAEN, UMR 950 Ecophysiologie Végétale, Agronomie et nutritions, Caen, France
[3] INRA, UMR 950 Ecophysiologie Végétale, Agronomie et nutritions, Caen, France
[4] Timac Agro International, St Malo, France
[5] Timac Agro Spain, Orcoyen, Spain
Email: alain.ourry@unicaen.fr

Oilseed rape (*Brassica napus*) is a S demanding crop, S-limitation resulting in a reduction of yield and of nutritional quality of seeds. Optimization of S fertilization requires to identify indicator of S nutrition that could be used early in the growth cycle.

In this study, we examined the effects of S-limitation on osmotic potential components of oilseed rape leaves. Plants were grown at vegetative stage and submitted to S-limitation (S+: 508.7 µM SO_4^{2-} or S−: 8.7 µM SO_4^{2-}) and were harvested at 0, 1, 2, 3, 7, 13, 24 and 34 days of S-limitation. Each plant was sampled as old leaves, new leaves emerging after S-limitation, roots and petioles.

Plant growth was maintained during the first 13 days of S-limitation as a result of massive internal sulfate mobilization mostly from leaves and its subsequent assimilation. This was at least partly compensated for by an accumulation of malate, nitrate, chloride and phosphate in leaves and to a lesser degree in roots. More surprisingly, leaf osmotic potential decreased after two days of S-limitation. Other compounds such as amino acids, soluble sugars and cations will be quantified in order to evaluate their contribution to the leaf osmotic potential. Additional data (^{15}N-nitrate uptake, nitrate reductase activity, transcript levels of sulfate and nitrate transporters) show that under S-limitation, osmotic potential is affected earlier that growth or N metabolism suggesting that field measurements of leaf ion contents, acting as osmoticum, could be used as early indicators for S fertilization management.

9) Elucidating the molecular components that allow *Salicornia* and *arcocornia* to thrive in high sulfate and sulfide levels

Assylay Kurmanbayeva, Aizat Bekturova, Moshe Sagi
Plant Stress Laboratory, The Albert Katz Department of Dryland Biotechnologies, French Associates Institute for Agriculture and Biotechnology of Drylands, Blaustein Institutes for Desert Research, Ben-Gurion University of Negev, Israel
Email: gizi@bgu.ac.il

Salicornia and *Sarcocornia* are extreme halophytes that grow on sea water with high sulfide and sulfate levels and have been recently introduced as new crops for extreme salt conditions such as in the level of Dead Sea water. We are exploring the mechanism/s that allows these plants to cope with sulfate and sulfide levels, which are toxic to many other plants. We grew the plants at different NaCl, sulfate and sulfide levels and we are scanning for relevant genes and proteins that might be related to plant resistance to those stresses.

10) The role of sulfite reductase in sulfite homeostasis

Dmitry Yarmolinsky, Galina Brychkova, Moshe Sagi
French Associates Institute for Agriculture and Biotechnology of Drylands, Blaustein Institutes for Desert Research, Sede Boqer Campus, Ben-Gurion University, Israel
Email: yarmod@post.bgu.ac.il

Using *Arabidopsis* and tomato plants with modified SiR expression, we invastigated the role of SiR in various metabolic processes in plants. We observed that resistance to ectopically applied SO_2/sulfite was a function of SiR expression levels and that plants with reduced SiR expression exhibited higher sensitivity than the wild type. The sulfite sensitive mutants accumulated sulfite and showed a decline in glutathione levels. In contrast, mutants that over-express SiR were more resistant to sulfite toxicity; exhibiting little or no damage. Resistance to high sulfite application was manifested by fast sulfite disappearance and increase in glutathione levels. The notion that SiR plays a role in the protection of plants against sulfite was supported by the rapid up-regulation of *SiR* transcript and activity within 30 min of sulfite injection into Arabidopsis and tomato leaves. Our results indicate that, in addition to participating in the sulfate assimilation reductive pathway, SiR also plays a role in sulfite homeostasis together with sulfite oxidase and the other members of the sulfite network.

11) Regulatory Network of SO_2 detoxification

Robert Hänsch[1], Domenica Hamisch[1], Dörte Randewig[2], Andrea Bräutigam[3], Andreas P. M. Weber[3], Cornelia Herschbach[2], Heinz Rennenberg[2], Ralf R. Mendel[1]
[1] Institut für Pflanzenbiologie, Technische Universität Braunschweig
[2] Institut für Forstbotanik und Baumphysiologie, Professur für Baumphysiologie, Albert-Ludwigs-Universität Freiburg
[3] Institut für Biochemie der Pflanzen, Heinrich-Heine-Universität, Düsseldorf
E-Mail: r.haensch@tu-bs.de

Abiotic stress occurs when plants are exposed to high concentrations of sulfur dioxide (SO_2) and its derivative sulfite. Physiological studies suggested sulfite oxidase (SO) as one important component of SO_2 detoxification. However, it is unknown to which extend plant fumigation with SO_2 triggers a specific transcriptional response. To address this question, we compared Arabidopsis wildtype (WT) and SO knock-out lines (SO-KO) facing the impact of 600 nL L^{-1} SO_2 using RNAseq to quantify absolute transcript abundances. These transcriptome data were correlated to sulfur metabolism related enzyme activities and metabolites obtained from the same samples in a physiological study. SO-KO plants showed first symptoms of leaf injury after fumigation that were not detectable in fumigated WT plants. Analogously, SO-KO exhibited remarkable and broad regulative responses at the mRNA level, especially in transcripts related to sulfur metabolism enzymes but also of those related to stress response and senescence. Our data provide evidence for a highly specific co-regulation between SO and sulfur related enzymes like APS reductase. Moreover, we could show that beside of SO a new player comes into business – apoplastic peroxidase (PRX). PRXs known from work of Pfanz and colleagues in the early 1990ies are able to detoxify sulfite. We could show that under SO_2-fumigation the mRNA of *prxcb* is 5fold up-regulated. This result implies that sulfite detoxification is of exceptional importance so that the plants have evolved a complex network of mechanisms and enzymes for controlling its level including regulation of stomata, generation of enzymes like SO and PRX for direct oxidation and use of the sulfur assimilation pathway for reductive detoxification.

12) The arbuscular mycorrhizal symbiosis influences sulfur starvation responses of *Medicago truncatula*

Daniela Sieh, Mutsumi Watanabe, Franziska Brueckner, Elmien Heyneke, Franziska Krajinski, Rainer Hoefgen
Max Planck Institute of Molecular Plant Physiology, Science Park Potsdam-Golm, Germany
E-Mail: hoefgen@mpimp-golm.mpg.de

Arbuscular mycorrhizal (AM) symbiosis is a mutualistic interaction that occurs between most vascular plants and fungi of the phylum Glomeromycota. The main benefit for both organisms in this relationship is an exchange of nutrients to the plant in return for plant-derived carbohydrates. In the past, the primary focus has been on mycorrhizal phosphate transfer, mainly because low phosphate concentrations in soils are often the main growth-limiting factor and known to be necessary to establish symbiosis. Evidence for symbiotic uptake pathways for additional nutrients emerged from the recent identification of a number of other nutrient transporter genes, which are also specifically expressed or induced in mycorrhized roots. In this study we were interested whether *in vivo* sulfate supply from the fungus to the plant does occur under conditions of sulfate starvation in addition to a mild phosphate starvation. Sulfur starvation leads to typical S starvation phenotype in *Medicago truncatula* Gaertn. with reduced thiol, protein and chlorophyll contents and consequently a reduced biomass in *M. truncatula*.

We investigated whether mycorrhizal colonization by the fungus *Rhizophagus irregularis* (previously termed *Glomus intraradices*) influences leaf metabolite composition and the expression of sulfur starvation-related genes when applying different sulfur and phosphate fertilization treatments to *M. truncatula*.

Single amino acids, as well as S-containing metabolites and ions, were analyzed in shoots of mycorrhized and non-mycorrhized plants grown under S starvation (−S) and S repletion (+S) as well as in plants fertilised with 1 mM phosphate (+P), which represents a mild phosphate starvation, and plants grown under strong phosphate starvation (−P). Primary metabolites of the S assimilation pathway like cysteine, methionine, glutathione, gamma-glutamylcysteine, sulfite, and also total protein content were correlated to the sulfate content in shoots. All metabolites displayed strong reductions under −S conditions which were slightly alleviated by mycorrhiza formation. This mainly resulted in increased biomass as additional sulfate was converted into growth. Leaf metabolite concentrations clearly showed that phosphate starvation has a greater impact than sulfur starvation on plant metabolism, with no demand for sulfur at strong phosphate starvation. However, when phosphate nutrition is high enough, mycorrhizal colonization reduces sulfur stress responses, as a result of symbiotic sulfur uptake.

We made use of a two-compartment system in which the plant's roots (in one compartment) were restricted to grow into a compartment, which only the fungus could reach. Application of ^{35}S to this "fungal compartment", confirmed that sulfate was taken up by the fungal hyphae and transported to the plant detected as radioactivity in the plant's leaves.

Similar to other plant species, sulfate deprivation also leads to the induction of sulfate transporters in *M. truncatula*. In this study nine putative sulfate transporter genes, which correspond well to the known classifications in other species were identified in *M. truncatula*. We also investigated mRNA accumulation levels in leaves and roots of mycorrhized and non-mycorrhized plants grown under the two different S concentrations (+S and −S) and at 1mM phosphate. Sulfur starvation leads to the induction of sulfur starvation-related genes and the myccorhizal provision of sulfate resulted in a less strong induction of these genes. Thus, the transcripts of marker genes of the S-assimilation pathway, adenosine-5′-phosphosulfate reductase (APR), and of the OAS-cluster gene of the ChaC-like protein were highly induced under sulfate starvation in sulfur starved non-mycorrhized plants while the induction level was lower in sulfur starved mycorrhized plants. It has to be noted that no additional sulfate source was provided but fungal hyphae mobilized sulfate more efficiently from the substrate than plant roots.

In this study we could show that mycorrhizal colonization of sulfur starved plants led to an increased accumulation of biomass and a shift in the metabolite pattern in the direction of that of sulfur-replete plants. We can assume that mycorrhizal colonization is able to reduce sulfur starvation responses in *M. truncatula*. This demonstrated the importance of mycorrhizal sulfur uptake for plant metabolism, when the plant's phosphate status is high enough for the plant to benefit from increased sulfate nutrition.

13) Compartment-specific changes in glutathione and ascorbate levels during high light stress in Arabidopsis

Elmien Heyneke[1], Nora Luschin-Ebengreuth[2], Maria Müller[2], Bernd Zechmann[2]
[1] Max-Planck-Institute of Molecular Plant Physiology, Golm, Germany
[2] University of Graz, Institute of Plant Sciences, Graz, Austria
E-Mail: bernd.zechmann@uni-graz.at

Excess light conditions represent a potential danger to the plant as it can lead to the accumulation of reactive oxygen species (ROS) in chloroplasts by overstraining the electron transport chain during photosynthesis and of hydrogen peroxide (H_2O_2) in peroxisomes as a result of glycolate oxidation in the photorespiratory pathway. If not detoxified ROS can leak into the

cytosol and other cell compartments where they are capable of oxidizing membrane components and proteins and can cause the degradation of nucleic acids, lipids, pigments, membranes, proteins, RNA, and DNA, causing mutation and eventually cell death. Ascorbate and glutathione are the most important antioxidants that are able to detoxify ROS. They occur in all cell compartments at high concentrations. Whereas an increase in ascorbate contents during high light conditions was commonly found with biochemical methods, unchanged levels of glutathione were observed during such conditions, indicating a more important role for ascorbate in the protection against high light stress. Nevertheless, all of these studies investigated ascorbate and glutathione levels in whole leaves and therefore the antioxidative defense situation on the subcellular level remained unclear. The aim of this study was to investigate subcellular changes in ascorbate and glutathione contents in Arabidopsis Col-0 plants during the exposure to high light (150 versus 700 μmol m^{-2} s^{-1}) in order to clarify the dynamic compartment specific protection of these key antioxidants against ROS produced during high light conditions.

On the subcellular level both ascorbate and glutathione were strongly increased in most cell compartments during high light conditions, as observed by transmission electron microscopy after immunohistochemical detection of ascorbate and glutathione. A very strong increase was detected in chloroplasts (104% for ascorbate and 190% for glutathione) after the exposure to light conditions of 700 μMol m^{-2} s^{-1} for 2 weeks. This data highlights the importance of both ascorbate and glutathione in the antioxidative protection against oxidative stress induced in this cell compartment during high light conditions. Both ascorbate and glutathione could also be detected inside the lumen of thylakoids of chloroplasts exposed to high light stress. This observation is interesting in respect to non-photochemical quenching which decreases the formation of ROS by dissipation of excess absorbed light as heat. One important mechanism for non-photochemical quenching is the formation of zeaxanthin to violaxanthin that is catalysed by the enzyme violaxanthin de-epoxidase. This enzyme is located inside the thylakoid lumen and uses ascorbic acid as a reductant. Additionally, ascorbate can be used as an alternative electron donor by photosystem II and I, which is especially important in situation of stress when the linear electron transport is impaired. Thus, the detection of ascorbate in the thylakoid lumen of plants exposed to high light conditions and the general increase inside the stroma of both antioxidants highlights the importance of high ascorbate and glutathione contents for the compartment specific protection of chloroplasts during high light conditions.

One very important aspect is the massive increase of ascorbate specific labeling in vacuoles during high light exposure (up to 395%). It has been proposed recently that ascorbate plays an important role in the detoxification of H_2O_2 that diffuses into vacuoles especially during environmental stress situations. In this cell compartment ascorbate helps to reduce phenoxyl radicals (created by oxidation of phenols by H_2O_2) and is oxidized to mono- and dehydroascorbic acid, which is then transported into the cytosol for reduction to ascorbic acid. Thus, it can be concluded that, in contrast to glutathione which did not occur in vacuoles, ascorbate plays important roles in vacuoles during excess light conditions most probably by direct or indirect detoxification of H_2O_2.

Summing up, we can conclude that in Arabidopsis wildtype plants the accumulation of ascorbate and glutathione especially in chloroplasts is an important mechanism to protect plants against ROS produced in this cell compartments during high light stress. Additionally, the accumulation of ascorbate in vacuoles of wildtype plants indicates an important role of this antioxidant in vacuoles for the detoxification of H_2O_2 leaking from peroxisomes and chloroplasts into the cytosol and vacuoles.

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14) The role of antioxidant metabolism during dark-induced senescence in Arabidopsis

Nora Luschin-Ebengreuth, Bernd Zechmann
University of Graz, Institute of Plant Sciences, Graz, Austria
E-Mail: bernd.zechmann@uni-graz.at

Senescence is a developmental program and highly regulated by specific genes. Senescing cells undergo drastic metabolic changes and organized degradation of cell structures. Antioxidants were shown to play an important role during senescence and many senescence-related genes were shown to be induced by reactive oxygen species (ROS). The equilibrium between the production and the scavenging of ROS is regulated by antioxidants as well as antioxidative enzymes. The aim of this study was to investigate the compartment specific importance of ascorbate and glutathione and related enzymes during dark induced senescence in *Arabidopsis thaliana* Col-0.

In this study senescence was induced by darkening of individual leaves, which has been shown to be an appropriated method to synchronize the senescence process in leaves. To further characterize the impact of the antioxidants ascorbate and glutathione in the regulation of senescence, the *Arabidopsis* mutants *vtc2-1* and *pad2-1*, deficient in ascorbate and glutathione, respectively were used in this study.

After initiating leaf senescence by dark treatment a yellowing of the leaves could be observed which correlated well with a decrease in pigment content of the leaves. Light microscopic investigations revealed a decrease of the number of chloroplasts per cell during senescence in wildtype plants and both mutant lines, already after 2 days of dark-treatment. Together with the decreased number of chloroplasts per cell, an overall reduction of size could be observed during senescence. A reduction of the size and number of chloroplasts has been observed in many plants species during senescence. In this study, prominent structures, diverse deposits and small vesicles, engulfed by single or double membranes could be observed in the cells during dark-induced senescence. These structures established throughout senescence processes in *Arabidopsis* wildtype, and the mutant lines *pad2-1* and *vtc2-1* to the same extent. Different compartments, deposits and vesicles were shown to be involved in degradation processes of cellular components.

A precise analysis of chloroplasts inner structure by transmission electron microscopy (TEM) revealed a massive degradation of senescing chloroplasts. When leaves were subjected to dark treatment starch grains were degraded within the first 2 days of treatment. Chloroplasts under control conditions contained an organized network of photosynthetic thylakoid membranes, starch grains and a number of small plastoglobuli droplets. During senescence thylakoid membrane system was found to decrease in size, which correlated with an increase in plastoglobuli area. Plastoglobuli are known to contain lipids derived from the degradation of thylakoids as well as chlorophyll, carotenoids, photosynthetic proteins. The degradation of thylakoid membranes is accompanied by the downregulation of photosynthesis during senescence. The degradation of chloroplasts was shown to be one of the most significant and earliest events during senescence and its degradation is a major source of nutrients which are recycled during leaf senescence.

Hydrogen peroxide strongly accumulated after 2 days of dark-treatment in the leaves and activities of dehydroascorbate reductase, glutathione reductase and catalase were decreased, whereas ascorbate peroxidase showed increased activities during dark-induced senescence. Ascorbate levels were unchanged during senescence, while glutathione content strongly decreased in all subcellular compartments 1 day after the beginning of darkness in all investigated plants. The increase of H_2O_2 correlated temporally with the reduction of glutathione contents, since H_2O_2 contents started to increase only after 2 days of treatment. Ascorbate and glutathione play a crucial role during senescence. Beside its role as antioxidant, the content and subcellular distribution of ascorbate and glutathione were shown to have regulatory function on ROS accumulation, gene expression and redox signaling. The rapid decrease of glutathione contents in all subcellular compartments by dark-incubation of individual leaves could serve as senescence-inducing signal, regulating senescence, via creating an oxidative burst by an increase of H_2O_2 contents, which is needed for senescence-progression.

Thus, we can conclude that the compartment specific decrease of glutathione rather than ascorbate contents contributes to the accumulation of H_2O_2, which is a well-known signal for triggering senescence.

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15) Cadmium sensitivity of pad2-1 and vtc2-1 mutants is correlated to lower subcellular glutathione rather than ascorbate contents

Barbara Koffler, Lisa Polanschütz, Bernd Zechmann
University of Graz, Institute of Plant Sciences, Graz, Austria
E-Mail: barbaraeva.koffler@edu.uni-graz.at

Toxic levels of cadmium (Cd) can cause alterations of the chloroplast ultrastructure, disturbs the synthesis of chlorophyll and carotenoids, leads to CO_2 deficiency due to stomatal closure and can cause several other metabolic disturbances. Indirectly Cd can cause oxidative stress and can lead to the accumulation of reactive oxygen species (ROS). Antioxidants such as ascorbate and glutathione can mitigate the effects of ROS by detoxifying them through the ascorbate-glutathione-cycle. Additionally, glutathione has the ability to bind to Cd directly and serves as a precursor of phytochelatins, which forms complexes with Cd and are then deposited in vacuoles. The aim of the study was to analyse the compartment specific distribution of ascorbate and glutathione over a time period of 14 days in Cd treated (50 µM and 100 µM) Arabidopsis Col-0 plants. To obtain a deeper insight into how possible limitations of ascorbate and glutathione contents affect the defense of plants against Cd we additionally analysed the importance of subcellular ascorbate and glutathione contents in mutants deficient in ascorbate and glutathione (*vtc2-1* and *pad2-1*, respectively). For this purpose we used a technique that visualizes glutathione and ascorbate in all cell compartments simultaneously in one experiment, which is based on immunogold-cytohistochemistry and computer-supported transmission electron microscopy.

In general, glutathione contents in wildtype plants followed recently proposed antioxidative stress models after treatment with 100 µM Cd in all cell compartments. Glutathione contents decreased during an initial alarming phase after 12 h (up to −84%), increased during a resistance phase between 48 h until 7d (up to 165%), and decreased during an exhaustion phase after 14 days of Cd-treatment (up to −45%). Compared to the wildtype both mutants showed strongly reduced glutathione contents (up to −90%) over the whole experiment after 100 µM Cd treatment. While ascorbate levels in wildtype plants and *vtc2-1* mutants followed the above described stress model after 100 µM Cd treatment, they were strongly reduced in the *pad2-1* mutant (up to −86%) throughout the experiment. After the exposure to 100 µM Cd both mutants showed earlier and stronger symptoms (96 h after treatment) like chlorosis and necrosis than the wildtype, which showed first symptoms 7 days after the treatment. 50 µM Cd treatment induced elevated glutathione contents in wildtype plants (up to 152%), decreased glutathione levels in the *pad2-1* mutant (up to −86%), whereas the *vtc2-1* mutant showed a typical bell shaped stress response curve over the whole experiment. Ascorbate contents after treatment with 50 µM Cd followed the above described antioxidative stress model in each plant. Symptom development remained absent in wildtype plants but both mutants showed first symptoms 7d after 50 µM Cd treatment. Although symptom development in the mutants appeared at the same time-point after Cd treatment the *pad2-1* mutant showed always stronger symptoms and larger necrotic areas than the *vtc2-1* mutant.

Wildtype plants showed a typical stress response curve with an immediate decrease in glutathione and ascorbate contents in all cell compartments, which indicates an excessive demand for antioxidants immediately after the exposure to 100 µM Cd. Such a reaction of plants seems likely as Cd has a high affinity to thiol groups and would bind to reduce glutathione in the cytosol after entering the cells. Additionally, glutathione is used for the production of phytochelatins, which are also involved in the detoxification of Cd. Considering that Cd enters the cytosol first and is then transported into the vacuoles it is interesting that both antioxidants decreased in all other cell compartments at similar rates. Thus, glutathione and ascorbate are either withdrawn from other cell compartments or transported from the origin of synthesis to the other cell compartments as they are used for Cd and ROS detoxification in the cytosol. The results also revealed that both mutants showed higher sensitivity to Cd treatment than the wildtype because of an earlier and stronger development of Cd-induced symptoms. The appearance of these symptoms is correlated with a strong decrease in glutathione contents in most cell compartments at these time-points and this indicates that low glutathione levels in the mutants were unable to protect plants against the toxic effects of Cd. The *pad2-1* mutant, a glutathione-deficient mutant which contains up to 90% less glutathione than wildtype plants, showed stronger symptom development than the *vtc2-1* mutant. This is another indication that Cd sensitivity is linked to low glutathione concentrations rather than changes in ascorbate contents.

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16) Metal toxicity and sulfur metabolism in Chinese cabbage

Luit J. De Kok[1], C. Elisabeth E. Stuiver[1], Freek S. Posthumus[1], Muhammad Shahbaz[1,2], Saroj Parmar[3] and Malcolm J. Hawkesford[3]
[1] Laboratory of Plant Physiology, Centre for Ecological and Evolutionary Studies, University of Groningen, Groningen, The Netherlands
[2] Biology Department and Program in Molecular Plant Biology, Colorado State University, Fort Collins, USA
[3] Plant Biology and Crop Science Department, Rothamsted Research, Plant Biology and Crop Science Department, Harpenden, Hertfordshire, UK
E-Mail: l.j.de.kok@rug.nl

Copper and zinc are essential nutrients for the plants; however, they become rapidly phytotoxic at elevated concentrations in the root environment. Exposure of Chinese cabbage to elevated Cu^{2+} (≥ 2 µM) or Zn2 + concentrations (≥ 5 µM) resulted in leaf chlorosis and subsequently in a loss of photosynthetic activity and a strongly reduced biomass production of both root and shoot. Cu is a redox-active metal, which has the potential to accelerate the formation of reactive oxygen species in plant tissue. However in Chinese cabbage, the Cu-induced chlorosis was most likely not due to the formation of reactive oxygen species but the consequence of a negatively affected chloroplast development. UV-A+B radiation and elevated Cu^{2+} concentrations had negative synergistic effects on biomass production, pigment content on the quantum yield of photosystem II.

The uptake and metabolism of sulfur and nitrogen were differentially affected at elevated Cu^{2+} or Zn^{2+} concentrations. Both Cu^{2+} and Zn^{2+} exposure resulted in an increased sulfate uptake by the roots and in enhanced total sulfur content of the shoot, which could be ascribed partially to an accumulation of sulfate. Moreover, exposure resulted in a strongly enhanced level of water-soluble non-protein thiols in the root, which only partially could be ascribed to a metal-induced synthesis of phytochelatins. The nitrate uptake by the root was decreased upon Cu^{2+} or Zn^{2+} exposure, demonstrating the absence of a mutual regulation of the uptake of sulfate and nitrate. There was no direct relation between the sulfur metabolite levels viz. total sulfur, sulfate and water-soluble non-protein thiols and the expression and activity of the sulfate transporters and the expression of APS reductase at elevated Cu and Zn concentrations. Apparently, the presumed signal transduction pathway involved in their regulation appeared to be overruled or bypassed at high tissue Cu and Zn levels. It is doubtful that the Cu^{2+} or Zn^{2+}-induced effects on the uptake and metabolism of sulfate have any adaptive significance in the detoxification of these metals in Chinese cabbage.

Elevated Zn^{2+} concentrations in the root environment did not only disturb the uptake, distribution and assimilation of sulfate, it also affected the uptake and metabolism of nitrate in Chinese cabbage. The uptake of nitrate appeared to be closely linked to the growth rate of the plant, even at toxic Zn levels. The total N content was strongly decreased in the shoot at toxic Zn concentrations. The decrease in total N in the shoot could only partly be ascribed to a decrease in nitrate content. In the root, however, the total N content remained unaffected at elevated Zn^{2+} concentrations.

17) Using H^+-selective microelectrodes to study the adaptive response of plant roots to sulfate deficiency

Martin Reich, Marten Staal, J. Theo M. Elzenga and Luit J. De Kok
Laboratory of Plant Physiology, University of Groningen, Groningen, the Netherlands
E-Mail: l.j.de.kok@rug.nl

The knowledge about the response of the sulfate uptake system of plants to sulfate deficiency on the level of gene expression has increased a lot during the last decades. However, there is still a need for a deeper understanding of the bio- and electrochemical processes involved in plant adaptation to sulfate deficiency. Ion-selective microelectrodes are a useful tool to answer questions in various fields of plant physiology, e.g. salt stress, calcium signaling, growth regulation and defense responses to pathogens. In plant nutrition ion-selective microelectrodes have been applied to study e.g. the uptake preference of plants for different nitrogen sources, and the uptake of calcium and potassium into wheat seeds. However, determination of sulfate uptake by plant roots using ion-selective microelectrodes was not carried out until now, probably also due to the lack of a liquid ion exchanger that is selective enough for sulfate over other anions. Sulfate is taken up by plant sulfate transporters via sulfate/proton-symporters and in the current case study the activity of sulfate transporters of plant roots was measured by using the non-invasive *Microelectrode Ion Flux Estimation* method (MIFE). Roots of seedlings of Chinese cabbage which were grown under sufficient nutrient conditions were compared with roots that had been sulfur deprived with the result that the latter showed a much higher proton influx in response to addition of $MgSO_4$. This indicated that the activity of the sulfate uptake system could be determined adequately by measuring proton fluxes using the MIFE technique. Sulfate-deficient plants acidified their rhizosphere via proton efflux, while sulfate sufficient plants did not. Furthermore the extent of the response to $MgSO_4$ addition to roots of sulfate-deficient plants correlated strongly with the root surface pH prior addition. This indicated that acidification might be an important component of the adaption to sulfate deficiency, as known for other nutrients such as phosphate and iron. This component might be an explanation for the discrepancies between the expression levels of sulfate transporters and the actual sulfate uptake capacity that are sometimes observed under sulfate deficiency. The involvement of the plasma membrane located ATPase in this adaptation will be further examined. Using MIFE, it was also possible to record root profiles of the sulfate import along the first seven millimeters from the root tip. Preliminary results suggest that there is no sulfate uptake taking place in the first 3.5 mm from the tip, but in the mature zone of the root. H^+-selective electrodes show promise as tool to further investigate adaptive responses of plant roots to sulfate deficiency, other than solely an up-regulation of the sulfate transporter genes.

18) Metabolic coordination of sulfur metabolism in *Brassica napus* by clock-controlled genes

Annekathrin Weese, Jutta Papenbrock, Anja Riemenschneider
Institute of Botany, Leibniz University Hannover
E-Mail: anja.riemenschneider@botanik.uni-hannover.de

Experiments done with *Arabidopsis* plants so far, such as results from microarrays, indicate that some key enzymes in sulfur metabolism are regulated diurnally and/or by the circadian clock. Understanding metabolic changes in plants and determining output-related function of clock genes could help in elucidating circadian-clock mechanisms underlying plant growth and development. The sulfur requirement of the plants fluctuate during plant development and vary between species differing in sulfur need for growth and the potential sink capacity of secondary sulfur compounds.

Several sulfur-containing metabolites are involved in pathogen defense mechanisms and are suggested as compounds to enhance defense (SED = sulfur enhanced defense). We analyzed whether in addition to genes involved in sulfur metabolism also the levels of sulfur-containing metabolites such as cysteine and glutathione were clock regulated. They could be released rhythmically to the apoplastic fluid or via the stomata and defend the plants against attacking pathogens.

Oilseed rape (canola) is one of the most important agricultural crop plants for oil and declared as renewable resource. Two different *Brassica napus* L. (oilseed rape) lines were analyzed on the transcriptomic and metabolomic level in light/dark and

free-running conditions. Different sulfur fertilization was applied to analyze the effects on pathogenesis-related compounds. Results from these experiments could help to optimize the use of fertilizer and if applicable reduce the amount of fungicides/pesticides.

19) The effect of the continuous light in combination with sulfur deprivation on the chlorophyll levels and carotenoids in young maize leaves

George Dilias, Styliani N. Chorianopoulou, Dimitris L. Bouranis
Plant Physiology Laboratory, Crop Science Department, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece
E-Mail: s.chorianopoulou@aua.gr

Light fulfills two significant roles in plant growth. At first, light drives photosynthesis by providing energy and second it is perceived by several photoreceptors, thus activating signal pathways. Continuous light changes plant physiology by affecting both roles, thus creating difficulties in identifying the factors that are responsible for injuries under such treatment. As far as we know, the effect of continuous light in combination with plant's nutritional status or nutritional deficiency on its physiology is poorly studied. In particular, there are no references with regard to sulfur deficiency. Towards this direction, the responses of *Zea mays* plants to light environment in combination with nutrition were studied in four treatments; C: normal photoperiod & complete nutrient solution, Cc: continuous light & complete nutrient solution, −S: normal photoperiod & nutrient solution without sulfur, −Sc: continuous light & nutrient solution without sulfur. Plants were grown for seven days under normal photoperiod condition and then the treatment was applied for 3 weeks. The photon flux density was not modified during this period. The effect of the above mentioned cases on growth was monitored via fresh mass measurement, whilst the corresponding effect on the photosystems antennas was followed by determining the extractable levels of Chl *a*, Chl *b* and carotenoids from leaf lamina or sheath, by means of dimethyl sulfoxide. Our results showed that the treatments affected the time of organ appearance as well as their presence itself. The ratio of Chl *a/b* as well as the ratio of carotenoids to total chlorophyll proved to be useful response indicators to each treatment. The dynamic of adjustments presented by the sheaths (Sh) were different than the corresponding ones presented by the laminas (L).

Under continuous light and complete nutrition, the influence focused on L_4, L_5, L_6, whilst L_7 did not occur. Sheaths appearance was not affected except for Sh_5. Injuries due to this condition focused on youngest leaf from L_4 onwards and Sh_4 onwards. The lamina overall average of Chl a/b ratio was 4.1 (an increase by 13.9%), whilst in sheaths it was 2.7 (decreased by 6.9%). The laminas average Car:Chl ratio was 2.1 (decreased by 4.5%), whilst in sheaths it was 3.1 (increased by 10.7%).

A two days delay was observed in laminas L_5, L_6, L_7 and sheaths Sh_2, Sh_3 during the treatment of sulfur deficiency under normal photoperiod. No injuries were caused in laminas. The average Chl *a/b* ratio of the laminas was 3.8 (increased by 5.6%), whilst the average one in sheaths was 2.6 (decreased by 10.4%). In laminas, the average of Car:Chl ratio was 2.5 (increased by 13.6%), whilst in sheaths the corresponding average was 2.9 (increased by 3.6%).

With regard to treatment with continuous light combined with sulfur deficiency, the appearance of organs took place at the same time as in control plants, with the exception of L_7 and Sh_2. This fact indicates that the deficiency eliminated the effect of continuous light. Aging and collapsing was observed at the oldest organs L_0, L_1, Sh_0, Sh_1. In laminas, the average value of Chl *a/b* ratio was 3.8 (increased by 5.6%), whilst in sheaths the average was 2.7 (decreased by 6.9%). At the end of the experiment, in laminas the average of Car:Chl ratio was 2.7 (increased by 22.7%), whilst in sheaths the corresponding average was 2.8 (as in control plants).

20) Aerenchyma formation in maize leaves during sulfate deprivation

Filippa Maniou, Styliani N. Chorianopoulou, Dimitris L. Bouranis
Plant Physiology Laboratory, Crop Science Department, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece
E-Mail: s.chorianopoulou@aua.gr

Aerenchyma is the term given to plant tissues containing enlarged gas spaces exceeding those commonly found as intracellular spaces. So far, aerenchyma formation under nutrient deficiencies and especially under nitrogen- or phosphorus- or sulfur deficiency has been reported only in the adventitious roots of maize by lysis of cortical cells. Seven-day-old maize plants were grown in a hydroponics setup for nineteen days under sulfate deprivation against plants grown under full nutrition and samplings were taken at day 17 and 26 from sowing (day 10 and day 19 of the deprivation respectively). Samples from the fresh laminas of the 2nd leaves were fixed and embedded in paraffin. Sections were received with microtome from the top, middle and base of the lamina and stained with safranin-fast green. The dry mass and water amount, the sulfate and total sulfur contents and the specific surface area of the 2nd leaf lamina and the transpiration rate of the plant were determined.

Under the circumstances we observed the presence of enlarged spaces in this lamina of the S-deprived plants, a fact that to our knowledge has not been reported so far. More specific, on the 10th day under the deprivation, the cross sections from the top of the 2nd leaf lamina of the S-deprived plants, revealed larger substomatal chambers compared to the control plants under full nutrition. In the middle of the S-deprived plants lamina-enlarged spaces appeared among the vascular bundles probably caused by lysis of mesophyll cells. These enlarged spaces stretched from the upper to the lower epidermis or between the stoma and the epidermis with equal frequency of appearance, whilst they appeared fewer times between the upper and the lower stoma. The percentage of the aerenchyma in relation to the total cross section area reached 4.9%. On the base of this lamina enlarge gas spaces appeared too, however to a very small extent, since the percentage of the aerenchymatous area was 0.3% of the total area of the section. On this day, the 2nd leaf of the S-deprived plants contained a significantly lesser amounts of sulfate, organic sulfur and total sulfur by 74%, 38% and 48% respectively compared to control plants. The S-deprived lamina's dry mass and water amounts were as in control. The specific surface area of the lamina (dry mass per lamina surface area) of the S-deprived plants was less by 19% compared to control plants. The S-deprived plants presented a larger transpiration rate by 28% than the control plants. These data indicate that on the tenth day of deprivation, aerenchyma may be formed in maize leaves in response to sulfur deficiency following the described pattern between the vascular bundles.

On the 19th day under the deprivation, such enlarged spaces appeared only in the middle of the lamina of the S-deprived plants 2nd leaf and the percentage of this aerenchyma reached just the 0.7% of the total cross section area. On this day, the 2nd leaf of the S-deprived plants presented less amounts of organic

and total sulfur by 68% and 63% respectively in comparison with control plants, whilst the corresponding percentage of the sulfate was 14% more than control plants. Furthermore, the S-deprived lamina presented more dry mass and water amount by 10% and 19% respectively. The specific surface area was as that of control plants and the transpiration rate of the S-deprived plants was decreased by 16% compared to control plants. It is concluded that somehow an alleviation process took place between the first and the second experimental day under the S-deprivation, a fact which to our knowledge has not been reported so far, too.

21) Metabolic state of Arabidopsis overexpressing SDI1

Fayezeh Aarabi, Hans-Michael Hubberten, Mutsumi Watanabe, Rainer Hoefgen
Max Planck Institute of Molecular Plant Physiology, Science Park Potsdam-Golm, Germany
E-Mail: hoefgen@mpimp-golm.mpg.de

O-acetyl-L-serine (OAS) provides the carbon backbone for cysteine synthesis and is assumed to positively regulate sulfur deficiency-responsive genes as its amount increases upon sulfur limitation. Therefore, mRNA accumulation of a number of sulfur responsive genes might be controlled by OAS. Recently this hypothesis was strengthened by global co-expression analysis under conditions of variable OAS levels with constant sulfur status. The function and the signaling cascade regulating the identified OAS cluster genes (*SDI-1*, *SDI-2*, *LSU1*, *LSU2*, *ChaC* and *SHM7*) are mostly unknown and need further elucidation. Functional characterization of SDI (sulfur-deficiency-induced 1 and 2) is the focus of this study as these candidate genes are prominently expressed upon sulfate deprivation, which is always coupled to OAS accumulation, and upon sulfate deprivation independent OAS accumulation. To promote our understanding of signaling processes and of the actual function of these genes, knock-out and double knock-out as well as overexpresser lines were produced. These lines are under investigation mainly employing transcriptomic and metabolomic approaches. GC/TOF measurements revealed significant increases of most amino acids in lines over-expressing SDI, most prominently alanine, asparagine and serine, as well as the polyamines putresine and spermidine. The levels of most sugars, sugar alcohols and organic acids (especially citrate, succinate, fumarate and malate) were significantly reduced in the same lines. While SDI double knock-out lines have reduced Cys levels, SDI1 overexpressers were found to have significantly elevated Cys levels and also accumulate more sulphate than WT plants under non sulphur limiting conditions. Besides the metabolic changes, SDI overexpresser lines display severe morphological phenotypes. Six-week-old plants grown under either shortday or longday conditions are much smaller in size than WT and they show delayed bolting and flowering and reduced seed production.

22) Making wealth out of sulfides and its consequences in Lavrion peninsula, Greece

Dimitris L. Bouranis[1], Kostantinos G. Serelis[2], Styliani N. Chorianopoulou[1]
[1] Plant Physiology Laboratory, Department of Crop Science, Agricultural University of Athens, Athens, Greece
[2] Mineralogy and Geology Laboratory, Department of Natural Resources and Agricultural Engineering, Agricultural University of Athens, Athens, Greece
E-Mail: bouranis@aua.gr

The history and the environmental issues of Lavrion are closely connected to sulfur and this connection was the subject of an excursion during the 4th Sulphyton Workshop. Lavrion is a town inhabited by more than 10,000 people, situated fifty five kilometers from Athens in the eastern part of Lavreotiki peninsula. Lavreotiki is located in the southeastern tip of Attica, it covers a surface of approximately 200 square kilometers where the places of Thorikos, Kamariza, Plaka, Botsaris Valley, Cavity Soureza Valley, Megala Pefka, Dimoliaki etc. are situated. Most of these places are remarkable centers of cultural and scientific tourism with ancient mines and ore washing plants, the Ancient Theatre of Thorikos, the natural National Park of Sounion, and the temples of Neptune and of Athena at Sounion. Lavreotiki was the center of considerable mining and smelting activities from ancient to recent times, due to its rich mineral wealth, which consists of argentiferous mineral deposits as polymetallic sulphides and iron ore. For this reasons, the history of Lavrion mines is directly connected with the history of Greece.

Many important metal ores are sulfides: argentite (silver sulfide), cinnabar (mercury sulfide), galena (lead sulfide), molybdenite (molybdenum sulfide), pentlandite (nickel sulfide), realgar (arsenic sulfide), stibnite (antimony sulfide), sphalerite (zinc sulfide), pyrite (iron disulfide), and chalcopyrite (iron-copper sulfide). There are mainly two types of mineralization in the substratum of Lavreotiki: a primary mineralization of mixed sulfide ores of basic metals, such as lead, zinc, iron and copper, which has been intensely exploited and a limited metallifery of Fe-Mn ores. A number of other sulfide minerals (e.g. Cu, As) and a variety of sulfur salts also take part in the ore mineral suite of the area. The accessory minerals that prevail are fluorite, calcite, barite, quartz and dolomite. Sulfide mineralization is mainly hosted within the carbonate formations.

The ancient Greeks extracted mainly silver and lead from the minerals of Lavrion, and especially from galena ore, with remarkable techniques. The galena ore was enriched in washing units by using water under pressure in order to remove the light and poor in metal parts of the ore. The enriched galena was converted to the oxide form (PbO + Ag) by the process called reduction roasting that produced lots of sulfur dioxide emissions in the atmosphere. The PbO + Ag chemical compound called "litharge" was directed into a furnace, where it melted with carbon (a process called reduction melting) and produced a PbAg alloy. The Pb-Ag alloy was next placed into a fireproof ceramic cup and heated in a specially constructed furnace were air was blowing under pressure. At 900°C the alloy melted and lead was oxidizing whilst silver was not oxidizing. Then the alloy formed two separate layers, a heavy one containing silver in the bottom of the cup and a lighter containing litharge in the top. Litharge was removing and the process was continuing until the silver in the bottom was pure (over 99%). The precious metal was then ready for coins construction.

The mining history of Lavrion began at Thorikos, a settlement to the north of Lavrion and one of the most ancient industrial areas in Europe. The peak of mining and smelting activities was between the 6th and 4th centuries BC, and especially during the 5th century BC, the Golden Age of Athens. After the 4th century BC the exploitation declined. Mining and smelting activities continued intermittently until the 1st century AD. During the Roman and Early Byzantine period (2nd century BC – 6th century AD) it was sporadic and of small scale. Lavrion area between the 6th and 19th century was abandoned, losing all of its old glory. During the period 1864–1989 mining and metallurgical activities exploited the remaining deposits and the huge piles of mineral and metallurgical wastes, rich in Pb, Zn, Cu, Fe and other mineral elements. Enormous quantities of wastes from these activities, were deposited in heaps around Lavrion area,

near the coastline, or dumped into the sea and heavily burdened the natural environment.

Nowadays, three main categories of these wastes occur in the Lavrion urban area: Slags, acid-generating sulfidic (pyritiferous) tailings and carbonaceous beneficiation tailings, exhibiting different geochemical and environmental characteristics. These wastes cover approximately 25% of the area (Fig. 1 and 2). Their subsequent redistribution by aerial, fluvial and anthropogenic processes has caused the contamination of surface residual soil and house dust. Lavrion has many peculiarities with respect to its contamination characteristics. The local population in Lavrion is exposed to multiple hazardous pollution sources, each of which contributes with a different intensity and through different pathways to the migration of contaminants and their final intake by humans. Extended pollution has been determined especially Pb, and As, whilst increased concentrations of heavy metals are present especially in the mineral excavation areas and in the processing and depositing waste areas. High concentrations of heavy metals are recorded in groundwater of Lavrion, too. These facts reveal the coexistence of physical and anthropogenic pollution in these areas with obvious adverse effects on humans, animals and plants. The molecular links between metals in the environment and plant sulfur metabolism have been reviewed. No phytoremediation actions have taken place in Lavreotiki. *Arundo donax* (giant reed, Poaceae) is widely spread in Lavreotiki peninsula. It is a fast growing, robust, invasive perennial grass which can easily adapt to different ecological conditions and grows in all types of soils; it is a highly pest-resistant crop and it produces high biomass (Fig. 3). Growth and photosynthesis of giant reed were not affected by high Cd and Ni soil content. For these reasons giant reed has been proposed as a potentially high-yielding nonfood crop, which displays many attractive characteristics for the production of biomass, of pulp and paper and of activated carbons.

Sulfur and Lavreotiki seem to be interwoven to one another. From the sulfurous ores to the sulfidic tailings through the centuries, a long chain of mining and metallurgical activities has led to a seriously contaminated land, starting from wealth that produced history, culture, philosophy and science, beyond the amazing, slavery-based mining and metallurgical technology.

Fig. 1. Olive trees grown on calcareous soil at the boundary between the National Park of Sounion and the outskirts of Lavrion near the old factory of the French metallurgical company; lots of heaps of metallurgical wastes have been deposited nearby, several of which are sulfidic tailings (photo: Dimitris Bouranis).

Fig. 2. Contamination of the golf of Thorikos with sulfidic metallurgical processing wastes of Komobil. These tailings (produced between 1953–1963) are characterized by high levels of Ag, As, Bi, Co, Fe, K, S, Sb, Sn and V (photo: Dimitris Bouranis).

Fig. 3. Giant reed (*Arundo donax*) grown nearby the sulfidic tailings that depicted in Fig. 2, very close to the seashore (left) and in a slightly contaminated calcareous soil of the area (right) (photo: Dimitris Bouranis).