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Regulation
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Signaling

editors: J.-C. Davidian - D. Grill - L.J. De Kok - I. Stulen - M.J. Hawkesford
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REGULATION, INTERACTION AND SIGNALING

Edited by J.-C. Davidian, D. Grill, L.J. De Kok, I. Stulen, M.J. Hawkesford, E. Schnug and H. Rennenberg

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PREFACE

This book contains the invited and contributed papers of the 5th Workshop on Sulfur Transport and Assimilation in Plants, a joined European Commission (COST Action 829) and OECD meeting hosted at the Ecole Nationale Supérieure Agronomique in Montpellier (France) from April 11 to 14, 2002. The meeting was co-organized by the ENSA-Montpellier (France), the University of Graz (Austria), the University of Groningen (The Netherlands), Rothamsted Research, (United Kingdom), Institute of Plant Nutrition and Soil Science, Braunschweig (Germany), the Agricultural Biotechnical Center of Gödöllö (Hungary), Albert-Ludwigs-University Freiburg (Germany) and the University of Chiba (Japan).

We are very pleased to dedicate this book to Prof. Dr. Christian Brunold, University of Bern, Switzerland. His views and research have significantly contributed to advanced understanding of the physiological, biochemical and molecular regulation of the sulfur metabolism pathway and its interactions with nitrogen metabolism. We also dedicate this book to Dr. Yolande Surdin-Kerjan, CNRS, Gif-sur-Yvette, France, whose outstanding studies on genetics and molecular biology have contributed to the identification of most of the genes involved in the regulation of the sulfur metabolism pathway of the yeast *Saccharomyces cerevisiae*.

Jean-Claude Davidian
Dieter Grill
Luit J. De Kok
Ineke Stulen
Malcolm J. Hawkesford
Ewald Schnug
Heinz Rennenberg

editors

Yolande Kergan

FOREWORD (I)

SULFUR METABOLISM

Yolande Surdin-Kerjan
Centre de Génétique Moléculaire, CNRS, 91198 Gif-sur-Yvette, France

I entered the "Ecole Nationale Supérieure de Chimie de Paris" with the intent of becoming a chemical engineer and then working in industry. After graduation I was confronted with two facts: chemical industry was hostile to the presence of women and anyhow, I was not good enough at it to consider the life with chemistry. I thus went to visit Dr. Françoise Labeyrie who was a friend of my family and whose laboratory was devoted to the study of cytochromes. It took her only four hours to persuade me that Biology was the research field to join. She introduced me to Dr. Huguette de Robichon-Szulmajster who just created her research group to study the metabolism of amino acids deriving from aspartate in *Saccharomyces cerevisiae*. Huguette wanted to use *S. cerevisiae* as a model organism in a world then almost completely devoted to *E. coli*. I was naive enough not to realize that, so this point did not bother me, but I must say that, during my thesis work, I moderately appreciated the remarks that I heard from time to time ("yeast ?, why not rhinoceros…"). So, in September 1962, I got a position in CNRS, and joined Huguette's laboratory.

It was only in 1969, after obtaining my Ph.D. that I switched to methionine metabolism the study of which had been initiated by Hélène Chérest (who still works with me). In 1974 Huguette died from a cancer without knowing how good had been her intuition to use yeast as a model organism. I had to take over and during the next 10 years, we characterized many genes involved in the sulfur amino acids metabolism. More particularly we isolated the first yeast strains impaired in sulfate transport. During these years also, I met Dominique Thomas who came in the laboratory as a student and stayed. I thank him for staying, for being so enthusiastic about the study of the regulation of transcription of the sulfur amino acids metabolism, which he turned into a very sophisticated model and for sharing my interest in metabolism.

In 1992, I met Jean-Claude Davidian. He phoned me to get our sulfate transporter mutants, wanting to clone the plant sulfate transporter genes. Unfortunately, I had lost them. He was disappointed but talked me into trying to isolate such mutants again. He thus spent several months in my laboratory and, together with Hélène Chérest, successfully characterized and cloned the genes encoding the two yeast sulfate permeases. Meanwhile, Smith, Hawkesford, Prosser and Clarkson published the isolation of a yeast mutant devoid of sulfate transport. I still think they were

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lucky, as their parental strain expressed only one active sulfate permease! Our paper, describing the isolation and characterization of the two sulfate permeases from *S. cerevisiae*, was published after theirs.

This is how I stepped into the world of plants. At the Montpellier meeting, it was a great pleasure for me to realize that our yeast mutants have been successfully used to clone several plant genes.

Sulfur amino acids metabolism in *Saccharomyces cerevisiae*

The biosynthetic pathway

Sulfur is an essential nutrient for all microorganisms. Extensive growth data has been accumulated showing that *S. cerevisiae* possesses various enzymatic systems that allow it to metabolize almost any sulfur source. In contrast to many other microorganisms, yeast is able to use various organic sulfur compounds, *i.e.* cysteine, methionine, homocysteine, glutathione, and *S*-adenosylmethionine (AdoMet) as a sole sulfur source. This is due to the organization of the sulfur pathway in yeast which allows the conversion of the main sulfur metabolites into the others. This pathway has been almost completely deciphered by my research group, using first biochemistry and classic genetics and later, the powerful reverse genetics methods. Finally, our work has also been greatly helped by the completion of the sequence determination of the yeast genome in 1996. To date, we have characterized more than 15 genes encoding enzymes of the sulfur amino acids pathway (Fig. 1).

In addition, we have shown that some mutants requiring methionine for growth bear mutations in genes encoding enzymes not directly related to the sulfur amino acids metabolism. These genes are *MET19, MET22* and *SOD1*, encoding glucose-6-phosphate dehydrogenase, 3',(2'),5'-bisphosphate nucleotidase and one superoxide dismutase, respectively, as well as the less well characterized *MET18* and *MET27* genes. It is still unclear why mutations within these genes result in methionine auxotrophy, but it suggests that some functions of sulfur amino acids remain to be uncovered (Thomas and Surdin-Kerjan 1997). Recently, we have also characterized the *FOL3* and the *MET7* genes encoding the dihydrofolate synthetase and the folylpolyglutamate synthetase respectively, both involved in the addition of glutamate tails to folate coenzymes (Cherest *et al*. 2000). The *fol3* and *met7* mutants have been used recently to characterize the corresponding plant genes (Ravanel *et al*. 2001).

Recycling of the products of the catabolism of AdoMet

Methionine is not only involved in protein synthesis but is also an essential determinant of the one carbon metabolism. Indeed, under its activated form, *S*-adenosylmethionine (AdoMet), it is the methyl donor in numerous transmethylation reactions of nucleic acids, proteins or lipids. Further, AdoMet serves as a precursor for the biosynthesis of polyamines and is one of the substrates used in a number of reactions, including vitamin biosyntheses and nucleotide modifications.

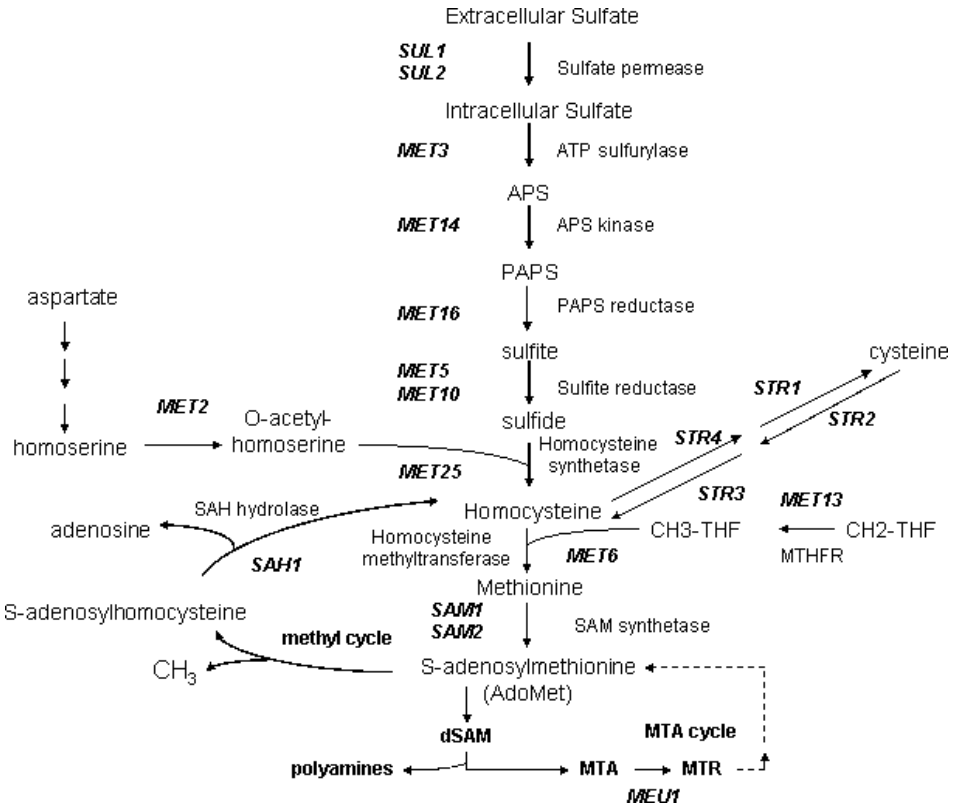

Fig. 1. Metabolism of sulfur amino-acids in *Saccharomyces cerevisiae*. APS, adenosine 5'-phosphosulfate; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; MTHFR, methylene tetrahydrofolate reductase; CH_3THF, methyl-tetrahydrofolate; CH_2THF, methylene tetrahydrofolate; MTA, methylthioadenosine; MTR, methylthioribose-1-phosphate; dSAM, decarboxylated AdoMet.

Given such ubiquitous functions, the equilibrium between methionine and AdoMet is thus expected to be of crucial importance for the overall cellular homeostasis. In eucaryotic cells, the methionine/AdoMet ratio was thought to be largely controlled through two recycling pathways that act on the products of AdoMet catabolism. The first one, called the methyl cycle, allows the conversion of S-adenosylhomocysteine, the by-product of all transmethylation reactions, into homocysteine which is next remethylated into methionine by the methionine synthase. The second one comprises a set of complex reactions that allow the direct synthesis of methionine from 5'-methylthioadenosine (MTA), a compound formed mainly during polyamine biosynthesis. In this pathway (called the MTA cycle), the ribose moiety of the adenosyl group gives rise to the four carbon skeleton of methionine while conserving the methylthiol group (Fig. 1).

Using molecular genetics, we have been able to prove that the methyl cycle as well as the MTA cycle are both active in *S. cerevisiae*. In addition, more recently, we have uncovered a pathway allowing the utilisation of *S*-methylmethionine by yeast, and we have shown that AdoMet can be used to directly methylate homocysteine, yielding methionine in what seems to be a futile cycle. However, this cycle is fully active when AdoMet is the sole sulfur source present in the growth medium (Thomas *et al*. 2000).

Transport of sulfur compounds

Transport systems allow yeast cells to extract virtually any sulfur compounds from their environment. Using specifically designed genetic screens, we have isolated and characterized: (i) the genes encoding the two sulfate transporters, Sul1p and Sul2p (Cherest *et al*. 1997). As I mentioned, the *SUL1* gene was isolated first by Smith *et al*. (1995); (ii) the two methionine transporters, Mup1p and Mup3p (Isnard *et al*. 1996); (iii) the AdoMet transporter, Sam3p (Rouillon *et al*. 1999); and (iv) the *S*-methylmethionine transporter, Mmp1p (Rouillon *et al*. 1999).

In addition to these highly specific transport systems, biochemical uptake assays have revealed the presence in yeast of less specific transport systems for most of the sulfur compounds.

Regulation of sulfur amino-acids biosynthesis

The first observations about the *MET* regulatory system were made in the 1970's. We showed that when wild type *S. cerevisiae* cells are grown in the presence of a high concentration of methionine (1 mM), a decrease of the synthesis of all the enzymes implicated in methionine biosynthesis is measured. Later, we were able to show that this negative regulation is acting at the transcription level and that the signal was AdoMet. Then, we identified several *cis*-acting regulatory sequences found upstream of the *MET* genes and demonstrated that the negative regulation resulted from the non-activation of the transcription of the genes (Thomas *et al*. 1989).

During the 1990s, we isolated most of the genes encoding the factors responsible for the transcriptional regulation the *MET* genes, *i.e*. Met4p, Met28p, Met30p, Met31p and Met32p. Meanwhile, Cbf1p, a protein that functions at both centromeres and *MET* gene promoters was characterized in several laboratories and its precise role in *MET* gene regulation was deciphered in my laboratory. All these factors were identified either by specific genetic screens or through specific molecular assays, such as one hybrid experiments.

The identification of so many factors was suggestive of an unanticipated complexity of the molecular mechanisms underlying the regulation of the *MET* gene network. We have been able to decipher the precise function of all these factors. Briefly, a unique transcriptional activator, Met4p, is recruited to the *MET* promoters trough the assembly of different large multiprotein complexes (Cbf1p-Met28p-

Met4p and Met4p-Met28p-Met31p (Met32p)) (Kuras *et al.* 1996; Kuras *et al.* 1997; Blaiseau and Thomas 1998) Indeed, the binding of Met4p to DNA varies from one gene to another. Once tethered to DNA, Met4p activates the transcription of the downstream gene owing to an unique acidic activation domain. When the cells are exposed to a high methionine concentration, Met4p is inactivated through the activity of the SCF^{Met30} ubiquitin ligase and *MET* gene transcription is turned off. SCF ubiquitin ligases are multi-protein complexes, that use substrate-specific adapter subunits termed F-box proteins (such as Met30p) to recruit substrates for ubiquitylation by a core apparatus, which is composed of the scaffold protein Cdc53/cullin, the RING finger protein Rbx1, the adapter protein Skp1, and the E2 enzyme Cdc34. Surprisingly, the consequence of Met4 ubiquitylation differs according to the growth conditions. In cells grown in minimal medium, Met4 ubiquitylation triggers its degradation by the 26S proteasome (Rouillon *et al*. 2000). In contrast, in rich medium, ubiquitylated Met4p is stable but unable to bind to the *MET* promoters while ubiquitylated Met4p is still capable of activating the AdoMet biosynthesis genes. Thus, ubiquitylation not only regulates the *MET* gene network by distinct degradation-dependent and -independent mechanisms, but also controls the differential recruitment of Met4p, thereby diversifying its activation specificity (Kuras *et al*. 2002).

I am very grateful to the editors and more particularly to Jean-Claude Davidian for partly dedicating this volume to me. And I am happy to share this honour with Dr. Brunold whom I have learned to know during these last three sulfur meetings and whose great knowledge of plant sulfur metabolism I have come to appreciate. This honour is a great reward and well compensates me for the hard work during all these years. I think that I have lived during a fantastic period of time during which, starting with a partially known biosynthetic pathway, we could end by formulating a recognized model for the regulation of transcription. This was greatly helped by our knowledge of the metabolism itself. With the modern techniques that can be now used for plant metabolism, I hope that the young scientists will live rewarding moments as I did.

Acknowledgements

I thank my friend Professor Pete Magee for editing this manuscript and for being always present to help me when needed, ever since we met in Huguette's lab in 1965.

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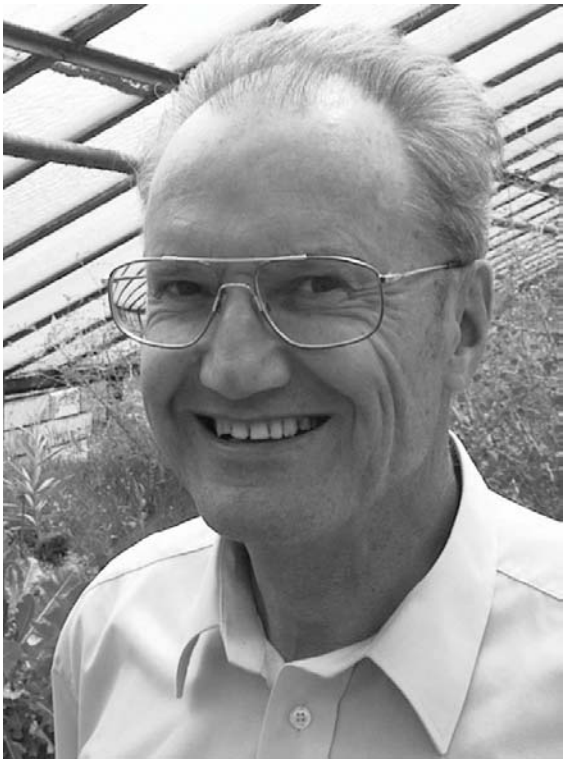

FOREWORD (II)

SULFUR NUTRITION AND SULFUR ASSIMILATION OF HIGHER PLANTS

Christian Brunold
IPS, University of Berne, Altenbergrain 21, 3013 Bern, Switzerland

H_2S as a starting point

I wish to thank the organizers of the 5th International Workshop on Sulfur Nutrition and Sulfur Assimilation in Higher Plants for their kind dedication of this distinguished volume containing many important contributions to the advancement of our knowledge of sulfur metabolism to Yolande Surdin-Kerjan and myself.

Sulfur metabolism was the main field of my scientific work since I started my Ph.D. thesis in 1969. At that time, my adviser, Prof. K.H. Erismann, suggested to find out, if higher plants could use electrons from H_2S instead of H_2O for producing reducing equivalents in photosynthesis. Since I was more interested in metabolism than in electron transport, I proposed to use H_2S as sulfur source and to analyze, if it had a regulatory effect on sulfate assimilation. At that time we knew that *Spirodela oligorrhiza* (Ferguson 1969), but also *Lemna minor*, the organism preferentially analyzed in Erismann's laboratory, used NH_4^+ rather than NO_3^- as a nitrogen source, when both ions were available in the nutrient solution. With this knowledge in mind, the obvious working hypothesis was that H_2S rather than SO_4^{2-} would be used as a sulfur source, when both were available. *L. minor* turned out to be cooperative, did exactly what we expected, and thus made it possible to finish the thesis in 1972. The results obtained can be summarized as follows: In *L. minor* gassed with 6 ppm H_2S, uptake and assimilation of sulfate were almost completely inhibited. H_2S taken up was partly directly used for cysteine formation, thus increasing the cysteine content (Brunold and Erismann 1975) and partly oxidized, which increased the sulfate content. The work with H_2S treated *L. minor* had several very positive consequences, since (i) I was offered a position at the Institute of Plant Physiology at the University of Berne, Switzerland, and could start an academic career, (ii) I got a grant from the Swiss National Science Foundation (SNF) for working in the laboratory of Jerry Schiff at Brandeis University, which led to an inseparable tie with the field of sulfur metabolism, and to my first publication in an international plant journal, (Brunold and Schiff 1976), (iii) I got into contact with Ineke Stulen and Luit de

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Kok, with whom I collaborated for almost 30 years (Westerman *et al.* 2001), (iv) I was contacted by Ahlert Schmidt for a successful collaboration (Brunold and Schmidt 1976), and (v) I met Dr. Bergmann, University of Cologne, Germany, who brought me into contact with Heinz Rennenberg, with whom I collaborated till the end of my scientific career (Rennenberg and Brunold 1994; Hesse *et al.* 2003). Looking back, I consider it a great privilege that Dr. Erismann allowed me to work in sulfur metabolism for my Ph.D. thesis.

Answers and questions

After studying the regulation and the localization of sulfate assimilation for many years, I now make the experience many scientists made before me: There are more open questions, than when I started. This situation can be well exemplified using adenosine 5'-phosphosulfate (APS) reductase (APR, Setya *et al.* 1996; Suter *et al.* 2000), formerly named APS sulfotransferase (Schmidt 1972), my favored enzyme:

1. Like sulfate transporters (Takahashi *et al.* 2000; Vidmar *et al.* 2000), APR is especially sensitive to a lack (Brunold *et al.* 2002) or a surplus of reduced sulfur (Brunold and Schmidt 1976; Westerman *et al.* 2001; Vauclare *et al.* 2002). There are several indications (Takahashi *et al.* 2000; Vidmar *et al.* 2000; Vauclare *et al.* 2002) that GSH plays a role in signaling the sulfur status of plants, however, the genetic sequences, the genes and the mechanisms involved, which lead to induction or repression of APR during lack and surplus of sulfur, respectively, are not known.

2. The assimilation of sulfate and nitrate is regulated reciprocally in a coordinate manner (Brunold *et al.* 2002). *O*-acetyl-L-serine (OAS) seems to play a role in this coordination (Neuenschwander *et al.* 1991; Harms *et al.* 2000), but the mechanisms involved are not known. This is in contrast to the situation in bacteria, where regulatory mechanisms involving N-acetyl-L-serine (NAS), an isomer of OAS, have been elucidated in great detail (Kredich 2000), which can give indications for analyzing plant systems. APR may be the enzyme of choice for this analysis, because it is very sensitive to regulatory signals and is easy and fast to measure.

3. APR is regulated by sugars (Kopriva *et al.* 1999) and several mechanisms, which might be involved, have been described (Hesse *et al.* 2003). Regulation by sugars is also important in other systems, therefore, checking proposed mechanisms (Smeekens 2000; Hesse *et al.* 2003) and possibly new ideas using APR as an indicator of the sugar status may result in contributions of general relevance in the field of plant sugar sensing.

4. Calculation of the flux control coefficient of APR in *A. thaliana* root cultures indicated that APR had a high flux control coefficient, but was not rate limiting (Vauclare *et al.* 2002). The question is, if corresponding calculations in other systems, especially in green tissues, result in similar values for APR and in addition give values for the flux control coefficients of other enzymes of sulfate assimilation. Such a mathematical analysis would greatly help to understand the enzymatic regulation of this pathway.

5. In maize leaves, APR is localized exclusively in bundle sheath cells, and cysteine formed there is transported into the mesophyll cells, the predominant cell type for GSH synthesis (Burgener *et al.* 1998). Several ideas for explaining this special localization have been discussed, but an unequivocal physiological explanation is still missing. The question is complicated by the fact that the localization of sulfate assimilation in bundle sheath cells as detected in some C_4 grasses is neither a prerequisite nor a consequence of C_4 photosynthesis, since in dicot C_4 *Flaveria* species, APR mRNA and protein were present at comparable levels in both types of cells (Koprivova *et al.* 2001).

6. It seems clear now that higher plants use APS as a sulfonyl donor in sulfate reduction, bacteria either use APS or adenosine 3'-phosphate 5'-phosphosulfate (PAPS), and in the moss *Physcomitrella patens* both APS and PAPS dependent sulfate assimilations coexist (Koprivova *et al.* 2002). The physiological, ecological and evolutionary causes for these different choices of sulfonyl donors are not known.

Scientists, Problems and Money

The interests of the scientists working in a certain field are changing with time, leaving some questions unanswered, focusing on others and possibly, but not necessarily, coming back to the old ones after some time for testing new ideas and new methods. It is therefore possible that at least some questions of the small selection presented above will never be answered, but answers to some of them will certainly be presented soon, because they are not only intriguing for me, but for many colleagues, too. At the same time I look forward to answers to interesting problems from other colleagues of the "sulfur family". I use this term, because over the years, the scientists working on sulfur metabolism of higher plants have developed into a community, in which the individuals do not only know and appreciate each other scientifically, but also personally. From my point of view, this development is mostly due to Ineke Stulen and Luit De Kok, who first initiated the Sulfur Workshops, and later on started COST action 829. This is my last occasion for thanking them for all what they did to promote research on sulfur metabolism. I consider it a great privilege that I got acquainted with both of them long ago, that I could learn a lot from them and that I could feel their sympathy and friendship.

At the beginning of my work on sulfur metabolism, I was not interested in the practical application of my findings, but in basic research. I wanted to know how sulfur assimilation was regulated and where it was localized. In my grant applications, of course, I always stressed that research on plant metabolism in general and on sulfate assimilation in particular was very important, because plants are the absolute basis for nourishing men, and sulfur metabolism was not only essential for the synthesis of storage proteins, but also for coping with many stress situations encountered by plants (Rennenberg and Brunold 1994). My grant applications were accepted, I do not know, however, if the granting committees were especially impressed by my general ideas about the importance of plant metabolism.

Today, more than 800,000,000 people are suffering from hunger, and the first symptoms of climate change with its stressful consequences for plants become evident. Therefore basic knowledge about plant metabolism is more important than ever, but plant scientists have now the additional obligation to contribute to solving practical problems. In this respect, COST actions and EU projects in general represent ideal platforms, and this will even be more the case, when COST is transformed into a system, in which all participating groups are subsidized with grant money. Up to now, granting COST actions was a Swiss specialty. This made the task of the Swiss coordinator easy to bring excellent Swiss groups together for working in a COST action and the future funding will also make the job of the European coordinator a more pleasant one. At the present time, I am happy that 5 Swiss research groups, funded by the Swiss Agency for Education and Science, are working in the "sulfur" COST 829 and that two Swiss groups are participating in a "sulfur" EU project. I am especially happy about the Swiss participation in this EU project, because my successor, Doris Rentsch, joined this excellent consortium of groups.

I would like to thank my friends Luit De Kok, Dieter Grill, Malcolm Hawkesford, Heinz Rennenberg, Kazuki Saito, Ewald Schnug, Ineke Stulen and Ervin Balazs for organizing this workshop. My special thanks go to Jean-Claude Davidian, because his friendship and his kind personality gave this event a touch of warm humanity.

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THE PLANT SULFATE TRANSPORTER FAMILY: SPECIALIZED FUNCTIONS AND INTEGRATION WITH WHOLE PLANT NUTRITION

Malcolm J. Hawkesford, Peter Buchner, Laura Hopkins and Jonathan R. Howarth
Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ,United Kingdom

Introduction

Uptake and transport of sulfate within the plant, including intercellular transport, long distance transport between organs and intracellular transport in and out of organelles is dependent on the activity of a family of sulfate transporters. The differential expression of these transporters during plant development and in response to environmental factors, particularly sulfate availability, optimizes the efficient management of S-resources. The size and diversity of the gene family (14 potential sulfate transporter (ST) genes have been identified in *Arabidopsis*) indicates a substantial complexity in the management of sulfate resources (Hawkesford 2000). Detailed expression and functional analysis is enabling the determination of roles of individual transporters; however mechanisms for integrating patterns of expression with nutritional status of the plant still remain elusive. This review, an update of the situation as presented in the proceedings of the previous Sulfur Workshop (Davidian *et al*. 2000), will outline the present status of the composition of the ST family, and summarize our current understanding of the regulation of expression in relation to nutrient status.

The sulfate transporter gene family

Genes for sulfate transporters were first isolated from *Neurospora crassa* (Ketter *et al*. 1991), *Saccharomyces cerevisiae* (Smith *et al*. 1995b) and from a number of mammalian systems (*e.g.* Hästbacka *et al*. 1994; Schweinfest *et al*. 1993; Markovich 2001). The cloning of plant sulfate transporters (Smith *et al*. 1995a, 1997) was achieved by heterologous phenotypic complementation of a yeast mutant (Breton and Surdin-Kerjan 1977; Smith *et al.* 1995b). In recent years, following the completion of the *Arabidopsis* genome sequencing project, and contributions from a number of laboratories, the total number of plant sequences determined is in excess of 40 from a variety of species (*e.g.* Bolchi *et al*. 1999; Heiss *et al*. 1999; Vidmar *et al*.

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1999, and summarized in Hawkesford and Wray 2000). A 'full' complement of 14 related sequences has been ascertained for *Arabidopsis* (Takahashi *et al*. 1996, 1997, 2000; Vidmar *et al*. 2000; Shibagaki *et al*. 2002; Yoshimoto *et al*. 2002). A phylogenetic tree of the protein sequences based on sequence similarity, following alignment, is presented in Fig. 1A. On the basis of sequence alone, the STs fall into 5 clusters, which are not related to species phylogeny, and for example, *Arabidopsis* STs occur in all of the clusters. The STs within the clusters have characteristics which lead to the designation of sub-types, referred to as Groups 1-5.

The STs in Group 1, which have been analyzed by expression in yeast, are characterized by a high affinity for sulfate (typically 1 - 10 μM). Expression and localization studies indicate that this group is responsible for initial uptake in the root, however not all members of the Group are root specific. Most of the STs in this group show a classical de-repression of expression under S-limiting conditions (Clarkson *et al*. 1983, and see Figs. 2 and 4), as exemplified by the *Brassica napus* STs, BnST1;1 and 1;2 (Fig. 1B). Three Group 1 *Arabidopsis* STs have been identified (see Takahashi *et al*. 2000; Vidmar *et al*. 2000; Shibagaki *et al*. 2002; Yoshimoto *et al*. 2002).

Within Group 2 there are two *Arabidopsis* examples, localized in the vascular tissues (Takahashi *et al*. 1997, 2000). AtSultr2;1 was localized in the xylem parenchyma cells of roots and leaves, the root pericycle and leaf phloem. In contrast, AtSultr2;2 was localized in root phloem and leaf vascular bundle sheath cells. The *B. napus* homologues, BnST2;1 and 2;2 were both induced by S-starvation in root tissues, and 2;1 was predominantly expressed in the stem and leaf (Fig. 1B). These patterns contrast with those found in *Arabidopsis* and advise caution when making generalizations from *Arabidopsis* alone. In contrast to the Group 1 STs, all Group 2 transporters examined to date, show a low (>0.1 mM) affinity for sulfate.

Group 3 has been referred to as the 'leaf group', based on initial localization of AtsSultr3;1-3 (Takahashi *et al*. 1999b, 2000) and the *Sporobolus stapfianus* ST which was isolated from shoot tissues (Ng *et al*. 1996). Consistent with this description, BnST3;1 is leaf/stem specific (Fig. 1B). Little more is known of the characteristics of Group 3, and expression of the other two *Arabidopsis* group members has not been demonstrated.

Group 4 STs have been reported to be plastid localized (Takahashi *et al*. 1999a), and by implication therefore are responsible for the essential step of import of sulfate into the plastid prior to reduction. More recent data utilizing a range of reporter constructs suggests that this transporter may be localized in the tonoplast (Hideki Takahashi, pers. comm.). The de-repression of expression of the *B. napus* homologue, BnST4;1 by S-starvation (Fig. 1B) would support a role in maximizing vacuolar unloading of stored sulfate under these conditions. It would also be consistent with maximizing uptake into the plastid.

A number of distantly related sequences, including two from *Arabidopsis* are placed in Group 5. These are truncated sequences lacking the usual long N- and C-terminal domains, which are thought to extend into the cytoplasm. BnST5;1 is expressed constitutively in all tissues examined with no effect of sulfur nutrition (Fig. 1B). The roles of any of the putative STs in Groups 3-5 remain to be confirmed, as

non have been expressed and characterized successfully in yeast or other heterologous systems.

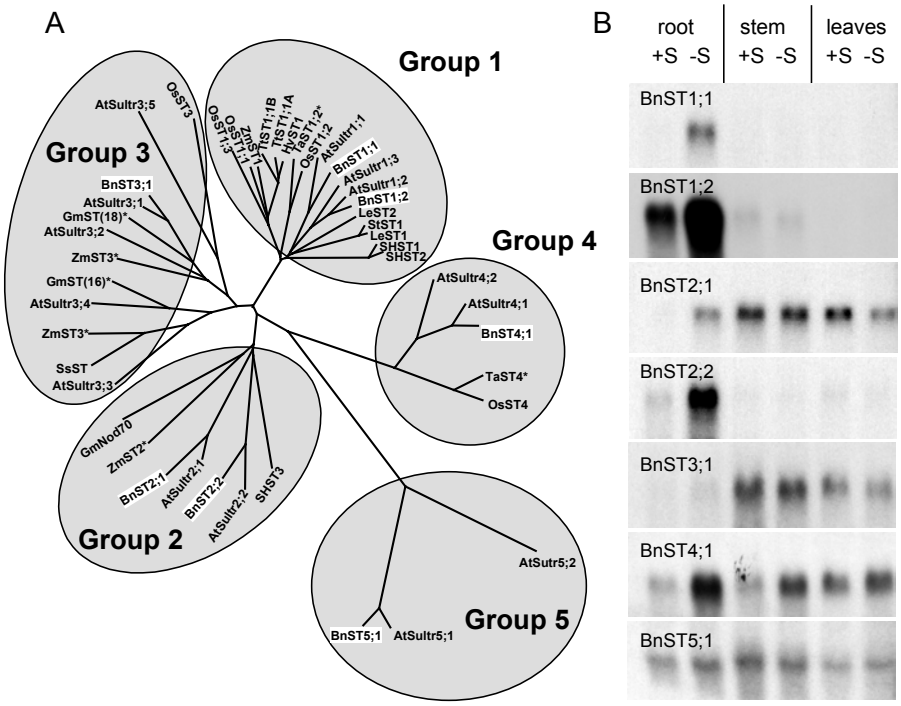

Fig. 1. Phylogenetic representation of the plant sulfate transporter amino acid sequences showing A: subdivision into 5 groups, and B: expression patterns of representatives of the groups from *Brassica napus*. Accession numbers: *Arabidopsis*: AtSultr1;1, AB018695; AtSultr1;2, AB042322; AtSultr1;3, AB049624; AtSultr2;1, AB003591; AtSultr2;2, D85416; AtSultr3;1, D89631; AtSultr3;2, AB004060; AtSultr3;3, AB023423; AtSultr3;4, B054645; AtSultr3;5, AB061739; AtSultr4;1, AB008782; AtSultr4;2, AB052775; AtSultr5;1, NP_178147; AtSultr5;2, NP_180139; *Triticum tauschii*: TtST1;1A and 1B, AJ238244 and AJ238245, respectively; *Hordeum vulgare*, X96431; *Brassica napus/juncea*: BnST1;1, AJ416460; BnST1;2, AJ311388; BnST2;1, unpublished; BjST, AJ223495; BnST3;1, unpublished; BnST4;1, AJ416461; BnST5;1, AJ311389; *Zea mays*: ZmST1-1, AF355602; *Stylosanthes hamata*: SHST1, X82255; SHST2, X82256; SHST3, X82454; *Sporobolus stapfianus*: SsST (X96761); *Lycopersicon esculentum*: LeST1, AF347613; LeST2, AF347614; *Solanum tuberosum*: StST1, AF309643; *Oryza sativa*: OsST1;1, AF493792; OsST4, AF493793; OsST3, BAB68064.1; OsST1;2 and 1;3 from Genome Database of Chinese Super Hybrid Rice. For all sequences marked with an asterisk (*), see DuPont patent application No. WO 00/04154. Alignments were performed using CLUSTAL W program (Thompson *et al*. 1994) version 1.7 and the tree was drawn using the Treeview32 program (Page 1996). Expression patterns were analyzed by Northern blotting, with equal (10 μg total RNA) loading of total RNA. 14 d old hydroponically grown plants were starved of sulfate for 3 d, where indicated (+/-S).

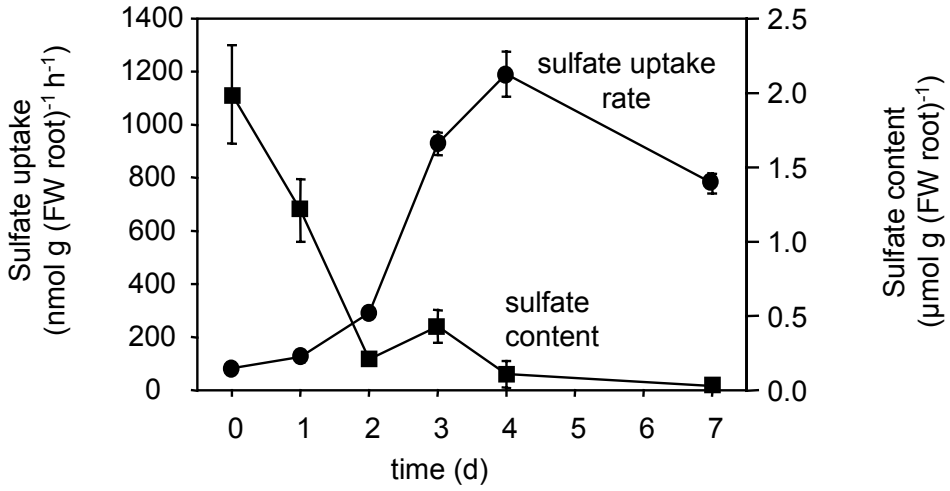

Fig. 2. The 'classical' de-repression experiment. Sulfate uptake capacity as measured by sulfate tracer influx from a 0.15 mM ^{35}S-sulfate solution over a 10 min period. Sulfate content measured by ion chromatography. Data adapted from Smith *et al.* 1997.

Regulation of expression: the role of S-compounds

Sulfate uptake and activity of other components of the assimilatory pathway, such as the ATP sulfurylase and APS reductase, are regulated by the nutrient status of the plant. The classically observed pattern of sulfate uptake capacity is of increased sulfate uptake capacity over a period of several days during limited S supply (Clarkson *et al*. 1983; Fig. 2, and see also Smith *et al*. 1997). Measured tissue concentrations of sulfate and reduced S-compounds such as cysteine and glutathione are decreased when there is no external supply of sulfur. This has led to the proposed model of substrate repression of expression (Fig. 3) by such S-containing compounds. When S supply is adequate, there is a large flux through the pathway and high levels of internal S-metabolites, including sulfate, sulfide, cysteine and glutathione (Fig. 3A). Any of these metabolites are candidates for the negative feedback acting on sulfate uptake. When S is limiting, there is no accumulation of the metabolites, pools become depleted and the negative feedback regulation no longer operates: the system is 'de-repressed' (Fig. 3B). A secondary consequence of these fluxes may be that the cysteine precursor, *O*-acetylserine (OAS), pool size may also fluctuate, being depleted when sulfur supply is adequate and accumulating when sulfide availability is limited.

Regulation of gene expression

The cloning of plant sulfate transporter genes has confirmed the expectation that the mechanism of this repression is clearly at the level of repression of gene expression

(Smith *et al*. 1995a, 1997) (Fig. 4). During the seven-day period of S-starvation the abundance of the mRNA for the barley *hvst1* transporter increases substantially (Fig. 4A). The cloned genes have facilitated the generation of antibodies to the transporter protein, which have been used to quantify the relative abundance of this protein: the ST protein, located in a root plasma membrane fraction, also increased over the time frame of the de-repression (compare Fig. 2 and Fig. 4C). Conversely re-supply of sulfate acts to decrease sulfate transport expression (Smith *et al*. 1997), with a rapid (within a few hours) decrease in both mRNA abundance (Fig. 4B) and ST protein in the plasma membrane (Fig. 4D). There was no evidence for internalized ST protein in membrane vesicles (data not shown) implying that there is a rapid turnover and degradation of the ST protein.

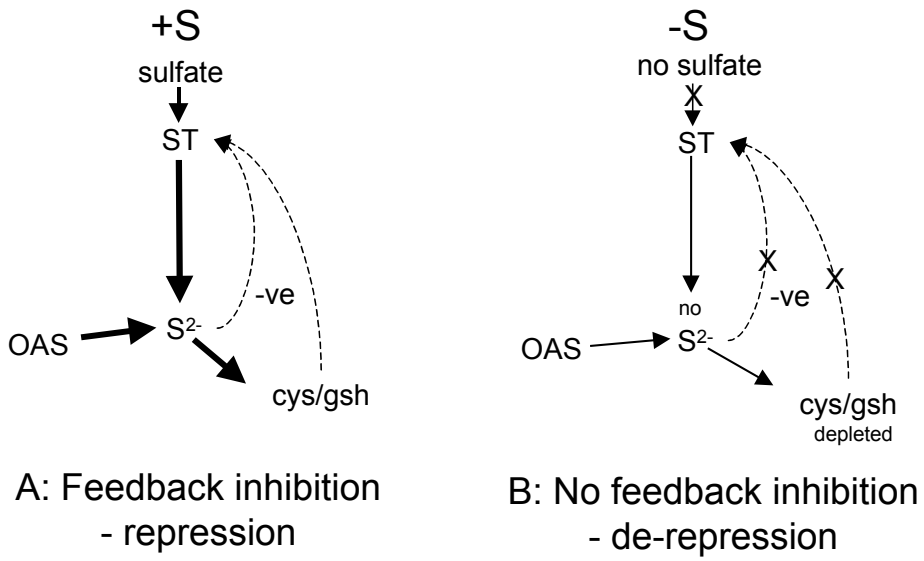

Fig. 3. Regulatory model of repression (A) and de-repression (B) of sulfate transporter gene expression effected by reduced S pools. Solid lines represent substrate fluxes, with line thickness indicating magnitude of flux. Broken lines indicate possible regulatory loops.

Regulation of expression: the role of OAS

As already noted, OAS may accumulate during S-starvation, and this may be involved in the signal transduction pathway as suggested for *Escherichia coli* (Kredich 1993). Exogenously supplied OAS induced ST gene expression and ST expression (Smith *et al*. 1997, and Fig. 5A) and it was concluded that OAS acts as an inducer of expression, as previously suggested for APS sulfotransferase in *Lemna minor* (Neuenschwander *et al*. 1991). As increased pools of cysteine and glutathione were measured, the inducer effect of OAS was greater than the repressive action of these compounds. An alternative explanation is that the over-supply of OAS as a

substrate for cysteine synthesis acts to drive a greater flux through the pathway. A consequence would be the depletion of S-metabolites required for cysteine synthesis, any of which may act as a positive signal. For example, demand for reduced S would lead to decreased sulfide pools (Fig. 5B) and therefore sulfide remains a likely candidate as a signal molecule, as postulated for *E. coli* (Kredich 1993).

ST gene expression is de-repressed by S-starvation in potato (Fig. 6). The mRNA pools increased over an 8 day period of S-starvation (Fig. 6A), although the capacity for sulfate uptake only transiently increased. In cereals, the increase in capacity does not continue indefinitely (see Fig. 2), although in both cases the mRNA abundance continues to rise. This may suggest an additional post-transcriptional regulation.

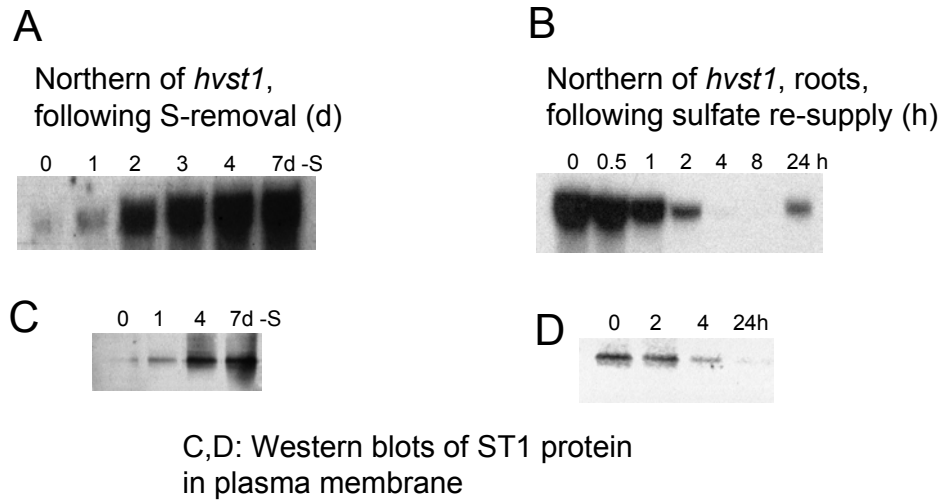

Fig. 4. De-repression (A, C) and repression (B, D) of sulfate transporters in barley. A, B: Northern blots using a barley cDNA probe to assess mRNA pools in barley roots (data from Smith *et al*. 1997). C, D: Western blots using a antibody to the C-terminal 150 amino acids of the HVST1 sequence probed against purified plasma membrane from hydroponically-grown roots of barley.

An increase in cellular OAS concentrations concurrent with the increase in mRNA pools would be expected from the regulatory model (Figs. 3 and 5), but this is not observed in potato (Fig. 6C). The large increase in OAS content after 8 d S-starvation is inconsistent with this model, and the accepted model of regulation of OAS synthesis in which the OAS pool has a direct influence on the state of association of serine acetyl transferase (SAT) and *O*-acetylserine (thiol)lyase (OAS-TL) complex (Droux *et al*. 1998; Hell and Hillebrand 2001). Elevated OAS disrupts complex stability, with the dissociated SAT being inactive, which prevents the excessive accumulation of OAS. To consolidate these two models, an increase in OAS concentration, which is sufficient to disrupt the complex, must also signal the need for increased gene expression of the ST. An added complexity may be that only

pools on individual subcellular compartments, particularly the cytosol, are relevant to the signal transduction pathway. As isoforms are known to exist in both plastid and cytosol, there may be quite different OAS pools and states of association of the complex. Extended S-deprivation is shown to lead to substantial OAS accumulation (Fig. 6C), which indicates that OAS pool regulation *via* the state of the SAT/OAS-TL complex is not sufficient under these circumstances.

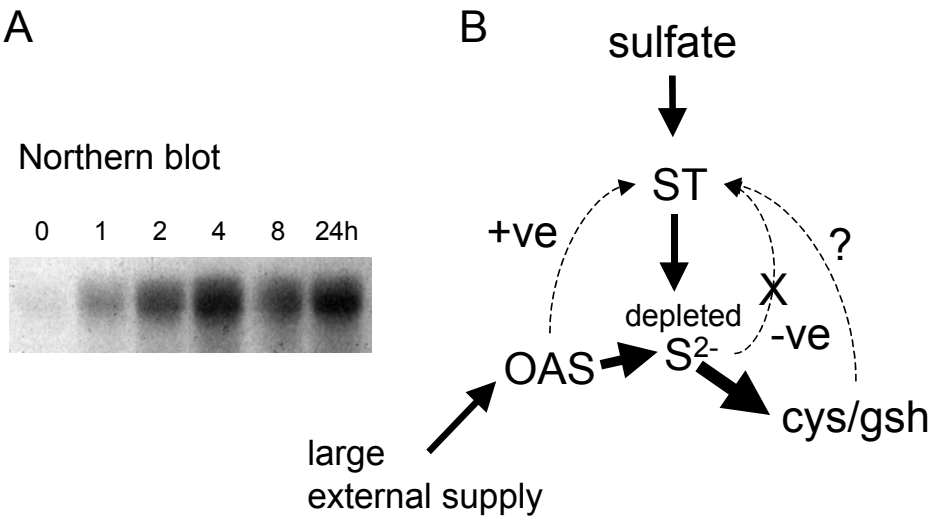

Fig. 5. OAS acts as an inducer of sulfate transporter gene expression. A: Northern blot (adapted from Smith *et al*. 1997) showing effect of 0.2 mM exogenously added OAS on mRNA abundance of *hvst1* over a 24 h period. B: Effect of externally added OAS on the regulatory model. Solid lines represent substrate fluxes with line thickness indicating magnitude. Broken lines indicate possible regulatory loops.

Shoot-root considerations

From Fig. 1B, it is apparent that a number of ST is regulated by S-status, at the level of mRNA abundance. Regulation of root expression is greatest, both for Group 1 STs principally involved in initial uptake, and for Group 2 STs involved in translocation. Although cysteine biosynthesis occurs in both roots and shoots, the major S-sink is likely to be in developing shoot tissue, and there is a clear requirement for a shoot to root signaling system. It has been suggested that a demand-driven signal from the shoots to the roots must mediate plant responses to S-availability (Lappartient and Touraine 1996). When sulfate availability to the roots is limiting, it is the root tissues which will be depleted of S-metabolites first (due to both lack of assimilation in the root and translocation of S to shoot tissues) prior to the leaf tissue depletion. Such a differential response is seen experimentally. Clearly the high sink demand of the shoot tissues acts to perturb all metabolite pools; however it is the

metabolite pools involved in cysteine synthesis in the root which have a direct effect on the transporter activity (and possibly other enzymes of the pathway).

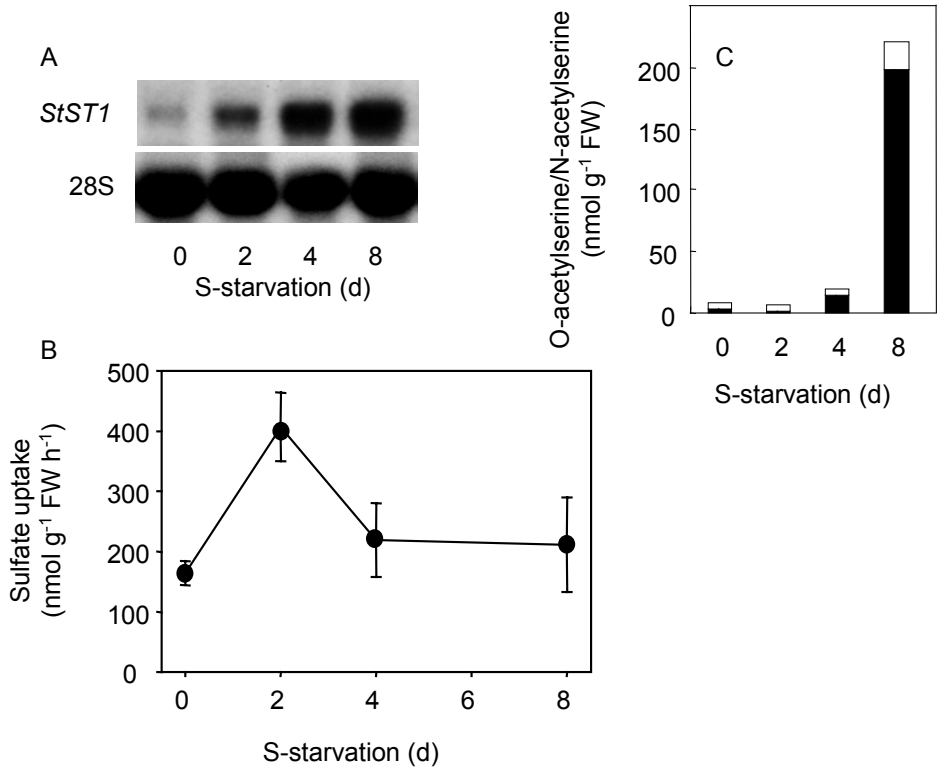

Fig. 6. Open questions: regulation of a high affinity sulfate transporter in potato (*Solanum tuberosum*). A: Northern blot showing mRNA pols of the StST1 sulfate transporter together with a parallel autoradiograph of the 28S rRNA to demonstrate equivalent loading. B: Sulfate uptake capacity as measured by sulfate tracer influx from a 0.15 mM ^{35}S-sulfate solution over a 10 min period. C: internal OAS (black bar) or the isomer, N-acetylserine (white bar), content as determined by GC-MS (Hopkins, Hesse, Höfgen and Hawkesford, unpublished data).

Open questions

Apparently a large number of diverse STs occurring within an individual plant are necessary to optimize the complex fluxes of sulfate involved in uptake, translocation, storage and assimilation. Up to 14 STs are known within one single plant species, *Arabidopsis thaliana*, however sulfate transport has yet to be confirmed for all of these, and some have yet to be confirmed as expressed genes. Expression of many of these STs is directly coupled to S-status of individual tissues. The signal transduction mechanisms linking gene expression to nutritional status remain elusive and

a priority for research. The identification of the critical metabolites and the transcription factors involved in the regulatory pathway remain a challenge for the future.

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MOLECULAR AND METABOLIC REGULATION OF SULFUR ASSIMILATION: INITIAL APPROACH BY THE POST-GENOMICS STRATEGY

Kazuki Saito
Graduate School of Pharmaceutical Sciences, Department of Molecular Biology and Biotechnology, Chiba University, CREST of Japan Science and Technology, Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan

Introduction – Sulfur metabolism in plants

Sulfur is an essential element in plant growth and plays roles in several aspects of plant cells (Leustek and Saito 1999): (1) It is a macronutrient used for synthesis of primary metabolites such as cysteine, methionine, vitamins, coenzymes and storage proteins. (2) It plays a role in redox cycle in cells as glutathione and thioredoxin. (3) It is essential for stress mitigation as glutathione, phytochelatins, and choline sulfate, etc. (4) Several important secondary metabolites and phytoalexins, *e.g.* camalexin, glucosinolates, and alliins etc, contain sulfur that is contributed for their characteristic biological activities. Research on sulfur metabolism comprises four aspects:

- Uptake and transport of the sulfate ion into cell and cell organelles
- Reduction of the sulfate ion and assimilation into a key organic sulfur-containing metabolite cysteine
- Derivatization from cysteine to a variety of sulfur-containing metabolites
- Degradation and re-utilization of sulfur-containing compounds

Retro-perspective of sulfur metabolism research

If one retraces the history of sulfur metabolism research, several stages can be distinguished chronically (Saito *et al.* 2000). The time until 1992 was one of basic physiology and biochemistry, in which metabolic pathways and the enzymes involved were elucidated by classical biochemical methodology. In 1992 molecular biology started with the first report for cDNA cloning of *O*-acetylserine (thiol)lyase (OAS-TL) (cysteine synthase) (Saito *et al.* 1992). Since this, almost all genes involved in sulfur metabolism have been isolated by molecular biological methods based on protein sequences of purified proteins and/or by genetic complementation in mutant microorganisms such as *Escherichia coli* and yeast (Saito 2000). This

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period was the time of 'gene hunting' until 2000. Since the completion of whole genome sequencing of a model plant species *Arabidopsis thaliana* in the end of 2000, plant science is taking a step forward to a new stage in the post-genomics eras. This new age can be called that of 'gene panning', identifying the specific function of genes from the numerous gene candidates whose functions are not confirmed. Research of sulfur metabolism is also involved in this big stream of modern biology. In this new era, sulfur metabolism will be studied through dynamic network of genes, transcripts, proteins and metabolites using *A. thaliana* as a model plant. Bio-diversity aspects for different plants and specific metabolism will be elucidated on the knowledge of the model plants. Post-genomics studies consist of three approaches of different levels:

- Transcriptomics – revealing a whole set of mRNAs accumulated in particular cells
- Proteomics – revealing a whole set of proteins accumulated in particular cells
- Metabolomics – revealing a whole set of metabolites accumulated in particular cells

Questions being addressed

In this new era, what questions should we address for better understanding of sulfur metabolism? Here are the questions and corresponding types of studies in the post-genomics eras.

- How many paralogous genes and proteins are present in a plant? => Genome
- Regulation at steady-state mRNA level => Transcriptome
- Modulation of enzyme activity => Proteome
- Feed-back regulation
- Protein-protein interaction
- Sub-cellular and C/N/S cross-talks
- Transcripts => Transcriptome
- Proteins => Proteome
- Metabolites => Metabolome
- Signal transduction => Proteome, metabolome, interactome
- Whole cellular and organ network => Systeome

How many genes related to sulfur assimilation in the *Arabidopsis* genome?

Figure 1 shows the pathway and enzymes of sulfur assimilation in plants. Sulfur assimilation from sulfate into cysteine is carried out by conjunction of two pathways; sulfate reduction and activation of serine. First, sulfate is taken up into cells and organelles. Sulfate is activated to adenosine 5'-phosphosulfate (APS), and then APS is reduced to sulfite. Sulfite is reduced to sulfide and then coupled with *O*-acetylserine to yield cysteine. *O*-Acetylserine is derived from serine. In total, six

proteins (sulfate transporter, ATP sulfurylase, APS reductase, sulfite reductase, serine acetyltransferase (SATase) and OAS-TL) are involved in sulfur assimilation.

By BLAST search on the *A. thaliana* genomic sequence, the number of putative genes encoding the enzymes for sulfate uptake and assimilation can be predicted (Table 1). Except for a single copy of the sulfite reductase gene, other genes comprise multiple copies in the *A. thaliana* genome. These multiple genes and encoded proteins may have different functions in cells, although some of them may be redundant. For instance, the proteins of a sub-family of OAS-TL (Bsas3) are indicated to act as β-cyanoalanine synthase rather than OAS-TL (Hatzfeld *et al.* 2000a; Maruyama *et al.* 2001; Warrilow *et al.* 2000).

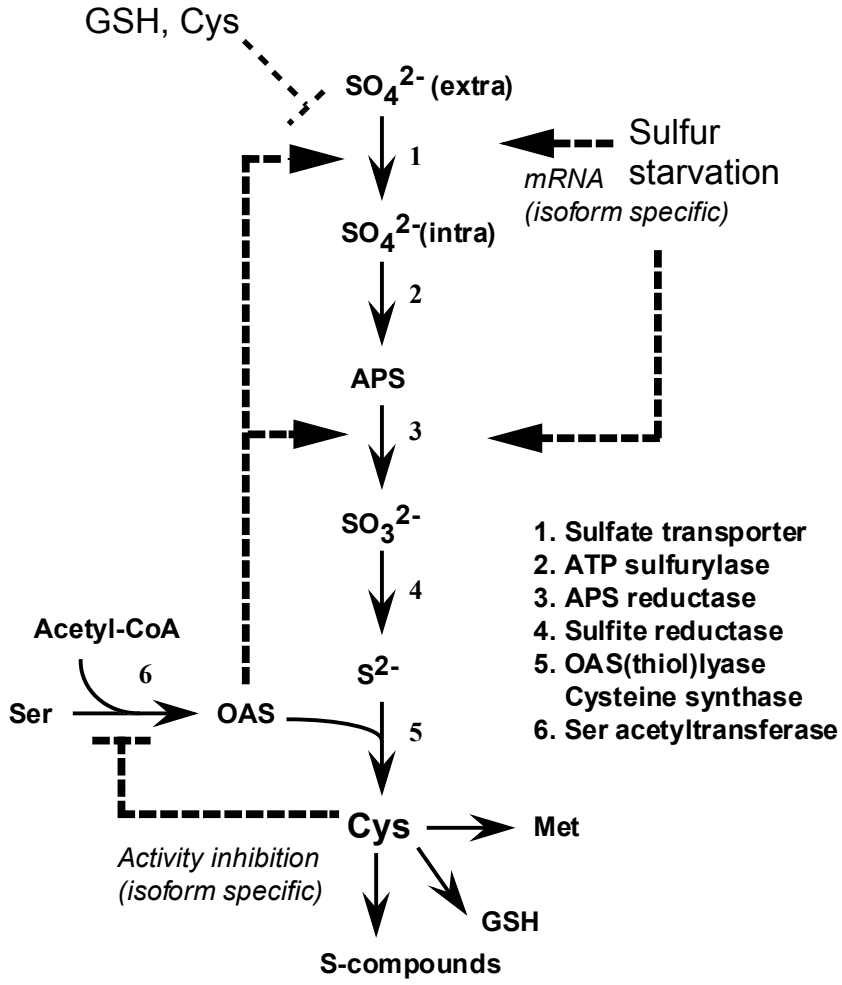

Fig. 1. Pathway, enzymes and regulation of sulfur assimilation into cysteine in plants.

Table 1. The number of paralogous genes encoding proteins for sulfur assimilation in *A. thaliana*.

Protein	Number
Sulfate transporter	12 (14)
ATP sulfurylase	4
APS reductase	3
Sulfite reductase	1
Serine acetyltransferase	5
O-Acetylserine (thiol)lyase	9

Sulfate transport

Sulfate transporters localized in the root plasma membrane mediate uptake of sulfate ion from external environments (soil or apoplast) to the symplastic system. Plasma-membrane transport systems for xylem loading in roots and unloading in leaves are also necessary. Inside cells, the presence of transport systems in chloroplast envelopes and tonoplast membranes is expected.

Predicted secondary structures of sulfate transporter proteins exhibited the presence of 12 membrane spanning domains, which are generally found in cation/solute symporters. In the *A. thaliana* genome, twelve or fourteen putative sulfate transporter genes have been identified (Yoshimoto *et al.* 2002). The expression of particular isoforms of sulfate transporter genes is tightly controlled by sulfate availability. The mRNAs coding for two high-affinity transporters, Sultr1;1 (Takahashi *et al.* 2000) and Sultr1;2 (Yoshimoto *et al.* 2002), and one low-affinity transporter, Sultr2;1 (Takahashi *et al.* 1997), were induced by sulfate starvation in roots. The *Sultr1;1* and *Sultr1;2* genes were expressed in epidermal and cortical cells of roots.

Sulfate reduction and cysteine synthesis

ATP sulfurylase is committed to the activation of sulfate to adenosine 5'-phosphosulfate (APS) (Fig. 1). ATP sulfurylase activity is detected in chloroplasts and cytosol. Indeed two cDNAs encoding chloroplastic and cytosolic isoforms were isolated from potato (Klonus *et al.* 1994). In *A. thaliana*, four cDNAs have been isolated, and all these seem to encode chloroplastic forms (Leustek *et al.* 1994; Logan *et al.* 1996). The cytosolic isoform is presumably produced from one of four genes by using a different translational start codon (Hatzfeld *et al.* 2000b). The step from APS to sulfite is catalyzed by a single enzyme termed as APS reductase. APS reductase is a GSH-dependent reductase, which used to be called APS sulfotransferase (Suter *et al.* 2000). Ferredoxin-dependent sulfite reductase catalyzes the 6-electron reduction of sulfite to sulfide (Yonekura-Sakakibara *et al.* 2000).

The final step of cysteine synthesis is the reaction incorporating the sulfide moiety into the β-position of alanine (Fig. 1). The amino acid moiety is derived from serine *via O*-acetylserine. Two enzymes, SATase and OAS-TL, are involved in this

step. Although the reduction of sulfate into sulfide takes place almost exclusively in chloroplasts, SATase and OAS-TL are localized in three major compartments of plant cells, *i.e.*, cytosol, chloroplasts and mitochondria (Lunn *et al.* 1990; Takahashi and Saito 1996; Ruffet *et al.* 1995; Noji *et al.* 1998).

Regulation through *O*-acetylserine and SATase

It is well known that sulfate uptake and assimilation activity is induced under sulfur-deficient conditions. This induction of activity is correlated with the inducible expression of a particular set of genes for sulfate transporter isoforms and APS reductase in *Arabidopsis* (Saito 2000). The question is what and how cellular signals are involved in this gene induction.

Involvement of O-acetylserine

O-Acetylserine is presumed to be a positive regulator (inducer) of sulfur assimilation either by direct gene induction or by indirect metabolic consequence. In fact, *O*-acetylserine induced the gene expression of particular isoforms of sulfate transporter in barley (Smith *et al.* 1997) and *A. thaliana* (Hatzfeld and Saito 2000), overriding the repressive effect of sulfur-rich nutritional conditions. The expression of APS reductase mRNA was also induced by *O*-acetylserine in *Arabidopsis* (Koprivova *et al.* 2000). In *Arabidopsis* and soybean cotyledons, the cellular *O*-acetylserine level increased under sulfur-starved conditions (Kim *et al.* 1999; Hirai *et al.* 2002). In contrast to the effect of *O*-acetylserine as a positive regulator, glutathione and cysteine are assumed to be negative regulators (repressors) for expression of genes regulated by sulfur status. By 'split-root' experiments, it was suggested that glutathione is a phloem-translocated signal molecule for repression of gene expression of the sulfate transporter and ATP sulfurylase (Lappartient *et al.* 1999). What mechanism is involved in the control of levels of regulators?

SATase gene expression

Five putative SATase paralogous genes are identified in the *A. thaliana* genome. The steady-state mRNA levels of some of these genes are regulated by sulfur status in a tissue-specific manner (Takahashi *et al.* 1997). Our recent comprehensive investigation indicated the more detailed expression pattern of specific isoforms of SATase genes under sulfur-deficient conditions (Kawashima *et al.* 2003).

Feedback inhibition on SATase activity

In *A. thaliana*, the cytosolic SATase is feedback-inhibited by cysteine at a physiological concentration (2 ~ 10 μM), but not the plastidic and mitochondrial forms (Noji *et al.* 1998), indicating the role of feedback inhibition as an important regulatory mechanism for *O*-acetylserine levels in cytosol. Because *O*-acetylserine is a

positive regulator for gene induction of sulfur assimilation, the significance of feedback regulation on cytosolic SATase is specially highlighted. The sites in a SATase responsible for feedback regulation were identified (Inoue *et al.* 1999).

The study using transgenic *A. thaliana* over-expressing a wild type SATase sensitive to cysteine-feedback inhibition and a mutated SATase insensitive to cysteine clearly indicated the importance of feedback regulation of SATase to control the level of *O*-acetylserine and cysteine *in vivo* (Noji and Saito 2002). As shown in Table 2, the levels of *O*-acetylserine and cysteine were enhanced in the transformants expressing the gene coding for feedback-inhibition insensitive SATase. The high SATase activities were observed in all transformants over-expressing the genes coding for wild and mutated SATases. The concentrations of glutathione were enhanced not only in the transformants expressing the mutated SATase gene but also those expressing the wild SATase gene. This could be due to the enhanced metabolic flow from *O*-acetylserine and cysteine to glutathione by the elevated SATase activity, although the steady-state level of cysteine is strictly controlled by feedback regulation in the transformants expressing the wild SATase gene.

The importance of feed-back regulation *in vivo* is further supported by the finding of correlation of recombinant SATase activity and cellular concentration of cysteine in *Allium tuberosum* (Urano *et al.* 2000). The sensitivity of SATase from *A. tuberosum* to cysteine is ca. 10-fold lower than those of *A. thaliana* and watermelon (Saito *et al.* 1995). This is correlated to the several-fold higher level of cysteine in *A. tuberosum* than *Arabidopsis* and tobacco, suggesting the key role of SATase feed-back regulation in determining cellular cysteine levels.

Table 2. Summary of transgenic studies over-expressing wild type and mutated SATases in *Arabidopsis*. The cDNAs encoding the wild type (feedback inhibition sensitive) SATase and a mutated (feedback inhibition insensitive) SATase from watermelon (Saito *et al.* 1995; Inoue *et al.* 1999) were expressed in transgenic *A. thaliana*. The SATase activity in cell-free extracts, and the levels of *O*-acetylserine, cysteine and glutathione, were determined in the transgenic plants. The data are shown in relative values compared to those of non-transformed control.

Transgene/localization	SATase activity	*O*-Acetylserine	Cysteine	Glutathione
Non-transformed	1	1	1.0	1.0
Wild-SATase/cytosol	111	2	0.9	2.0
Wild-SATase/plastid	58	11	1.4	5.3
Mutant-SATase/cytosol	113	62	7.1	4.3
Mutant-SATase/plastid	36	79	6.4	8.4

Modulation of SATase activity by Ca^{2+}

Considering the fact that the cytosolic SATase is regulated by cysteine feedback inhibition, this feedback inhibition may be further controlled by cytosolic signal(s) induced by change of sulfur status. Recent studies have indicated that cytosolic Ca^{2+} concentration increases transiently with sulfate deprivation in *Arabidopsis* (Inoue *et al.* unpublished). In addition, Ca^{2+} can bind to cytosolic SATase and partially in-

crease the activity under the inhibited condition by physiological level of cysteine, indicating a new role of Ca^{2+} in desensitizing SATase from cysteine inhibition. This suggests that cytosolic Ca^{2+} finely modulates the feedback regulation of SATase and eventually the cytosolic *O*-acetylserine level.

Bi-enzyme complex of SATase and OAS-TL

Another regulatory mechanism is also proposed. The bi-enzyme complex is formed with SATase and OAS-TL (Saito *et al.* 1995; Bogdanova and Hell 1997). However, the ratio of the two enzymes in chloroplasts is a large excess of OAS-TL, indicating that only a fraction of OAS-TL associates with SATase (Droux *et al.* 1998). The bound form of OAS-TL, which showed a dramatically decreased catalytic activity, seems to modulate the activity SATase in the enzyme complex. The free form of OAS-TL is presumably responsible for the actual catalytic function of cysteine synthesis. Since excess *O*-acetylserine dissociates the SATase-OAS-TL complex to increasing free OAS-TL availability, cysteine synthesis is enhanced. The dissociation and association of the bi-enzyme complex certainly also contributes to the regulation of cysteine synthesis.

Transcriptomics of sulfur assimilation: DNA array study

For the comprehensive analysis of gene expression under conditions of sulfate starvation and supplementation of *O*-acetylserine, transcriptomics analysis using DNA macro-array was conducted for 13,000 non-redundant EST clones from *A. thaliana* (Hirai *et al.* 2003). 216 genes were induced in leaf tissue by 2-day sulfate starvation; and 104 genes were induced in leaf tissue by addition of *O*-acetylserine under sulfate sufficient conditions. Among them, the same 40 genes are induced by both conditions, suggesting positive correlation in genes induced by short-term (2 days) sulfate starvation and by addition of *O*-acetylserine. These genes induced by both conditions were of a variety of functions related to metabolism, putative nucleic-acid binding proteins and of unknown function. However, almost no correlation was observed in the genes induced by short-term and long-term (3 weeks) sulfate starvation. Different sets of genes were induced in leaves and roots of starved plants.

Metabolomics: biochemical panning of T-DNA activation lines of *Arabidopsis*

To link metabolite patterns and functions of genes directly, screening of *A. thaliana* T-DNA activation-tagged lines for accumulation of high level of metabolites related to sulfur metabolism is being conducted (Awazuhara *et al.* 2003). We are applying a metabolite profiling system by a combination of capillary electrophoresis, HPLC and biological screening. Approximately 9,000 activation-tagged lines are being assayed for over-accumulation of anions (sulfate, nitrate and phosphate), amino acids,

sugars, thiols (cysteine, γ-glutamylcysteine, glutathione and phytochelatins) and flavonoids.

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METABOLIC REGULATION OF CYSTEINE SYNTHESIS AND SULFUR ASSIMILATION

Rüdiger Hell
Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK, Leibniz-Institut), Corrensstrasse 3, 06466 Gatersleben, Germany

Introduction

The biosynthesis of cysteine marks the central position between reduction and actual metabolization in the sulfate assimilation pathway of plants (reviewed by Leustek *et al.* 2000; Saito 2000). Following sulfate reduction, cysteine synthesis uses free sulfide and *O*-acetylserine (OAS) to form the first stable and non-toxic reduced sulfur compound. The reaction intermediate OAS is synthesized by serine acetyltransferase (SAT) from serine and acetyl-coenzyme A. *O*-acetylserine (thiol)lyase (OAS-TL, also named cysteine synthase) then catalyzes the insertion of sulfide in a β-elimination reaction. Both enzymes are associated in the cysteine synthase complex and are located not only in plastids but also in mitochondria and the cytosol.

Reduced sulfur is distributed exclusively from cysteine into downstream metabolic pathways. The importance and position of cysteine formation is therefore comparable to the fixation of ammonia in glutamine by glutamine synthetase. Such steps are generally suitable targets of regulatory mechanisms for flux control in pathways; indeed, sulfate and nitrate assimilation are connected *via* the intermediate OAS (Brunold *et al.* 2003). The evidence for genetic, allosteric and metabolic control mechanisms of cysteine synthesis will be reviewed in the following chapters.

Genetic regulation of cysteine synthesis by supply and demand

The sulfur status of a plant is defined by the ratio of supply and demand. While the overall requirement for sulfur is defined by the relative growth rate during the vegetative state, locations of enhanced demand exist in growing tissues like tips of roots and shoots and in developing fruits and seeds. Consequently, sulfur deficiency is typically detected first in young leaves and only later in mature organs (Hell and Hillebrand 2001). These requirements for reduced sulfur are reflected by spatial and temporal gene expression and activities of the encoded proteins of sulfur metabolism. Enhanced demand for reduced sulfur can also arise as part of the stress response program of plants. In particular conditions that stimulate glutathione synthesis will increase the demand for cysteine (Gullner and Kömives 2001). Since many

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plant defense compounds contain reduced sulfur, the supply of sulfate also forms the bases of the so-called sulfur induced resistance of crop plants to fungal pathogens (Schnug *et al.* 1995; Podlesna *et al.* 2003; Jost *et al.* 2003).

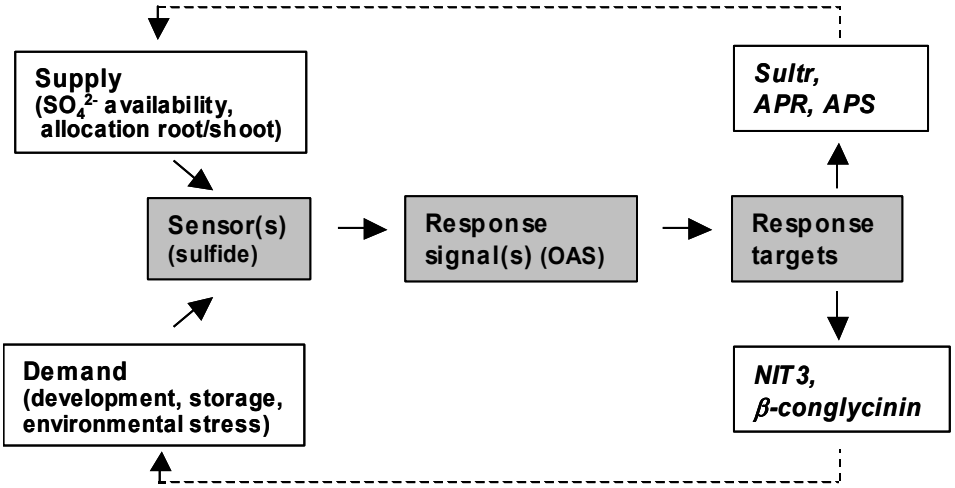

Fig. 1. Schematic representation of regulatory elements in the circuit of sulfate supply, demand and reduced sulfur homeostasis. In this cellular model the target genes (*Sultr* = sulfate transporter genes) are just starting to be revealed by multiparallel expression analysis. Additional factors act at the whole plant level and may use different signals and sensors.

At the whole plant level the uptake of sulfate by the roots may be driven by the demand of the shoot as the major site of sulfur assimilation. For communication between these organs the ratio between glutathione and sulfate transported in the phloem is discussed as signal (Herschbach *et al.* 2000). At the level of individual cells the responses to increased demand and limiting supply of sulfur may be mediated by different signaling pathways or simply converge in the maintenance of cellular thiol homeostasis and are therefore likely to induce overlapping responses (Fig. 1). Sulfate deprivation rapidly induces the expression of genes encoding sulfate transporter proteins of the plasmalemma, resulting in an increase of the capacity for uptake as well as of the affinity for sulfate (Smith *et al.* 1997; Hawkesford *et al.* 2003). The genes encoding activation and reduction steps that follow sulfate uptake are expressed in most cells at a basal level, but can be correspondingly regulated by changing sulfate supply and demand. The amplitude at the mRNA level however is smaller, and often even less effect at the protein and activity levels is observed when compared to sulfate transport. ATP sulfurylase (*APS)* and particularly adenosine 5'-phosphosulfate reductase (*APR)* mRNAs can be induced 3- to 4-fold under such conditions (Logan *et al.* 1996; Takahashi *et al.* 1997), whereas only small changes are reported for sulfite reductase (Bork and Hell 2000). Interestingly, the response

of genes encoding cysteine synthesizing enzymes to sulfate limitation and the corresponding enzyme activity changes are rather small and almost negligible (Hell *et al.* 1994; Hell 1998; Hesse *et al.* 1999). Thus, genes encoding sulfate transporters and APR exhibit the strongest transcriptional responses. These are the same response genes identified in feeding experiments with OAS (Smith *et al.* 1997; Clarkson *et al.* 1999; Koprivova *et al.* 2000; Brunold *et al.* 2003). It should be noted, however, that other environmental factors such as light and nitrogen nutrition or stress factors like heavy metals and herbicides may act differently on the transcription of genes in the pathway that respond only weakly to sulfate limitation. An example is cytosolic OAS-TL from *Arabidopsis* that is up-regulated by heavy metals (Domínguez-Solís *et al.* 2001). The overall aim of these adaptive mechanisms is to sense sulfate deficiency and trigger response reactions before appearance of severe deficiency symptoms (Hell 1998). It is concluded that genetic responses to changes in sulfur supply and demand are significant for sulfate transport, activation and reduction. They are probably triggered by metabolite activation/repression of gene expression, but are of minor relevance for the expression of genes of cysteine synthesis.

Allosteric regulation by cysteine feedback inhibition

Modulation of SAT activity has been suggested as a major element of flux control in cysteine synthesis (Saito 2000). This model is based on the remarkable observation that in *A. thaliana* the cytosolic SAT isoform is strongly feedback inhibited by cysteine, whereas the plastid and mitochondrial SAT isoforms are rather insensitive to cysteine (Noji *et al.* 1998). An inhibition of 50 % (IC_{50}) activity was achieved for the cytosolic SAT isoforms from watermelon (*Citrullus vulgaris*) and *A. thaliana* at 1.8 and 2.9 µM cysteine, respectively (Saito *et al.* 1995; Noji *et al.* 1998). Since OAS is presumably a positive regulator of sulfur metabolism genes (see section below), it was suggested that cytosolic OAS levels are controlled *via* cysteine-mediated feedback inhibition of cytosolic SAT in response to sulfate availability. Feedback-insensitive isoforms in the organelles would be required to allow independent cysteine formation in this hypothesis (Saito 2000).

On the other hand, the degree of SAT feedback-sensitivity may vary between plant species. A plastid SAT isoform from spinach (SAT56) revealed a IC_{50} of 7.6 µM cysteine (Noji *et al.* 2001), while a cytosolic (SAT7) and an organelle-localized (SAT1) SAT from tobacco (*Nicotiana tabacum*) both showed IC_{50} values of about 50 µM cysteine (Wirtz and Hell 2003). In pea (*Pisum sativum*) the cytosolic SAT activity was not inhibited by cysteine at all, while the SAT activities in isolated chloroplasts and mitochondria displayed IC_{50} values of 33 and 283 µM, respectively (Droux *et al.* 1998a). It should also be considered that most of the above cited data were determined using non-purified or recombinant enzymes with N-terminal fusions. In addition to these *in vitro* data some *in vivo* experiments are difficult to explain with the hypothesis of allosteric regulation of cytosolic SAT as primary mechanism. First, when the abiotic defense response of plants is induced by heavy metals or xenobiotics in order to form glutathione, an increased flux and accumula-

tion of cysteine can be observed (Rüegsegger and Brunold 1992; Farago *et al.* 1994). Second, fumigation of plants with H_2S results in enhanced levels of cysteine and is eventually sufficient to meet the sulfur demand of the plant (De Kok *et al.* 2000). Third, over-expression of feedback-sensitive as well as insensitive SAT from *E. coli* and *A. thaliana* in transgenic plants leads to elevated cysteine levels (Blasczyk *et al.* 1999; Harms *et al.* 2000; Noji and Saito 2002; Wirtz and Hell 2003). An accumulation of cysteine despite feedback inhibition could only be explained if the subcellular cysteine pools were strictly separated. To date only few SATs have been thoroughly investigated and data from compartment-specific SAT isoforms from more plant species are required to unequivocally establish the role of allosteric inhibition. It should also be considered that additional control mechanisms such as phosphorylation could overrule feedback inhibition.

Role of *O*-acetylserine in regulation of cysteine synthesis

The reaction intermediate OAS is synthesized from serine and serves as acceptor for sulfide. Research on OAS was hampered for a long time by the problematic biochemical analysis, not the least because of its rapid conversion to N-acetylserine under alkaline conditions. Table 1 gives an overview of OAS concentrations in response to sulfate availability. In summary, OAS appears to be somewhat less abundant than cysteine in various species and organs, but while cysteine concentrations generally decrease under short- and long-term sulfur limitation, OAS increases.

Table 1. Concentrations of OAS in plants. The sulfate supplies used in the individual experiments are simplified as +S and –S. Note that the time course of sulfate limitation varies considerably between these experiments.

Plant species	Plant organ	OAS (nmol/g fresh weight)		Analytical method	Reference
		+ S	- S		
Tobacco	cell culture	120	-	^{14}C-labelling	Smith (1977)
Soybean	cotyledon	5	36	HPLC	Kim *et al.* (1997)
A. thaliana	leaf	2	56	HPLC	Awazuhara *et al.* (2000)
A. thaliana	silique	10	60	HPLC	Kim *et al.* (1999)
A. thaliana	cell culture	0.3	3.8	HPLC	Wirtz and Hell, unpublished
Potato	whole shoot	3 – 6	220	mass spectroscopy	in: Hawkesford *et al*. (2003)

Feeding studies with *L. minor* (Neuenschwander *et al.* 1991) and chloroplasts from transgenic tobacco over-expressing OAS-TL (Saito *et al.* 1994) demonstrated that OAS could limit cysteine synthesis. Addition of OAS to barley roots (Smith *et al.* 1997), *Zea mays* cell cultures (Clarkson *et al.* 1999) and *Arabidopsis* plants (Ko-

privova *et al.* 2000; Brunold *et al.* 2003) induced mRNA steady state levels and protein activities of sulfate transport, *APS* and *APR*. External concentrations of 1 mM OAS were sufficient to over-rule the otherwise repressing effect of sufficient sulfate in the medium. These findings suggest that OAS is directly or indirectly involved in the control of sulfate assimilation as a positive regulator. This role apparently is not restricted to primary sulfate metabolism but extends to a large number of genes. For example, OAS feeding induced the expression of a nitrilase gene (*Nit3*) that is involved in glucosinolate degradation in *Arabidopsis* (Kutz *et al.* 2002). DNA micro-array analysis comparing sulfate sufficient, deficient and OAS fed *Arabidopsis* plants revealed 104 up-regulated mRNAs of which 40 % were identical between the sulfate deficiency response and the effect of OAS feeding (Saito 2003). Closest analysis of the effect of OAS on transcription is provided by identification of promoter sections of the *Arabidopsis Nit3* gene (Kutz *et al.* 2002) and the soybean β-conglycinin gene (Kim *et al.* 1999; Ohkama *et al.* 2003) that are sufficient to confer sulfate deficiency and OAS responsiveness to reporter genes. These data suggest that OAS is not only an intermediate in cysteine synthesis but also a potential element of signal transduction to maintain the sulfur status of the plant.

Biochemical and allosteric properties of the cysteine synthase complex

In contrast to earlier assumptions the function of the cysteine synthase complex consists not in metabolite channeling of OAS (Cook and Wedding 1977; Droux *et al.* 1998b). The reason is the allosteric inactivation of OAS-TL in the complex, forcing OAS to leave the reaction. It was therefore suggested that bound OAS-TL acts more as a regulatory subunit by stabilization and activation of SAT rather than enzymatically, whereas the free OAS-TL dimers serve cysteine synthesis (Droux *et al.* 1998b).

Indeed, SAT activity apparently relies on the association with OAS-TL. SAT has never been found in plant or bacterial protein preparations without OAS-TL. When SAT is released from the complex *in vitro*, it rapidly looses activity (Droux *et al.* 1998b; Wirtz *et al.* 2001). Maximal SAT activity for cysteine production requires a 400-fold excess of OAS-TL activity (Ruffet *et al.* 1994; Droux *et al.* 1998b). This ratio correlates with the 354-fold and 306-fold, respectively, excess of OAS-TL to SAT activity in chloroplasts of spinach and pea (Ruffet *et al.* 1994; Droux *et al.* 1998a).

In vivo protein-protein interaction studies using the yeast two-hybrid system provided data on the intensity of the interactions for the *Arabidopsis* enzymes and revealed a central domain responsible for SAT/SAT interaction (Bogdanova and Hell 1997; Jost *et al.* 2000). The C-terminal domain of SAT alone was sufficient for interaction with OAS-TL and mapped precisely with the predicted catalytic transferase domain (Bogdanova and Hell 1997). The observation that cysteine synthase complex formation could be a prerequisite for full SAT enzymatic activity is supported by *in vitro* and *in vivo* results with mutated SAT from *Arabidopsis* mitochon-

dria (Wirtz *et al.* 2001). When SAT was inactivated at a histidin residue in the catalytic center, it was still able to bind to OAS-TL. A three-dimensional computer model of the bifunctional catalytic and interaction domain of SAT corroborated this observation (Wirtz *et al.* 2001).

The dissociation of the cysteine synthase complex by millimolar concentrations of OAS was first observed for the *S. typhimurium* complex and a plant complex mixed from *Arabidopsis* and spinach subunits *in vitro* (Kredich *et al.* 1969; Droux *et al.* 1998b). The precise concentration-dependent dissociation kinetics that defines OAS as a potential regulatory effector were recently determined for the recombinant cysteine synthase complex from *Arabidopsis* mitochondria (Berkowitz *et al.* 2002). An equilibrium dissociation constant of 57 µM OAS and a dissociation rate constant of 77 µM OAS with a strongly cooperative Hill-constant suggest that the equilibrium of complex association/dissociation can be significantly and rapidly shifted (Berkowitz *et al.* 2002). Fluctuations of cellular OAS concentrations in this range have been observed in response to sulfate limitation (Table 1). They could thus control the rate of cysteine synthesis *via* reversible protein-protein interaction that determines abundance and activity of complex-bound SAT. A stabilization of the complex *in vitro* by addition of 1 mM sulfide to the complex from *S. typhimurium* and *Arabidopsis* completely prevents the dissociation by OAS (Kredich *et al.* 1969; M. Wirtz and R. Hell, unpublished), although the physiological relevance of the applied sulfide concentrations is unclear.

Metabolite sensing and regulation by the cysteine synthase complex

Starting with initial observations (Droux *et al.* 1998b; Hell 1998), the available evidence was integrated into a working hypothesis to explain the function of the cysteine synthase complex in the regulation of cellular cysteine homeostasis (Hell and Hillebrand 2001; Hell *et al.* 2002). This model refers to cellular, but not whole plant, regulation and defines the cysteine synthase complex as a sensor of the sulfide status of the plant (Fig. 2). During sufficient sulfate supply the cysteine synthase complex is fully associated, *i.e.* all SAT is bound to OAS-TL and surrounded by free and active OAS-TL dimers. SAT in the complex is active and produces OAS that diffuses into the solution because of the inactivity of bound OAS-TL. OAS-TL dimers catalyze the formation of cysteine from OAS and sulfide that is provided by the constitutively expressed proteins of sulfate uptake and reduction. This way OAS concentrations are kept low, allowing for partial repression of sulfate assimilation genes; sulfide may stabilize the complex to sustain OAS formation and to avoid accumulation of toxic levels of free sulfide.

In situations of sulfate limitation the supply of sulfide will drop and OAS concentrations will start to increase, because one substrate for cysteine synthesis in lacking. This has two consequences: (1) OAS accumulation above the threshold dissociation of about 50-80 µM OAS dissociates the cysteine synthase complex in the absence of sulfide. SAT is released and rapidly loses activity, reducing further consumption of acetyl-coenzyme A. (2) Elevated OAS concentrations trigger the de-

repression of genes of sulfate transport, *APS* and *APR*. Thus, the affinity and capacity for sulfate uptake at the plasmalemma increases and sulfate becomes available for assimilation again. Sulfide is produced and immediately reacts with OAS with the help of active OAS-TL dimers. When OAS concentrations begin to decrease, the complex can re-associate and activate SAT. The reduction of OAS is resumed, but now at a rate that is adjusted to the sulfide status of the cell. Simultaneously, the sulfate assimilation genes become repressed (by cysteine or glutathione). In this model the cysteine synthase complex actually synthesizes no cysteine but is part of a sensor system that regulates the flux of primary sulfur metabolism in a cell.

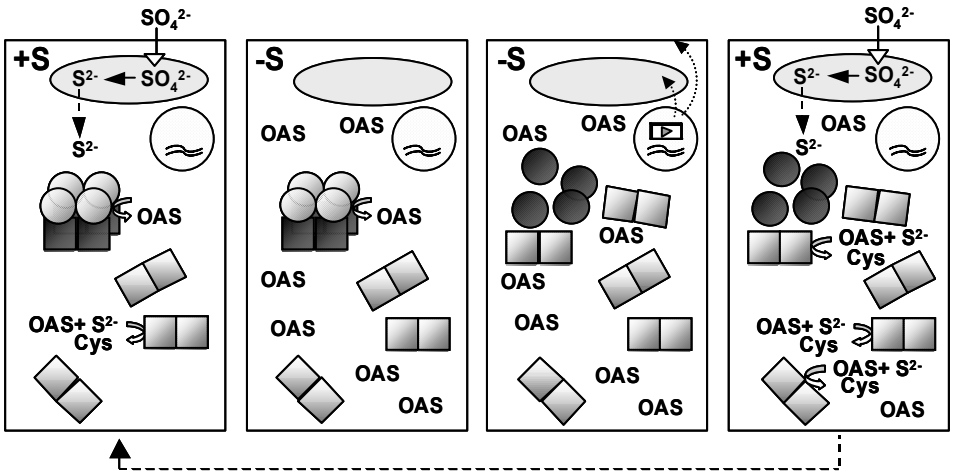

Fig. 2. Dynamic model of the cysteine synthase complex in sensing of the sulfide status of a cell and triggering of the rate of sulfate uptake and reduction. Reversible protein-protein interaction of the complex is only shown for the cytosolic compartment. The oval and dotted circles represent plastid and nucleus, respectively. Shaded circles stand for SAT, shaded squares show OAS-TL subunits. Light shading indicates active enzymes, dark shading indicates inactive enzymes. Derepression of genes and targeting of gene products to plasmalemma and plastid is represented by dotted arrows.

It should be cautioned that not all correlations between OAS fluctuations, sulfate transport capacity and sulfate transporter expression are kinetically in line (Hawkesford *et al.* 2003). However, allosteric regulation by cysteine feedback inhibition of SAT (Saito 2000) would also be compatible with the model, since this mechanism acts downstream of the key components sulfide and OAS, whereas the sensing mechanism serves as a trigger for upstream regulation. In addition the cysteine synthase model fits with most physiological situations of sulfur metabolism. For example, sulfur nutrition *via* H_2S would stabilize the complex to maximize OAS production for cysteine synthesis as is indeed observed in whole plant experiments (De Kok *et al.* 2000). Exogenous feeding of OAS would potentially result in partial complex dissociation, but at the same time the induced sulfate assimilation genes

generate more sulfide that stabilizes the complex, allowing for elevated cysteine levels (Neuenschwander *et al.* 1991). Similarly, over-expression of SAT in transgenic plants would produce more complex-bound SAT and consequently more OAS and cysteine formation. This was indeed observed (Blasczyk *et al.* 1999; Harms *et al.* 2000; Noji and Saito 2002; Wirtz and Hell 2002), whereas over-production of OAS-TL without feeding of precursors or challenging treatments had no or only limited effect on cysteine contents (Saito *et al.* 1994; Domínguez-Solís *et al.* 2001). These experiments indicate that the model is in agreement with whole plant regulation of sulfur supply and demand and that the system potentially functions in all cell types capable of sulfate assimilation.

Conclusions

Cysteine synthesis represents the transition from the reductive part of sulfate assimilation to the metabolization of reduced sulfur and is therefore a prospective target for flux regulation of the entire pathway. The signals from changes in sulfate supply and demand for reduced sulfur strongly affect the expression of sulfate transporter genes but become less significant along the reduction pathway including cysteine synthesis. To explain the response signals at the cellular level, a sensor model is suggested, where the reversible equilibrium of protein-protein interactions within the cysteine synthase complex are affected by OAS and sulfide that dissociate or stabilize the complex.

A number of questions remain to be answered before full verification of the sensor model. First, it is unclear whether the plastid and cytosolic cysteine synthase complexes have different roles in regulation of cysteine synthesis. Second, the model predicts a decline of the OAS accumulation rate during prolonged sulfate starvation due to SAT inactivation; this has not been observed so far, but no information on subcellular OAS compartmentation is available. Third, the mechanism and the degree of inactivation of free SAT are unclear; options are conformational changes, post-translational modification or degradation. Fourth, this cellular mechanism has to be integrated into whole plant regulation of sulfur metabolism. However, the proposed model is in agreement with physiological scenarios that are known to modify the flux of reduced sulfur.

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REGULATION OF SULFATE UPTAKE: FROM MOLECULAR BASES TO WHOLE PLANT INTEGRATION - LESSONS FROM RESEARCH ON THE REGULATION OF NITRATE UPTAKE

Bruno Touraine, Timothy J. Tranbarger and Patricia Nazoa
Université Montpellier II, Laboratoire des Symbioses Tropicales et Méditerranéennes, UMR 1063 UM2/IRD/CIRAD/INRA/ENSAM, CC 002, Place E. Bataillon, 34095 Montpellier cedex 5, France

Introduction

A regular remark at sulfur meetings and in books about sulfur nutrition is that the sulfate assimilation pathway shares many similarities with the nitrate assimilation pathway. Moreover, the end product of sulfur assimilation pathway is cysteine, an amino acid that links metabolically the two assimilatory pathways. Due to this interconnection and because the S/N ratio is maintained within narrow limits, the two pathways must be co-regulated to some extent. However, probably due to the quantitative importance of nitrogen in plant mineral nutrition and the high impact of nitrogen fertilizer utilization in agriculture, the research effort to understand NO_3^- uptake has been much greater than the effort devoted to SO_4^{2-} uptake. As a consequence, the physiological knowledge of the processes differs both in terms of the quantity of data available and the level of detail. Although molecular approaches have been more or less successfully applied to ion transport of higher plants during the last decade, there are differences between the progress made in our knowledge of the molecular basis of NO_3^- and SO_4^{2-} uptake.

In the present chapter we will focus on certain features of NO_3^- uptake in order to discuss some specific successes or difficulties encountered in the studies designed to identify and characterize plant NO_3^- transporters and to understand how NO_3^- uptake is regulated by the nutritional demand of the plant. Based on this description, we will question whether this overview can provide some lessons for SO_4^{2-} uptake studies.

Physiological background: the transport systems

The rate of ion absorption in root cells typically is the balance of the influx from apoplasm to cytoplasm, and the efflux in the reverse direction. The occurrence of

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such futile cycles across the plasma membrane has been demonstrated for both NO_3^- and SO_4^{2-} ions (Cram 1983; Lee and Clarkson 1986). The long series of kinetic studies performed with the short-term (*ca.* 9.5 min) radioisotope ^{13}N or the heavy isotope ^{15}N all showed that the recycling of NO_3^- ions across the plasma membrane is especially rapid (see Touraine *et al.* 2001 for review). Overall, nitrate studies show the quantitative importance of the efflux component for NO_3^- uptake, and this might well be also the case for SO_4^{2-} uptake. The occurrence of a large efflux theoretically provides an increased capacity for controlling ion uptake (the diminution of uptake rate could be achieved either by decreasing the influx or by increasing the efflux). However, much of the regulation of net NO_3^- uptake in response to changes in environmental factors like nutrient availability, light, temperature, are accounted for by changes in NO_3^- influx (*e.g.* Muller *et al.* 1995). On the other hand, NO_3^- efflux is enhanced by physical manipulations of the roots to the extent that a net loss of NO_3^- from the tissues can occur for several minutes (Aslam *et al.* 1996). The disturbance does not need to be severe to provoke this effect and the response of NO_3^- efflux is rapid and transient. The general opinion is that the overall nitrogen nutrition is controlled *via* the influx regulation, while the efflux may have a role as a signal in a general stress response pathway. However, it is important to point out the lack of our understanding of this basic feature of ion uptake that may be especially important for NO_3^- and SO_4^{2-} ions, and consequently the need for this question to be addressed. When tracer studies developed, the transport rate-concentration relationship was investigated for influxes specifically, including those of SO_4^{2-} and NO_3^-. Although similar studies have been performed for SO_4^{2-} (Bell *et al.* 1994), the studies on NO_3^- uptake by Yaeesh Siddiqi and Anthony Glass (*e.g.* Siddiqi *et al.* 1990, 1991) are certainly those which pushed the utilization of tracer experiments for the understanding of ion transport to their limits. These studies are especially important because they led to a classification of the transport systems which is still used for characterization of the transporters identified by molecular approaches. NO_3^- influx involves three transport systems: the inducible high affinity transport system (iHATS) accounts for the main part of NO_3^- uptake at low external NO_3^- concentrations but needs induction by NO_3^- ions; a constitutive high affinity transport system (cHATS) that is present even in plants deprived of NO_3^- for several days; a low affinity transport system (LATS) which makes a significant contribution to NO_3^- influx only at concentrations above 1 mM. Both HATS follow saturable kinetics with K_m values in the 10 µM range, while LATS presents no saturation up to 50 mM. A HATS and a LATS have been distinguished for SO_4^{2-}, but no iHATS has been found. Among the transport systems responsible for ion uptake in plants, the inducibility by the ion itself is unique for NO_3^- influx. The physiological meaning for this process is not understood, but it has been widely used to fish for NO_3^- transporters by looking at proteins, and more recently genes, that are induced after a few hours exposure to NO_3^-.

Transporters: What proteins (and genes) correspond to the transport systems?

At the moment, no information is available about NO_3^- and SO_4^{2-} efflux transport proteins and, therefore, we will specifically focus on the influx systems. The physiological studies have led to the identification of so-called transport systems that contribute to NO_3^- and SO_4^{2-} influx. The identification of the molecular entities, *i.e.* the transporters, for these transport systems has been a major challenge for many groups working on mineral nutrition in plants during the past two decades. Early attempts by researchers involved the isolation of plasma membrane hydrophobic polypeptides which increased in abundance during the induction of ion influx in root cells. The abundance of some polypeptides was higher in the plasma membrane fractions isolated from the roots of NO_3^--induced plants (Ageorges *et al.* 1996) or sulfur-deprived plants (Massonneau *et al.* 1997), but none of these membrane polypeptides have been isolated and their role in NO_3^- or SO_4^{2-} influx has not been demonstrated.

In the 1990s, various molecular genetic approaches were used to isolate genes that encode NO_3^- and SO_4^{2-} transporters from higher plants and to characterize their function. In this section, we will try to compare these approaches and to highlight their advantages and disadvantages.

Complementation of yeast mutants

The complementation of yeast mutants defective in ion uptake with plant cDNA libraries (usually from *A. thaliana*) has been successfully applied to the search for genes coding for SO_4^{2-} transporters (Smith *et al.* 1995, 1997) using *S. cerevisiae* mutants lacking SO_4^{2-} uptake capacity (Davidian *et al.* 2000). Furthermore, heterologous expression of plant cDNAs coding for SO_4^{2-} transporters provides a powerful tool for functional characterization. First of all, it is relatively simple to check that the gene product is actually involved in the uptake process itself by measuring the incorporation rate of labelled ions. In addition, the availability of complemented yeast mutants allowed kinetic characterization of the SO_4^{2-} transporters, which resulted in the classification of the plant SO_4^{2-} transporters into categories based on their different affinities for SO_4^{2-} (*e.g.* Vidmar *et al.* 2000a). As for NO_3^- transporters, the difficulty is that most yeast species, including *S. cerevisiae*, are unable to transport NO_3^- or grow in the absence of reduced N. Therefore, the yeast complementation strategy has not been used successfully with plant NO_3^- transporters. Here, it is worth mentioning the work by Vidmar *et al.* (1999) that led to a totally unexpected result. These authors designed a heterologous bacterial expression system in order to isolate plant NO_3^- transporters, and a series of clones complemented with a cDNA barley root library was able to grow on NO_3^-. However, none of these clones corresponded to a NO_3^- transporter, while a cDNA identical with the *Hvst1* gene coding for a high-affinity SO_4^{2-} transporter (Smith *et al.* 1997) was cloned. The level of *Hvst1* specificity for SO_4^{2-} *vs.* NO_3^- has not been determined, but in any case it shows the limit that one may encounter for cloning or characterizing plant transporters using heterologous complementation methodology.

Functional characterization in planta

Though chlorate is a rather poor analogue of NO_3^- for uptake processes (Kosola and Bloom 1996; Touraine and Glass 1997), plants do take up ClO_3^- and an early screening for ClO_3^- resistance in *Arabidopsis* led to the isolation of one mutant which is defective in ClO_3^- uptake (Oostindier-Braaksma and Feenstra 1973). This *chl1* mutant was subsequently characterized (Doddema and Telkamp 1979; Scholten and Feenstra 1986), and at this point, the mutation seemed to impair the NO_3^- LATS but also affected the absorption of other ions. This phenotype was later found in a T-DNA tagged mutant collection leading to the identification of the *CHL1* gene (Tsay *et al.* 1993), now renamed *Nrt1.1*. The gene product has been characterized as a low-affinity NO_3^- transporter by expression in *Xenopus oocytes* (Huang *et al.* 1996). However, some characteristics of *CHL1* were difficult to reconcile with the physiological characteristics associated with NO_3^- LATS (Touraine and Glass 1997), and a series of reports that further characterized the *chl1* mutants resulted in a more complex view of the CHL1 function. To summarize: CHL1 is responsible for most of ClO_3^- uptake, it mediates NO_3^- influx *via* a LATS but its deletion may be compensated for by other yet to be discovered low affinity transporters (Touraine and Glass 1997), and it is also involved in HATS under certain growth conditions (Wang *et al.* 1998). All together, these results led to the proposal that CHL1 is a "dual affinity NO_3^- transporter" (Liu *et al.* 1999). In fact, the situation has turned even less clear since the *CHL1* gene has been shown not to be located in the tissues where NO_3^- uptake takes place (Guo *et al.* 2001). Globally, the results of the CHL1/NRT1.1 studies reflect the difficulties and limits of approaches to isolate and functionally characterize ion transporters from higher plants based upon resistance against a toxic analogue of the ion. Furthermore, several laboratories have tried to apply modified chlorate resistance screens in order to isolate other NO_3^- transporters, but to our knowledge none of these attempts succeeded. The *Nrt2* family of NO_3^- transporter genes has been discovered in several higher plant species (Trueman *et al.* 1996; Quesada *et al.* 1997; Amarasinghe *et al.* 1998; Filleur and Daniel-Vedele 1999; Zhuo *et al.* 1999) by virtue of sequence homology with the *A. nidulans crnA* (Unkles *et al.* 1991) and *C. reinhardtii Nrt2* (Quesada *et al.* 1994) genes. Thereafter, functional characterization *in planta* was done by a reverse genetic approach. An *Arabidopsis* T-DNA mutant affected in *Nrt2.1* and *Nrt2.2* genes is severely impaired in the NO_3^- HATS but not in the LATS (Filleur *et al.* 2001). Since the expression of *Nrt2.1* is induced by NO_3^-, NRT2.1 is likely be the transporter responsible for the iHATS.

Finally, the utilization of genetic and reverse genetics to identify NO_3^- transporter genes and to determine their function *in planta* has shown both the usefulness and the limits of these approaches. In *Arabidopsis*, only two NO_3^- transporter genes have been studied to any extent. Although *Nrt2.1* makes a major contribution to NO_3^- influx in roots, genetic approaches failed to isolate a mutant altered in this gene. By contrast, genetic screening succeeded in isolating plants carrying mutations in the *Nrt1.1* gene, but in this case the gene product does not make a major contribution to NO_3^- uptake and its real function is still unclear. Similarly to what

was achieved with chlorate, recently selenate has been used as a SO_4^{2-} toxic analogue in order to identify *Arabidopsis* mutants deficient in SO_4^{2-} transport. The identification and characterization of a class of selenate resistant mutants (*sel1*) that are altered in selenate and SO_4^{2-} transport have been reported recently (Shibagaki *et al.* 2002). The *sel1* mutants contain lesions in the *Sultr1.2* gene which encodes a root SO_4^{2-} transporter involved in the uptake of this anion. Selenate could thus be a better analogue for SO_4^{2-} than chlorate is for NO_3^-. If so, the genetic approach to identify SO_4^{2-} transporters based upon selenate resistance may be more fruitful than the strategy to identify NO_3^- transporters based upon chlorate resistance.

Expression studies

Once putative NO_3^- or SO_4^{2-} transporter genes have been cloned, the steady-state accumulation of the corresponding transcripts has been investigated. Especially in the case of the putative NO_3^- transporter genes *Nrt1* and *Nrt2*, a large number of papers report such Northern blot experiments (Tsay *et al.* 1993; Trueman *et al.* 1996; Quesada *et al.* 1997; Amarasinghe *et al.* 1998; Krapp *et al.* 1998; Zhou *et al.* 1998, 1999; Filleur and Daniel-Vedele 1999; Lejay *et al.* 1999; Vidmar *et al.* 2000b; Gansel *et al.* 2001). Together these studies show that the accumulation of both *Nrt1* and *Nrt2* transcripts was very low in plants grown in a NO_3^--free medium, was rapidly induced by NO_3^- and increased with light, while only the *Nrt2* transcript accumulation was de-repressed by nitrogen starvation and repressed by treatments increasing the plant's N status level (*e.g.* ammonium or amino acid feeding). The broad variety of conditions used by the different groups to investigate the expression of *Nrt2* in *A. thaliana* has provided a large quantity of data that support the hypothesis that *Nrt2* is a major NO_3^- transporter and suggest that it is regulated at the transcriptional level. However, the Northern blot experiments reported were generally not able to distinguish between the members of the *Nrt2* family. Recently, using a *Nrt2.1* gene specific probe we have shown that the *Nrt2.1* mRNAs are inversely correlated to glutamine and not to other amino acids, suggesting a physiological role specifically for glutamine (Nazoa *et al.* 2003). Furthermore, the results obtained using semiquantitative RT-PCR in two independent studies (Orsel *et al.* 2002; Okamoto *et al.* 2003) suggest that all the *Nrt2* genes except *Nrt2.6* and *Nrt2.7* are under negative regulation in plants grown under high N conditions.

Promoter::reporter gene fusions

Surprisingly enough when considering that the first *Nrt1* and *Nrt2* genes were isolated respectively 10 and 7 years ago, only very recently the localization of their expression has been studied. In both cases, however, these investigations brought unexpected results which support the importance of the promoter::reporter gene fusion approach. *Nrt1.1* (*CHL1*) fusions of the promoter with *GFP* or *GUS* showed that its expression is mainly located in the tips of both primary and lateral roots and is very low in the epidermis and cortical parenchyma where NO_3^- uptake occurs (Guo *et al.* 2001). Even more surprising, a strong activity of the *CHL1* promoter was

found in young leaves and floral buds. In addition, the growth of nascent root and shoot organs was reduced in *chl1* mutants. The *CHL1* gene, therefore, seems to have an essential function in early organ development during vegetative and reproductive growth, but not in the primary nitrate nutrition of the plant. Finally, thirty years after the isolation of the first *chl1* mutant from *Arabidopsis*, and ten years after the cloning of the *CHL1* gene, Guo *et al.* (2002) in a recent review paper concluded that "*Nrt1.1* supports the growth of these cells, but its precise role is still a mystery". One can add that the identity of the transporters responsible for NO_3^- LATS is still a mystery.

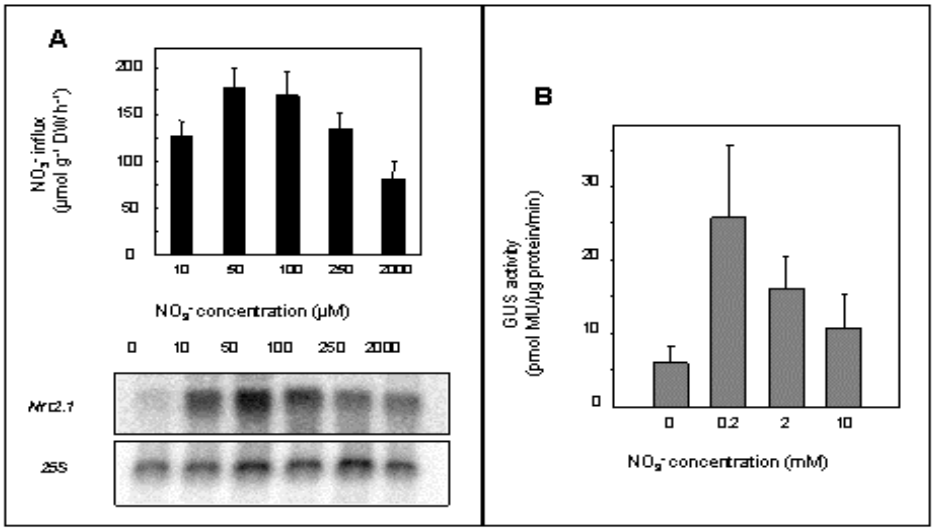

Fig. 1. Effects of NO_3^- concentration on NO_3^- influx and *Nrt2.1* transcript amounts in the roots of *Arabidopsis thaliana* (A) and GUS activity in the roots of transgenic *A. thaliana* plants expressing a *Nrt2.1* promoter-*GUS* chimeric gene (B). Figures drawn from Nazoa *et al.* (2003).

The GUS activity extracted from root systems of *Arabidopsis* transgenic plants carrying a *pNrt2.1*::*GUS* chimeric gene was induced by NO_3^-, down-regulated by increasing NO_3^- concentrations (Fig. 1), repressed by amino acids and up-regulated by sucrose and increasing (Nazoa *et al.* 2003). At the tissue level, the *Nrt2.1* promoter drives expression mainly in root epidermis and cortex. However, an unexpected result was that this promoter targets gene expression specifically to older root parts, but not to the apices or elongation zones of the roots. On the other hand, these latter zones absorbed NO_3^- at a rate similar to that measured in older root parts, suggesting that other transporters are expressed in younger root parts. However, no information is available to envisage whether the NO_3^- uptake in young root tissues involves another transporter of the NRT2 family, or a transporter of another family. Finally, using the *pNrt2.1*::*GUS* transgenic plants, we showed that the expression of

Nrt2.1 is regulated during the plant life cycle (especially, declining at floral stem emergence) in correlation to the changes in NO_3^- iHATS.

Finally, the recent results obtained on the two NO_3^- transporter genes unambiguously recognized in *Arabidopsis* using a promoter::reporter gene fusion approach have required a reappraisal of the models derived from a simple fitting of the first data from Northern analysis to the physiological framework. We think that such approaches are a priority to decipher the function of ion transporters. Although the cloning of SO_4^{2-} transporters is somewhat more recent than that of NO_3^- transporters, fusion of SO_4^{2-} transporter promoters with *GUS* or *GFP* reporter genes has already been included in several studies (*e.g.* Takahashi *et al.* 2000; Shibagaki *et al.* 2002; Yoshimoto *et al.* 2002). This approach should certainly be extended to all SO_4^{2-} transporters identified in *Arabidopsis* for comparison.

Regulation of nitrate and sulfate uptake

There remain many unanswered questions concerning the regulation of NO_3^- and SO_4^{2-} uptake at the more integrated and more detailed ends of the question. At the whole plant level, evidence exist that a systemic regulation operates resulting in element homeostasis, but many important aspects are unresolved, especially the identity of the regulatory signals translocated between organs. At the mechanistic level, there is evidence that a transcriptional regulation of NO_3^- and SO_4^{2-} transporter genes occurs but the regulatory elements and the transduction pathways are not identified. Furthermore, regulations at other levels (translational, post-translational) are also likely to exist, but no clear experimental data are available. We will not make an exhaustive overview of this important topic here, but rather, we will address the question of the systemic regulation and phloem signalling as an example of the gaps in our knowledge.

The regulation processes of SO_4^{2-} and NO_3^- uptake in the whole plant present many similarities. In both cases, the capacity of absorption is high and transporters are normally under repression to adjust the actual uptake rate to the nutritional requirements. Only under elemental deficiency is this repression completely eliminated and the transporters expressed at their highest level. Split root experiments have shown that underlying systemic regulations involve phloem-translocated signals to convey the information about nutritional demand to the roots. However, the identification of these signals is complicated by the difficulty of collecting sieve sap and the impossibility of changing experimentally the sieve sap composition and examine the consequences on root uptake.

Some evidence indicates that phloem-translocated amino acids could act as inter-organ signals responsible for the regulation of NO_3^- uptake by plant N demand (Imsande and Touraine 1994). In brief, the lower the N demand, the higher amino acid concentration in the sieve sap, the stronger the repression of NO_3^- influx in roots. However, a key question for the model is still unresolved: do the changes of the N demand level actually result in changes of the amino acid concentration in the phloem sap? This question has been addressed once in *Ricinus communis* (Tillard *et*

al. 1998), a species that provides the exceptional possibility to collect the sieve sap from severed stems. Split root grown plants fed with an N free solution on one side exhibited no significant change in the amino acid composition of the sieve sap compared to plants fed with NO_3^- on both sides of the root system. However, the plants did absorb NO_3^- at a higher rate. These results thus do not support the model of regulation of NO_3^- uptake by amino acid translocation. However, the sieve sap velocity could likely be affected by cutting the sieve tubes and it is still feasible that amino acid translocation rates are different in N-deficient and N-replete plants. On the other hand, circumstantial evidence in favor of the N cycling regulation model has been obtained by quantifying nitrogen flows in plants under various nutritional conditions. For instance, feeding spruce shoots with atmospheric NO_2 led to an increase in N assimilatory metabolism in shoots, an increase in N transport from shoot to root, and a down-regulation of NO_3^- uptake (Muller *et al.* 1996).

Again fewer experiments have been reported on the regulation of SO_4^{2-} uptake than on the regulation of NO_3^- uptake. Globally, a model much resembling to the amino acid model of NO_3^- uptake regulation has been proposed for SO_4^{2-} uptake regulation (Lappartient and Touraine 1996; Lappartient *et al.* 1999). In this model, glutathione (GSH) would play the role of amino acids. Although this model is generally accepted, another regulation process seems to operate to regulate SO_4^{2-} uptake in higher plants. This second pathway involves *O*-acetylserine (OAS) as a positive regulator (Smith *et al.* 1997). It has been proposed that GSH would repress the OAS pathway, which ultimately exerts a positive effect on the expression of SO_4^{2-} transporters (Saito 2000). However, there is no strong experimental data available to determine whether indeed these two regulatory pathways interact or whether they are independent.

At the mechanistic level, a rather large number of reports on NO_3^- or SO_4^{2-} transporter expression have been published. However, these studies have been limited to Northern blot experiments so far, and correlations between the transcript levels and ion uptake rates shed no light on the mechanisms involved. Clearly, more refined promoter analyses should now be designed in order to identify regulatory elements and decipher the transduction pathways. Besides such targeted approaches, one can also use more global approaches based on functional genomic. An example is provided by a transcriptome analysis of NO_3^--induced genes that were made using micro-arrays containing cDNA clones corresponding to about 5500 genes of *Arabidopsis* (Wang *et al.* 2000). In this study, most of the known NO_3^--induced genes appeared, together with a few genes encoding possible regulatory proteins, enzymes and proteins with unknown functions. This indicates that NO_3^- sensing is not involved in NO_3^- uptake regulation only, but also in a variety of mechanisms. This conclusion is supported by the fact that the changes in the expression pattern of 126 transcription factors from *A. thaliana* roots triggered by changes in nitrogen availability correlated with the changes in root architecture rather than with those in nitrate uptake (Tranbarger *et al.* 2003). Transcriptome methodology provides a very promising approach to identify new regulatory genes, but it is not routine yet and the large number of data released makes the analysis difficult.

Conclusions

Overall, the comparison of what has been done to characterize the transporters involved in NO_3^- and SO_4^{2-} uptake and to decipher the mechanisms involved in their regulation shows that despite more effort and a greater number of publications on NO_3^- uptake, many aspects are still a mystery. A large part of this void of our understanding is due to a lack of feasible methodologies. A very good example of this is provided by the identification of the phloem-translocated signals that integrate the nutritional demand of the plant and control ion uptake. This question appears as a major challenge to be addressed, but unfortunately no technical solution has been envisaged at the moment. The gaps in our knowledge to fit scattered molecular data with physiological background are certainly easier to resolve. However, they probably need to shift the research effort from some approaches to others. For instance, it is somewhat surprising that only two NO_3^- transporters have been characterized so far in *A. thaliana* and that there is so little evidence to decipher the mechanisms involved in their transcriptional regulation, while there are so many reports of the changes in their transcript levels. Finally, despite the fewer number of researchers working in the field of sulfur molecular biology, it seems that the investigations may be more exhaustive, making a larger use of some molecular techniques likes heterologous expression and promoter::reporter gene fusion. After a rapid overview of recent advances in NO_3^- uptake, the lessons to draw may rather be to think about the unexpected difficulties that can be encountered with seemingly logical approaches (*e.g.* functional characterization *in planta* using knock-out mutants) and to envisage the appropriate approaches to investigate the underlying mechanisms rather than be limited to correlation studies.

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INTERACTIONS BETWEEN SULFUR, NITROGEN AND CARBON METABOLISM

Christian Brunold[1], Peter Von Ballmoos[1], Holger Hesse[2], David Fell[3] and Stanislav Kopriva[4]

[1]IPS, University of Berne, Altenbergrain 21, 3013 Bern, Switzerland; [2]Freie Universität Berlin, Institut für Angewandte Genetik, Albrecht-Thaer-Weg 6, 14195 Berlin, Germany; [3]School of Biological & Molecular Sciences, Oxford Brookes University, Headington,Oxford OX3 0BP, UK; [4]Institute of Tree Physiology, University of Freiburg, Georges-Köhler-Allee 53, 79085 Freiburg, Germany

Introduction

Under ecological conditions, plants are confronted with an environment changing continuously with respect to physical, chemical and biotic parameters (Brunold *et al.* 1996). In this situation they have developed strategies resulting in optimal use of the environmental resources needed for growth, respiration and propagation, but also for coping with stress and repair (Fig. 1; Rennenberg and Brunold 1994; Brunold *et al.* 1996). Among these strategies, coordination of carbon, nitrogen and sulfur assimilation is especially important, because these pathways are not only dependent on the varying availability of mineral nutrients and CO_2, but also on changes in light energy (Fig. 1). These assimilatory pathways interact for the formation of products and are regulated in coordinate manner (Brunold 1993; Leustek *et al.* 2000; Saito 2000). It is the aim of this article to highlight these interactions and to focus on possible links and signals (Fig. 1).

O-Acetyl-L-serine links sulfate, nitrate and carbon assimilation

Cysteine synthesis from *O*-acetyl-L-serine (OAS) and sulfide is the most important reaction linking sulfur, nitrogen and carbon assimilation (Hawkesford and Wray 2000). Sulfide is formed in a reaction sequence starting with sulfate, taken up from the external solution *via* specific transporters. Sulfate is activated to adenosine 5'-phosphosulfate (APS) by ATP sulfurylase (ATPS). APS reductase (APR) reduces APS to sulfite (Leustek *et al.* 2000; Suter *et al.* 2000). Sulfite is either used for sulfolipid synthesis (Sanda *et al.* 2001) or further reduced to sulfide by sulfite reductase. Finally, OAS (thiol) lyase (OAS-TL) combines sulfide with OAS, thus forming cysteine. OAS is synthesized by serine acetyltransferase (SAT) from acetyl-CoA and serine, produced preferentially in photorespiration (Noctor *et al.* 1997). Cys-

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teine is thus a product of carbon, nitrogen and sulfur assimilation. It is mostly used for protein and methionine synthesis, but also for GSH formation and synthesis of coenzymes (Leustek *et al.* 2000).

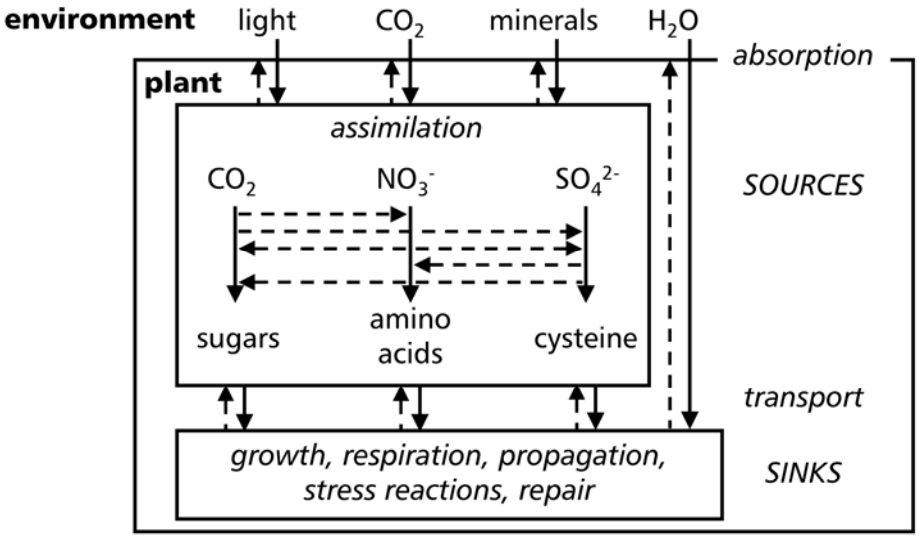

Fig. 1. Absorption of resources from the environment, assimilation of carbon, nitrogen and sulfur and transport of the products to the sinks, where they are used as indicated (according to Brunold *et al.* 1996). Fluxes, ——▶ ; regulatory interactions, - - -▶

SAT is subject to feedback inhibition by cysteine. Saito's group (Noji *et al.* 1998) reported that in *A. thaliana* this inhibition could only be detected for cytoplasmic SAT, which was decreased by 50 % at a cysteine concentration of 5 µM.

SAT is only active in a complex with OAS-TL (Droux *et al.* 1998; Hell and Hillebrand 2001; Wirtz *et al.* 2001). Increased OAS levels caused this complex to dissociate, resulting in inactive SAT. Thus OAS regulates its own formation.

In bacteria, N-acetyl-L-serine (NAS; Kredich 2000), an isomer of OAS, regulates sulfate assimilation. Since OAS may also be involved in regulating plant sulfate assimilation (Saito 2000), the OAS-measurements of Fujiwara's group in leaves (Awazuhara *et al.* 2000) and siliques (Kim *et al.* 1999) of *A. thaliana* are of great interest. The OAS level was high at high external nitrate and low external sulfate, whereas at high external sulfate, the OAS level was low.

Supplying OAS to barley roots (Smith *et al.* 1997) and *Zea mays* cell cultures (Clarkson *et al.* 1999) induced the expression of a sulfate transporter, and increased sulfate uptake, ATPS activity and the cysteine pool. OAS also induced APR (Neuenschwander *et al.* 1991; Koprivova *et al.* 2000). The increase in cysteine and

GSH after addition of OAS leads to the prediction that increasing OAS internally by overexpressing SAT should induce increased cysteine and GSH levels. Indeed, overexpression of a bacterial SAT cDNA in tobacco and potato increased the levels of SAT mRNA and activity, and also of cysteine and GSH (Blaszczyk *et al.* 1999; Harms *et al.* 2000).

Interactions induced by sulfur deficiency

In the green alga *Dunaliella salina* sulfur deprivation increased ATPS activity and decreased the activity of nitrate reductase (NR) and PEP carboxylase, whereas no effect was detected on carbonic anhydrase (Giordano *et al.* 2000). In sulfur deficient tobacco leaves (Migge *et al.* 2000) NR mRNA and activity were decreased, whereas asparagine, glutamine and arginine accumulated. Decreased NR activity at increased internal amino acid levels was detected in other systems (Suter *et al.* 1986; Coruzzi and Bush 2001; Prosser *et al.* 2001), corroborating their signaling function for NR expression.

Sulfur deficiency induced sulfate transporters and enzymes of assimilatory sulfate reduction. Pioneering work was done in Filner's laboratory using tobacco cell cultures (Reuveny and Filner 1977). When these cells were cultivated in the absence of a sulfur source, the activity of ATPS increased to a high level, indicating that under normal sulfur availability this enzyme was repressed. In *Lemna minor* cultivated without a sulfur source, protein content decreased and APR activity increased. ATPS did not change in activity, indicating that in *Lemna* APR was more susceptible to regulation than ATPS (Brunold *et al.* 1987). In a more recent experiment with *A. thaliana* cultivated under sulfur deficiency, Saito's group (Takahashi *et al.* 1997) discovered that the mRNA of a sulfate transporter and of APR accumulated in the roots, whereas SAT mRNA preferentially accumulated in leaves. Addition of selenate to sulfate sufficient medium increased the sulfate uptake capacity, tissue sulfate and the abundance of two sulfate transporter mRNAs in *A. thaliana* roots (Takahashi *et al.* 2000). Concomitant decrease of the tissue thiol content suggested a role of GSH as a repressor for these transporters. Indeed, simultaneous addition of cysteine and buthionine sulfoximine, an inhibitor of GSH synthesis, abolished the repressive effect of cysteine on a sulfate transporter (Vidmar *et al.* 2000). Correspondingly, the accumulation of mRNAs for ATPS and APR in *A. thaliana* exposed to Cd (Heiss *et al.* 1999) can be best explained by assuming that the low GSH levels due to phytochelatin synthesis were repressing the expression of the two enzymes less than the normal levels.

Interactions induced by excess sulfur

Plants exposed to high external sulfate or gaseous sulfur compounds like SO_2 or H_2S are characterized by increased levels of internal sulfate, cysteine and GSH, and decreased levels of the enzymes of sulfate assimilation (Brunold *et al.* 1987;

Westerman *et al.* 2001; Tausz *et al.* 2002; Vauclare *et al.* 2002). Pumpkin leaf disks exposed to very high levels of sulfate even emitted H_2S, indicating that more sulfur was reduced than could be assimilated (Rennenberg 1983). Addition of OAS to the leaf disk medium rapidly ended H_2S emission. This result shows that the acceptor for reduced sulfur was not available in sufficient quantities and indicates that sulfate assimilation and OAS formation were not completely coordinated right after the transfer to high sulfate.

In *Brassica oleracea* exposed to H_2S, thiols increased in the leaves, but not in the roots (Westerman *et al.* 2001). Correspondingly there was a decrease in APR activity in the leaves but not in the roots. Accumulating GSH rather than cysteine was the signal for excess sulfur (Lappartient *et al.* 1999). This idea was tested with root cultures of *A. thaliana* (Vauclare *et al.* 2002). Cysteine added to the culture medium alone decreased APR mRNA, protein and activity. In the presence of BSO, cysteine had no effect, but when GSH was added additionally, APR mRNA, protein and activity were decreased again. ATPS and the other enzymes of sulfate assimilation were not affected, showing that they were less susceptible to regulation than APR. Uptake of radioactive sulfate and incorporation of label into cysteine, GSH and protein, however, were decreased corresponding to APR activity, indicating that changes in APR activity have a great effect on the flux through sulfate assimilation. This effect can be quantified by estimating the flux control coefficient C^J_E. It indicates to what extent the flux *J* through a pathway changes with a change in enzyme *E* (Fell 1997). The sum of all flux control coefficients of all enzymes involved in a pathway is equal to 1. If an enzyme has a flux control coefficient of 1, it is rate limiting. Normally, flux control coefficients are lower. For APR, the flux control coefficient can be calculated according to:

$$C^J_{APR} = \frac{\Delta \ln J}{\Delta \ln E_{APR} + \varepsilon^{APR}_{APS} \Delta \ln APS}$$

Using the values for J, E and APS estimated in the presence and absence of GSH in the root medium, values between 0.57 and 0.92 resulted from this calculation (Vauclare *et al.* 2002) for the pathway from internal sulfate, excluding transport. The transport step of external sulfate into the roots was more inhibited by GSH than the assimilation of internal sulfate (Vauclare *et al.* 2002), indicating a dominant role of transport in regulating sulfate assimilation. For the pathway from external sulfate it is likely that the control coefficient of transport is greater than 0.5 and that of APR is accordingly reduced to below 0.5. The relatively high flux control coefficient of APR makes it likely that overexpression of this enzyme should increase the pool size of cysteine and GSH. Such an effect was indeed detected in the leaves *A. thaliana* (Fig. 2) transformed with *Lemna minor* APR (Suter *et al.* 2000). The increase in APR activity in line 7 seems to have saturated the concentration change in cysteine and GSH, so that further increases in activity have no further effect. Future work aimed at manipulating thiol contents in plants (Höfgen *et al.* 2001) should take

this phenomenon into account together with considerations about flux control coefficients (Vauclare *et al.* 2002).

In maize leaves, the signal for down-regulation of sulfate assimilation may rather be cysteine than GSH (Bolchi *et al.* 1999). This can be explained by the fact that GSH is preferentially synthesized in the mesophyll cells, whereas cysteine is formed in the bundle sheath cells (Burgener *et al.* 1998). Obviously, this differentially localized synthesis makes cysteine a better suited signal than GSH in this system.

Interactions induced by the nitrogen source

Nitrogen deficiency is characterized by decrease in NR, low levels of amino acids and decreased synthesis of proteins (Haller *et al.* 1986; Melzer *et al.* 1989; Coruzzi and Bush 2001). Omission of a nitrogen source from the medium of *Lemna minor* rapidly decreased NR, but also the activity of ATPS and APR (Brunold and Suter 1984). The decrease in NR activity can be explained by the fact that this enzyme is only induced in the presence of nitrate (Coruzzi and Bush 2001). The decrease of the activity of APR and ATPS may be due to low levels of amino acids, especially OAS. In *A. thaliana*, nitrogen deficiency also resulted in decreased APR mRNA, protein and activity (Koprivova *et al.* 2000).

When nitrogen was offered to plants in form of ammonium, carbon skeletons were preferentially channeled away from carbohydrate pools into amino acids and proteins (Brunold and Suter 1984). The decrease in NR activity induced by ammonium may be explained by a signaling effect of accumulating amino acids or ammonium itself (Migge *et al.* 2000; Coruzzi and Bush 2001). It is tempting to assume that the increase in APR activity was based on a positive signal originating from an increased level of amino acids, especially OAS. An experiment with root cultures of *A. thaliana* shows that a positive signal originating from OAS (Fig. 3) can override the negative one from thiols (Vauclare *et al.* 2002). Addition of OAS to the culture medium of roots, reduced in APR activity due to the presence of cysteine, rapidly increased this enzyme activity. OAS also induced an increase in APR activity in the controls (Fig. 3), corroborating the signaling strength of OAS (Neuenschwander *et al.* 1991; Koprivova *et al.* 2000).

Interactions induced by carbon assimilation

Many nuclear encoded genes for plastidic localized enzymes are light regulated (Hesse *et al.* 1999; Kaiser *et al.* 1999) or follow circadian rhythms. Also APR activity and the flux through sulfate assimilation are controlled by light (Neuenschwander *et al.* 1991; Kocsy *et al.* 1997; Kopriva *et al.* 1999). APR activity of maize and *A. thaliana* changes according to a diurnal rhythm in shoots and roots with a maximum during the light period (Kocsy *et al.* 1997; Kopriva *et al.* 1999). In *A. thaliana,* APR mRNA starts to accumulate already four hours before light onset, demonstrating that the enzyme is not controlled by light alone, but also by an internal signal.

Indeed, feeding of sucrose or glucose after a prolonged dark period of 38 h, which resulted in a very low level of APR, led to increased levels of APR mRNA, protein and enzyme activity in the roots, indicating that sugar availability could be the internal signal (Kopriva *et al.* 1999; Hesse *et al.* 2003). The mechanisms by which plants sense sugars and initiate signal transduction are unclear, even though sensing of glucose by hexokinase, sensing at the entry in the cytosol and direct sensing are the preferred hypotheses (Jang *et al.* 1997; Koch *et al.* 2000; Smeekens 2000). Since sucrose also induced NR (Cheng *et al.* 1992; Klein *et al.* 2000), the effect of carbohydrates on APR activity could be triggered by accumulated products of nitrate assimilation. Indeed, addition of OAS had an even faster effect on APR activity, protein and mRNA. When both glucose and OAS were applied, APR activity detected after 6 h was higher than the sum of the values obtained with glucose or OAS alone, indicating that glucose was not acting on APR expression *via* OAS. This idea was tested by using *A. thaliana* cultivated without a nitrogen source for 3 days under day night cycles and then for 38 h in the dark as before. Under these conditions, addition of glucose induced an increase in APR activity, enzyme and mRNA. NR activity was not affected. Obviously this enzyme was only induced by glucose in the presence of nitrate. It seems reasonable to assume that N-deficiency also caused low levels of amino acids, especially OAS, and that glucose induced APR directly and not *via* OAS (Hesse *et al.* 2003). The hypothesis of a carbohydrate-dependent expression of APR was corroborated in experiments with *Lemna minor* (Kopriva *et al.* 2002). Both APR and NR activity and mRNA decreased in plants cultivated without CO_2. The decrease in enzyme activity was prevented, when sucrose was added to the culture medium at the time of CO_2 withdrawal. When *Lemna* was cultivated again in atmospheric air after a 24 hour pre-treatment without CO_2, both APR and nitrate reductase activity and mRNA increased to control levels within 24 hours. The same increase was detected after addition of sucrose or glucose to the medium of *Lemna* cultivated in CO_2-free atmosphere, demonstrating that carbohydrates are involved in coordinating carbon assimilation with nitrate and sulfate assimilation.

Conclusions

A summary of the results discussed and a working hypotheses based on these results can be best presented using a scheme by Davidian *et al.* 2000 (Fig. 4).

1. During sulfur deficiency, enzymes of carbon assimilation and NR are repressed. Sulfate transporters and enzymes involved in sulfate assimilation like ATPS and APR are derepressed, probably due to accumulating amino acids, especially OAS, which functions as positive signals, and a decreased level of GSH, which does not repress the formation of the enzymes of sulfate assimilation. Increased OAS dissociates the complex between SAT and OAS-TL, thus inhibiting additional OAS formation.

2. Under sulfur excess, increased levels of cysteine and GSH are detected. Cysteine functions as a feedback inhibitor of cytoplasmic SAT, GSH represses sulfate

transport, APR and ATPS. Both regulatory mechanisms decrease sulfate assimilation.

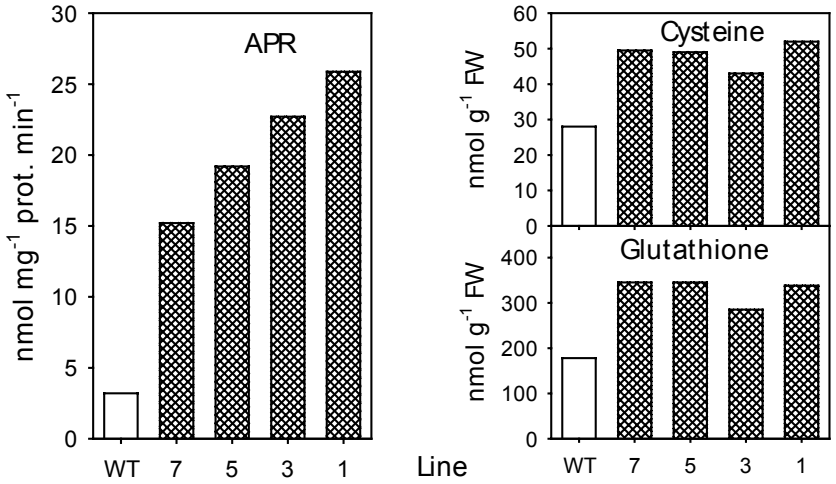

Fig. 2. Effect of overexpression of APR from *Lemna minor* in *Arabidopsis thaliana* on APR activity and contents in cysteine and GSH in the leaves. APR cDNA was isolated according to Suter *et al.* (2000). Measurement of APR activity was done according to Koprivova *et al.* (2000), cysteine and GSH were determined according to Harms *et al.* (2000). Mean values of 2 measurements are presented.

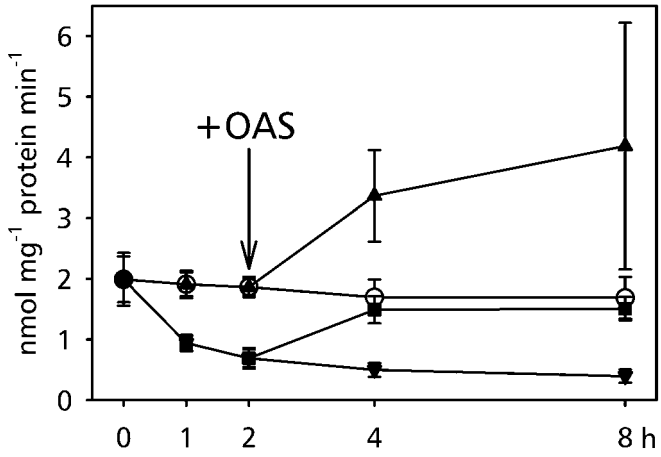

Fig. 3. Effect of the combined feeding of *Arabidopsis thaliana* roots cultures with 0.5 mM cysteine and 0.5 mM OAS on APR activity. The roots were cultivated without (O) or with (▼) cysteine. OAS was added 2 h later to the controls (▲) and the medium containing cysteine (■). The experiment was done according to Vauclare *et al.* (2002). Mean values ± SD from 4 measurements are presented.

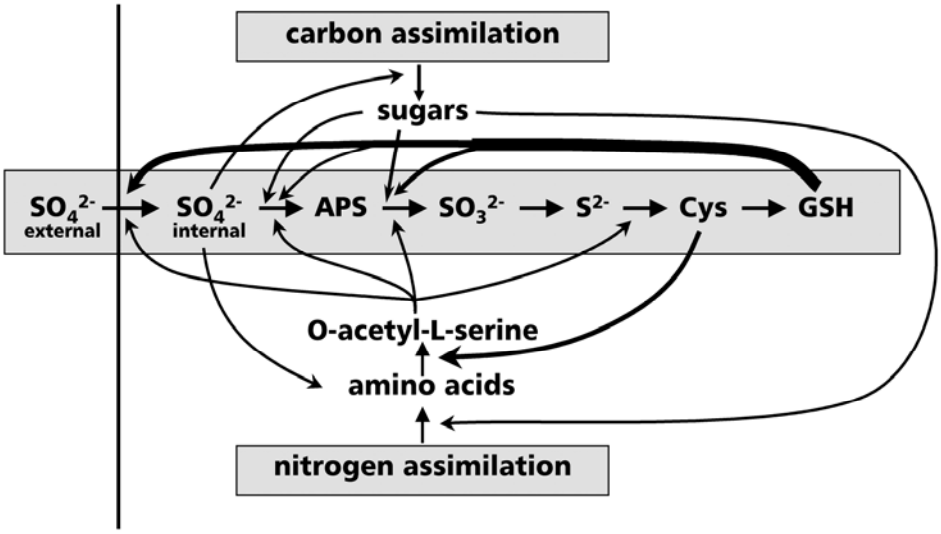

Fig. 4. Interactions between sulfur, nitrogen and carbon assimilation.

3. Under N-deficiency, OAS and sulfate reduction enzymes are at a low level, thus reducing the formation of sulfur amino acids, which are not used in this situation.

4. When ammonium is available as a nitrogen source, more carbon skeletons are channeled into amino acid and protein synthesis. Amino acids and probably also OAS are high under these conditions and induce enzymes of sulfate assimilation, especially APR. Increased APR activity increases the flux through sulfate assimilation, thus providing additional sulfur amino acids for increased protein synthesis.

5. Under conditions of low sugar availability, enzymes of sulfate and nitrate assimilation like NR, ATPS and APR are at low levels, thus reducing the rate of nitrate and sulfate assimilation in a situation where carbon skeletons are less available for accepting reduced nitrogen or sulfur. Addition of carbohydrates induces APR expression

All interactions discussed may be considered as strategies aimed at adapting sulfur and nitrogen to carbon assimilation and to changing needs of plants and changing resources available in the environment. Future work will establish the molecular mechanisms involved and the exact functions of amino acids, GSH and sugars in signaling.

Acknowledgements

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S-METHYLMETHIONINE AND THE INTERFACE BETWEEN SULFUR AND ONE-CARBON METABOLISM

Sanja Roje and Andrew D. Hanson
Horticultural Sciences Department, University of Florida, Gainesville, FL 36611-0690, USA

Introduction

Sulfur and one-carbon (C_1) metabolism intersect at methionine and its activated derivative *S*-adenosylmethionine (AdoMet) as methyl groups pass through these compounds *en route* between folate-mediated C_1 metabolism and methylated products (Fig. 1) (Giovanelli *et al.* 1980; Hanson and Roje 2001). C_1 metabolism – especially methyl metabolism – is very active in higher plants, in part because they contain high levels of methylated compounds, such as lignin, alkaloids, and betaines (Hanson and Roje 2001). Moreover, plants are unique in having the photorespiratory pathway – to which C_1 metabolism is central (Cossins 2000; Douce and Neuburger 1999).

While plant C_1 metabolism has much in common with that of other organisms, it also has special features. As in other organisms, plants metabolize serine, glycine, formate and other molecules to generate specific C_1 derivatives of the coenzyme tetrahydrofolate (THF) that are then interconverted between different oxidation states – from 10-formyl-THF (most oxidized) through 5,10-methenyl- and 5,10-methylene- to 5-methyl-THF (most reduced) (Fig. 1). These interconversions of C_1 substituted folates form the core of C_1 metabolism, from which C_1 units are withdrawn by anabolic reactions. The largest anabolic flux is the use of 5-methyl-THF to convert homocysteine to methionine, which is incorporated into proteins or converted to AdoMet, the donor for methylation reactions. Other anabolic reactions are use of 10-formyl-THF to synthesize purines and formylmethionyl-tRNA (for translation initiation in organelles), and use of 5,10-methylene-THF to produce thymidylate and pantothenate. In the activated methyl cycle, the *S*-adenosylhomocysteine (AdoHcy) formed after transmethylations is first hydrolyzed to homocysteine and adenosine by AdoHcy hydrolase. The homocysteine is then remethylated by 5-methyl-THF to give methionine, and the adenosine is recycled to adenylates.

The special features of plant C_1 metabolism include several novel reactions and enzymes (Hanson *et al.* 2000). This article reviews two such novelties, and suggests how they may be connected. The first is the enzyme that produces methyl groups,

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5,10-methylene-THF reductase (MTHFR), which has special properties in plants. The second novelty is the presence of *S*-methylmethionine (SMM) and of a futile cycle – the SMM cycle – in which it participates (Fig. 1).

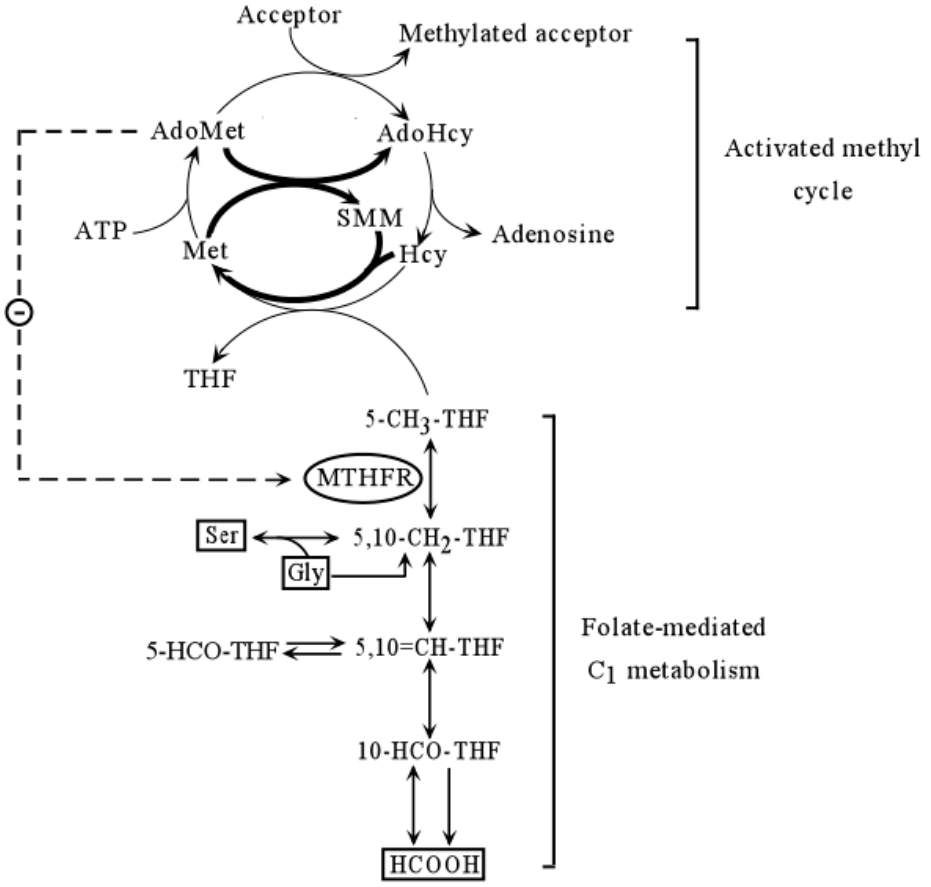

Fig. 1. The main reactions of plant C_1 metabolism and their relation to methionine metabolism. The principal sources of C_1 units are boxed. The reactions of the SMM cycle are bolded. The dotted line denotes a negative feedback loop whereby AdoMet regulates its own synthesis; this loop is missing in plants but is present in other eukaryotes. THF, tetrahydrofolate; 10-CHO-THF, 10-formyl-THF; 5-CHO-THF, 5-formyl-THF; 5,10=CH-THF, 5,10-methenyl-THF; 5,10-CH_2-THF, 5,10-methylene-THF; 5-CH_3-THF, 5-methyl-THF; Hcy, homocysteine; Met, methionine.

Methylenetetrahydrofolate reductases in plants and other eukaryotes

In mammals and yeast, the conversion of 5,10-methylene-THF to 5-methyl-THF is catalyzed by MTHFRs that are NADPH-dependent and allosterically inhibited by

AdoMet (Jencks and Matthews 1987; Roje *et al.* 2002). The AdoMet sensitivity has been considered to be a key regulatory feature (Bagley and Selhub 1998; Matthews *et al.* 1998), although there has been no direct evidence for the existence of feedback regulation of AdoMet levels *in vivo*. The mammalian enzyme has been shown to comprise an N-terminal catalytic domain, and a C-terminal regulatory domain containing the binding site for AdoMet (Matthews *et al.* 1998). Yeast MTHFRs have the same bipartite structure (Raymond *et al.* 1999; Roje *et al.* 2002).

The primary structure of plant MTHFRs is similar to those of the mammalian and yeast enzymes but the plant enzymes are NADH-dependent and insensitive to AdoMet (Roje *et al.* 1999). The difference in coenzyme requirement probably renders the reaction in plants reversible (Roje *et al.* 1999). The AdoMet-insensitivity also has important implications. Specifically, if the AdoMet feedback loop in other organisms is as important as commonly supposed, its absence in plants raises the question of what regulates AdoMet levels in plants.

To determine whether the AdoMet feedback loop is indeed important *in vivo*, we constructed a chimeric MTHFR by fusing the N-terminal catalytic domain of the yeast MTHFR Met13p to the C-terminal (AdoMet-insensitive) domain of the *Arabidopsis* enzyme AtMTHFR-1 (Roje *et al.* 2002). The chimeric enzyme proved to be fully functional *in vitro*. As anticipated, it uses NADPH (like its yeast parent) and is AdoMet-insensitive (like its plant parent). When the chromosomal copy of *MET13* (the principal MTHFR gene in yeast) was replaced by the chimeric MTHFR, the cells grew normally but accumulated 140-fold more AdoMet and seven-fold more methionine than the wild type (Roje *et al.* 2002). This finding provides a clear *in vivo* demonstration that the AdoMet sensitivity of MTHFR controls metabolic flux to Met and AdoMet in non-plant organisms. It also substantiates the question raised above, *viz.*: How, in the absence of AdoMet feedback on MTHFR, do plants regulate AdoMet levels? This question is underscored by the report that plants lack another negative feedback on AdoMet synthesis that is present in other eukaryotes – inhibition of AdoMet synthetase by AdoMet (Schröder *et al.* 1997).

S-Methylmethionine – a unique plant metabolite

SMM is a compound unique to plants that has been found in all major angiosperm groups, including monocots and dicots (Giovanelli *et al.* 1980; Paquet *et al.* 1995). This indicates that SMM has been present since very early in angiosperm phylogeny and originated at least 130 million years before the present (Crane *et al.* 1995). An even older origin is probable, since SMM has also been reported to be synthesized in liverworts, mosses, and gymnosperms (Pokorny *et al.* 1970). SMM is produced by the plant-specific enzyme methionine *S*-methyltransferase (MMT) through the essentially irreversible, AdoMet-mediated methylation of methionine (Giovanelli *et al.* 1980; James *et al.* 1995). An opposing reaction, in which SMM is used to methylate homocysteine to yield two molecules of methionine, is catalyzed by the enzyme homocysteine *S*-methyltransferase (HMT) (Giovanelli *et al.* 1980). Unlike MMT, HMTs also occur in bacteria, yeast, and mammals, enabling them to catab-

olize SMM of plant origin, and providing an alternative to the methionine synthase reaction as a means to methylate homocysteine (Shapiro and Yphantis, 1959; Neuhierl *et al.* 1999; Thomas *et al.* 2000). The widespread occurrence in nature of HMT enzymes to metabolize SMM is a further indication that SMM is an evolutionarily ancient metabolite.

MMT cDNAs have been cloned and characterized from *Wollastonia biflora* (*Asteraceae*), *Arabidopsis*, maize (Bourgis *et al.* 1999) and barley (GenBank accession no. AB028870). The *W. biflora* MMT was cloned *de novo* using amino acid sequence data from purified enzyme, and the other sequences were then identified by homology. MMT is a novel type of methyltransferase that is apparently the product of an ancient gene fusion event. It comprises an N-terminal methyltransferase domain and a C-terminal region with homology to transaminases and other pyridoxal 5'-phosphate (PLP)-dependent enzymes but lacking the catalytically essential PLP-binding lysine residue (Bourgis *et al.* 1999). It is not known whether the C-terminal domain has a catalytic or a regulatory function. Deduced MMT polypeptides lack obvious targeting signals and, consistent with this, the enzyme has been shown to be localized in the cytosol in *W. biflora* (Trossat *et al.* 1996). Both *Arabidopsis* and maize have single MMT genes (Bourgis *et al.* 1999).

Following the cloning of an HMT from *Escherichia coli* (Neuhierl *et al.* 1999), HMTs were recently cloned from *Arabidopsis* and maize by homology, and characterized (Ranocha *et al.* 2000, 2001b). Searches of *Arabidopsis* and maize expressed sequence tag collections and the *Arabidopsis* genome identified three different HMT-coding sequences from *Arabidopsis* (AtHMT-1 to 3), and four from maize (ZmHMT-1 to 4). Phylogenetic analysis of these sequences indicated that they comprise two subfamilies that pre-date the separation of monocots and dicots, corroborating the phytochemical evidence for an ancient origin of SMM (see above). One subfamily contains AtHMT-1 and ZmHMT-1; the other contains the rest. When the three HMTs from *Arabidopsis* were expressed in *E. coli*, analysis of their biochemical properties revealed that AtHMT-1 is strongly inhibited by methionine, whereas the other two HMTs (which are members of a phylogenetically separate subfamily) are methionine-insensitive (Ranocha *et al.* 2000, 2001b). This finding raises the possibility that methionine sensitivity of HMTs is important for the control of flux through the HMT reaction, and that different HMTs have different functions. Analysis of the expression levels of HMTs in *Arabidopsis* organs revealed that different members of the family are prevalent in different organs. Interestingly, seeds, which have the highest methionine content of all organs, predominantly express the methionine-insensitive AtHMT-2. Like MMTs, HMT proteins appear to lack targeting signals and preliminary evidence suggests that the enzyme activity is located in the cytosol (Ranocha *et al.* 2000).

The functions of SMM are only partially understood. In morning glory flower buds it appears to act as a reserve of methionine moieties (Hanson and Kende 1976). A role as a storage form of methionine is also supported by evidence for a metabolically inactive 'storage' pool of SMM in leaves of *Arabidopsis* (Ranocha *et al.* 2001b) and the grass *Spartina alterniflora* (Kocsis *et al.* 1998). Further evidence for a storage role is that methionine feeding, and mutations or transgenes that increase

free methionine levels, result in SMM accumulation (Datko and Mudd 1984; Inaba *et al.* 1994; Kim *et al.* 2002).

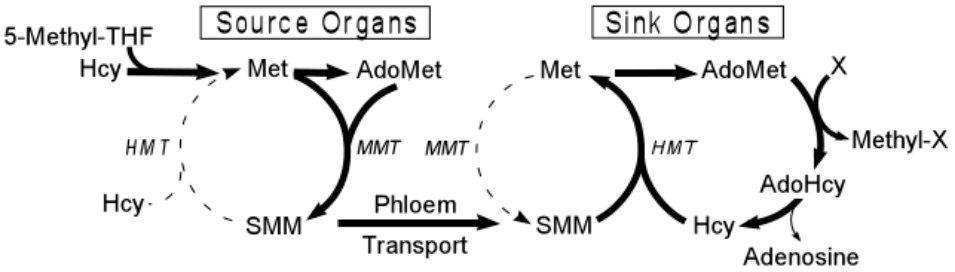

Fig. 2. Long-distance transport of methionine and methyl moieties *via* movement of SMM in the phloem. Major fluxes are bolded. SMM is formed in source organs such as leaves and moves in the phloem to sink organs such as seeds, where it is recycled to methionine. This methionine can be used to produce AdoMet for methylation reactions.

SMM is also known to serve as a vehicle for the long-distance transport of methionine and methyl groups (Fig. 2). This role is supported by qualitative evidence that SMM is translocated in the phloem of various dicot and monocot species, and by quantitative evidence that SMM moving *via* the phloem provides around half the sulfur needed by developing wheat grains (Bourgis *et al.* 1999). It should be noted that when SMM is made in one organ, translocated, and then recycled to methionine in another organ, the actions of MMT and HMT are separated in time and space (Fig. 2). This situation should be distinguished from the futile cycling of SMM to be discussed below. The contribution of SMM to long-distance sulfur transport is probably less in many species than in wheat (Bourgis *et al.* 1999), and may vary with developmental stage and sulfur nutrition (Sunarpi and Anderson 1997; Fitzgerald *et al.* 2001). It is therefore still unclear how crucial SMM is to inter-organ sulfur fluxes in angiosperms as a whole.

In the special cases of angiosperm species that synthesize and accumulate the osmoprotectant dimethylsulfoniopropionate (DMSP), SMM has been shown to be the first intermediate in the DMSP synthesis pathway. This has been established both for the dicot *W. biflora* (Hanson *et al.* 1994) and for the monocot *Spartina alterniflora* (Kocsis *et al.* 1998). Surprisingly, the steps after SMM are not the same in these two species (Rhodes *et al.* 1997; Kocsis and Hanson 2000), which indicates that their DMSP synthesis pathways have independent evolutionary origins. In connection with DMSP synthesis *via* SMM, it is important to note that this is a rare secondary pathway, and so cannot explain the distribution of SMM throughout the angiosperms.

Besides general roles in methionine storage and transport, and a special role in DMSP synthesis, it has been suggested that SMM could be essential in plants as a methyl donor for an unknown reaction or reactions (Giovanelli *et al.* 1980). While intriguing, this hypothesis is hard to test by biochemical means since we have no

idea of what SMM-dependent reactions to look for. It has consequently not been investigated.

The mysterious *S*-methylmethionine cycle

When MMT and HMT act simultaneously, together with the reactions catalyzed by AdoMet synthetase and AdoHcy hydrolase, they constitute a cycle known as the SMM cycle (Mudd and Datko 1990). Each turn of this cycle consumes and then regenerates two methionines while converting ATP to adenosine, pyrophosphate, and phosphate; it is therefore futile and, in effect, short circuits the activated methyl cycle (Fig. 1). Surprisingly, there is good evidence that in angiosperms this cycle operates continuously throughout the plant. This evidence includes radiotracer data on methionine and SMM metabolism (Mudd and Datko 1990; Ranocha *et al.* 2001b), Northern analysis and enzyme assays (Ranocha *et al.* 2001b), and digital gene expression profiles (Hanson *et al.* 2000; Ranocha *et al.* 2001b). Also surprisingly, the SMM cycle has been found to consume large amounts of AdoMet. Thus, calculations based on ^{35}S radiotracer data indicate that the cycle consumes 50 % of the AdoMet produced by mature *Arabidopsis* leaves (Ranocha *et al.* 2001b) and 7 % of that formed in *Lemna* fronds (Mudd and Datko 1990).

The function of the futile and seemingly wasteful SMM cycle is long-standing mystery (Giovanelli *et al.* 1980; Mudd and Datko 1990). There are two extant hypotheses. The first and older of the two is that the cycle serves to maintain the pool of free methionine in the event of an overshoot in the conversion of methionine to AdoMet (Mudd and Datko 1990). It does this by providing a way to convert methionine moieties trapped in AdoMet back to free methionine. Since a stable supply of free methionine is essential for protein synthesis, this hypothesis assigns a vital role to the SMM cycle; we will refer to it as the 'overshoot' hypothesis. The second hypothesis was generated in the course of *in silico* modeling work, and incorporates recent findings on methionine and AdoMet metabolism (Ranocha *et al.* 2001b). It posits that the SMM cycle is a major way in which plants achieve short-term regulation of AdoMet level, the mechanisms that other eukaryotes use to do this being absent. In this hypothesis the cycle provides a way to dispose of AdoMet that is surplus to the current requirement for methylation reactions; we will therefore refer to it as the 'overflow' hypothesis.

In silico modeling of the *S*-methylmethionine cycle

In order to estimate the flux through the SMM cycle and to test hypotheses about its function, we developed a computer model of methionine metabolism in mature *Arabidopsis* rosette leaves, based on data from radiotracer experiments and on metabolite contents (Ranocha *et al.* 2001b). The model was then used to simulate the consequences of eliminating the SMM cycle by ablating SMM synthesis. Eliminating the SMM cycle *in silico* caused AdoMet to accumulate to about three times its

original level, but had very little effect on the size of the metabolic pool of free methionine. AdoMet accumulation eventually came to a stop in the model because the expanding AdoMet pool drove up the rates of AdoMet utilization, spontaneous racemization, and decomposition until these processes were in balance with AdoMet synthesis. This modeling work thus showed that the SMM cycle greatly influences the AdoMet pool size, which is consistent with the 'overflow' hypothesis.

Two related scenarios were used to test the Mudd and Datko (1990) 'overshoot' hypothesis. In both scenarios, we simulated an overshoot in methionine → AdoMet conversion by instantaneously transferring almost all (93 %) of the metabolically active methionine pool to the AdoMet pool. In one case the SMM cycle was left intact; in the other it was deleted and the AdoMet synthesis rate was reduced by the amount consumed by SMM synthesis. In both simulations, the metabolic methionine pool quickly returned to its original size, with only slightly different half-times (10 min when the cycle was present, and 13 min when it was not). With the SMM cycle present, the AdoMet pool also came rapidly back to normal, with a half-time of 10 min. However, with no SMM cycle the AdoMet pool reverted to normal much more slowly, with a half-time of 47 min or more. The resilience of the metabolic methionine pool is explained in large part by the stabilizing effects of a storage (*i.e.* metabolically inactive) pool of methionine, and by the methionine input from protein turnover. These two features – a storage methionine pool and a modest rate of protein turnover (about 0.5 % day^{-1}) – are supported by the experimental data used to construct the model and are reasonable in the light of many other metabolic modeling studies in plants (Morgan and Rhodes 2002). Overall, these *in silico* results indicate that the SMM cycle does not stabilize the methionine pool, but has a major role in controlling short-term fluctuations in AdoMet levels. These results therefore support the 'overflow' rather than the 'overshoot' hypothesis.

In summary, the modeling results suggest that the SMM cycle is at least a partial answer to the question 'How, in the absence of AdoMet feedback on MTHFR, do plants regulate AdoMet levels?' While controlling metabolite levels by futile cycles is energetically inefficient it should be noted that this function for futile cycles has been invoked many times (Fell 1997) and that it may often be necessary for plants to sacrifice efficiency for expediency (Charles-Edwards 1975). In this connection it is interesting that the kinetics of retroinhibition of MTHFR by AdoMet – a mammalian and yeast 'alternative' to the SMM cycle – are quite slow (Jencks and Matthews 1987). As noted in the Introduction, plants have very active C_1 metabolism because of their high content of methyl groups and because they have photorespiration. It is therefore conceivable that for plants, a slow response to changes in demand for AdoMet synthesis would be a disadvantage and that they are spared this disadvantage by the SMM cycle.

Methionine *S*-methyltransferase knockouts

In order to directly test the roles of SMM and its cycle, we have recently isolated insertional MMT mutants of *Arabidopsis* and maize, which – as noted above – both

have single *MMT* genes (Ranocha *et al.* 2001a). The MMT mutants have very little or no capacity to produce SMM and consequently have no SMM cycle, but grow, flower, and set seed normally under laboratory growth conditions. These observations essentially rule out an indispensable role for SMM in plants as a methyl donor – a hypothesis that has been refractory to testing by biochemical approaches (see above).

Preliminary analysis of the pools of free methionine, threonine, other amino acids, thiols, and AdoMet in *Arabidopsis* have indicated that MMT ablation increases AdoMet levels about six-fold, but has otherwise little effect. These data provide further support for the 'overflow' hypothesis, *i.e.* that the SMM cycle serves to control AdoMet level. They do not favor the 'overshoot' hypothesis, *i.e.* that the cycle prevents depletion of the free methionine pool.

It is at first sight surprising that AdoMet-accumulating *mmt* mutants lack a severe metabolic phenotype and grow normally, because AdoMet is a substrate for many crucial metabolic reactions and is implicated in regulation of the biosynthesis of aspartate-family amino acids (Chiang *et al.* 1996; Galili and Höfgen 2002). However, yeast is known to accumulate AdoMet in the vacuole (Schlenk 1965). There is no evidence that wild type plants grown in normal conditions have a significant vacuolar storage pool of AdoMet (Ranocha *et al.* 2001b) but it is entirely possible that excess AdoMet produced by mutants – or by plants supplied with methionine – is compartmented in the vacuole. Consistent with this possibility, the high levels of AdoMet reported in *Arabidopsis mto1* mutants (90-fold above wild type) and in *Lemna* exposed to methionine (11-fold above controls) are not associated with major growth effects (Datko and Mudd 1984; Inaba *et al.* 1994).

In connection with compartmentation of AdoMet, it is noteworthy that *mmt* mutants do not show major changes in threonine level. The prevailing view of regulation in the aspartate amino acid family is that the level of AdoMet controls flux partitioning at the branch point between threonine and methionine synthesis by activating the chloroplast enzyme threonine synthase, which competes with cystathionine γ-synthase for the common intermediate *O*-phosphohomoserine (Giovanelli *et al.* 1989; Galili and Höfgen 2002). This view stems from the strong allosteric activation of threonine synthase by AdoMet that is observed *in vitro* (Curien *et al.* 1998; Laber *et al.* 1999) but the role of threonine synthase activation in regulating flux to threonine *in vivo* is not yet clear and there are some contrary findings (Galili and Höfgen 2002). If AdoMet activates threonine synthase, then AdoMet and threonine levels should in the simplest case be positively related. However, *Arabidopsis* transgenics with suppressed AdoMet synthesis accumulate threonine, while those that accumulate methionine (and presumably AdoMet) as a result of cystathionine γ-synthase overexpression do not (Kim *et al.* 2002). If *mmt* mutants accumulate AdoMet but show no threonine accumulation, it would be another indication that the role of threonine synthase activation *in vivo* is less crucial than currently supposed, perhaps because excess AdoMet does not enter chloroplasts where threonine synthase is located.

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GENETIC AND MOLECULAR ANALYSIS OF PHYTOCHELATIN BIOSYNTHESIS, REGULATION AND FUNCTION

Christopher S. Cobbett
Department of Genetics, University of Melbourne, 3010, Australia

Introduction

Among a variety of adaptive responses to heavy metal toxicity in plants, induction of the heavy metal-binding peptides, phytochelatins (PCs), is one of the best characterized. PCs are synthesized enzymatically and, because PCs themselves contain cysteine residues and PC-metal complexes contain sulfide, they are in some circumstances an important sink for plant sulfur. In recent years considerable progress towards understanding the biosynthesis, regulation and function of PCs has been achieved. In particular, through the characterization of mutants in model organisms such as the yeast *Schizosaccharomyces pombe*, in which PCs were first identified, and the plant *Arabidopsis thaliana*. In the following article reviewing this progress I focus on the genetic and subsequent molecular characterization of aspects of PC expression and function.

Phytochelatins

PCs are a family of related peptides with the structures, $(\gamma\text{-GluCys})_n\text{-Gly}$ (where $n > 1$). The Glu and Cys residues in PCs are linked through a γ-carboxylamide bond demonstrating these peptides, unlike the Class I and II MTs, are not encoded directly by genes but are the products of a biosynthetic pathway. These peptides have been referred to as cadystins (from *S. pombe*), poly-$(\gamma\text{-EC})_n$G peptides, Cd-binding peptides, and PCs and are broadly classified as Class III MTs.

A number of structural variants of PCs have been identified in different species (see Rauser 1999). For example, some plants, particularly legumes, contain both GSH and a related compound called homoGSH (γ-GluCys-β-Ala). On exposure to Cd these plants synthesise an analogous family of PCs, homoPCs $[(\gamma\text{-GluCys})_n\text{-}\beta\text{-Ala}]$. In maize, $(\gamma\text{-GluCys})_n$-Glu, and in *Agrostis* and rice, $(\gamma\text{-GluCys})_n$-Ser, have been detected, while the variant, $[(\gamma\text{-GluCys})_n]$ has also been found in Cd-binding complexes from maize and some yeasts. These variants were all identified in heavy

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metal-binding complexes. Presumably, they function in physiologically similar ways.

PCs have been identified in a wide variety of plant species including monocots, dicots, gymnosperms and algae (see Rauser 1999). In addition, PCs have been described in various fungal species, including *S. pombe* and *Candida glabrata*, and in marine diatoms. *S. pombe* and *A. thaliana* have been used most extensively as models for the molecular genetic characterization of PC biosynthesis and function. PCs themselves have not been directly identified from an animal species. However, the recent identification of PC synthase genes in some animal species has led to the conclusion that PCs are not, as previously thought, essentially a plant-specific metal detoxification mechanism (see below).

Phytochelatin biosynthetic pathway

The structural analysis of PCs indicates they are derived from GSH (or, in some cases, a related compound). This has been confirmed through various physiological, biochemical and genetic studies (see Rauser 1995, 1999). The level of GSH decreases on the induction of PCs in plant cell cultures and the exposure of whole plants or plant cell cultures to an inhibitor of GSH biosynthesis, buthionine sulfoximine (BSO), also inhibits the induction of PCs and/or confers hypersensitivity to metal ions. Furthermore, the effects of BSO on PC biosynthesis in cell cultures can be reversed by the addition of GSH. These physiological studies have been confirmed by the demonstration that mutants of both *S. pombe* and *A. thaliana* which have defects in genes encoding GSH biosynthetic enzymes are deficient in PCs and are hypersensitive to metal ions, emphasising the link between GSH and PC biosynthesis (see Cobbett and Goldsbrough 2002). A schematic illustration of the genes and functions contributing to PC biosynthesis and sequestration in plants and yeasts is shown in Fig. 1.

GSH is synthesized from its constituent amino acids in a two-step pathway catalyzed successively by the ATP-dependent enzymes, γ-glutamylcysteine synthetase (GCS) and glutathione synthetase (GSHS). In plants the GSH biosynthetic enzymes have been purified and some studies have suggested the existence of different isozymes of each in chloroplastic and cytosolic fractions (see Noctor *et al.* 2002). This has been interpreted as indicating the existence of separate GSH biosynthetic pathways in these two cellular compartments. Genes encoding these enzyme activities were first cloned from *Arabidopsis* by functional complementation of *E. coli* and *S. cerevisiae* GSH-deficient mutants (May *et al.* 1998). For each of GCS and GSHS in *Arabidopsis*, only a single gene has been identified and both enzymes are predicted to have chloroplast targeting sequences. Homologous sequences have been subsequently isolated from various other plants. The cDNA (or genomic DNA) sequences encoding these enzymes described in the databases generally include a plastid transit sequence. Thus, notwithstanding the apparent identification of the GSH biosynthetic activities in both cytosolic and plastid compartments, the subcellular localization of the pathway is yet to be resolved, and the possibility that GSH

is exclusively synthesized in the plastid must be considered. In view of this possibility greater consideration needs to be given to mechanisms of GSH transport across the plastid membrane.

GSH-deficient mutants have been identified in *Arabidopsis* (Fig. 1). The *cad2-1*, Cd-sensitive mutant is partially PC-deficient and contains only 20 - 40 % of wildtype levels of GSH (Cobbett *et al.* 1998). The mutant has only 40 % of wildtype GCS levels and has a 6 bp in-frame deletion in an exon of the GCS gene. A more extreme GCS mutant, *rml1* (*rootmeristemless1*) has less than 2 % wild type levels of GSH (Vernoux *et al.* 2000). This suggests that the *cad2-1* mutant gene product is partially active and that there is unlikely to be a second gene encoding GCS activity. Interestingly, the *rml1* mutant has an unexpected phenotype whereby it fails to develop a root following germination (Cheng *et al.* 1995). Wildtype seedlings germinated in the presence of BSO, an inhibitor of GCS, have an identical appearance to *rml1* indicating the chemical inhibition of GCS activity can phenocopy the *rml1* mutant. In a further study, transgenic *Arabidopsis* plants in which GCS expression was suppressed through antisense expression had GSH levels as low as 2 - 3 % of wildtype (Xiang *et al.* 2001). Only in antisense plants with an extreme reduction in GSH levels was an effect on growth, including root growth, evident. However, since the generation of transgenic plants required root growth, no severely affected, *rml1*-like lines were identified. The *rml1* phenotype is due to an apparent lack of root cell division post-germination and studies in synchronized tobacco cell cultures indicate depletion of GSH blocks the cell cycle (Vernoux *et al.* 2000). This implies an unexpected role for GSH in controlling root growth and has led to speculation that GSH may be an important signal by which root growth is modulated in response to environmental stress. The *rml1* mutation is an amino acid substitution and it is not clear whether it is a complete loss-of-function mutation. However, preliminary analysis of a GCS T-DNA insertion mutant indicates a lethal phenotype suggesting the *rml1* mutant retains some residual activity (N. Cairns and C. Cobbett, unpublished results).

Grill *et al.* (1989) identified a PC biosynthetic activity from cultured cells of *Silene cucubalis*. This enzyme synthesized PCs from GSH, catalyzing the successive appearance of PC_2, PC_3 and PC_4. The reaction was presumed to involve the transpeptidation of the γ-GluCys moiety of GSH onto a second GSH molecule to form PC_2 or onto a PC molecule to produce an extended PC oligomer. This γ-GluCys dipeptididyl transpeptidase (EC 2.3.2.15) has been named PC synthase.

The PC synthase gene was first identified through the isolation of Cd-sensitive, PC-deficient, *cad1*, mutants of *Arabidopsis* (Howden *et al.* 1995). The *cad1* mutants, in contrast to *cad2-1*, have wildtype levels of GSH and, consistent with this, lack PC synthase activity. Thus *CAD1* was presumed to be the structural gene for PC synthase and the *CAD1* gene was isolated using a positional cloning strategy (Ha *et al.* 1999). In parallel studies an *Arabidopsis* cDNA (*AtPCS1*) (Vatamaniuk *et al.* 1999), which is identical to *CAD1*, and a similar cDNA from wheat (*TaPCS1*) (Clemens *et al*. 1999) were identified through their ability to confer Cd-resistance when expressed in *S. cerevisiae*. Targeted deletion mutants of a similar sequence identified in *S. pombe* are also Cd-sensitive and PC-deficient, confirming the analo-

gous function of the two genes in the different organisms. Assays of the activity of the *Arabidopsis* and *S. pombe* gene products, purified as epitope-tagged derivatives or expressed in *E. coli*, demonstrated each was sufficient for GSH-dependent PC biosynthesis *in vitro*.

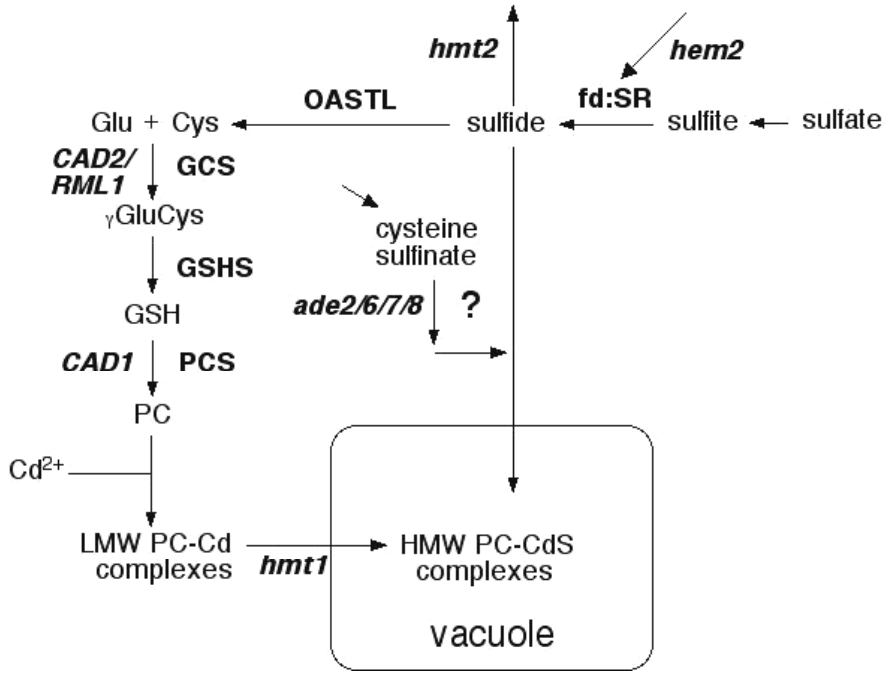

Fig. 1. Genes and functions contributing to PC biosynthesis and sequestration in plants and yeasts. Genetic loci are shown in italics: *CAD1* and *CAD2/RML1* are in *Arabidopsis*; *hmt1*, *hmt2*, *ade2, ade6, ade7,* and *ade8* are in *S. pombe*; and *hem2* is in *C. glabrata*. Mutants in each of these loci are Cd-sensitive. Enzyme abbreviations are: fd:SR, ferridoxin:sulfite reductase; OAS-TL, *O*-acetylserine (thiol)lyase; GCS, γ-glutamylcysteine synthetase; GSHS, glutathione synthetase; PCS, phytochelatin synthase. *Hmt2* encodes a mitochondrial sulfide:quinone oxidoreductase. *Hem2* encodes porphobilinogen synthase involved in the biosynthesis of siroheme, a cofactor for sulfite reductase. *Hmt1* encodes a vacuolar membrane PC-Cd transporter. LMW, HMW; low and high molecular weight, respectively. The details of the pathway/s by which sulfide is incorporated into PC-Cd complexes, indicated by ?, are poorly understood.

Despite the observation that the *cad1-3* mutant of *Arabidopsis* made no detectable PCs after prolonged exposure to Cd, a second gene, *AtPCS2*, was evident from the genome sequence. This gene has since been shown to encode a functional PC synthase (Cazale and Clemens 2001). Characterization of the, presumably specialized, physiological role of this gene will depend upon the identification and analysis of a *pcs2* mutant. PC synthase genes have now been identified in a variety of plants.

Surprisingly, since PCs themselves have not been detected in animal species, a functional PC synthase gene has also been described in the nematode, *Caenorhabditis elegans* (Clemens *et al.* 2001; Vatamaniuk *et al.* 2001), and the slime mould, *Dictyostelium discoideum* (C. Cobbett; unpublished data).

Regulation of PC biosynthesis

In view of the demand for cysteine and GSH for PC biosynthesis, observations that genes in the GSH biosynthetic pathway are regulated in response to Cd exposure are not surprising. Mechanisms regulating the expression of GCS include induction of transcription by heavy metals and jasmonic acid, a translational regulatory mechanism responding to oxidative stress and possible post-translational regulation. These have been reviewed recently by Noctor *et al.* (2002).

Expression of PCs themselves is regulated by exposure to heavy metals (Rauser 1995, 1999; Zenk 1996). Early studies used *S. pombe* and plant cell cultures to investigate the kinetics of induction. In medium containing trace levels of only essential heavy metals, low levels of PCs can be detected. On exposure to Cd, PCs can be detected within minutes with a concomitant decrease in GSH levels. Continued exposure leads to the successive accumulation of progressively larger PC oligomers. A wide range of metal ions, including Cd, Ni, Cu, Zn, Ag, Sn, Sb, Te, W, Au, Hg, Pb and Bi cations and arsenate and selenite anions, induce PC biosynthesis *in vivo*. Direct activation of PC synthase appears to be the primary determinant of PC biosynthesis. Using plant cell cultures PC biosynthesis is independent of *de novo* protein synthesis. Studies with purified PC synthase have confirmed the requirement for metal ions for activity *in vitro* (Vatamaniuk *et al.* 2000; Oven *et al.* 2002). The enzyme appears to be constitutively expressed and in *Arabidopsis* levels of *AtPCS1/CAD1* mRNA are not influenced by exposure to a range of heavy metals (Vatamaniuk *et al.* 2000). In contrast, *TaPCS1* expression in wheat roots is induced on exposure to Cd (Clemens *et al.* 1999), suggesting that in some organisms regulation of PC synthase activity may involve multiple mechanisms.

There is some debate about the mechanism by which PC synthase is activated by such a wide range of metal ions. Early models for the activation of PC synthase assumed a direct interaction between metal ions and the enzyme but raised the question of how the enzyme might be activated by such a wide range of metals. With the cloning of PC synthase genes, the expression and purification of different enzymes has led to more comprehensive investigations of the mechanisms of enzyme activation and catalysis. Nonetheless, two recent studies have led to different interpretations.

The data of Vatamaniuk *et al.* (2000) suggested that, in contrast to earlier models of activation, metal binding to the enzyme *per se* is not responsible for catalytic activation. AtPCS1 binds Cd ions at high affinity (Kd = 0.54 ± 0.20 μM) and high capacity (stoichiometric ratio = 7.09 ± 0.94). However, it has a much lower affinity for other metal ions, such as Cu, which are equally effective activators (Vatamaniuk *et al.* 1999, 2000). Modelling studies showed that in the presence of physiological

concentrations of GSH and μM concentrations of Cd, essentially all of the Cd would be in the form of a GSH thiolate suggesting that free Cd would be unlikely to be the activator. They also showed that *S*-alkylglutathiones can participate in PC biosynthesis in the absence of heavy metals indicating metal ions were not an absolute requirement for activation and suggesting a model whereby blocked glutathione molecules are both the substrate and the activator for PC biosynthesis. In contrast, the study by Oven *et al.* (2002) found *S*-methyl-GSH activated AtPCS1 to a very limited extent and that this activation was completely inhibited in the presence of Cd ions. This difference was ascribed to the use of different buffers in the reactions. Under their chosen reaction conditions, Oven *et al.* (2002) demonstrated the presence of thiols as an essential requirement for PC synthase activation. Interestingly, these authors found that different combinations of thiols and metal ions influenced activity to greatly different extents indicating the characteristics of the metal-thiolate complexes are likely to be important in *in vivo* activation of the enzyme.

PC-Cd complexes are sequestered to the vacuole

In *S. pombe* sequestration in the vacuole is an integral aspect of PC detoxification of Cd as demonstrated by studies of the *hmt1*$^-$ mutant. This mutant is Cd-sensitive and unable to form HMW PC-Cd complexes. Molecular studies demonstrated *Hmt1* encodes a member of the family of ATP-binding cassette (ABC) membrane transport proteins which is located in the vacuolar membrane (Ortiz *et al.* 1992, 1995) (Fig. 1). The HMT1 protein was required for the ATP-dependent transport of PCs in the absence of Cd or PC-Cd complexes into vacuolar membrane vesicles. The mechanism was not dependent on the proton gradient established across the vacuolar membrane by the vacuolar proton-ATPase. In *Saccharomyces cerevisiae*, YCF1, is also a member of the ABC family of transporters, transports both GSH-conjugates and $(GSH)_2Cd$ complexes to the vacuole and plays a significant role in Cd detoxification (Li *et al.* 1997).

Sequestration of PCs to plant vacuoles has also been observed. In mesophyll protoplasts derived from tobacco plants exposed to Cd, almost all of the Cd and PCs accumulated was confined to the vacuole (Vogeli-Lange and Wagner 1990). An ATP-dependent, proton gradient-independent activity capable of transporting both PCs and PC-Cd complexes into tonoplast vesicles derived from oat roots has also been identified biochemically (Salt and Rauser 1995). Although a recent inventory of the ABC transporter protein genes in the *Arabidopsis* genome (Sanchez-Fernandez *et al.* 2001) did not identify obvious immediate homologues of *Ycf1* and *Hmt1*, some members of this gene family have been characterized. AtMRP3, for example, can transport GS-conjugates of cadmium and might be considered a functional homologue of YCF1 (Tommasini *et al.* 1998). However, a functional homologue of HMT1 has not yet been identified in plants.

PC-Cd complexes are stabilized by sulfide

Since Cd has been used in many of the physiological studies of metal detoxification in *Schizosaccharomyces pombe* and in plants and is an effective inducer of PCs, many of the studies of the structure of PC-metal complexes formed *in vivo* have concentrated on those formed with Cd. Native Cd-binding complexes can be resolved on the basis of size using gel-filtration chromatography. In most cases, in extracts of plant cell cultures or tissues exposed to Cd, a single broad peak is observed. However, in extracts of *S. pombe* and of some plants two peaks of Cd-binding complexes (LMW and HMW) have been resolved (see Rauser 1995, 1999; Cobbett 2000).

In both fungi and plants, labile sulfide can also be detected in PC-Cd complexes. In *S. pombe* and *C. glabrata*, HMW complexes contain both Cd and acid-labile sulfide. Those complexes with a comparatively high ratio of S^{2-}/Cd consist of aggregates of 20-A-diameter particles which contain a CdS crystallite core coated with PCs (Dameron *et al.* 1989). The presence of sulfide in the HMW complexes increases both the stability of the complex and the amount of Cd per molecule. Similarly in plants, where HMW and LMW complexes have been separated, the ratio of S^{2-}/Cd was higher in the HMW complex compared with the LMW complex.

The characterization of Cd-sensitive mutants of *S. pombe* which are deficient in HMW complexes has provided genetic evidence for the importance of sulfide in the function of PCs. One such mutant was found to have a mutation affecting adenine biosynthesis. Subsequent genetic analysis demonstrated that different single or double mutants deficient in steps in the adenine biosynthetic pathway (Fig. 1) lacked HMW complexes (Speiser *et al.* 1992). The enzymes encoded by these genes catalyze the conversion of aspartate to intermediates in adenine biosynthesis. However, in addition, this pathway can also utilize cysteine sulfinate, a sulfur-containing analog of aspartate, to form other sulfur-containing compounds and it has been suggested that these may be intermediates or carriers in the pathway of sulfide incorporation into HMW complexes (Juang *et al.* 1993).

Cd-sensitive mutants isolated in *S. pombe* and *Candida glabrata* have identified additional functions which are probably also important in sulfide metabolism. In *S. pombe* the *hmt2* mutant hyperaccumulates sulfide in both the presence and absence of Cd. The *HMT2* gene encodes a mitochondrial sulfide:quinone oxidoreductase which was suggested to function in the detoxification of endogenous sulfide (Vande Weghe and Ow 1999) (Fig. 1). The role of HMT2 in Cd tolerance is uncertain, but one possibility is to detoxify excess sulfide generated during the formation of HMW PC-Cd complexes after Cd exposure. In *C. glabrata* the *hem2* mutant is deficient in porphobilinogen synthase involved in siroheme biosynthesis (Hunter and Mehra 1998) (Fig. 1). Siroheme is a cofactor for sulfite reductase required for sulfide biosynthesis. This deficiency may contribute to the Cd-sensitive phenotype. However, additional studies are required to establish the precise influence of this pathway on PC function. Furthermore, although sulfide appears to play an important role in PC function in plants, corresponding mutants have not yet been identified.

Conclusions

Through the genetic analysis of PC biosynthesis and function using the model organisms *S. pombe* and *A. thaliana* a number of important functions have been identified. Some functions in *S. pombe* for which counterparts would be expected to exist in plants but have not yet been identified at the genetic (or even the molecular) level. Further studies in *Arabidopsis* are likely to achieve this end. In addition, there undoubtedly remain unexpected aspects of the biosynthesis and metabolism of GSH and PCs, particularly in the area of regulation. The use of further genetic approaches to explore these aspects using these same model organisms is to be encouraged.

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PHYSIOLOGICAL AND ENVIRONMENTAL SIGNIFICANCE OF SELENIUM

Danika L. Leduc and Norman Terry
Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102, USA

Introduction

Selenium (Se), a group VI metalloid with valencies of 2-, 0, 2+, 4+ and 6+, forms a variety of inorganic and organic compounds. At low concentrations, Se is a requisite micronutrient for animals (Rayman 2000), but acts as a potent toxin at only slightly higher doses. High Se diets have led to the loss of hair, teeth, and nails, fatigue, and even death in humans (American Medical Association 1989). Animals who ate Se-contaminated plants suffered from 'alkali disease', 'blind staggers,' birth defects, and sterility (Moxon 1937; Eisler 1985; Lemly and Smith 1987; Sorenson 1991).

Selenium is a paradox to researchers: some work to increase their population's dietary intake of Se, while others try to remove it from contaminated soil and water. Here, we discuss how the field of sulfur metabolism serves as a stepping-stone for understanding Se metabolism and finding ways of enhancing Se phytoremediation.

Selenium assimilation in higher plants

Because of their chemical similarity, plants take up, transport, and metabolize Se and S using closely parallel mechanisms, although a Se requirement in plants has not been established (Fig. 1; Schwarz and Foltz 1957; Rotruck *et al.* 1973; Anderson and Scarf 1983; Lauchli 1993; Terry *et al*. 2000). Plants are classified by their ability to tolerate and accumulate Se. Primary hyperaccumulators, such as *Astragalus* species and *Stanleya pinnata*, grow only on seleniferous soil and accumulate Se (mostly as detoxified, organic forms) to 1000's of ppm (Trelease and Beath 1949). Secondary accumulators, including the *Brassicaceae* family and *Allium* species, grow on soils of varying Se concentration and accumulate lower concentrations of Se (100s of ppm) (Rosenfield and Beath 1964). Most crop and forage plants are non-accumulators, unable to tolerate excess selenium.

Selenium is most likely metabolized *via* the S assimilation pathway, which reduces inorganic forms of S to organic compounds such as cysteine (Cys) and methionine (Met) (Butler and Peterson 1967; Brown and Shrift 1981; Lauchli 1993; Terry *et al*. 2000). S and Se analogs compete for many enzymatic steps, and Se

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analogs of S products have been identified, such as the Se-amino acids selenocysteine (SeCys) and selenomethionine (SeMet) (Ng and Anderson 1979; Brown and Shrift 1980; Eustice *et al*. 1980; Burnell 1981; Bottino *et al*. 1984; Guo and Wu 1998; Dawson and Anderson 1989).

Sulfate (SO_4^{2-}) permeases actively transport selenate (SeO_4^{2-}), usually the predominant form of soluble, bioavailable soil Se, into plant roots (Fig. 1A; Ulrich and Shrift 1968). High SO_4^{2-} concentrations directly inhibit SeO_4^{2-} uptake by plants, since both compete for uptake by the same transporter (Leggett and Epstein 1956; Zayed and Terry 1992). Sulfur starvation conditions instead trigger increased expression of SO_4^{2-}/SeO_4^{2-} transporters and S/Se metabolism enzymes, increasing SeO_4^{2-} uptake (Zayed and Terry 1992; Bolchi *et al*. 1999). Selenite (SeO_3^{2-}) seems to be taken up passively (Asher *et al*. 1967; Arvy 1993). The mechanism of organic Se (*e.g.* SeMet) uptake has not been elucidated, but appears to be metabolically driven (Abrams *et al*. 1990).

SeO_4^{2-} is transported *via* the xylem to the shoot without chemical modification (Asher *et al*. 1967). SeO_4^{2-} assimilation begins in the chloroplasts, where most S/Se assimilation enzymes are located (Ng and Anderson 1979). First, ATP sulfurylase (ATPS) reduces SeO_4^{2-} to SeO_3^{2-} (Fig. 1B; Shaw and Anderson 1972; Dilworth and Bandurski 1977). ATPS catalyzes the synthesis of adenosine-5'-phosphoselenate (APSe), an energy-rich mixed anhydride, from ATP and SeO_4^{2-} (Brown and Shrift 1982; Lauchli 1993; Terry *et al.* 2000). In fact, *in vitro* experiments showed that ATPS has a greater affinity for SeO_4^{2-} than SO_4^{2-} (Shaw and Anderson 1972; Dilworth and Bandurski 1977; Burnell 1981). Indian mustard overexpressing ATPS from *Arabidopsis thaliana* (APS1) had increased SeO_4^{2-} assimilation, providing *in vivo* evidence that ATPS activates SeO_4^{2-} (Leustek *et al.* 1994; Pilon-Smits *et al.* 1999). Whereas SeO_4^{2-}-supplied plants mainly accumulate SeO_4^{2-} in their tissues, SeO_4^{2-}-supplied APS Indian mustard accumulated mostly organic Se, having overcome the barrier of SeO_4^{2-} reduction (de Souza *et al*. 1998; Pilon-Smits *et al.* 1999).

The mechanism of assimilatory reduction of APSe has not been established. In SO_4^{2-} reduction, APS reductase (APR) transforms APS to SO_3^{2-} in a glutathione(GSH)-dependent step (Fig. 1C; Leustek and Saito 1999; Saito 1999; 2000; Leustek *et al*. 2000; Höfgen *et al*. 2001). However, APR has not yet been shown to reduce APSe (Bick and Leustek 1998; Saito 1999; 2000; Terry *et al*. 2000). In another model, APSe is reduced non-enzymatically using two molecules of GSH, *via* the intermediates GS-SeO_3^{2-} and selenodiglutathione (GS-Se-SG) (Fig. 1D; Anderson and Scarf 1983; Anderson 1993). GS-Se-SG may be reduced to selenol (GS-SH) non-enzymatically with GSH or by GSH reductase (GR). GR is thought to then catalyze the NADPH-dependent reduction of GS-SH to GSH-conjugated selenide ($GS\text{-}Se^-$) (Ng and Anderson 1979; Anderson and Scarf 1983). Sulfite reductase reduces SO_3^{2-} to sulfide (Bork *et al*. 1998; Yonekura-Sakakibara *et al*. 1998; Nakayama *et al*. 2000), but has has not yet been shown to reduce SeO_3^{2-}.

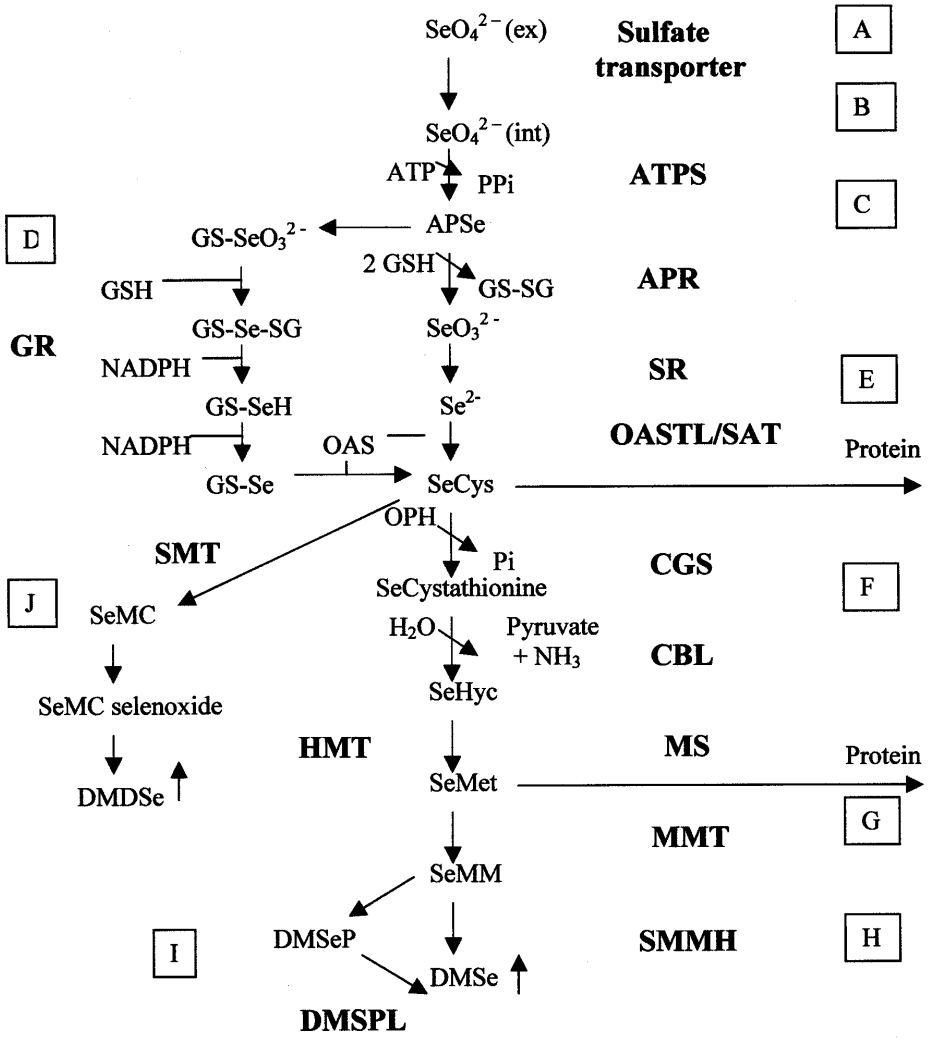

Fig. 1. Proposed selenium assimilation pathways in different plant species and under different conditions. Letters refer to the relevant section of the text, which contains more detailed descriptions of the enzymes, their reactions, and products. Figure adapted from Berken *et al.* (2002).

Cysteine synthase (CS) converts selenide (Se^{2-}) to SeCys (Fig. 1E), which can be incorporated into protein, transformed along the S assimilation pathway, or metabolized to other organic Se compounds. CS is an enzyme complex comprised of serine acetyltransferase (SAT) and *O*-acetylserine (thiol)lyase (OAS-TL) (Kredich 1987). SAT transfers an acetyl group from acetyl-CoA to serine to form OAS. OAS-TL

then incorporates GS-Se^- into OAS to form SeCys (Tsang and Schiff 1978; Ng and Anderson 1979).

In vitro evidence (Giovanelli *et al*. 1980; Ravanel *et al*. 1998) suggests that Se-Met synthesis is analogous to Met biosynthesis, consisting of three reactions catalyzed, in turn, by cystathionine γ-synthase (CGS), cystathionine β-lyase (CBL), and methionine synthase (MS; Fig. 1F; Terry *et al*. 2000). CGS combines SeCys and *O*-phosphohomoserine to form Se-cystathionine. Spinach and *Astragalus racemosus* CGS had higher affinities for SeCys than Cys (Dawson and Anderson 1988; 1989). CBL cleaves Se-cystathionine into Se-homocysteine (SeHcy), pyruvate, and ammonia (Hell 1997). Pea CBL has similar affinities for cystathionine and Se-cystathionine (McCluskey *et al*. 1986). MS transfers a methyl group from methyltetrahydrofolate to SeHcy to form SeMet. Alternatively, homocysteine methyltransferase (HMT) might synthesize SeMet using either *S*-adenosyl-methionine (SAM) or *S*-methylmethionine (SMM) as a methyl donor (de Souza *et al*. 2000a).

Plants produce volatile selenium compounds such as dimethylselenide (DMSe) and dimethyl diselenide (DMDSe). Se-methylMet (SeMM) is a precursor of DMSe in cabbage (Lewis *et al*. 1974). SAM-dependent methionine *S*-methyltransferase (MMT) transfers a methyl group from SAM to SeMet to produce SeMM (Fig. 1G; James *et al*. 1995; Pimenta *et al*. 1998; Bourgis *et al*. 1999; Tagmount *et al*. 2002). SMM hydrolase (SMMH) cleaves SMM to form dimethylsulfide (DMS) and may also use SeMM as a substrate (Fig. 1H; Hattula and Granroth 1974; Lewis *et al*. 1974; Giovanelli *et al*. 1980; Gessler and Bezzubov 1988). Under some conditions, plants may produce DMS *via* dimethylsulfinopropionate (DMSP; Fig. 1I; Dacey *et al*. 1987; Mudd and Datko 1990; Hanson *et al*. 1994; Kocsis *et al*. 1998). In these plants, SMM is deaminated and decarboxylated yielding the DMSP-aldehyde, which is further oxidized to DMSP by a dehydrogenase (Trossat *et al.* 1996, 1998; Vojtechova *et al*. 1997; Kocsis and Hanson 2000). DMSP lyase may then release volatile DMS (Dacey *et al*. 1987; Ansede and Yoch 1997). The role of these enzymes in DMSe formation has not been established. Se-accumulating plants that accumulate Se-methylselenocysteine (MetSeCys), a non-protein amino acid, volatilize Se as DMDSe. MetSeCys may be converted to Se-methyl selenocysteine selenoxide (SeMC selenoxide) (Fig. 1J; Evans *et al*. 1968; Lewis 1971). SMC sulfoxide yields dimethyl disulfide (DMDS) upon acid hydrolysis and thermal degradation (Ostermayer and Tarbell 1959; Kubec *et al*. 1998).

Toxicity and tolerance

Although chemically similar, S and Se analogs are not entirely interchangeable. In some enzymes, such as GSH peroxidase, SeCys, encoded by the stop codon, UGA, rather than Cys, is required for activity (Rotruck *et al*. 1973; Chamber and Harrison 1987; Hatfield *et al*. 1992). However, lack of discrimination against SeCys and SeMet in peptide bond formation, translation initiation, and methylation reactions, may lead to Se toxicity (Brown and Shrift 1981). For instance, disulfide bonds between Cys residues lock proteins in the unique conformation needed for enzyme

activity. The longer and weaker diselenide bonds between SeCys residues could alter protein structure. Further, the pKa of the Cys thiol group is usually around 8, but that of SeCys is only about 4, a significant difference for enzyme active sites (Huber and Criddle 1967). SeCys cytotoxicity may also result from its ability to generate free radicals (Spallholz 1994). Misincorporation of SeMet may not alter enzyme activity (Boles *et al*. 1991). *E. coli* β-galactosidase retained normal activity even when 75 Met residues were replaced with SeMet (Huber and Criddle 1967). Instead, SeMet may act as an ecotoxin, a bioavailable, stable, and transferable form of Se easily metabolized to the more cytotoxic SeCys (Fan *et al.* 2002).

Se-hyperaccumulators discriminate between Se and S analogs to reduce misincorporation of selenoamino acids into their proteins. Some hyperaccumulators divert Se from incorporation into proteins by methylating SeCys to form MetSeCys, which can be stored as γ-glutamylMetSeCys or methylated to DMDSe (Virupaksha and Shrift 1963; Nigam and McConnell 1969; Lewis *et al*. 1974; Brown and Shrift 1981; Neuhierl and Böck 1996; Neuhierl *et al*. 1999). Others reduce Se incorporation in their proteins by accumulating another non-protein amino acid, Se-cystathionine (Virupaksha and Shrift 1963; Peterson and Butler 1967). Since these plants continue to synthesize Met, their CBL must discriminate against the Se substrate to block SeMet synthesis.

Phytoremediation

High levels of Se in the environment arise from anthropogenic activities but also occur naturally in soils derived from shale rock. Agricultural irrigation leaches Se from soil into the subsurface drainage water, which collects in evaporation ponds (Presser and Ohlendorf 1987). As the water evaporates, Se concentrations build up to toxic levels, possibly leading to environmental disasters similar to those at the Kesterson Reservoir in California (Skorupa 1998). Industrial sources of Se include oil refineries, electric utilities, and production of electronics, steel, glass, ceramics, pigments, paints, pharmaceuticals, rubber, fungicides, and insecticides (Kerr 1988).

Although similarities between Se and S lead to Se toxicity, they can also be exploited for use in Se remediation. Phytoremediation uses plants and their associated microbes to remove, contain, or render harmless environmental pollutants. In general, phytoremediation approaches cost less than traditional technologies and reduce the amount of hazardous waste for disposal (Glass 1999). Phytoremediation is particularly amenable to Se cleanup since plants are able to phytoextract and phytovolatilize Se as they do S (Terry *et al*. 2000). Phytoextracted Se accumulates in plant tissue, which can be harvested and removed from the site (Kumar *et al*. 1995). Even more attractive is the ability of plants to volatilize Se in non-toxic forms, thereby removing Se from the local ground ecosystem (McConnell and Portman 1952; Hansen *et al*. 1998; Terry *et al*. 2000).

Constructed wetlands efficiently remove dilute Se from large volumes of water, such as industrial wastewater and agricultural drainage water (Hansen *et al*. 1998). Wetland sediments act as a biogeochemical filter in which plants and microbes ex-

tract and volatilize Se. A 36-hectare constructed wetland at the Chevron oil refinery in Richmond, CA removed 89 % of Se from the 10 million L/day of wastewater (Hansen *et al.* 1998). A second wetland comprised of 10 quarter-acre cells of different plant species removed up to 85 % of the inflow of Se from agricultural drainage water in Corcoran, CA (Terry 1998; 2000). Wetlands are an almost ideal solution to the problem of Se-contaminated wastewater. However, since a majority of the Se removed is retained in the sediment, Se can build up to toxic levels, forcing retirement of the wetland (Lin and Terry 2000).

Increasing Se volatilization from wetlands would decrease the amount retained in the wetland sediment. Due to their large biomass, plants have great potential for Se volatilization. The ability to volatilize Se is determined by a species' Se tolerance and ability to efficiently metabolize Se to volatile forms (Terry 2000). The superior volatilizing ability of *Salicornia bigelovii*, for example, correlates with its ability to metabolize Se to organic forms (Lee *et al*. 2001). Altering plant Se tolerance or metabolism through conventional breeding or genetic engineering is a promising method for increasing Se volatilization (Terry *et al*. 2000). Genetic engineering approaches rely upon thorough knowledge of the relevant biochemical pathways and genes. Knowledge of plant S metabolism has greatly aided work in Se phytoremediation,

Genetic engineering can overcome rate-limiting steps in the Se volatilization pathway or confer new metabolic capabilities (Terry *et al*. 2000). For example, overexpressing the ATPS gene from *Arabidopsis* in Indian mustard, overcame the barrier to SeO_4^{2-} reduction (Pilon-Smits *et al*. 1999). Although Se hyperaccumulators naturally remove Se from the soil, many grow too slowly for effective phytoremediation. Transferring unique genes from Se hyperaccumulators to fast-growing, high-biomass plants using genetic engineering could create highly effective Se-phytoremediating plants (Terry *et al*. 2000).

Conclusions

The remediation of Se-contaminated water and soil remains a very real and difficult problem, requiring new and innovative approaches. Phytoremediation offers a promising solution. Genetic engineering, coupled with elucidation of the unique biochemical capabilities of Se-tolerant organisms, could greatly optimize this approach.

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HEAVY METAL REGULATION OF CYSTEINE BIOSYNTHESIS AND SULFUR METABOLISM RELATED TO STRESS IN *ARABIDOPSIS THALIANA* TRICHOMES

Cecilia Gotor, José R. Domínguez-Solís, Gloria Gutiérrez-Alcalá, M. Carmen López-Martín, Leticia Calo and Luis C. Romero

Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC-Universidad de Sevilla, Avenida Américo Vespucio s/n, 41092 Sevilla, Spain

Introduction

Cysteine biosynthesis is the final step in the sulfate assimilation pathway and is catalyzed by the *O*-acetylserine (thiol)lyase enzyme (OAS-TL). This enzyme incorporates sulfide into the *O*-acetylserine molecule, which is produced by an acetylation reaction of serine catalyzed by the serine acetyltransferase enzyme (SAT). Both OAS-TL and SAT activities have been demonstrated to be localized in three cellular compartments involved in protein synthesis, the cytosol, plastids and mitochondria (Hawkesford and Wray 2000; Saito 2000). However, the contribution of each isoform to a particular metabolic pathway is still under investigation.

Cysteine is the precursor molecule for the synthesis of glutathione (GSH), the predominant non-protein thiol in plants. The reduced form of glutathione is synthesized in two ATP-dependent steps: first, γ-glutamylcysteine (γ-EC) is synthesized from glutamate and cysteine, catalyzed by γ-glutamylcysteine synthetase (γ-ECS); second, glycine is added to the C-terminal end of γ-EC in a reaction catalyzed by glutathione synthetase (GS). Glutathione has important roles acting as a mobile pool of reduced sulfur, in the regulation of plant growth and development, and as antioxidant in stress responses (Noctor *et al.* 1998). GSH is also involved in the detoxification of xenobiotics and cytotoxins by targeting them into the vacuole. These compounds are conjugated with GSH by the action of glutathione *S*-transferase (GST) and are transported through the tonoplast by specific glutathione *S*-conjugate pumps (Rea *et al.* 1998).

Besides, glutathione is implicated in the plant responses to toxic levels of heavy metals, as it is the precursor for the synthesis of phytochelatins (PCs), the thiolate peptides involved in the detoxification of cadmium and some other heavy metals. PCs form a family of structures with increasing repetitions of the γ-EC dipeptide followed by a terminal Gly, and are synthesized from GSH in a reaction catalyzed

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by the PC synthase enzyme in response to heavy metal exposure. PCs bind toxic heavy metal ions through their thiol groups serving as ligands, and these complexes are transported into the vacuole by an ABC type transporter. In this way, phytochelatins remove the toxic metals from the cellular machinery (Cobbett 2000).

In previous work we isolated from *Arabidopsis*, the *Atcys-3A* gene that codes for the cytosolic isoform of *O*-acetylserine (thiol)lyase (Barroso *et al*. 1995). This gene is regulated by different abiotic stress conditions (Barroso *et al*. 1999), and is highly expressed in trichomes (Gotor *et al*. 1997). *Arabidopsis* trichomes are unicellular characteristic three-branched structures derived from the epidermal cell layer, with unknown function (Marks 1997). However, evidences are appearing of possible roles of trichomes in plant responses to various stress conditions. In this work, we further investigate our initial observations, mainly the heavy metal regulation of *Atcys-3A* gene and the physiological role of trichomes related to sulfur metabolism.

Heavy metal regulation of cysteine biosynthesis

When *Arabidopsis* plants are subjected to 50 μM $CdCl_2$, a 7-fold induction of *Atcys-3A* gene expression is observed in leaves 18h after treatment when compared with non-treated plants. This increase in transcript abundance is also detectable at the cellular level by *in situ* hybridization. OAS-TL enzyme activity also increases 2.5-fold with the same kinetic as the mRNA level increase (Domínguez-Solís *et al*. 2001). It is well established that cadmium induces the biosynthesis of phytochelatin peptides from GSH (Cobbett 2000), which needs cysteine as a precursor thiol molecule. In concordance, our results suggest that plants are responding to heavy metal exposure by inducing the cysteine biosynthesis rate. Upon cadmium treatment, GSH content increase is clearly observed with similar kinetic as described above, reaching the maximum level after 18 h with 50 μM $CdCl_2$ (Domínguez-Solís *et al*. 2001). An increased expression of genes involved in sulfur assimilation, glutathione and phytochelatin synthesis in response to cadmium treatment has been shown in *Arabidopsis* (Xiang and Oliver 1998) and other plant species, *e.g.* in *Brassica juncea* (Heiss *et al*. 1999). All these data indicate that plants respond to cadmium toxicity by inducing the genes responsible for cysteine, glutathione and phytochelatin synthesis.

To check the functional role of Atcys-3A protein in the mechanisms developed by plants against cadmium stress, we have overexpressed the gene in *Arabidopsis* plants. Full-length *Atcys-3A* cDNA was fused in sense orientation to cauliflower mosaic virus 35S promoter to obtain a strong and constitutive expression of the gene. This construction was introduced in a binary vector containing the kanamycin resistance marker, and the resulting plasmid was used to transform *Arabidopsis* plants *via Agrobacterium* using the infiltration method. Ten independent transformed lines in addition to several control lines obtained by overexpression of the GUS gene were used for further analysis. Analysis of the genomic DNAs of the different lines shows a great diversity in the number of insertions of the transgene. Several lines contain a unique insertion when compared with the wild type gDNA,

whereas others show up to 8 - 9 insertions. Northern blot analysis also shows divergent transcript abundance levels between the transformed lines compared with the control line. Some lines accumulate more *Atcys-3A* mRNA than the control line, *e.g.* up to 9-fold increase in the line 10-10, whereas the level of *Atcys-3A* expression in other lines is lower than the expression observed in the control line. Curiously, there is a correlation between the number of transgene insertions and the amount of transcript abundance. Thus, line 1-2 is the one with the largest number of insertions and shows the lowest level of gene expression, suggesting that a possible gene silencing mechanism may have occurred in this line.

We have performed a cadmium tolerance test of the different transformed lines, by germinating seeds on solid MS medium containing 250 μM $CdCl_2$ and let them grow for 15 days. Wild type seeds failed to germinate and the transformed lines with lower levels of *Atcys-3A* mRNA barely germinated and developed chlorotic leaves. On the contrary, the transformed lines with higher levels of transcript such as lines 1-3, 10-1, 10-2, 10-4 and 10-10, are able to germinate and produce green leaves (Fig. 1). Concomitant with these phenotypic observations, the Cd-resistant lines showed an increase in cadmium accumulation in leaves when compared with control lines (Domínguez-Solís *et al.* 2001). Our results suggest that increased cysteine availability is responsible for cadmium tolerance. By increasing the OAS-TL mRNA, the cysteine synthesis machinery within the transformed plants seems to be able to supply the required thiol precursor for PC synthesis (Cobbett 2000). However, we still do not have data about PC content in the transformed plants. Similar results have been obtained in transgenic tobacco plants expressing a rice OAS-TL gene, resulting in a high level production of sulfur-containing compounds that detoxify cadmium (Harada *et al*. 2001). In *B. juncea*, the limiting step for cadmium tolerance seems to be GSH biosynthesis instead of cysteine availability, since overexpression of γ-EC synthetase enhances the tolerance to a similar level we obtained in the present study (Zhu *et al*. 1999).

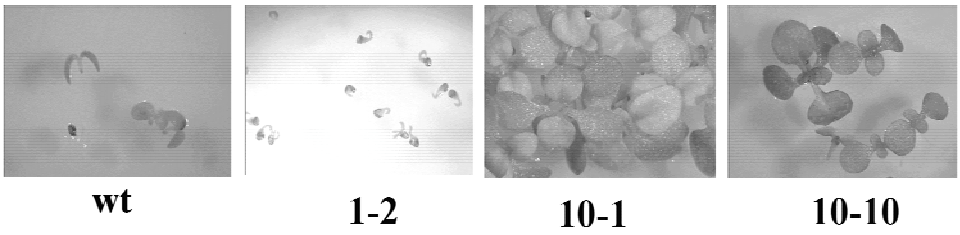

Fig. 1. Cadmium tolerance of the transgenic plants overexpressing the *Atcys-3A* gene. Seeds from wild type and the different transgenic lines were grown on solid MS medium containing 250 μM $CdCl_2$ for 15 days. Seedlings were visualized with an Olympus microscope.

Involvement of trichomes in detoxification processes

Previous studies with in other plant species suggested trichomes as a possible location of cadmium accumulation in leaves (Salt *et al.* 1995; Choi *et al* 2001). To investigate the involvement of *Arabidopsis* trichomes in cadmium detoxification, we have performed some nuclear microscopy techniques using the facilities of the National Center of Accelerators (Sevilla, Spain). Figure 2 shows typical results that we routinely obtain by the combination of secondary-electron (SEM), proton backscattering (RBS) and proton induced X-ray emission (PIXE) techniques on an *Arabidopsis* leaf. The PIXE technique allows the analysis of various elements above sodium in the periodic table with high sensitivity. Using this technique we can obtain distribution maps of the different elements, as it is shown for potassium and calcium. Calcium is highly abundant in the middle and upper parts of trichome cells while potassium is most abundant in the epidermal leaf surface (Fig. 2). Upon cadmium treatment, PIXE analysis shows a preferential accumulation of cadmium in a very restricted area of the trichome, underneath its branching point (data not shown). Similar distributions of zinc and cadmium inside the trichomes have been observed in the hyperaccumulator *Arabidopsis halleri* (Küpper *et al*. 2000).

We have collected PIXE spectra from trichomes and epidermal cells and quantified the concentrations of various elements in these cells from plants untreated or treated with cadmium. As shown in Table 1, the main point of Cd accumulation is inside the trichome, where the concentration is 10-fold higher that is found in the epidermis. Interesting, the concentrations of phosphorus and sulfur increase significantly in the trichome upon cadmium treatment (Table 1). Further work is needed to elucidate whether P and S are involved in the sequestration of Cd in the trichome. This result highlights the important physiological significance of trichome cells as their possible role as sinks during metal detoxification.

It is well established that cadmium detoxification involves the chelation of the heavy metal by a family of peptide ligands, the phytochelatins (Cobbett 2000). If *Arabidopsis* trichomes are involved in the plant responses to metal toxicity, a high capacity for the synthesis of the precursors thiol molecules, cysteine and glutathione, should be required in this cell type. In this sense, we have analyzed by *in situ* hybridization the expression of the genes involved in cysteine biosynthesis, *Atcys-3A* and *Sat-5*, which encode *O*-acetylserine (thiol)lyase and serine acetyltransferase, respectively (Barroso *et al*. 1995; Ruffet *et al*. 1995). We have also extended this study to the genes involved in GSH biosynthesis, *Gsh1* and *Gsh2*, which encode γ-EC synthetase and GSH synthetase, respectively (May and Leaver 1994; Ullmann *et al*. 1996). All four genes are highly expressed in leaf trichomes of *Arabidopsis* through the different stages of trichome development and their mRNA accumulate at high levels (Gutiérrez-Alcalá *et al*. 2000). These results suggest that cysteine and GSH biosynthesis pathways are highly active in this cell type. The reduction and assimilation of sulfate into cysteine has been assigned to photosynthetically active plastids, but in recent years it has become evident that nonphotosynthetic tissues contribute to the pathway (Hawkesford and Wray 2000; Saito 2000). Because trichomes do not contain green plastids, sulfate may be reduced to sulfide in non-green

plastids, or else intermediates in the pathway may be provided by other epidermal cells.

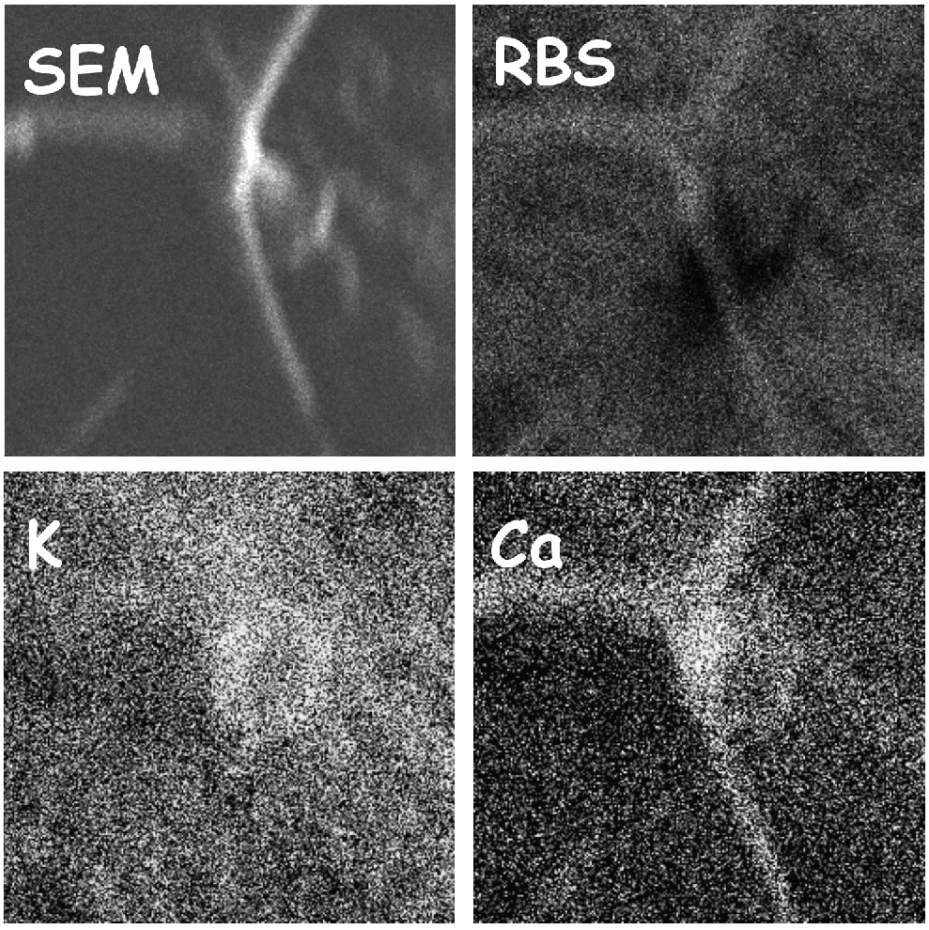

Fig. 2. Secondary-electron (SEM), proton backscattering (RBS), potassium (K) and calcium (Ca) distribution maps revealed by PIXE (proton induced X-ray emission) of a cadmium treated-*Arabidopsis* leaf surface with a emerging trichome. PIXE energy spectra from trichome and epidermal cells.

To address the question of GSH biosynthesis in *Arabidopsis* trichomes, we have measured the GSH content in the cytoplasm after conjugation with monochlorobimane (MCB) by confocal laser scanning microscopy, a technique that allows quantitative measurements in intact tissues. MCB is cell permeant and nonfluorescent until conjugated to thiol groups, becoming fluorescent and membrane impermeant. In plant tissues, this conjugation is enzymatically catalyzed by a glutathione *S*-

transferase, which confers specificity for GSH (Meyer *et al.* 2001). The formed conjugate is transported actively into the vacuole by a glutathione translocator of the ABC type. To prevent this transfer to the vacuole, and avoid a dilution of the fluorescent signal to the detection threshold of the technique, an inhibitor of ATP synthesis is included (Fricker *et al.* 2000). In trichomes, GSH-conjugate signal is localized in a thin layer of cytoplasm underneath the plant cell wall, in cytoplasmic strands crossing the vacuole and around the nucleus (Fig. 3). For comparison with other leaf cells, we have also detected the conjugate in the cells that are at the base of the trichome (basement cells) and in epidermal cells. In both cases, a fluorescent signal is observed in the cytoplasm below the plant cell wall (Fig. 3).

Table 1. Concentrations of various elements in trichome and epidermal cells untreated and treated with 2.5 mM $CdCl_2$, quantified by PIXE analysis. Means and standard deviations of five replicate experiments are shown. All values show significant differences ($p < 0.05$) from the sample without Cd treatment, except those indicate with the symbol *.

Element	Trichome (-Cd) Conc (ppm)	Trichome (+Cd) Conc (ppm)	Epidermis (-Cd) Conc (ppm)	Epidermis (+Cd) Conc (ppm)
P	9250 ± 735	43788 ± 411	6259 ± 168	4113 ± 167
S	5226 ± 352	21082 ± 216	7873 ± 59	13172 ± 233
Cl	35227 ± 418	32714 ± 200	267 ± 21	17613 ± 418
K	45712 ± 2357	95955 ± 480	42657 ± 168	46552 ± 1067
Ca	195204 ± 9979	100616 ± 557	21868 ± 700	12352 ± 237
Mn	959 ± 52	966 ± 20*	22 ± 10	41 ± 5
Fe	35 ± 19	84 ± 22	56 ± 6	16 ± 4
Zn	n.d.	207 ± 15	67 ± 9	68 ± 7*
Cd	n.d.	8691 ± 922	n.d.	862 ± 287

For comparison with other leaf cells, we have also detected the conjugate in the cells that are at the base of the trichome (basement cells) and in epidermal cells. In both cases, a fluorescent signal is observed in the cytoplasm below the plant cell wall (Fig. 3).

Appropriate calibration of the fluorescence intensity against standards allows us to quantify the concentration of cytosolic GSH under identical conditions. Considering that the volume of trichome cells is 200 fold-higher that of other epidermal cells, the total GSH content of trichomes is more than 300-fold higher than in other measured cells (Gutiérrez-Alcalá *et al*. 2000) being the concentration range from

100 to 800 µM. These studies demonstrate that the glutathione biosynthesis pathway is highly active in trichomes, indicating the physiological importance of this cell type, related to a specific function. Up to now, the unicellular trichomes in *Arabidopsis* have been considered dispensable because mutants lacking trichomes (*gl1* mutants) grow normally (Marks 1997). From our data, we suggest that trichomes likely function efficiently in the detoxification of xenobiotics *via* GSH conjugation and sequestration in the vacuole, and also in the detoxification of heavy metals as a site of metal accumulation. Considering the big size of the trichome cells, and that the vacuole is about 90 - 95 % of the total volume, each trichome has a substantial storage capacity of toxic molecules.

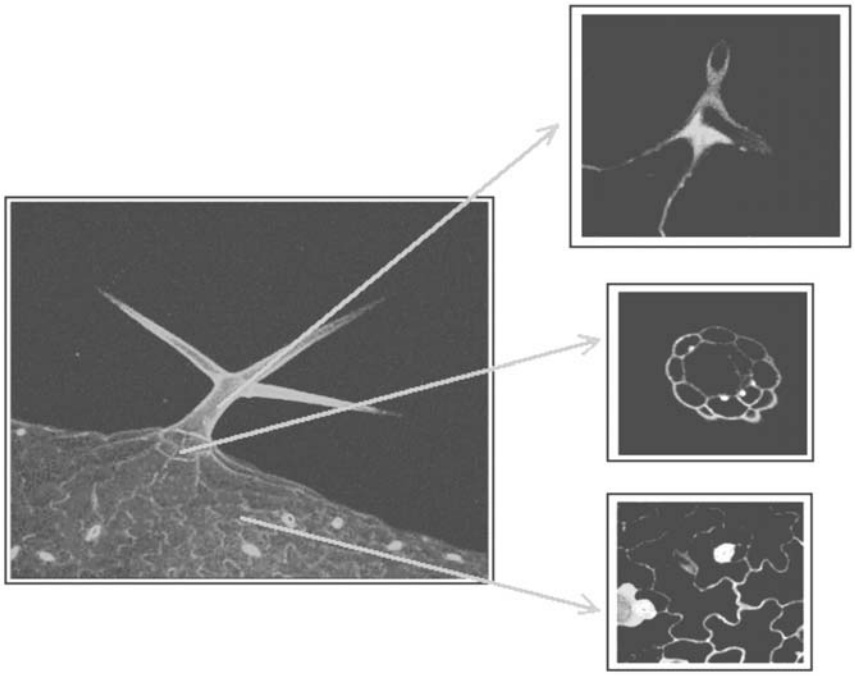

Fig. 3. Measurement of cytoplasmic GSH levels by confocal laser-scanning microscopy. GSH was visualized in trichome (upper), basement (middle) and epidermal (lower) cells of *Arabidopsis* after *in situ* conjugation to give a fluorescent conjugate.

Conclusions

Plants are exposed during their vital cycle to adverse environmental conditions that negatively affect growth, development or productivity. Among these conditions that cause plant damage is the presence of toxic compounds as heavy metals or herbicides. Plants develop defense mechanisms to deal with these adverse conditions and one of these responses involve the synthesis of thiol-containing molecules.

We have demonstrated in *Arabidopsis* that the plant responds to cadmium toxicity by inducing the synthesis of the thiol precursor molecule cysteine required for phytochelatins synthesis. It is well established that these peptides play an essential role in the detoxification of cadmium *via* a chelation process. By increasing the availability of cysteine, an enhanced cadmium tolerance is achieved due to a higher capacity for Cd accumulation in leaves. The main site of cadmium sequestration is a very restricted area of the *Arabidopsis* trichomes, where is located the vacuole. Considering that the volume of the trichome is 200-fold higher than any other epidermal cell and the vacuole occupies the 90 - 95 % of this volume, each trichome has a substantial storage capacity. *Arabiopsis* trichomes also functions efficiently in the detoxification of cytotoxins and xenobiotic compounds acting as a sink. Besides its considerable volume, this cell type has the highest GSH content of all epidermal cells. Our data highlights that the mechanism operating in trichomes is GSH conjugation of xenobiotics and sequestration of the conjugates into the vacuole.

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ROLES OF GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES IN REMEDIATION OF POLLUTED SOILS BY TRANSGENIC POPLARS

Tamás Kömives[1], Gabor Gullner[1] and Heinz Rennenberg[2]
[1]Plant Protection Institute, Hungarian Academy of Sciences, Herman Otto 15, H-1022 Budapest, Hungary; [2]Albert-Ludwigs-Universität Freiburg, Institut für Forstbotanik und Baumphysiologie, Georges-Köhler-Allee 53, D-79085 Freiburg, Germany

Introduction

Phytoremediation uses plants for cleaning up of environments contaminated with organic or inorganic pollutants by removing, sequestering, or chemically decomposing the pollutant (for recent reviews, see Kömives and Gullner 2000; Kramer and Chardonnens 2001; Salt *et al.* 1998).

Environmental fate of pollutants in the plant-soil system is determined by a highly complicated set of chemical, biochemical, physical and biophysical reactions, elements of which may play significant roles in determining the ultimate success of the solution for a particular pollution problem by phytoremediation (Kömives and Gullner 2000). Efficiency of phytoremediation depends on a) the ability of the plant to take up the contaminant without suffering toxic effects and b) the rate at which the contaminants are available for plant uptake (Kramer and Chardonnens 2001; Salt *et al.* 1998).

Considerable variation in ability to take up and tolerate environmental pollutants exists between plant species. Only those plants having the appropriate biochemical pathways are effective for phytoremediation purposes. In spite of numerous successful phytoremediation attempts involving both inorganic and organic pollutants, rates of de-pollution are often insufficient when the plants are chosen from wild species growing on polluted sites, or, alternatively, when crop plants are used, that are selected with specific characteristics determined by the particular environment and pollutant. Unfortunately, naturally selected wild biotypes usually produce little biomass and lack the agronomic technologies of production as available commercially crop genotypes were bred for traits markedly different from the needs of phytoremediation. However, remediative capacity of plants can be significantly increased by genetic manipulation: if it is known which pathway is involved in the uptake and detoxification of the pollutant, overexpression of the rate-limiting enzyme(s) may significantly improve the rate of the phytoremediation process. Therefore, genetic

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modification of high biomass plants, such as poplars (*Populus* spp.), provides a promising strategy for designing highly efficient phytoremediation technologies. (Doty *et al*. 2000; Gullner *et al*. 2001; Kramer and Chardonnens 2001; Li *et al*. 2001; Zhu *et al*. 1999).

Poplars in phytoremediation

Several lines of evidence indicate that poplar trees may be highly suitable for phytoremediation purposes. Their high biomass production, root depth, as well as their resistance to different types of environmental stress makes them good candidates for uptake and removal of organic and inorganic chemicals from polluted soils (Kömives and Gullner 2000). Poplars are known to take up and detoxify several inorganic and organic pollutants such as atrazine (Burken and Schnoor 1997), mercury (Rugh *et al*. 1998), selenium (Pilon-Smits *et al*. 1998), trinitrotoluene (Thompson *et al*. 1998) and trichloroethylene (Gordon *et al*. 1998).

Role of sulfhydryl groups in phytoremediation

The ability of sulfhydryl-containing compounds to protect cells against a wide range of chemicals is well established. Molecules containing two or more sterically close sulfhydryl groups are especially efficient chelators of several heavy metals. For example, the chemical dimercaptosuccinic acid and its vicinal dithiol structural analogues are used as antidotes in chronic and acute metal poisonings of humans to bind and to remove the toxicant (Lasky *et al*. 2001). In plants, the tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) and related sulfhydryl-containing endogenous compounds participate in numerous redox and nuclophilic substitution reactions (Noctor *et al*. 1998). Elevated GSH levels were found in plants exposed to a wide range of environmental stresses, chemicals and microbial infections (Kömives and Gullner 2000; Mauch and Dudler 1993). The increased production of GSH contributes to the antioxidative protection of plant cells against oxidative stress caused by various environmental factors (Noctor *et al*. 1998). GSH, as the metabolic precursor of the heavy metal chelating, thiol-rich oligopeptide phytochelatins (PCs), plays an important role in the heavy metal detoxification in plants (Cobbett 2000). Thus, overexpression of γ-glutamylcysteine synthetase (γ-ECS, the rate-limiting regulatory enzyme in the biosynthesis of GSH; Noctor *et al*. 1998) activity in Indian mustard plants resulted in improved phytochelatin biosynthesis and, hence, reduced sensitivity towards cadmium (Zhu *et al*. 1999). Transgenic poplars overexpressing γ-ECS exposed to cadmium contained higher levels of phytochelatins and accumulated higher amounts of cadmium in their roots than wild type plants (Rennenberg and Will 2000).

Thiol-rich proteins (metallothioneins, MTs) are also important biological chelators of metals (Salt *et al*. 1998; Li *et al*. 2001). Once a metal ion is bound with PCs and MTs, a stable complex is formed. Once complexed, there is a reduction in tox-

icity of the metal and it becomes unavailable (or less available) for binding to sulfhydryl group-containing essential biological components.

PCs are synthesized from GSH (Cobbett 2000) in an enzyme (phytochelatin synthase) catalyzed reaction powerfully up-regulated by traces of heavy metal ions in the cytosol. In addition, coordinated changes of gene expression occur for several sulfur assimilation enzymes in response to an increased demand for cysteine during PC synthesis (Cobbett 2000).

MTs, on the other hand, are gene-encoded, low-molecular-weight, cysteine-rich proteins. MTs are induced by copper and have high affinity for this metal. Transgenic plants, with increased ability to synthesize MTs were found to take up and tolerate higher concentrations of toxic metals (Li *et al*. 2001).

The redox state of the plant cell and the availability of reducing agents are important factors in cellular defense against chemical stress. The toxicity of such metals and metalloids as chromium, mercury, selenium and arsenic can be lessened in plants by chemical reduction of the element and/or by its incorporation into organic compounds (Bizily *et al*. 2003; de Sousa *et al*. 2002; Pilon-Smits *et al*. 1999). Some of the enzymes participating in the reduction require a low molecular weight thiol-organic cofactor (*e.g.* cysteine, 2-mercaptoethanol) for *in vitro* activity and may require sulfhydryl-bound substrates (Rugh *et al*. 1998).

Transgenic poplars with increased sulfhydryl content

In plant tissues, organic pollutants are transformed by a wide variety of chemical/biochemical metabolic reactions. Metabolism of a pollutant is involved in determining sensitivity/tolerance between plant species and has been found to play an essential role in the development of stress-resistant plants. Biotransformation reactions of xenobiotics are generally referred to as *Phases I* and *II*, where *Phase I* includes oxidation of xenobiotics and *Phase II* deals with the conjugation of *Phase I* products. In plants, oxidative metabolism in the *Phase I* system is usually mediated by cytochrome P-450 mixed function oxygenases. In the *Phase II* systems, activated hydrophobic xenobiotics are converted to more hydrophilic forms *via* conjugation with sugars or GSH (Kömives and Gullner 2000).

Poplar plants have an efficient *Phase II* detoxificiation system consisting of the endogenous cosubstrate, GSH (Noctor *et al*. 1988) and the enzyme system GSH *S*-transferase (GST, Gullner *et al*. 2001) that catalyzes the metabolism of potentially toxic electrophilic compounds *via* GSH conjugation (Eq. 1):

$$\mathrm{GSH + X\text{-}R \xrightarrow{GST} GS\text{-}R + X\text{-}H} \qquad \text{(Eq. 1)}$$

in which X-R is the toxicant (with X as the leaving group) and GS-R is the GSH conjugate. GSH conjugates are generally much less toxic and more water-soluble than the original electrophilic molecules (Kömives and Gullner 2000).

Recently poplar plants were transformed to overexpress bacterial γ-ECS (Noctor *et al*. 1998). The transformed poplars contain higher levels of GSH and its precursor γ-L-glutamyl-L-cysteine (γ-EC), than the wild type (Noctor *et al*. 1996), and show elevated phloem and xylem transport of these thiols as compared to wildtype plants (Herschbach *et al.* 1998). The transgenic poplar lines overexpressing γ-ECS activity and the wild type plants have the same growth rate and phenotypic appearance (Noctor *et al*. 1996, 1998), and foliar GST activity (Gullner *et al*. 2001).

Use of transgenic poplars in phytoremediation of chloroacetanilide herbicides

Chloroacetanilide herbicides are widely used for the control of annual grasses and broad-leaf weeds in a variety of major crops such as maize and soybeans. Contamination of some soils with these herbicides has become a serious environmental problem: acetochlor, alachlor and metolachlor (Table 1) are common contaminants in agricultural settings and at pesticide formulation and manufacturing sites. For example, in the soil of a 45-hectare sludge dump site, belonging to a herbicide producing chemical plant at Lake Balaton, Hungary, acetochlor and propachlor were detected at concentrations above the recommended field application rates (1-3 kg a.i. ha^{-1}).

Since conjugation with GSH or its homologue homoglutathione has long been established as the major metabolic reaction by which these herbicides are detoxified in higher plants (Jablonkai and Hatzios 1991), the use of wild type and transgenic poplar hybrids was considered when a phytoremediation project was planned to clean up this site.

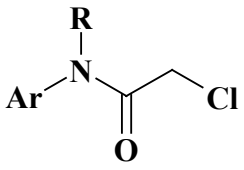
Little information is available about the phytoremediation of soils contaminated by chloroacetanilide herbicides (Kömives and Gullner 2000; Gullner *et al*. 2001). A general obstacle in phytoremediation was finding appropriate plant species that can tolerate greater concentrations of chloroacetanilides in soils.

Laboratory studies show that detached poplar leaves exposed to chloroacetanilide herbicides *via* uptake through the petiole respond with a rapid loss of chlorophyll, and young poplar trees respond with retarded shoot development and decreased root- and shoot fresh weights (Kömives *et al*. 2001; Gullner *et al*. 2001). Transgenic poplars overexpressing γ-ECS are significantly more tolerant against the herbicides than wild type plants (Gullner *et al*. 2001). Poplars detoxify chloroacetanilides *via* GSH-conjugation (Kömives *et al*. 2001). Therefore, it can be reasonably concluded that the elevated γ-EC and GSH level of transgenic poplar plants results in rapid herbicide degradation and, as a consequence, in elevated herbicide tolerance. In addition, the increased inducibility of foliar contents of these non-protein thiols as well as of GST by chloroacetanilide herbicides, may also contribute to the elevated herbicide tolerance of transgenic lines (Gullner *et al*. 2001).

The role of GSH in determining poplar tolerance against injury by chloroacetanilide herbicides was further supported by experiments with L-2-oxothiazolidine-4-carboxylic acid (OTC, a precursor of L-cysteine, Kömives *et al*. 2001). OTC increased GSH content in poplar (*Populux tremula* x *Populus alba*) leaves in a con-

centration-dependent manner: treatments with higher concentrations of OTC led to higher GSH levels in the leaves (Fig. 1). In addition, OTC (applied together with the herbicide or as a 24-h or 48-h pretreatment) protected poplar leaves against acetochlor injury (data not shown). Herbicide damage caused by acetochlor to poplar leaves pretreated with different concentrations of OTC for 48 h correlated closely and negatively with the GSH levels in OTC-treated plants ($r = -0.889$, significant at the $p < 0.05$ level).

Table 1. Chemical structures of the chloroacetanilide herbicides studied their phytotoxicity to *Populus nigra* leaves (ED_{50}), and their ability to induce GST activities.



Compound	**Ar**	**R**	ED_{50}[1] μM	GST activity[2] Control%
Acetochlor	2-methyl-6-ethylphenyl	ethoxymethyl	85 ± 21	241 ± 33
Alachlor	2,6-diethylphenyl	methoxymethyl	144 ± 16	201 ± 39
Butachlor	2,6-diethylphenyl	butoxymethyl	not applicable	139 ± 12
Dimethenamid	2,4-dimethyl-3-thienyl	2-methoxy-1-methylethyl	109 ± 19	188 ± 28
Metazachlor	2,6-dimethylphenyl	1-pyrazolylmethyl	105 ± 22	215 ± 36
Metolachlor	2,6-diethylphenyl	2-methoxy-1-methylethyl	138 ± 7	274 ± 44
Propachlor	Phenyl	2-propyl	426 ± 95	115 ± 17

[1]Based on loss of chlorophyll content (determined after 96 h); [2]Determined after a 72-h exposure to herbicides at 50 μM (butachlor was applied as a saturated solution), and by using CDNB as substrate. GST activity in the control leaves was 210 ± 27 μmol g FW^{-1} min^{-1}.

Based on the findings of the laboratory studies, a 10-year phytoremediation project has been launched at the Lake Balaton site heavily polluted with chloroacetanilide herbicides. In November 2001, poplar trees belonging to 11 hybrids (100 young trees of each) were planted at the contaminated site. During the project, soil and groundwater pollution will be continuously monitored, and the effects of OTC, as well as the phytoremediation potency of genetically modified poplars with enhanced GSH synthesis activity (Noctor *et al.* 1998) under field conditions, will be investigated.

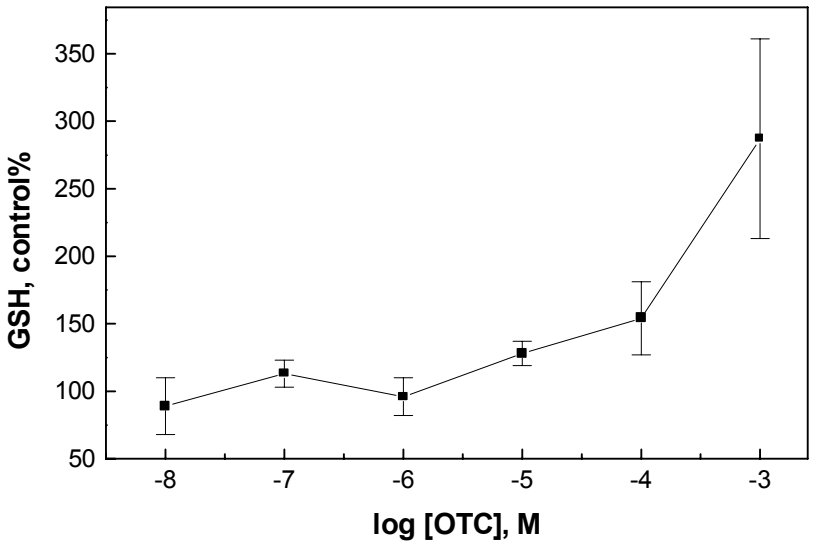


Fig. 1. Effects of L-2-oxothiazolidine-4-carboxylic acid (OTC, 48 h exposure) on the level of glutathione (GSH) in poplar leaves (GSH content in untreated control leaves was 214 ± 29 nmol g fresh weight^{-1}).

Future perspectives

The full potential of the GSH-based *Phase II* plant detoxification system can be exploited only, if the uptake (*Phase I*) and disposal processes (*Phase III*) are efficient. For example, transgenic plants with increased ability to secrete metal-chelating products are more efficient in taking up and accumulating of metals in the aerial tissues (Tesfaye *et al*. 2001). In addition, in transgenic tobacco plants a calmodulin-binding protein (involved in metal uptake across the plasma membrane) has been found to modulate tolerance and accumulation of lead (Arazi *et al*. 1999), and an *Arabidopsis* blue copper-binding protein was confirmed to ameliorate Al toxicity (Ezaki *et al*. 2001).

Cytochrome P-450 enzymes are of crucial importance to bioactivate recalcitrant hydrophobic pollutants into chemically reactive electrophilic compounds that readily form conjugates with GSH. Engineering of transgenic plants expressing certain cytochrome P-450 isoenzymes is also important for crop breeding with useful traits including phytoremediation (Doty *et al*. 2000; Inui *et al.* 2001).

Plants lack the excretion systems of animals. In plant cells, toxic metabolites and pollutants are sequestered into the vacuole. This *Phase III* type process is an active one and is catalyzed by membrane-bound ATP-driven pumps. Recent studies have shown the existence of a *Phase III* system that is involved in the elimination of potentially toxic GSH conjugates from the cytosol (Klein *et al*. 2000). In addition,

tobacco plants expressing an *Arabidopsis* calcium exchanger antiporter, accumulated more Ca^{2+}, Cd^{2+}, and Mn^{2+} and were more tolerant to elevated Mn^{2+} levels (Hirschi *et al*. 2000).

Conclusions

Phytoremediation research using poplar trees has made significant strides in recent years by taking advantage of the spectacular developments in molecular biology and genetic engineering. As a result, our knowledge of the factors that determine the efficacy of phytoremediation has expanded greatly. It became evident that pollutant phytotoxicity is determined by a highly complicated sequence of events, elements of which may play a significant role in promoting or antagonizing plant tissue damage, depending on the plant-pollutant system. Phytoremediation efficiency seems to be strongly influenced by the ability of the plant to escape deleterious concentrations of the toxic form of the pollutant and the active oxygen species that might be generated in the treated tissue. The key role of the cytochrome P-450 monooxygenase and GSH-related detoxification system in the biotransformation of organic pollutants in some tolerant plants, and the importance of the antioxidant systems to counteract peroxidative damage, has been clearly established. However, much is yet to be learned about these systems in poplar, especially, with respect to their specificity and their mechanisms of induction. *In vivo* and *in vitro* studies to follow pollutant-induced changes in poplar biochemistry, biophysics and molecular biology provide us with intriguing challenges for further research. Attention is now being focused on the development and use of transgenic poplar plants specifically tailored for the bioremediation of organic pollutants and heavy metals. Given the existing advances in plant molecular biology, the use of transgenic poplars in the daily practice of phytoremediation could soon become a reality.

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SULFUR NUTRITION OF DECIDUOUS TREES AT DIFFERENT ENVIRONMENTAL GROWTH CONDITIONS

Cornelia Herschbach
Albert-Ludwigs-University Freiburg, Institute of Forest Botany and Tree Physiology, Chair of Tree Physiology, Georges-Köhler-Allee 053/054, 79085 Freiburg i.B., Germany

Introduction

In comparison to herbaceous plants, the sulfur nutrition of deciduous trees exhibits some peculiarities. These originate from the special growth characteristics of perennial plants, the long distance between the shoot and the roots, as well as the long-life span of deciduous trees. Similar to herbaceous plants, deciduous trees take up sulfate from the soil solution by the roots and load the sulfate taken up into the xylem (Herschbach and Rennenberg 2001b). In the xylem, sulfate and also reduced sulfur, are transported in the transpiration stream into the shoot. There, the sulfur must be taken up into the leaves. The sulfate in the leaves is either stored in the vacuole (Bell *et al.* 1995a,b) or reduced to sulfide in the chloroplast (Brunold 1990). The reduced sulfur may then be used for protein synthesis. A surplus of oxidized and reduced sulfur may be exported out of the leaves to the organs which are sinks for sulfur. Although APS reductase was found to be active in the tissues along the trunk (Herschbach 2003), these are sinks for reduced sulfur for sulfur storage in the parenchyma cells of the wood and the bark (Herschbach and Rennenberg 2001b). Reduced sulfur and sulfate is transported in the phloem up to the roots. As proposed for herbaceous plants, the sulfur allocation in the phloem from the shoot to the roots may signal the sulfur state of the tree (Herschbach *et al.* 2000). The demand driven control model of sulfur nutrition proposed GSH as the signal from the shoot to the roots not only to regulate sulfate uptake, but also to regulate sulfate reduction in the roots (Lappartient and Touraine 1996; Lappartient *et al.* 1999). However, in deciduous trees the APS reductase activity in lateral roots does not exceed ca. 20% of the activity determined in leaves (Herschbach 2003). Since a phloem-to-xylem exchange of reduced sulfur seems not to proceed when trunk tissues of deciduous trees are sinks for sulfur, reduced sulfur in the xylem must originate from the root's sulfate assimilation (Herschbach and Rennenberg 2001a,b). Except during the spring, when sulfur from storage tissues of the trunk is mobilized, sulfur loading into the xylem along the entire trunk is an important process for the sulfur supply to the

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shoot (Herschbach and Rennenberg 1996, 2001b). These findings led to the assumption that the contribution of the roots sulfate assimilation to the sulfur budget of the whole tree cannot be disregarded.

As a consequence of the long-life span trees must adapt not only carbon and nitrogen nutrition, but also sulfur nutrition to a changing environment. In most cases a combination of several environmental factors may influence the nutrition of trees. The present review provides an overview of environmental effects on sulfur nutrition of deciduous trees and relates these findings to the demand driven control model.

Effects of sulfur availability and water supply

The most important environmental factors of the pedosphere on growth and development of plants are the general nutritional state as well as the water content of the soil. Short-term deprivation of sulfate led to reduced sulfate contents in leaves and fine roots of non-mycorrhizal beech grown in hydroponic culture solution (Kreuzwieser *et al.* 1997). Reduced sulfur contents, however, were not affected during three days of sulfur deprivation (Table 1). The sulfate uptake rate increased, whereas xylem loading of sulfate remained unchanged with standard nitrogen nutrition (Kreuzwieser *et al.* 1996). Additional sulfate supply led to increased Cys and GSH contents of the leaves, whereas they remained unaffected in other tissues (Kreuzwieser *et al.* 1997). Both uptake and xylem loading of sulfate were reduced under these conditions (Kreuzwieser *et al.* 1996). If the demand driven control model of sulfur nutrition was applied, it seems impossible that GSH is the signal to reduce sulfate uptake and xylem loading of sulfate under these conditions, because GSH contents in fine roots remained unchanged. This view is supported by the finding that in excised beech roots GSH did not influence sulfate uptake, and even increased xylem loading of sulfate (Kreuzwieser *et al.* 1996). If beech roots were associated with mycorrhiza, then the observed effects of sulfur deficiency disappeared (Table 3). Obviously, mycorrhization has a compensatory effect on the sulfur nutrition of beech. Sulfate uptake and xylem loading of sulfate in beech also changed with nitrogen nutrition and especially depended on the nitrogen form supplied (Kreuzwieser *et al.* 1996). For example, at enhanced nitrogen fertilization sulfate uptake increased, but not xylem loading of sulfate (Table 1). A well balanced sulfur state was observed in poplar under long-term enhanced sulfur fertilization (Herschbach and Rennenberg 2001a). Only in the xylem sap were GSH and sulfate contents enhanced, although the total reduced sulfur content was unaffected (Table 1). Even if sulfate uptake was enhanced under these conditions, the sulfur nutrition of the whole plant seemed to be well adjusted to the demand.

The effect of drought on sulfur nutrition was investigated in the green summer *Q. robur*. In non-mycorrhizal pedunculate oak trees, export of sulfur out of mature leaves completely disappeared at high negative leaf water potential (Schulte *et al.* 1998). It may be expected that under these conditions, phloem transport and therefore sulfur transport to the roots ceased. However, GSH contents in fine roots were

unaffected (Schulte 1998) and, in agreement to the demand driven control model sulfate uptake remained unchanged (Seegmüller 1998). In addition, xylem loading of sulfate was diminished, probably due to a disturbed water transport at the soil-root interface. Apparently, an interrupted nutrient exchange between the shoot and the roots seemed to be the most important effect on sulfur nutrition of *Q. robur* exposed to drought.

Table 1. Effects of pedospheric environmental factors on sulfur nutrition of non-mycorrhizal deciduous trees.

	Nutrition				Water content	
	Short-term sulfate depletion	Short-term enhanced sulfate fertilization	Long-term enhanced sulfate fertilization	Enhanced nitrogen supply	Drought	Flooding
Tree species	Beech	Beech	Poplar	Beech	*Q. robur*	Poplar
GSH (leaves)	=[2]	↑[2]	=[4]	n.d.	↓[6]	=[9]
Cys (leaves)	=[2]	↑[2]	=[4]	n.d.	n.d.	↑[9]
APS reductase (leaves)	n.d.	n.d.	n.d.	n.d.	n.d.	=[9]
Export out of mature leaves	n.d.	n.d.	n.d.	n.d.	↓[8]	n.d.
GSH (phloem)	=/↓[3]	=/↑[3]	=[4]	=[5]	n.d.	↑[9]
SO_4^{2-} (phloem)	n.d.	n.d.	=[4]	=[5]	n.d.	↑[9]
GSH (fine roots)	=[2]	=[2]	=[4]	n.d.	=[6]	=[9]
Cys (fine roots)	=[2]	=[2]	=[4]	n.d.	n.d.	↑[9]
APS reductase (fine roots)	n.d.	n.d.	n.d.	n.d.	n.d.	↓[9]
SO_4^{2-} uptake	↑[1]	↓[1]	n.d.	↑[1]	=[7]	n.d.
Xylem loading of SO_4^{2-}	=[1]	↓[1]	n.d.	=[1]	↓[7]	n.d.
GSH (xylem)	n.d.	n.d.	↑[4]	n.d.	n.d.	n.d.
SO_4^{2-} (xylem)	n.d.	n.d.	↑[4]	n.d.	n.d.	n.d.

[1]Kreuzwieser *et al.* 1996; [2]Kreuzwieser *et al.* 1997; [3]Kreuzwieser 1997; [4]Herschbach and Rennenberg 2001a; [5]Schraml *et al.* 2002; [6]Schulte 1998; [7]Seegmüller 1998; [8]Schulte *et al.* 1998; [9]Herschbach 2003; n.d., not determined.

The contrasting environmental scenario to drought is flooding. Under these conditions the GSH content and the activity of APS reductase in mature leaves was not affected, whereas the GSH content in phloem exudates increased and the activity as well as the mRNA of the APS reductase in fine roots completely disappeared (Herschbach 2003). Since Cys but not GSH increased in fine roots, GSH seems not to be the signal regulating sulfate reduction in fine roots during flooding. During flooding primary metabolism of the roots changed from aerobic to anaerobic dis-

similation processes and, consequently, ATP production per mol of glucose decreased (Brändle 1996). Therefore, it cannot be excluded that Cys from protein break-down is involved in the regulation of APS reductase (Bick *et al.* 2001). On the other hand, a reduced ATP availability may also be responsible for the knockout of the APS reductase activity under these conditions.

Effects of air pollution

Sulfur containing air pollutants are taken up by plants (De Kok 1990, Rennenberg and Herschbach 1996) and can be used as sulfur sources (De Kok *et al.* 1997, 2000; Westerman *et al.* 2000, 2001). Regulation of sulfur nutrition in the presence of atmospheric H_2S was investigated in poplar (Herschbach *et al.* 2000). As in herbaceous plants, GSH increased in leaves, but also in phloem exudates and in fine roots (Table 2), but in poplar sulfate uptake remained unaffected. According to the demand driven control model, xylem loading of sulfate decreased, although neither sulfate nor reduced sulfur in the xylem changed. Because transpiration was not influenced by H_2S exposure (Herschbach *et al.* 2000) the sulfate content in the xylem was probably refilled by phloem-to-xylem exchange of sulfate which was demonstrated for poplar (Hartmann *et al.* 2000).

Table 2. Effects of atmospheric environmental factors on sulfur nutrition of deciduous trees.

	H_2S exposure	Short-term elevated pCO_2	Short-term elevated pCO_2	Lifelong elevated pCO_2
Tree species	Poplar (non-mycorrhizal)	*Q. robur* (non-mycorrhizal)	*Q. ilex* (mycorrhizal)	*Q. ilex* (mycorrhizal)
GSH (leaves)	↑[1]	=[4]	↓[5]	↓[5]
Cys (leaves)	↑[1]	n.d.	↓[5]	↓[5]
APS reductase (leaves)	n.d.	n.d.	↓[5]	=[5]
Export out of mature leaves	n.d.	↑[2]	n.d.	n.d.
GSH (phloem)	↑[1]	n.d.	↓[5]	↓[5]
SO_4^{2-} (phloem)	=[1]	n.d.	n.d.	n.d.
GSH (fine roots)	↑[1]	=[4]	=[5]	↑[5]
Cys (fine roots)	=[1]	n.d.	=[5]	=[5]
APS reductase (fine roots)	n.d.	n.d.	↓[5]	=[5]
SO_4^{2-} uptake	=[1]	=[3]	n.d.	n.d.
Xylem loading of SO_4^{2-}	↓[1]	=[3]	n.d.	n.d.
GSH (xylem)	=[1]	n.d.	n.d.	n.d.
SO_4^{2-} (xylem)	=[1]	n.d.	n.d.	n.d.

[1]Herschbach *et al.* 2000; [2]Schulte *et al.* 1998; [3]Seegmüller *et al.* 1996; [4]Schulte 1998; [5]Schulte *et al.* 2002; n.d., not determined.

With respect to the global CO_2 enrichment of the atmosphere, changes in tree biomass have frequently been discussed (*c.f.* Saxe *et al.* 1998; *c.f.* Norby *et al.* 1999). An enhanced biomass production can only be achieved if all nutrients are present in sufficient amounts. This question has been mainly investigated and discussed for nitrogen (*c.f.* Stitt and Krapp 1999). An enhanced biomass production, however, needs enhanced protein synthesis which not only depends on nitrogen, but also on sulfur nutrition. For the effects of elevated pCO_2 on regulatory mechanisms it is important to distinguish between short-term exposure to elevated pCO_2 which allows immediate metabolic adaptation and life-long elevated pCO_2 which allows genetic adaptation. Short-term exposure to elevated pCO_2 of non-mycorrhizal *Q. robur* trees enhanced sulfur export out of mature leaves (Schulte *et al.* 1998), but the GSH content of the leaves was unaffected (Schulte 1998). The higher sulfur export did not result in a higher GSH content of fine roots (Schulte 1998) and sulfate uptake as well as xylem loading of sulfate remained unchanged (Seegmüller *et al.* 1996, Table 2). In another study, short-term elevated pCO_2 caused several changes in mycorrhizal *Q. ilex* at comparable growth conditions (Schulte *et al.* 2002). Reduced sulfur decreased, probably due to the diminished APS reductase activity in the leaves, which resulted in a diminished reduced sulfur content of phloem exudates (Table 2). In fine roots, reduced sulfur contents were not changed, but APS reductase activity also decreased. Under these conditions, reduced sulfur contents did not correlate with APS reductase activity. Because reduced nitrogen decreased simultaneously, it may be assumed that a reduced precursor availability, probably OAS for Cys synthesis, may be responsible for the reduction in APS reductase activity (Hawkesford and Wray 2000). Nevertheless, biomass production of the shoot and the roots were enhanced under these conditions (Polle *et al.* 2001). Life-long exposure to elevated pCO_2 however, recovered the APS reductase activity to the level of control oaks at ambient pCO_2, although reduced sulfur contents in leaves and phloem exudates remained diminished. Apparently, the activity of the APS reductase adapts to life-long elevated pCO_2. It can not be excluded that the enhanced reduced nitrogen content in the leaves under life-long elevated pCO_2, also triggers the higher APS reductase activity.

Effects of mycorrhization in combination with sulfur nutrition, water supply and elevated pCO_2

In the field, the roots of deciduous trees are always inhabited by fungi in a symbiosis called mycorrhiza. Nutrient and water availability can be improved by this symbiosis due to the formation of hyphae which tap new sources from a greater soil area (*c.f.* Smith and Read 1997). In particular nitrogen and phosphorus is affected (*c.f.* Smith and Read 1997), but little is known for sulfur. The influence of mycorrhiza on sulfur nutrition of trees was investigated with beech and *Q. robur*. Mycorrhization of beech did not effect the sulfur nutrition of beech at normal sulfur fertilization (Table 3), but several changes were found in *Q. robur*. Sulfur export out of mature leaves decreased (Schulte *et al.* 1998), but GSH in phloem exudates increased (Ren-

nenberg 1999). A higher sulfate and GSH content in the xylem was observed (Rennenberg 1999) that may originate from a higher xylem loading (Seegmüller *et al.* 1996). Apparently, mycorrhization improved the cycling of sulfur in *Q. robur* without effects on the reduced sulfur content in leaf and root tissues (Schulte 1998). The higher GSH content in phloem exudates connected with higher xylem loading of sulfate and unchanged sulfate uptake, contradicts the demand driven control model.

Table 3. Effects of mycorrhization on the sulfur nutrition of deciduous trees at different environmental growth conditions.

	Normal sulfate fertilization	Short-term deprivation of sulfur	Short-term enhanced sulfate supply	Ambient pCO_2	Ambient pCO_2 and drought	Elevated pCO_2
Tree species	Beech	Beech	Beech	*Q. robur*	*Q. robur*	*Q. robur*
GSH (leaves)	=[3]	=[3]	↑[3]	=[7]	=[7]	=[7]
Cys (leaves)	=[3]	=[3]	↑[3]	n.d.	n.d.	n.d.
APS reductase (leaves)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Export out of mature leaves	n.d.	n.d.	n.d.	↓[5]	↓[5]	=[5]
GSH (phloem)	=[4]	=/↓[4]	=/↑[4]	↑[8]	n.d.	n.d.
SO_4^{2-} (phloem)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GSH (fine roots)	=[3]	=[3]	=[3]	=[7]	=[7]	=[7]
Cys (fine roots)	=[3]	=[3]	=[3]	n.d.	n.d.	n.d.
APS reductase (fine roots)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SO_4^{2-} uptake	=[1,2]	=[1,2]	=[1,2]	=[6]	=[9]	=[6]
Xylem loading of SO_4^{2-}	=[1,2]	=[1,2,4]	↑[1,2,4]	↑[6]	↓[9]	↑[6]
GSH (xylem)	n.d.	n.d.	n.d.	↑[8]	n.d.	n.d.
SO_4^{2-} (xylem)	n.d.	n.d.	n.d.	↑[8]	n.d.	n.d.

[1]Kreuzwieser *et al.* 1996; [2]Kreuzwieser and Rennenberg 1998; [3]Kreuzwieser *et al.* 1997; [4]Kreuzwieser 1997; [5]Schulte *et al.* 1998; [6]Seegmüller *et al.* 1996; [7]Schulte 1998; [8]Rennenberg 1999; [9]Seegmüller 1998

Although sulfate uptake did not change in beech (Morrison 1963) or oak (Seegmüller *et al.* 1996), the kinetic characteristics of the low affinity uptake system changed due to mycorrhization in beech (Kreuzwieser *et al.* 1996; Kreuzwieser and Rennenberg 1998). The K_M increased and V_{max} decreased. Apparently, mycorrhization led to a down-regulation of sulfate uptake at high sulfate supply and may improve sulfate uptake at low sulfate supply in beech. This was demonstrated after short-term sulfur depriviation. In contrast to non-mycorrhizal beech roots (Kreuzwieser *et al.* 1996), mycorrhizal beech roots showed no effects on sulfur nutrition due to sulfate deprivation (Kreuzwieser and Rennenberg 1998). Mycorrhization however, prevented the reduction of sulfate uptake and even increased xylem loading of

sulfate of beech roots at enhanced sulfate fertilization (Table 1, 3). Obviously, mycorrhization affects sulfate uptake in a compensating way and interacts with the regulation of sulfate uptake.

During strong drought stress, mycorrhization was not able to prevent the reduction in sulfur export out of mature leaves or the reduction in xylem loading of *Q. robur* (Schulte *et al.* 1998). If drought was applied to the seedlings at elevated pCO_2, drought effects on sulfur nutrition were no longer observed (Table 1,3). Apparently, elevated pCO_2 counteracts drought effects on sulfur nutrition. Elevated pCO_2 also prevented the increase in sulfur export out of mature leaves mediated by mycorrhization of *Q. robur* roots, but enhanced xylem loading of sulfate was still observed (Table 2, 3). Since the latter was also found at ambient pCO_2 this effect may be attributed to the mycorrhizal symbiosis.

Conclusions

It is evident from the presented data that sulfur nutrition of deciduous trees can be influenced in different ways due to environmental factors. The changes observed were not always coherent with the demand driven control model of sulfur nutrition with GSH acting as the signal. Hence, other specific physiological changes at the whole plant level must be considered in relation to environmental factors.

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IMPACT OF VOLATILE SULFUR COMPOUNDS ON WINE QUALITY

Doris Rauhut
State Research Institute Geisenheim, Department of Microbiology and Biochemistry,Von-Lade-Strasse 1, D-65366 Geisenheim, Germany.

Introduction

Volatile sulfur(S)-containing compounds have a significant role in the flavor of wines. This is related to their high volatility, reactivity and potency at very low concentrations. Some of the S substances are necessary for the wine quality, while others are the cause of strong objectionable flavors (rotten eggs, cooked cabbage, cauliflower, burnt rubber etc.), even at extremely low concentrations (*e.g.* H_2S, methanethiol (MeSH), ethanethiol (EtSH)). Certain thiols contribute to the typical sensory impression of grape varieties like Chenin blanc, Sauvignon blanc, Scheurebe etc. (Augustyn *et al.* 1982; Darriet and Dubourdieu 1992; Darriet *et al.* 1991, 1993; Marais 1994; Murat *et al.* 2001a,b). In wines produced from these varieties very varied aromatic nuances are noticeable (blackcurrant, passion fruit, boxtree, grapefruit etc.).

For a long time, it is known that the formation of volatile S compounds is affected by the organic and inorganic S-containing substances and pesticides in grapes and musts. Other factors are the nutrient level in grapes and musts and the yeast metabolism during fermentation (Glowacz *et al.* 1999; Henschke and Jiranek 1991, 1993; Jiranek *et al.* 1995a,b; Rauhut 1993, 1996; Rauhut and Kürbel 1996; Rauhut *et al.* 1996, 1997a,b, 1998a,b, 1999a,b,c, 2000a,b; Rankine 1963, 1964, 1968; Vos and Gray 1979). An overview of factors that influence the formation of off-flavors caused by S compounds is given in Fig. 1 (Bernath 1997; Keck 1989; Lavigne-Cruége 1996; Maujean 1989, 2001; Rapp *et al.* 1984, 1985; Schütz and Kunkee 1977; Wenzel and Dittrich 1978; Wenzel *et al.*1980).

Over the last twenty years, due to the progress in the instrumental techniques for analyzing aroma compounds like mild concentration techniques and highly sensitive and selective methods, it has become possible to identify, detect and quantify the odor-active S compounds in traces in must and wine (Baumes *et al.* 1986; Chatonnet 1993; Chatonnet *et al.* 1992; Drawert and Rapp 1968; Peppard 1988; Rauhut 1996; Rauhut *et al.* 1998b). Afterwards an extensive research followed to enlighten the formation of S substances during the wine making process and to get more knowledge of their sensory properties and their contribution to wine quality.

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In the following a survey is given of the formation and importance of volatile S compounds to the aroma of wine.

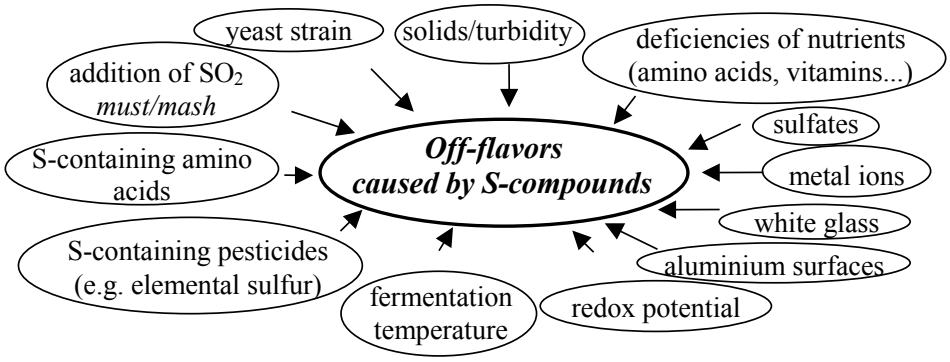

Fig. 1. Influence of different factors on the formation of off-flavors caused by S compounds in wine.

Influence of S-containing pesticide residues on the occurrence of off-flavors in wine

Sulfur residues in the must, which originate from the spraying of sulfur as fungicide, can be reduced during the fermentation by yeast metabolism to H_2S. H_2S concentrations exceeding the threshold value cause an undesirable off-flavor in wine that is reminiscent of rotten eggs (Wenzel and Dittrich 1978; Wenzel *et al.* 1980).

The development of sulfurous off-flavors in bottled wine from residues of acephate, the active ingredient of an insecticide, has resulted in severe economic loss in some of the Western European wine making countries since the end of the 1970s. Acephate (*O*,*S*-dimethyl-N-acetyl-amido-thiophosphate) is hydrolyzed *via* S-containing intermediates to methanethiol and additional metabolites. These reactions depend upon the concentration of residue, wine pH and the time and temperature of wine storage. Methanethiol and its oxidation product, dimethyl disulfide, have an unpleasant smell and are responsible for the sulfurous off-flavor caused by acephate. Therefore insecticides with the active ingredient acephate are no longer recommended in wine growing regions (Rauhut *et al.* 1986; Rauhut 1990, 1996; Maujean 2001). Cabras *et al.* (1987) and Casanova and Guichon (1988) demonstrated that CS_2 is a degradation product of ethylenbisdithiocarbamates, a common fungicide used in vineyards.

Influence of assimilable nitrogen and other nutrients in grape musts and yeast S metabolism on the formation of disagreeable volatile S compounds in wine

In northern, cool climate wine growing regions the amino acid content in grapes or must was not restricted or a critical factor in the wine producing process up to now. Since the mid-1980s, there has been noticed that in musts of grapes from vineyards with a normally sufficient N-fertilization the free assimilable nitrogen (N) for yeasts is often only half of the amount, which was found in musts during the mid-1970s. At the same time an increase in the occurrence of disagreeable S compounds, off-flavors in wine and problems in the course of fermentation was observed. A decrease in N-fertilization and a changing in the soil management system (*e. g.* green cover vineyards) are the main causes for a deficiency of assimilable nitrogen in the grapes. Further effects are low rainfall during the vegetation period, amount of storage products, vine distance, vintage, and time of vintage (Löhnertz 1988; Löhnertz *et al.* 2000; Prior 1997).

The majority of all volatile S substances in wine is produced during the alcoholic fermentation by the wine yeast (*Saccharomyces cerevisiae*). An accelerated H_2S production during fermentation leads to a higher formation of other volatile S compounds. Normally, H_2S is produced from inorganic S sources (mainly sulfate) in the grape must by the sulfate reduction sequence in yeasts to provide a reduced S precursor for the biosynthesis of S-containing amino acids. Consequently, its information in wine is related to both, S and N metabolism by yeasts. Under nutritionally sufficient conditions, H_2S is normally matched to biosynthetic demand through tight metabolic regulation. A nitrogen deficiency leads to an overproduction of H_2S and other disagreeable volatile S compounds. The intensity and the sensory impression of the resulting off-flavors depend on the qualitative and quantitative composition of the volatile S substances in the wines. The formation of S compounds is also influenced by different requirements of commercial yeast strains for certain amino acids and their capability to produce aroma-active S substances. It could be demonstrated that yeast strains differ in their formation of S compounds (Fig. 2; Rauhut and Kürbel 1994; Rauhut *et al.* 1995, 2000a,b).

Reoccurrence of off-flavors after filling and storage of wine

A reoccurrence of off-flavors in wines during storage after treatment and bottling is related to a release of unpleasant volatile compounds from non-volatile or volatile precursors like the hydrolysis of thioacetic acid esters to thiols and acetic acid (Fig. 3). This depends on the chemical equilibrium. Therefore an off-flavor can increase or reoccur. In comparison to the thioacetic acid esters, the mercaptanes have very low threshold values (> 40 mg l^{-1} and < 2 µg l^{-1}, respectively).

Consequently traces of the mercaptanes are sufficient to induce a sulfur off-flavor. In the presence of oxygen, the mercaptanes can be oxidized to disulfides, which are also odor active S compounds. Moreover, disulfides are not removable by a copper sulfate treatment. High amounts of thioacetic acid esters can be seen as an

indicator for a possible reoccurrence of the aroma defect after a copper fining. A treatment with copper sulfate has no influence on the concentration of the thioacetic esters, because copper ions mainly react with H_2S and thiols (Rauhut 1996; Rauhut *et al.* 1996; Tanner 1969).

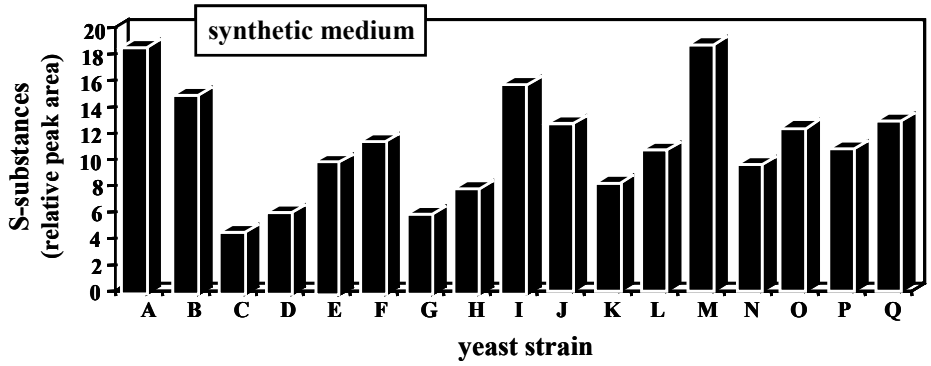

Fig. 2. Influence of the yeast strain on the formation of S substances (Rauhut *et al.* 1997a).

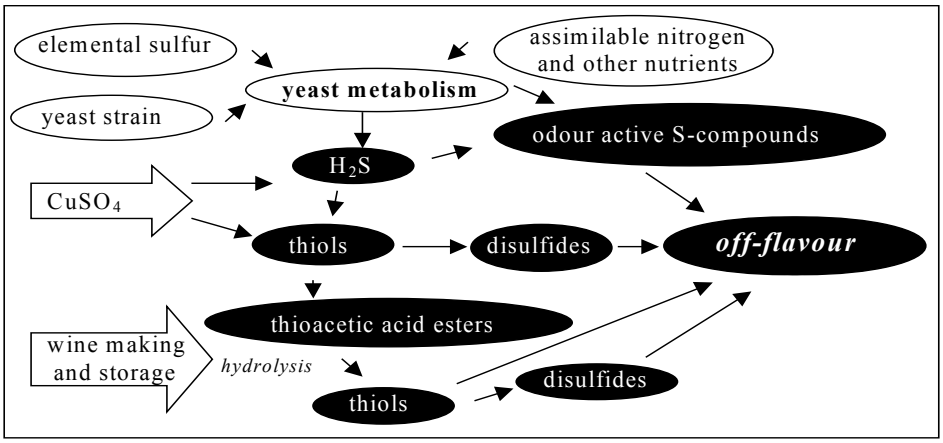

Fig. 3. Formation of off-flavors during wine making and storage (Rauhut *et al.* 1996).

Differences in the composition of S substances in wines with and without organoleptic defect

The analysis of commercial wines with and without sulfurous off-flavor showed significant differences between the combination and concentration of the S compounds. In wines with a sulfurous off-flavor, higher concentrations of methanethiol

(MeSH), ethanethiol (EtSH), dimethyl disulfide (DMDS), methylethyl disulfide (MeSSEt), diethyl disulfide (DEDS), MeSAc and EtSAc were detected.

Significant differences in the concentrations of higher boiling S compounds in wines with and without an aroma defect can also be seen in Fig. 4 (Rauhut 1996). The names of the marked substances and their RI values are listed in Table 1. Fig. 3 shows a chromatogram produced from the analysis of an extract from a 1991 Riesling wine without off-flavor (see left) and a chromatogram of the extract of a 1993 Riesling wine with a very strong off-flavor (see right). In the wine with off-flavor a high content of MeSAc (52 $\mu g\ l^{-1}$, RI 1051) and EtSAc (135 $\mu g\ l^{-1}$, RI 1101) was measured. In wines without a sensory complaint MeSAc was determined in a concentration below 5 $\mu g\ l^{-1}$ and EtSAc was not detectable.

Bernath (1997) measured higher concentrations of 2-methyl-3-furanthiol and 2-methyl-3-hydroxythiophene in wines with an off-flavor. Especially the thiol is a very odorous compound with a flavor like roasted meat.

The intensity and the sensory impression of the resulting off-flavors depend on the qualitative and quantitative composition of volatile S substances in the wines (Rauhut 1996; Rauhut *et al.* 1996). Furthermore the aroma impression is influenced by synergistic and antagonistic effects of all aroma compounds.

S Substances and their role in the atypical aging flavor of wines

Very often a reduced sulfur flavor defect is accompanied with an "atypical aging" (ATA) in wines. The cause is a lack of nutrients (mainly assimilable nitrogen) in the fermented grape must and/or the use of a yeast strain that requires high amounts of nutrients. In wines with both off-flavors the ATA taint is often masked by the present S compounds. In those wines the ATA off-flavor can be detected after a copper fining. Recent research work demonstrated that certain S substances (*e.g.* methional (3-methylthiopropanal)) are involved in the development of ATA off-flavors (Rauhut 1996; Rauhut *et al.* 2001; Rauhut and Kürbel 2002).

Furthermore there are indications that methional can be a precursor of dimethyl trisulfide (DMTS) in aged wines (Rauhut and Kürbel 2002). DMTS is a very powerful aroma compound with an odor threshold value 0.1 - 0.2 $\mu g\ l^{-1}$ and an awful scent.

Apart from other factors (*e.g*. viticultural treatment, stress conditions during the vegetation period and fermentation, low nutrient levels in the musts etc.) a low antioxidative potential after fermentation in combination with SO_2-addition is discussed as cause for the off-flavor development. Gessner *et al.* (1998) demonstrated that the addition of ascorbic acid before SO_2 treatment can diminish the amount of 2-aminoacetophenone and prevents or slows down the development of ATA taint. Therefore Riesling musts with very low assimilable nitrogen concentration were subject to pre-and/or post-fermentation additions of different antioxidants like ascorbic acid and tannins and were fermented with different commercial yeast strains (*Saccharomyces cerevisiae*). The results demonstrated that ascorbic acid diminished 2-aminoacetophenone and also methional. Glutathione addition to musts

and wines after fermentation in concentrations > 50 - 60 mg l^{-1} can increase the formation of sulfur compounds and the development of off-flavors (Rauhut *et al.* 2001).

Table 1. Main volatile S substances in a wine concentrate[1)]

Retention time (min)	Linear retention-Index [2)]	Sulfur component
4.34	1051	Thioacetic acid-*S*-methyl ester
4.82	1080	Dimethyl disulfide
5.02	1101	Thioacetic acid-*S*-ethyl ester
8.22	1220	Diethyl disulfide
9.11	1260	Unknown
11.17	1323	Di-tert-butyl disulfide (internal standard I)
16.14	1456	3-(Methylthio)-propanal (Methional)[3)]
18.90	1525	4,5 Dihydro-2-methylthiophene-3(2H)-one[3)]
20.15	1555	unknown, R-SH
20.55	1567	3-(Methylthio)-propionic acid ethyl ester[3)]
23.04	1628	Acetic acid-3-(methylthio)-propyl ester
26.22	1707	3-(Methylthio)-1-propanol (Methionol)[3)]
28.18	1758	cis-Tetrahydro-2-methylthiophene-3-ol[3)]
28.82	1771	3-(Ethylthio)-1-propanol (Ethionol)[*3)]
30.02	1805	trans-Tetrahydro-2-methylthiophene-3-ol[3)]
34.77	1929	2-Methyl-benzothiazole (internal standard II)
35.26	1943	Benzothiazole
43.21	2180	Unknown
43.56	2188	Unknown
50.94	2417	Unknown

[1)]Some S compounds are added for calibration and illustration; [2)] Linear retention index on Carbowax 20M (Rauhut *et al.* 1996); [3)] In accordance with the new rules for the international nomenclature of chemical compounds (IUPAC), the prefic "methyl-sulfanyl" must replace the prefix "methylthio" and "ethyl-sulfanyl" must replace the prefix "ethylthio".

Volatile S substances and their contribution to the typical aroma of wine

At the beginning of the 1980's it was indicated that certain volatile thiols contribute to characteristic aromas of Chenin and Sauvignon wines (Augustyn *et al.* 1982; Marais 1994).

The first thiol identified in Sauvignon wines was 4-mercapto-4-methyl-pentan-2-one. This substance has a strong odor of boxtree and broom and is very powerful (odor threshold value 0.1 – 0.8 ng l^{-1} in water and in a model solution). It has a significant contribution to the characteristic nuances observed in Sauvignon wines with concentrations close to 40 ng l^{-1} (Darriet *et al.* 1991, 1993; Darriet and Dubourdieu 1992; Murat *et al.* 2001a,b).

Other compounds which contribute to the aroma of Sauvignon wines are 4-methylthio-4-methylpentan-2-one, 3-mercaptohexyl-acetate, 3-mercaptohexan-1-ol (according to new IUPAC- rules the prefix "sulfanyl" must replace the prefix "mer-

capto"). These compounds were also detected in young Merlot, Cabernet Sauvignon and Cabernet Franc wines. Especially 3-mercaptohexanol strengthens the cooked fruit and blackcurrant character of red wines.

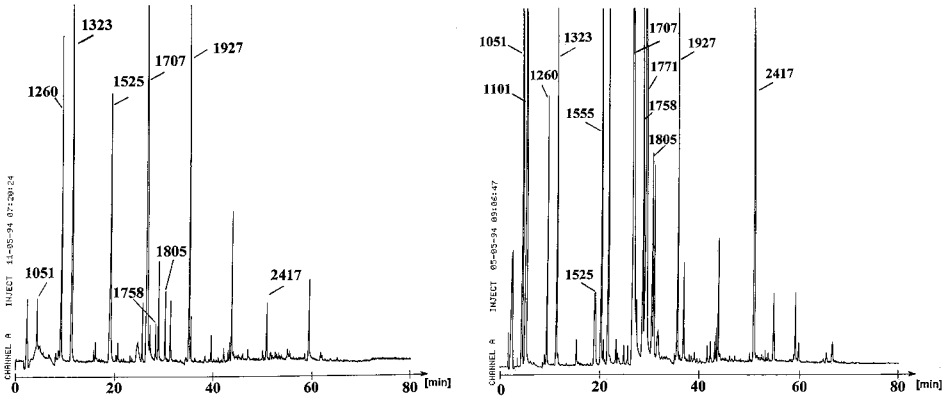

Fig. 4. Chromatograms of a wine without organoleptic aroma defect (left) and with organoleptic defect (right) caused by S substances (see Table 1 for peak identification, Rauhut 1996).

It could be demonstrated that 3-mercaptohexanol, 4-methyl-4-mercapto-hexanol, 4-methyl-4-mercaptopentan-2-one and 4-mercaptopentan-2-ol exist in the must in the form of *S*-conjugates with cysteine. The aroma compounds are revealed during alcoholic fermentation. The intensity of the release depends on the strain of *Saccharomyces cerevisiae* used for fermentation (Darriet and Dubourdieu 1992; Darriet *et al.* 1991, 1993; Murat *et al.* 2001a,b). Dubourdieu and Lavigne-Cruége (2002) proposed that the adding of 10 mg l^{-1} glutathione to Sauvignon blanc wine during bottling prevents the color from yellowing, dissipation of the varietal aroma, and the wine's tendency towards developing aging defects. Beloqui and Bertrand (1995) and Beloqui *et al.* (1995) found higher levels of bis (2-hydroxyethyl)disulfide in wines of *Vitis labrusca* and hybrids.

Conclusions

The presence or absence of volatile sulfur compounds in wines can have a significant impact on wine quality. Specific S-containing substances often are responsible for imparting the characteristic pleasant taste and aroma of wine. Excess concentrations of some of those S compounds or the presence of unexpected S compounds can be responsible for an undesirable odor or taste and can indicate product degradation.

Even very low concentrations of S compounds can greatly affect the flavors of wines, and because their presence is noticeable at very low, sub-ppb or sub-ppt lev-

els, accurate detection and quantification is an important quality control step and necessary for research work in this field.

Future research on sulfur and wine quality should be focused on the S demand and S transport of vines. There are still large gaps on the effect of S metabolism and influence of inorganic or organic S sources on vine growing and on quality of grapes and vines. It is important to know more about the role of glutathione in vines, grapes, musts and wine. Furthermore the influence of S metabolism on plant resistance should be studied.

Other interesting research fields are the identification of non-volatile S-containing precursors in certain grapes of different varieties, the selection of appropriate yeast strains of *Saccharomyces cerevisiae* to optimize the formation and release of S substances and studies on biochemical regulation of the S and N metabolism of yeasts and lactic acid bacteria during malolactic fermentation.

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IMPORTANCE OF SULFUR FOR THE QUALITY OF BREADMAKING WHEAT AND MALTING BARLEY

Fang-Jie Zhao and Steve P. McGrath
Agriculture and Environment Division, IACR-Rothamsted, Harpenden, Hertfordshire AL5 2JQ, United Kingdom

Introduction

In Europe, S emissions from man-made sources and S deposition to land have decreased dramatically over the last 30 years. For example, UK S emissions in 1999 were only 18 % of the level recorded in 1970 (National Expert Group on Transboundary Air Pollution 2001). Atmospheric S deposition is now <10 kg ha^{-1} in most arable areas in the UK, which is insufficient to meet the requirements for S by most crops. Although the decreases in S emissions and deposition are beneficial to the environment as a whole, the decreased S inputs have resulted in widespread S deficiency in agricultural crops. In recent years, the occurrence of S deficiency in UK crops appears to be even more widespread than predicted in the mid 1990s (McGrath and Zhao 1995), due to a larger than expected decrease in S deposition.

Sulfur nutrition affects not only crop yields, but also crop quality. Although crop quality is genetically determined to a large extent, environmental factors also play a significant role. Availability of N and S, and more importantly, the balance between the two nutrients, can have a large impact on the quality of many crop produces. Quality requirements are diverse, and dependent on the end uses. Sulfur can effect the nutritional value, taste and flavor, processing quality, or appearance of produce. Because methionine is often the limiting essential amino acid in legume grains (Friedman 1996), S deficiency has been shown to lower the nutritional values of grain legumes (*e.g.* Eppendorfer and Eggum 1995). Sulfur is a constituent of many secondary metabolites, which have a strong influence on taste and flavor. Examples are glucosinolates in *Brassica* crops and *S*-alk(en)yl-L-cysteine sulfoxide (ACSO) in onions. It is well established that S supply greatly influences the contents of glucosinolates in *Brassica* crops (*e.g.* Zhao *et al.* 1994; Schnug 1997) and of ACSO in onions (*e.g.* Randle *et al.* 1995). In sugar beet, S deficiency can lead to accumulation of α-amino-N in the storage roots, particularly when N supply is abundant, resulting in a poorer quality of beet roots (Bell *et al.* 1995). In cereals, S availability influences the composition of storage proteins and thus the processing quality. The effects of S on the quality of breadmaking wheat and malting barley are reviewed in this paper.

Sulfur Transport and Assimilation in Plants, pp. 133-143
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Effects of sulfur on wheat quality

Among all cereal proteins, only wheat gluten exhibits the important rheological properties of elasticity (strength) and extensibility (viscosity). It is these properties that are exploited in the diverse uses of wheat flour. Wheat dough that has high elasticity and reasonable extensibility is good for making bread. Highly extensible dough is good for biscuits, and dough with intermediate properties is used for making flat breads or noodles. Visco-elasticity of wheat dough is determined by the composition of prolamins, which are a group of proteins soluble in alcohol/water mixtures, and account for about 50 % of the total N in wheat grain (Shewry 1995). Prolamins can be further divided into monomeric gliadins and polymeric glutenins. Glutenins polymers consist of high molecular weight (HMW) and low molecular weight (LWM) subunits. Prolamin polypeptides differ greatly in the content of cysteine residues, and can therefore be classified into the S-poor, S-rich and HMW glutenin subunits (Fig. 1) (Shewry *et al.* 1997). Disulfide bonds, formed between the sulfhydryl groups of cysteine residues, play a key role in determining the structure and properties of wheat proteins (Frater *et al.* 1960; Wall 1971). Monomeric gliadins form only intra-chain disulfide bonds, or no disulfide bonds in the case of ω-gliadins, and contribute mainly to the viscosity of dough. In contrast, polymeric glutenins have both intra-chain and inter-chain disulfide bonds and contribute mainly to dough elasticity (Shewry and Tatham 1997).

Because different storage proteins vary greatly in terms of the content of S-containing amino acids, it is not surprising that the composition of proteins is influenced substantially by the S availability. The effect of S was demonstrated clearly by earlier researches in Australia. Wrigley and co-workers showed that S deficiency in wheat resulted in increases in the relative proportions of HMW glutenin subunits and the S-poor ω-gliadins, and concurrent decreases in the relative proportions of the S-rich groups, including LMW glutenin subunits, α-, β- and γ-gliadins, LMW albumins and globulins (Wrigley *et al.* 1980, 1984; Fullington *et al.* 1987). More recently, MacRitchie and Gupta (1993) showed that a decrease in grain S concentration was associated with an increase of the ratio of HMW/LMW subunits of glutenins. This in turn shifted the molecular weight distribution of glutenin polymers to higher M_r, because the HMW glutenin subunits are predominantly present in the high M_r polymers. Although the effects of S on the synthesis and accumulation of HMW subunits and LMW subunits of glutenins were opposite to each other, the net result of S deficiency was to decrease the total amount of polymeric proteins (glutenins) in the flour, because the LMW subunits are the major components of glutenin. This was confirmed by recent studies in the UK (Zhao *et al.* 1999a,b). In general, additions of S fertilizer did not affect the total protein content in grain, but resulted in larger amounts of gel protein, which are predominantly glutenins (Fig. 2). Increasing evidence indicates that the quantity of glutenin or glutenin macropolymer is more closely linked to breadmaking quality than subunit composition (Weegels *et al.* 1996). The effect of S on breadmaking quality may be largely due to its effect on the quantity of glutenin.

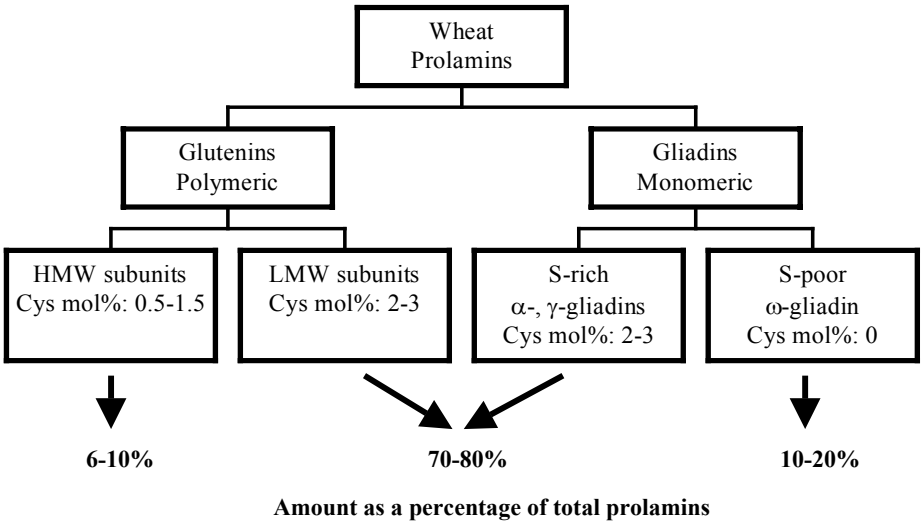

Fig. 1. Composition of wheat prolamins.

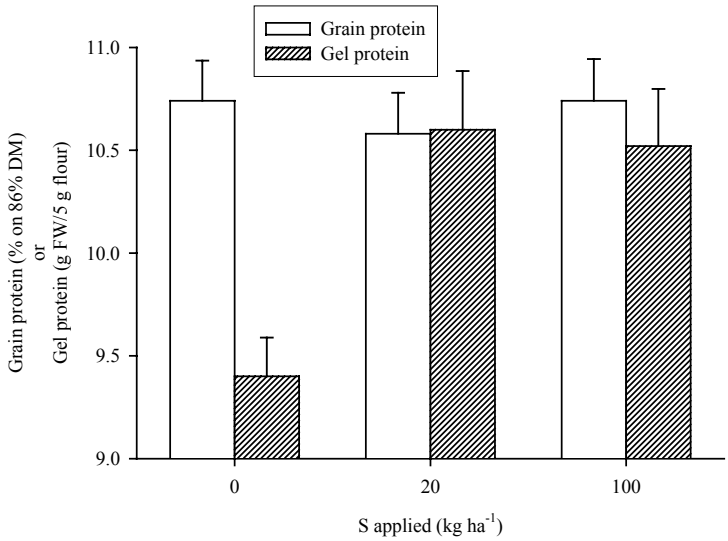

Fig. 2. Effects of S on grain protein content and gel protein content.

Sulfur nutrition of wheat has a profound effect on the rheological properties of dough (Moss *et al.* 1981, 1983; Zhao *et al.* 1999a, b). In general, increasing S concentration in wheat grain is associated with increasing dough extensibility. This positive relationship was observed for three breadmaking cultivars which differed considerably in dough rheology (Fig. 3). In contrast, dough resistance tends to in-

crease with increasing N/S ratio in grain (Fig. 3). The influence of S on dough rheology is consistent with the effect of S on the composition of prolamins described above. The positive effect of S on dough extensibility is probably due to increases in the quantity of low M*r* glutenins and some gliadins, whereas decreased dough resistance in response to S may be linked to a decrease of the ratio of HMW/LMW glutenin subunits and a shift of glutentin polymer to lower M_r (MacRitchie and Gupta 1993; Zhao *et al.* 1999a).

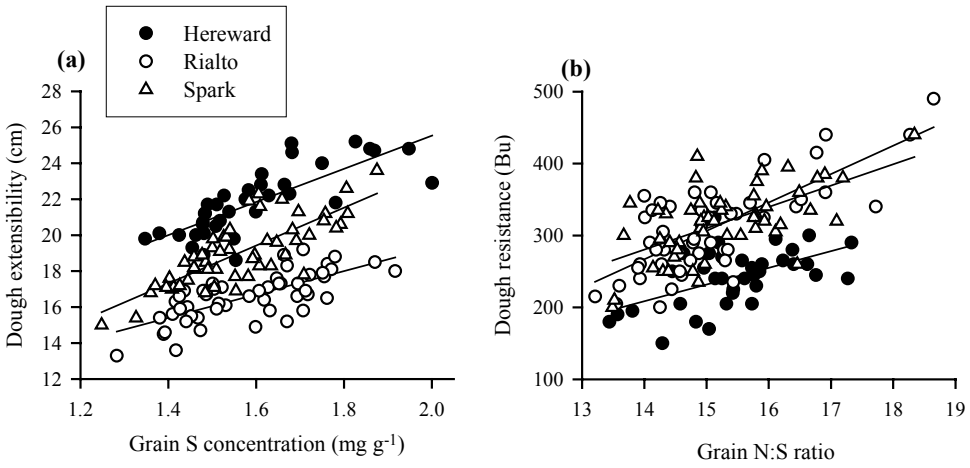

Fig. 3. Correlations between grain S concentration and dough extensibility (a), and between grain N/S ratio and dough resistance (b), in three breadmaking varieties of winter wheat (Zhao *et al.* 1999a).

Loaf volume is probably the most important parameter for breadmaking quality. Byers *et al*. (1987) showed that wheat grain produced from a low S treatment in a sand culture experiment, having a S concentration of 0.8 mg g^{-1} and a N/S ratio of 31, was completely unsuitable for making bread. Moss *et al.* (1981, 1983) reported a significant positive effect of S on the loaf volume of bread made from a multipurpose soft wheat variety, but no significant improvement with a hard bread wheat variety. In ten field experiments conducted in the UK between 1995 and 1998, loaf volume was significantly increased by S in six experiments (Zhao *et al.* 1999a,b). This series of experiments also tested the effect of applying 50 kg ha^{-1} extra N on top of the normal rate of 180 kg N ha^{-1}. The effect of this extra N on loaf volume was significant only in one experiment, even though grain protein content was increased in all experiments. Figure 4 shows the responses of loaf volume to S and N in one of the experiments. When data from different sites and years were combined, it was found that loaf volume correlated more strongly with grain S concentration than with grain N concentration. Responses of breadmaking quality to S addition

have also been obtained in Germany. Schnug *et al.* (1993) showed that an application of 46 kg S ha^{-1} increased loaf volume by 6 %.

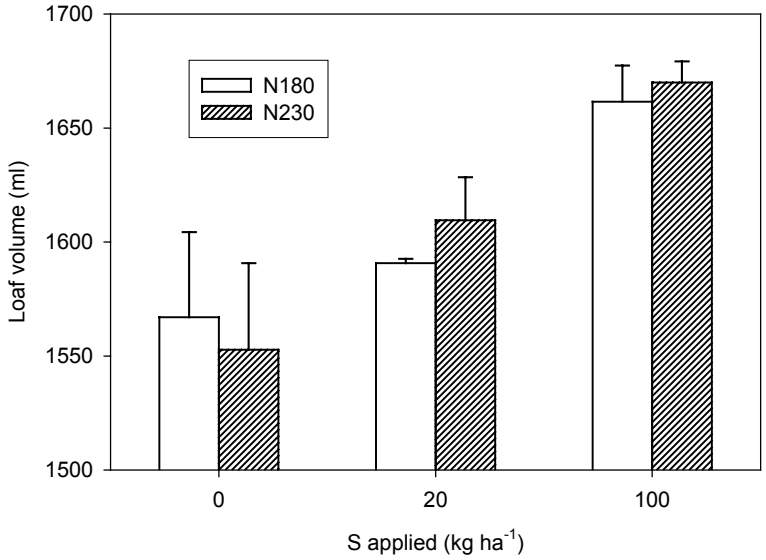

Fig. 4. Effect of S applications on loaf volume at two different N rates (in kg ha^{-1}). The experiment was conducted in 1996 in the Northeast England, using the winter wheat variety Hereward (Zhao *et al.* 1999b).

Effects of sulfur on yield and malting quality of barley

Malting quality of barley and brewhouse performance are assessed in terms of ease of processing and flavor characteristics (Palmer 1989). During malting of barley grain, large molecular weight components of the endosperm cell walls, the storage proteins and starch granules are hydrolyzed (modified) enzymatically, rendering them more soluble in hot water during mashing. Variability in malting quality is due to factors such as the rate of enzyme synthesis during germination, the composition of the endosperm, and the packing of starch granules (Palmer 1989). Hot water extraction of malt reflects the degree of endosperm modification, and is therefore an important parameter of malting quality. It has long been known that malting quality is adversely affected by high contents of protein in barley grain (Bishop 1930). Barley prolamins (hordeins) account for 30 - 50 % of the total N in grain (Shewry *et al.* 2001). Similar to wheat prolamins, hordeins can be separated into three groups: S-poor C hordein, S-rich B and γ hordeins, and HMW D hordein which contains intermediate amount of S. Studies by Smith and Lister (1983) and Skerritt and Janes (1992) showed a strong and negative correlation between hot water extract of malt and the gel protein fraction, which comprised mainly D hordein and some B hordein. Howard *et al.* (1996) studied malting quality of three barley cultivars grown under five nitrogen regimes in two seasons. They found that hot water extract of

malt correlated inversely with the content of D hordein, and the relationship was independent of cultivar (Fig. 5). However, a more recent study showed that neither presence or absence of D hordein, nor gel protein content related to differences in malting performance in six pairs of near isogenic barley lines (Brennan *et al.* 1998). The lack of impact of D hordein may be because other factors (*e.g.* endosperm structure) were more dominant and influential.

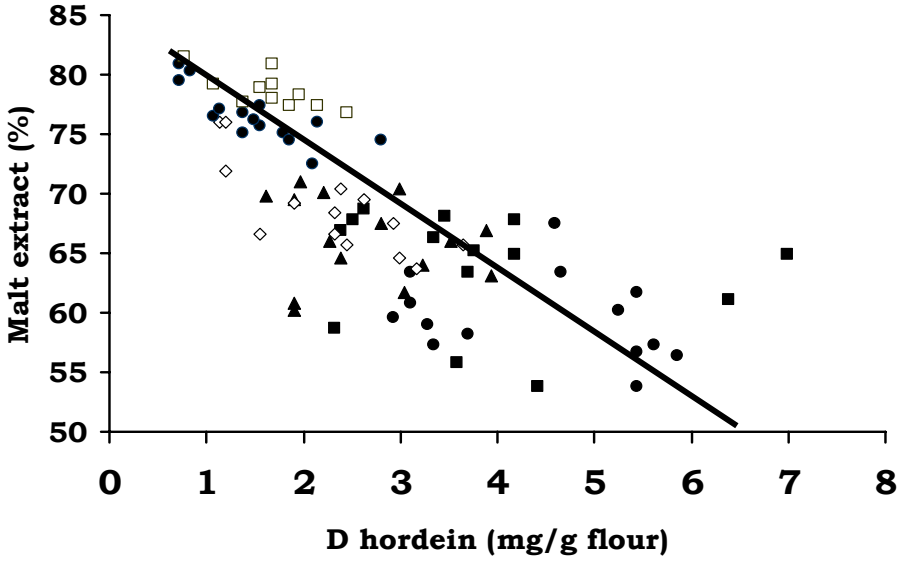

Fig. 5. Relationship between malt extract and D hordein content. Different symbols represent different cultivars or growing seasons (Howard *et al.* 1996).

Little is known about the influence of S availability on malting quality of barley. However, S supply does have a significant influence on the composition of hordeins in barley grain. For example, Shewry *et al.* (1983) showed that S starvation resulted in a marked increase in the proportion of S-poor C hordein, and a concurrent decrease of the S-rich B hordein in barley grain. It is not clear whether the changes in hordein composition would impact on malting quality.

In a field experiment at a S-deficient site, grain yield of a winter malting barley variety (Regina) was increased significantly by applications of S fertilizers (Fig. 6). The average yield of all +S treatments was 1.1 t ha^{-1} higher than that of the S0 treatment, representing a 34.9 % yield increase. Grain N concentration ranged from 1.6 to 2.0 % on a dry weight basis. It is clear that applications of S decreased grain N concentration significantly ($P < 0.001$; Fig. 6). Grain N concentration was 1.95 % in the S0 treatment, whereas in all +S treatments grain N concentrations were below the value 1.8 %, which is required for malting purposes. Despite a reduction in grain N concentration, total N uptake in grain was actually increased by the S treatments.

This suggests that the effect of S on grain N concentration was probably due to a dilution of N in the crop, which resulted from a growth stimulation and yield increase in response to S, particularly with a relatively low dose of N fertilizer used in this experiment (120 kg ha^{-1}). Results from this experiment indicate that S applications not only increased yield, but also may benefit malting quality by decreasing grain protein content.

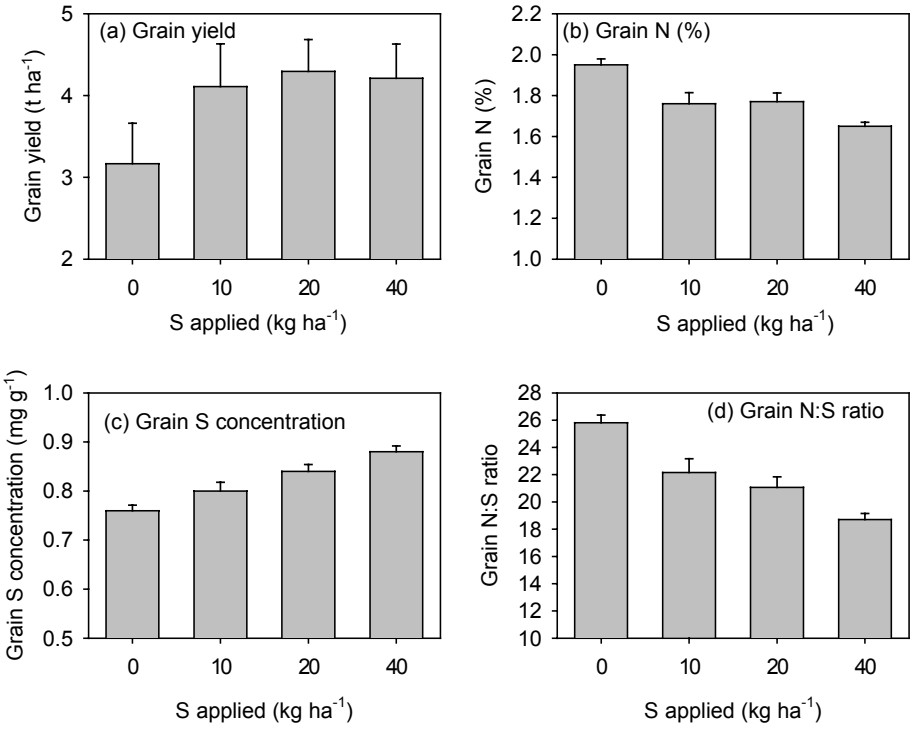

Fig. 6. Effects of S applications on yield, grain N and S concentrations, and N/S ratio in winter malting barley.

Dimethyl sulfide (DMS) is an important flavor constituent of lager beers (Palmer 1989). DMS is produced from the precursor *S*-methylmethionine (SMM) by yeast during fermentation or by thermal breakdown in hot wort. It remains to be investigated whether S deficiency in malting barley leads to insufficient SMM levels in malts, thus affecting the flavor of beers.

Sulfur supply and grain S concentration

The concentration of S in wheat and barley grain usually varies between 1 and 1.8 mg g^{-1} (Withers *et al.* 1993; Zhao *et al.* 1995). Most of the S in grain is bound in

proteins, with <10 % being present as sulfate. The effects of S fertilization on the concentration of S in wheat grain were investigated in seven field experiments in two growing seasons in the UK (Fig. 7). Soil available S in the top 60 cm profile varied from 7 to 63 kg ha^{-1}. However, there was no correlation between soil available S and grain S concentration in the control treatment (nil S). Applications of S up to 100 kg ha^{-1} increased grain S concentration by 10 - 50 %. The largest increases were obtained at the low yielding (4 - 6 t ha^{-1}) Woburn site, whereas the increases in grain S were moderate at the other sites which were high yielding (8 - 10 t ha^{-1}). These results suggest that factors other than S supply also influence the concentration of S in grain.

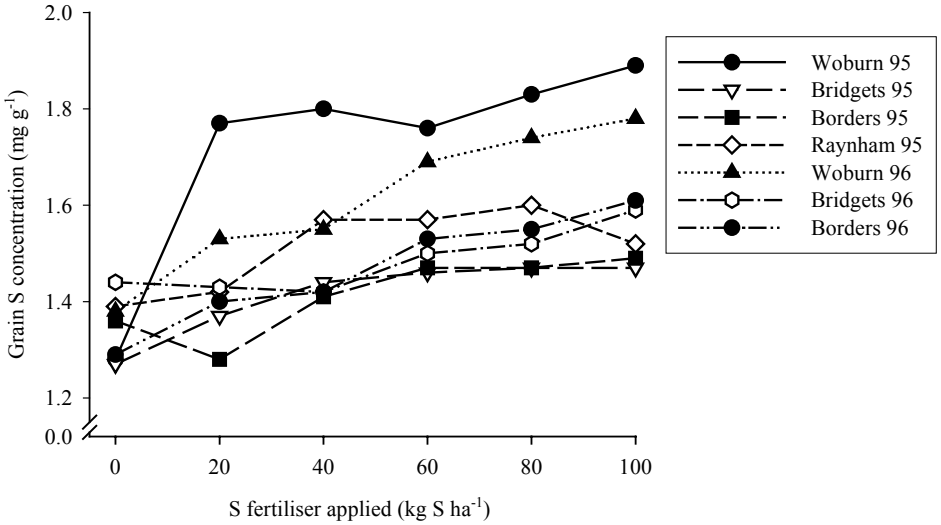

Fig. 7. Responses of grain S concentration of winter wheat to S fertilizer applications.

Sulfur has an intermediate mobility within plant tissues. The re-distribution of S from vegetative tissues to wheat grain is considerably less than for N and P. This is reflected by a harvest index (the proportion of a nutrient in the shoots that occurs in the grain) for S in wheat of between 0.4 - 0.5, compared to 0.7 - 0.8 for N and P (Hocking 1994). Eriksen *et al.* (2001) showed that, under the conditions of sufficient N and S supplies, the pool of S in leaves decreased by an average of 28 % during the grain filling period in spring barley, whereas the leaf N pool decreased by approximately 70 %. In a hydroponic study using different ratios of stable isotopes $^{34}S/^{32}S$, Monaghan *et al.* (1999) found that, at maturity, wheat grain derived 14, 30, 6 and 50 % of its S from the uptake during the following successive growth stages: between emergence and early stem extension, between stem extension and flag leaf emergence, between flag leaf emergence and anthesis, and after anthesis, respectively. The results indicated that the pre- and post-anthesis S uptakes were equally important for the S accumulation in grain, and imply that S availability would have

to be maintained at a sufficient level throughout the whole period of wheat growth to achieve an adequate accumulation of S in grain that is required for quality. This conclusion is supported by results from two further experiments conducted by the authors of this paper. In the first experiment, the effects of applying 30 kg S ha^{-1} were compared at different growth stages on the concentration of S in wheat grain under field conditions. Late applications were found to increase grain S more than early applications. In the second experiment conducted under greenhouse conditions, the effect of withholding S supply after anthesis on grain S accumulation was investigated. Even though withholding S supply after anthesis did not result in a loss of grain yield, the concentration of S in grain was decreased by approximately 50 % compared to the treatment that had a constant S supply both before and after anthesis.

Conclusions

Different groups of storage proteins in wheat and barley grains contain contrasting amounts of S. Therefore, S availability affects the composition of these proteins. This effect has a substantial impact on the rheology of wheat dough. Additions of S fertilizers have been shown to improve breadmaking quality. Although S supply also influences the composition of barley storage proteins, the effects of S on malting quality of barley remain to be investigated. Sulfur has an intermediate mobility within plant tissues. Although S can be re-distributed from vegetative tissues to grain, it has been shown that S uptake after anthesis is also important for maintaining a high concentration of S in grain.

Acknowledgements

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EFFECT OF N AND S FERTILIZATION ON THE GLUCOSINOLATE CONTENT OF LEAVES AND ROOTS OF BROCCOLI SEEDLINGS

Alfredo Aires, Rosa Carvalho, Eduardo Rosa and Fernanda Maria Pereira
Universidade de Trás-os-Montes e Alto-Douro, Department of Plant Science and Agricultural Engineering, Apartado 1013, 5001-911 Vila Real, Portugal

Brassicaceae contain secondary sulfur compounds, glucosinolates (Gls), which have been reported to have health protecting effects. Broccoli sprouts contain relatively high levels of 4-methylsulfinylbutyl, a precursor of sulforaphane, which as potent inducer of Phase 2 enzymes may function as a cancer preventive (Zhang *et al*. 1992; Fahey *et al*. 1997). S and N nutrition of plants have a significant effect on the level and composition of Gls (Rosa *et al*. 1997, Schnug and Haneklaus 1993). Zhao *et al*. (1994) showed significant interactions between N and S on the yield, quality and glucosinolate composition, indicating that the balance between the N and S supply played an important role in the Gls biosynthesis.

The effect of N and S fertilization on Gls content of the leaves and roots of broccoli seedlings was measured. The impact of various levels and combinations of N (0, 45.5, 91 mg l^{-1}) and S (0, 14.6, 29.2 mg l^{-1}) fertilization on Gls were tested in broccoli seedlings. Seeds of broccoli cv. *Marathon* were sown on 2 cm thick rockwool in Petri dishes (14 cm diameter) and watered with the nutrient solution. The photoperiod was 14 h, the photon fluence rate was 340 μmol m^{-2} s^{-1} (PAR) at 30/15º C day/night temperature regime and 70 % relative humidity. Seedlings were harvested 11 days after sowing, homogenized in liquid nitrogen and freeze-dried. The plant material was powdered and stored until analytical analysis. Gls were extracted and quantified as described by Heaney and Fenwick (1980) and Spinks *et al.* (1984), respectively. Gls was expressed in μmol g^{-1} dry weight. Data were analyzed with ANOVA.

The results showed that total Gls level in the leaves was significantly higher ($P<0.001$) than that in the roots. The major Gls present in the leaves were 4-methylsulfinylbutyl and 3-methylsulfinylpropyl representing 48.2 and 20.4 % of total Gls content. In roots, 2-phenethyl and 4-methylthiobutyl were the 2 major Gls, representing 38.5 and 25.5 % of the total Gls content (Table 1). It was noted that when no N fertilization was applied but S was increasing up to 29.2 mg l^{-1}, the total Gls levels in the leaves were increased. However, at maximum N levels (91 mg l^{-1}), an opposite trend was noted, which suggests that above 45.5 mg l^{-1} of N increasing levels of S have a detriment effect on Gls content.

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The results also showed a difference between the aliphatic, indolic and aromatic groups. The aliphatic group clear follows the total Gls trend whilst the indolic and aromatic group showed opposite trend, *e.g.* for the highest N fertilization (91 mg l^{-1}) with no S or moderate amount (14.6 mg l^{-1}) the indolic and aromatic group showed the highest levels of glucosinolates.

Table 1. Glucosinolate content (μmol g^{-1} dry weight) in broccoli sprouts (*B. oleracea* var. *italica* cv. Marathon) as affected by S and N (mg l^{-1}) fertilization.

Levels of S and N fertilization	Glucosinolates[1]							
	GI	GR	HGB	GE	GB	PE	NGB	Total
Leaves								
S_0N_0	4.83	11.65	1.04	2.47	1.81	2.43	0.47	24.7
$S_{14.6}N_0$	5.06	11.05	2.00	3.41	1.78	1.93	0.50	25.7
$S_{29.2}N_0$	8.32	19.37	2.02	3.59	1.27	1.39	0.30	36.3
$S_0N_{45.5}$	7.77	18.07	1.40	3.38	1.21	1.72	0.32	33.9
$S_{14.6}N_{45.5}$	4.86	12.35	0.91	2.25	1.37	2.66	0.64	25.0
$S_{29.2}N_{45.5}$	6.21	15.73	1.56	3.91	2.07	3.58	0.68	33.8
S_0N_{91}	5.98	14.10	1.20	4.16	2.42	3.51	0.67	32.0
$S_{14.6}N_{91}$	5.01	11.53	1.17	3.54	2.31	3.50	0.58	27.6
$S_{29.2}N_{91}$	4.74	11.42	0.97	2.40	1.81	2.32	0.51	24.2
Roots								
S_0N_0	0.83	2.06	1.54	6.63	1.19	11.61	4.73	28.6
$S_{14.6}N_0$	0.73	1.79	1.72	7.28	1.49	12.73	5.89	31.6
$S_{29.2}N_0$	0.89	2.21	1.36	6.96	1.20	10.48	4.21	27.3
$S_0N_{45.5}$	0.52	1.29	1.12	4.94	0.82	9.74	3.36	21.8
$S_{14.6}N_{45.5}$	0.66	1.75	1.49	9.29	1.52	14.67	4.24	33.6
$S_{29.2}N_{45.5}$	0.49	1.30	1.26	6.87	2.57	13.31	4.87	30.7
S_0N_{91}	1.16	2.57	1.44	8.25	2.30	10.23	4.91	30.9
$S_{14.6}N_{91}$	0.74	1.92	1.24	8.08	1.90	8.99	4.59	27.5
$S_{29.2}N_{91}$	0.79	1.86	1.62	9.00	2.51	12.18	5.73	33.7

[1]Glucosinolates: GI, 3-methylsulfinylpropyl; GR, 4-methylsulfinylbutyl; HGB, 4-hydroxyindol-3-ylmethyl; GE, 4-methylthiobutyl; GB, indol-3-ylmethyl; PE, 2-phenylethyl; NGB, 1-methoxyindol-3-ylmethyl

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SCREENING OF MUTANTS ACCUMULATING HIGH LEVELS OF ANIONS AND THIOLS FROM ACTIVATION-TAGGED LINES OF *ARABIDOPSIS THALIANA*

Motoko Awazuhara[1], Akiko Hayase[1], Masatomo Kobayashi[2], Masaaki Noji[1], Mami Yamazaki[1], Motoaki Seki[3], Kazuo Shinozaki[3] and Kazuki Saito[1,4]
[1]Department of Molecular Biology and Biochemistry, Graduate school of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan[2]; BioResource Center, RIKEN, Koyadai 3-1-1, Tsukuba, Ibaraki 305-0074, Japan; [3]Plant Molecular Biology, RIKEN, Koyadai 3-1-1, Tsukuba, Ibaraki 305-0074, Japan; [4]CREST, JST(Japanese Science and Technology Corporation), Japan

In higher plants, sulfate is incorporated and assimilated into various organic sulfur compounds, such as thiols. Recently, almost all genes encoding the enzymes of the sulfur assimilation pathway were isolated and characterized (Saito 2000). However, the factors involved in the regulation of this pathway have not been identified. In this study, to elucidate those factors or genes, we have initiated phytochemical screening of *Arabidopsis thaliana* T-DNA activation-tagged lines for accumulation of high levels of metabolites related to sulfur assimilation using high-throughput metabolite profiling systems.

For this screening, we used T-DNA activation tagged lines of *Arabidopsis* carrying the activation tagging vector pPCVICEn4HPT, which has 4 repeats of the enhancer regions of the CaMV 35S promoter, and a hygromycin resistant gene as a selection marker (Hayashi *et al.* 1992). We expected that some of those activation tagged lines show altered levels of metabolites, which resulted from increased levels of transcription by the effects of enhancers. We aimed to isolate such mutants with altered metabolite levels and identify enhancer-tagged genes in those mutants. In this study, by using activation tagged line for screening, we expected to obtain novel and interesting gain-of-function type mutants which have not been isolated from EMS mutants or T-DNA tagged lines. Especially, we expected that novel key enzymes, regulatory factors and signal proteins of the sulfur-metabolic pathways would be identified.

In this screening, we used a high throughput metabolite profiling system by combination of capillary electrophoresis (CE) and HPLC analysis. Metabolites were extracted from rosette leaves of individual plants with 0.01N HCl. For CE analysis, to remove the proteins, the extracts were filtered through a membrane with witch only molecules smaller than 5000Da can pass. The CE used in this experiment is

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Agilent HP3D system (Agilent technologies GmbH, Waldbronn, Germany) and the analysis was conducted following the instruction of the equipment. By this method, the metabolite profiling was successful for about 20 compounds, including inorganic anions, organic acids and amino acids (Fig. 1). Since, thiols could not be detected by CE, we analyzed them by fluorescent HPLC (Kuzuhara *et al*. 2000). For the HPLC analysis, the same extracts used for CE were reduced by DTT, and then thiols in the samples were labelled at the SH residues with a fluorescent reagent, monobromobimane (Molecular Probes, Inc. OR. USA). Using this HPLC system, cysteine, γ-glutamylcysteine and glutathione could be detected. The analysis by CE and HPLC took 30 min for each sample.

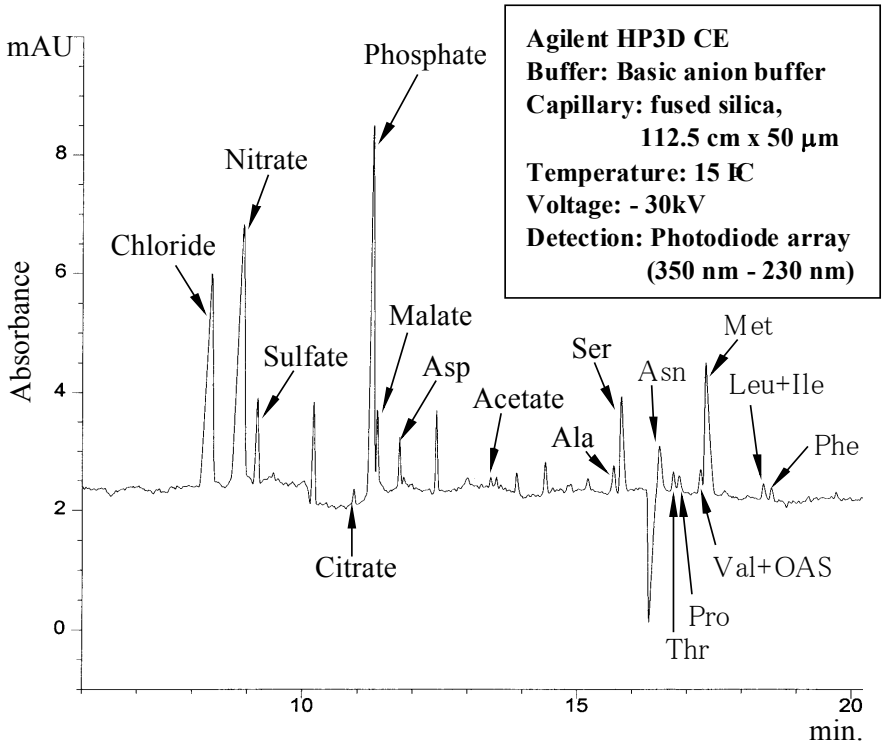

Fig. 1. Typical electropherogram of leaves of wild type *Arabidopsis* plants growing for 3 weeks. Metabolites were extracted with 0.01N HCl. The extract was filtered to remove protein and subjected to CE analysis.

Approximately 8,600 activation-tagged lines of *A. thaliana* have been generated. For the first screening, we used T2 plants of T-DNA activation-tagged lines as batches containing 20 independent lines. The seeds were sown on Murashige-Skoog (MS) solid medium containing hygromycin. Three weeks after sowing, the rosette leaves of about 18 plants in each batch were harvested separately and subjected to

metabolite profiling. So far, the metabolite profiles of more than 5000 activation tagged-lines have been analyzed, and several interesting candidates have been selected in the first screening.

In addition to the screening by direct metabolite profiling, the biological screening was started, by using biological inhibitors such as cadmium, ethionine, selenocystine and selenate *etc*. In the screening, plants have been selected, which can grow better in the presence of these inhibitors. There is a possibility that the plants resistant to these inhibitors accumulate high levels of thiols, methionine and so on. This would be a promising approach to identify genes encoding regulatory factors and key enzymes, because the enhanced expression of those genes can result in increased metabolite accumulation.

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COMPARISON OF SULFUR DEFICIENCY INDICATORS IN WINTER OILSEED RAPE

Ursula Balestra[1], Mechteld Blake-Kalff[2], J.-C. Lavanchy[3], A. Keiser[4], H.-R. Pfeifer[3] and D. Pellet[1]

[1]Swiss Federal Research Station for Plant Production, Changins, Nyon, Switzerland; [2]Agriculture and the Environment Division, IACR-Rothamsted, Harpenden, UK; [3]Mineral Analysis Center, Institute of Earth Sciences, BFSH 2, Lausanne, Switzerland; [4]Swiss College of Agriculture, Zollikofen, Switzerland

Sulfur deficiency may cause severe yield reduction in oilseed rape (*Brassica napus*) and can be detected by plant analysis (total S, N/S ratio, malate/sulfate ratio, Blake-Kalff *et al.* 2001). Depending on the severity of S deficiency, macroscopic symptoms are observed, as leaf chlorosis and deformations, pale yellow color of petals. However, macroscopic symptoms do not appear systematically or appear too late during crop development to apply corrective S fertilization.

The goal of our study was to test a bio-indicator able to show early deficiency symptoms during crop development and to test it with mostly used diagnostic indicators.

In field experiments, the bio-indicator of S deficiency consisted in oilseed rape plants grown on various spots of reduced area in a field. Indicator plants were drilled 3 weeks later than the oilseed rape crop (Delayed Drilling, DD). Contrasting S regimes were applied to both the *Brassica* crop (Normal Drilling, ND) and the indicator plants. Nitrogen fertilization was 160 kg N ha^{-1}; leaf samples were taken at crop growth stage ranging from GS 57 to GS 61. Total sulfur in plant samples was quantified by X-ray spectroscopy, total N by Kjeldahl, and the malate/sulfate ratio by ion chromatography, according to Blake-Kalff *et al.* (2000). Leaf chlorophyll content was measured with a N tester (Hydro).

Critical S_{total} values of 0.4 and 0.5 % in young expanded leaves were established for, respectively 90 % and 95 % of maximum yield in conditions typical for Switzerland. Data (not shown) were collected in five different field trials, at crop growth stage ranging from GS 57 (flower buds raised above leaves) to GS 61 (begin of flowering). Critical values were somewhat lower, but in the same range as published data (Blake-Kalff *et al.* 2000; Schnug and Haneklaus 1998).

No macroscopic symptoms of S deficiency (measured by leaf chlorophyll content, Table 1) were observed in the crop (ND plants), in absence of S fertilization, although plant analysis suggested that the crop was S deficient. A statistically significant 10 % yield increase in response to S fertilization (data not shown) confirmed the indication of plant analysis. In bio-indicator plants, however, characteristic mac-

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roscopic S-deficiency symptoms were visible in the absence of S fertilization and quantified by leaf chlorophyll (Chl) content (Table 1). S-deficiency diagnosis was confirmed in this case by plant analysis (Table 1). Among the S-deficiency indicators, S_{total} and N/S ratio gave similar information about plant sulfur status, while malate:sulfate ratio detected a mild S deficiency, in S-fertilized plants. This is common with this indicator, particularly when analysis is performed close to the flowering stage. According to our S_{total} critical values (mentioned above in text), none of the treatments with S fertilization were S-deficient (at the 90 % level).

Results showed that bio-indicator plants were more sensitive to S deficiency than the crop. This is probably why delayed drilling made it possible to detect macroscopic symptoms of S deficiency, while the crop was characterized by hidden S deficiency.

We concluded that visual symptoms of S deficiency in field bio-indicator plants give an accurate information about S status of oilseed rape crop.

Table 1. Influence of S fertilization on leaf chlorophyll content (Chl) and S status of oilseed rape plants (ND, GS 61 begin of flowering) or bio-indicator plants (DD, GS 59, first flower buds raised), measured with various S-deficiency diagnostics ± SE (n = 4).

	Oilseed rape crop (ND)				Bio-indicator plants (DD)			
S supply (kg ha^{-1})	S_{total} (mg g^{-1})	N/S ratio	malate/ sulfate ratio	Chl (unit free)	S_{total} (mg g^{-1})	N/S ratio	malate/ sulfate ratio	Chl (unit free)
0	4.1 ± 0 2	13.7 ± 0.6	10.2 ± 1.2	657 ± 25	3.0 ± 0.4	19.8 ± 3.1	14.7 ± 4.9	550 ± 32
40	6.1 ± 0.6	9.3 ± 1.1	3.2 ± 1.0	659 ± 10	4.4 ± 0.3	12.6 ± 1.0	4.5 ± 1.0	639 ± 18
80	6.0 ± 0.9	6.5 ± 0.7	1.4 ± 1.0	663 ± 17	5.9 ± 0.6	9.9 ± 1.5	2.1 ± 1.0	675 ± 28
Critical values	<4.6	>10	>1.5		<4.6	>10	>1.5	

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FURTHER INVESTIGATION ON THE ROLE OF THE PROLONGED LINKER SEQUENCE IN PLANT SULFUR TRANSFERASES

Michael Bauer and Jutta Papenbrock
Institut für Botanik, Universität Hannover, Herrenhäuserstr. 2, D-30419 Hannover, Germany

Sulfur transferases/rhodaneses are a group of enzymes widely distributed in plants, animals, and bacteria that catalyze the transfer of a sulfur atom from a donor molecule to a thiophilic acceptor substrate (Westley 1973). Sulfur transferases (STs) consist of two globular domains (N-terminal and C-terminal) of nearly identical size and conformation connected by a short linker sequence which is prolonged in ST sequences from plants (Fig. 1). The C-terminal domain contains the active site cysteine residue. In plant sequences the connecting linker sequence between both domains is quite long in comparison to sequences from prokaryots and other eukaryots. We would like to elucidate the role of the prolonged linker and each single domain of STs from *Arabidopsis thaliana*.

The N-terminal and the C-terminal domains without and including the linker of ST1 from *Arabidopsis* (Acc. no. CAB64716) were expressed in *E. coli* with a 6xHis-tag at the N-terminus, purified by nickel-affinity chromatography and used for enzyme activity determinations. Enzyme assays were done as described using 3-mercaptopyruvate as sulfur donor and cyanide as sulfur acceptor (Papenbrock and Schmidt 2000).

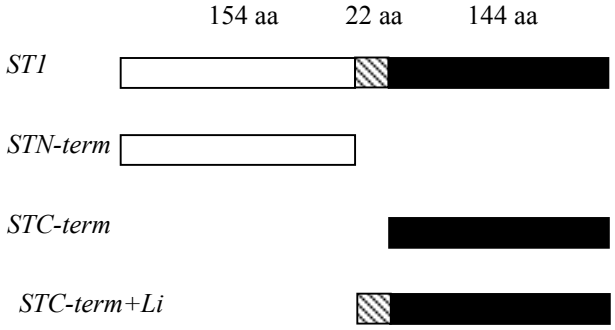

Fig. 1. The *Arabidopsis* ST1 protein consists of two domains, an N-terminal (STN-term) and a C-terminal domain (STC-term). Both are connected by a prolonged linker. Li, linker.

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The N-terminal domain of ST1 (STN-term) alone did not show enzyme activity at all whereas the activity of the C-terminal domain of ST1 (STC-term) without and including the linker sequence was very low but anyway well measurable (Fig. 2). To investigate the possibility of reconstitution the full activity compared with the activity of the complete recombinant ST1, increasing amounts of the STN-term were added to the STC-term without and including the linker. Increasing amounts of the STN-term protein resulted in increased enzyme activities for STC-term without and STC-term including the linker sequence. With regard to the specific activity of the whole ST1 enzyme the levels were much lower (Burow *et al*. 2002). Interestingly, the combination of the STN-term with the STC-term including the linker resulted in lower specific activities than the interaction with the STC-term without the linker. When thiosulfate was used as substrate neither the STC-term including nor the STC-term without the linker did show any activity.

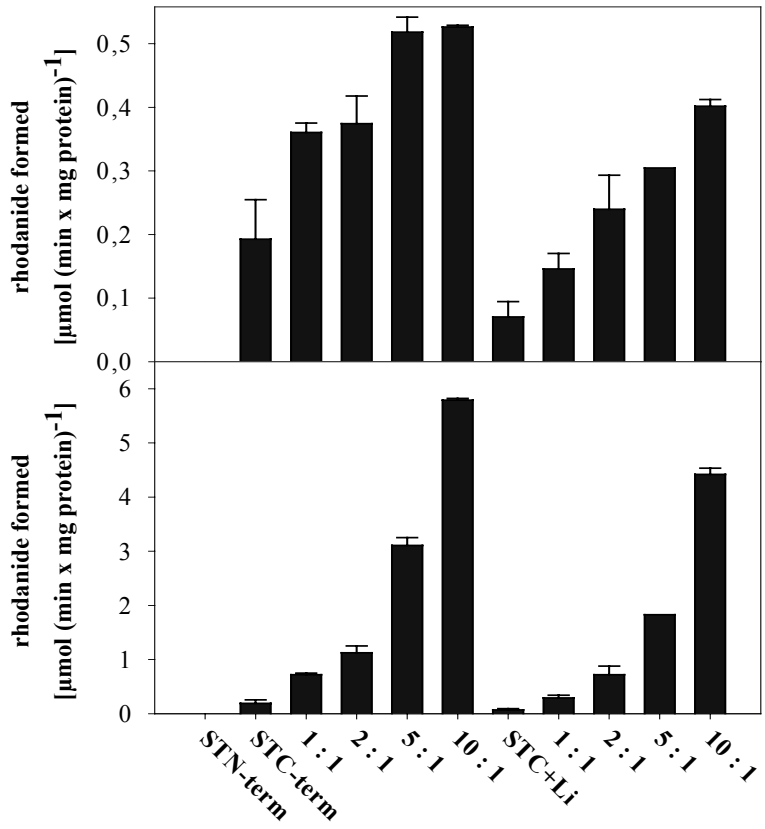

Fig. 2. The N-terminal domain was combined with the C-terminal domains without and including the linker in increasing mass-to-mass ratios and used for enzyme activity measurements as described. Sulfur donor: 3-mercaptopyruvate; sulfur acceptor: cyanide. (A) The specific enzyme activity was calculated on the basis of the total protein or (B) on the STC-term protein content.

The enzymatic activity of the *Arabidopsi*s ST1 resides in the C-terminal domain, which contains the active site cysteine residue, but is boosted by the N-terminal domain and the linker peptide in the full-length enzyme. The presence of about two times more N-terminal protein molecules might protect the active site cysteine residue better than equal numbers of molecules. On account of this the role of the prolonged linker remains unclear because the specific activities were even lower in the combination experiments. Probably neither 3-mercaptopyruvate nor thiosulfate are metabolized in the plant organism by STs. A larger substrate might need the extended protective hydrophobic environment of the prolonged linker sequence in plants.

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APPLICATION OF BIACORE TECHNOLOGY FOR THE ANALYSIS OF THE PROTEIN INTERACTIONS WITHIN THE CYSTEINE SYNTHASE COMPLEX

Oliver Berkowitz[1], Markus Wirtz[1], Alexander Wolf[2], Jürgen Kuhlmann[2] and Rüdiger Hell[1]

[1]Institute of Plant Genetics and Crop Plant Research (IPK), 06466 Gatersleben, Germany; [2]Max-Planck-Institute for Molecular Physiology, 44227 Dortmund, Germany

Cysteine biosynthesis represents the exclusive entry of reduced sulfur into plant metabolism. The formation of cysteine is initiated by the production of the intermediate *O*-acetylserine (OAS) catalyzed by serine acetyltransferase (SAT). Sulfide is then incorporated into cysteine by *O*-acetylserine (thiol)lyase (OAS-TL) through the consumption of OAS. Both SAT and OAS-TL together form the cysteine synthase complex (CSC) but no channeling of OAS can be observed. SAT is fully active only in the complex with high affinity for the substrates and becomes unstable and inactive upon complex dissociation. In contrast, OAS-TL is active only as the free dimer with high affinities for the substrates but inactive in the complex (Droux *et al*. 1998; Wirtz *et al*. 2001). A regulatory model integrates the metabolic control of cysteine biosynthesis by reversible dissociation of the CSC with the observed de-repression of genes encoding for sulfate transporters (Hell *et al*. 2002). Upon sulfur starvation sulfide is lacking and OAS accumulates to a CSC dissociating threshold. Thereby SAT becomes inactive, preventing further serine and acetyl-CoA consumption. In parallel re-supply of sulfate through induced sulfate transporters is initiated. After sulfate reduction the OAS level drops through OAS-TL activity and the CSC can re-associate.

To analyze the protein interaction within the CSC and the possible regulatory role of OAS quantitatively, we applied surface plasmon resonance based technology with the BIAcore system (Myszka 1997). In this system the association and dissociation of proteins on the biosensor surface results in an increase and decrease, respectively, of the signal which is visualized as a sensorgram in real time.

Immobilization of 6xhis-SAT on the sensor chip surface to 1000RU was performed by injection to the Ni-loaded NTA biosensor surface and interaction analysis was carried out by applying OAS-TL concentrations of 20 to 200nM. Injections of saturating amounts of OAS-TL led to a molar ratio of 2:1 for SAT and OAS-TL monomers. This might indicate steric blocking of 6xhis-SAT bindings sites for OAS-TL due to the attachment to the chip surface. But also the recently reported

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quarternary structure for the *E. coli* SAT homomer as a dimer of trimers (Hindson *et al*. 2000) might be a explanation. For the determination of the association and dissociation rate constants sensorgrams were analyzed by fitting the data to the equations for a simple A+B ⇔ AB model. Experimental data for the association phase showed only minor deviations from obtained fits in the range of only approx. 2 % of the overall amplitude for the residuals (Fig. 1A). Data fits of the dissociation phase of SAT/OAS-TL interaction showed an independence of the OAS-TL concentration as expected. Fits revealed slightly higher deviations from the experimental data at around 5 % of overall amplitude for the residuals (Fig. 1B) but still provided a good determination of the dissociation rate constant. The observed deviation from the model can be explained by intermediate steps in the dissociation. Analysis of SAT and OAS-TL interaction lead to the determination of the equilibrium dissociation constant K_D at about 25 nM. We also analyzed the effect of OAS concentration on the CSC dissociation. Concentrations of only 1 µM OAS showed already a clear effect with the strongest changes in dissociation rate in the lower physiological range at approximately 80 µM OAS (Berkowitz *et al*. 2002).

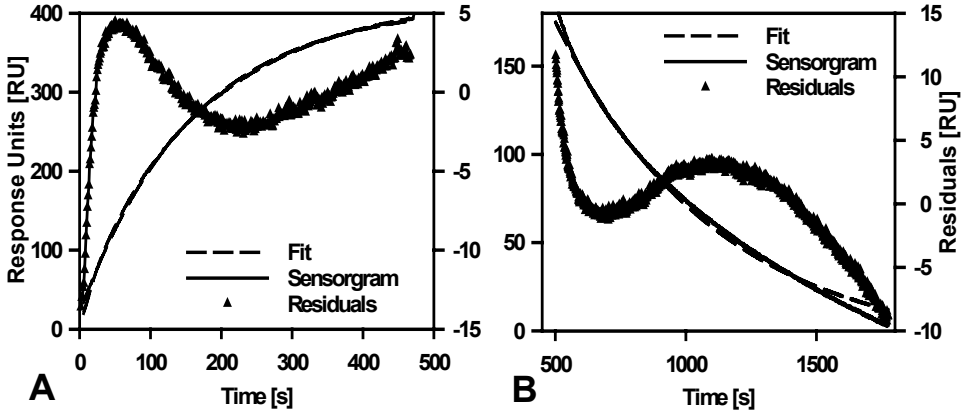

Fig. 1. Analysis of the association and dissociation of SAT and OAS-TL. Shown are the sensorgrams of experimental data (—), fits to this data according to the equations of the A+B ⇔ AB Model (---) and the residuals plot (▼) for the association (A) and dissociation (B) of SAT/OAS-TL (note the different scales for data/fits and residuals). Residuals in the range of 2 % and 5 % of overall amplitude in association and dissociation phase, respectively, demonstrate the correctness of the model.

The presented results provide the quantitative basis for the function of the CSC as a molecular switch that controls sulfate assimilation and cysteine biosynthesis, supporting the current regulatory model (Hell *et al*. 2002). The monitoring of the sulfur status of the cell by the CSC adjusts the enzyme activities of SAT and OAS-TL to the availability of sulfate through the modulation of its dissociation state.

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INFLUENCE OF SULFUR AVAILABILITY ON NON-PROTEIN THIOLS CONTENTS IN TRANSGENIC TOBACCO LINES PRODUCING BACTERIAL SERINE ACETYLTRANSFERASE

Anna Błaszczyk and Agnieszka Sirko
Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawinskiego 5A, 02-106 Warsaw, Poland

Transgenic tobacco lines overproducing either wild type or mutated (insensitive to the feedback inhibition by cysteine) form of *Escherichia coli* serine acetyltransferase (SAT) in either chloroplasts or the cytosol have been described previously (Blaszczyk *et al.* 1999, 2002). Significant differences in enzymatic activities and chemical composition were observed between the transgenic SAT-overproducers and control plants. The conclusion from these results was that the observed effects depended rather on the targeting of the bacterial enzyme but not on the SAT isoform used. In an attempt to investigate further the differences between various SAT-overproducing transformants and the wild type plants an additional approach has been taken in this study. We investigated the influence of sulfur nutrition (both deficit and surplus) on the level of the non-protein SH-containing compounds in three groups of plants: (i) the control wild type tobacco (*Nicotiana tabacum* cv. LA Burley 21), (ii) transgenic CE-20 line producing wild type *E. coli* SAT targeted to the cytosol, (iii) transgenic RCEM-18 line producing mutated *E. coli* SAT targeted to the chloroplasts.

Three individuals of T_1 generation of each analyzed line were grown hydroponically for 3 weeks in the medium containing 1 mM sulfate. Subsequently, the plants were transferred for either 2 or 6 days to the following media: (i) the control conditions (1 mM sulfate), (ii) sulfur deficit (lack of any sulfur source) for either 2 or 6 days, and (iii) sulfur excess (10 mM). The plant tissue material was collected separately from young leaves, mature leaves, roots and stems, and analyzed.

The results of the thiols assay in various plant organs are shown in Fig. 1. The strongest effects of sulfur nutrition were observed in the roots and the young leaves of all analyzed plant lines. However, upon 6 days of sulfur starvation the significant drop of thiols levels was observed in all parts of the plants. In the normal conditions the thiols contents in the roots of the control plants was quite low. In contrast, the roots of plants that belong to both transgenic lines had strongly increased levels of thiols. This is probably due to the choice of promoter used for the expression of SAT that enables the strongest transgene expression in the root tissues. Statistically

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significant differences in the distribution of thiols in response to sulfur nutrition between control plants and SAT-overproducers might suggest that the excess of SAT affects the plant intrinsic regulatory mechanisms responsible for maintaining the appropriate balance of sulfur in various organs. However, it is necessary to emphasize that although the average data are from the assays of three independent plants, the experiment has been performed with the single transgenic lines from each group. Therefore, for more conclusive results it would be necessary to confirm these pilot experiments with a larger number of independent transformants from each groups.

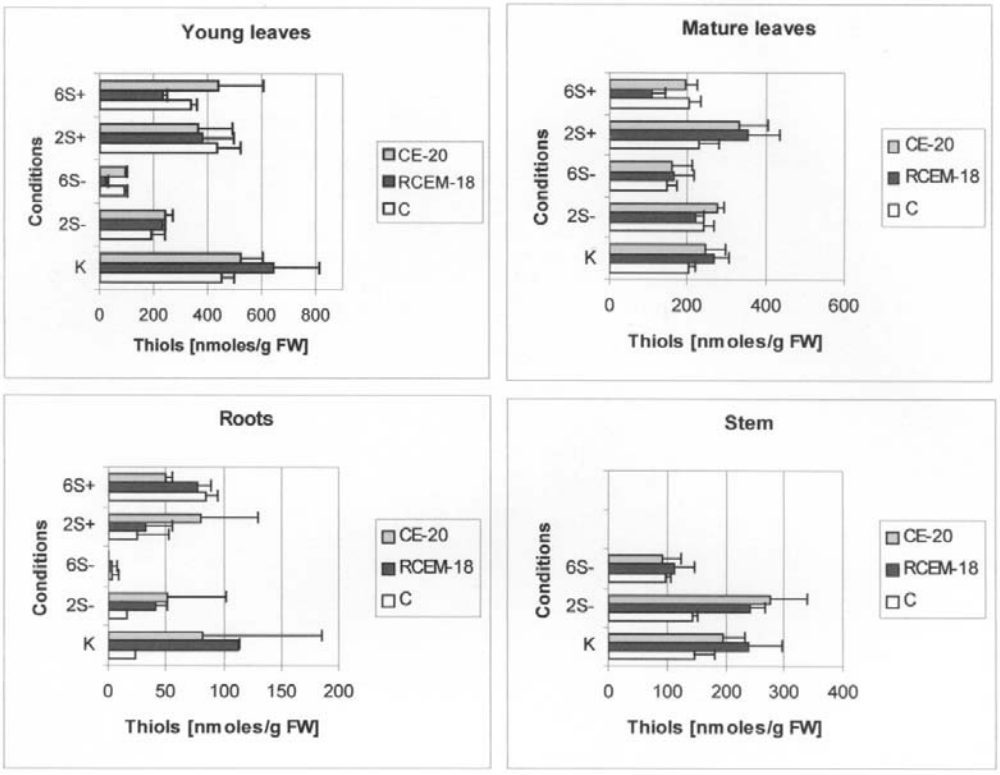

Fig. 1. Levels of low-molecular-weight non-protein SH-containing compounds in the organs of hydroponically grown plants. The samples were collected from plants grown in the control conditions (K), sulfur deficit for 2 days (2S-) or 6 days (6S-), and sulfur surplus for 2 days (2S+) or 6 days (6S+). The results are average of the three independent individuals of T_1 generation of each line. For each individual plant the assay had been done twice. C- control, parental line (LA Burley 21).

Acknowledgements

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INFLUENCE OF THE SULFUR SUPPLY ON THE (ISO)ALLIIN CONTENT IN LEAVES AND BULBS OF *ALLIUM CEPA* AND *ALLIUM SATIVUM*

Elke Bloem, Silvia Haneklaus and Ewald Schnug
Institute of Plant Nutrition and Soil Science, Federal Agricultural Research Centre (FAL), Bundesallee 50, 38116 Braunschweig, Germany

Onions (*Allium cepa* L.) and garlic (*Allium sativum* L.) were one of the earliest cultivated crops and are popular in folk medicine since the middle age. Onions promote digestion, cure mild burns and asthma. Garlic is part of phytopharmaceuticals taken against arteriosclerosis and high blood pressure. One of the active compounds is the sulfur (S) containing (iso)alliin. The alliin content is an important quality criteria of the fresh product and when used as phytopharmaceutical. The recommended daily intake of alliin in form of garlic is 4 - 12 mg. It was the aim of this study to investigate the influence of the S supply on the (iso)alliin content of onions and garlic.

A greenhouse trial with onions and garlic was carried out. The plants were grown in Mitscherlich pots containing 8 kg of sand. S was applied in the rates 0, 50 and 250 mg pot^{-1}. Additionally a field trial with onions was conducted with S application rates of 0, 30, 60 and 90 kg ha^{-1} S. The (iso)alliin content in leaves and bulbs was determined by HPLC according to Hoppe *et al.* (1996).

Table 1. (Iso)alliin content in leaves and bulbs of onions and garlic grown in the greenhouse under different S levels.

	Sulfur fertilization	1st sampling		2nd sampling	
	[mg S pot^{-1}]	Leaf	Bulb	Leaf	Bulb
		Alliin content [mg g^{-1}]			
Garlic	0	12.8	4.2	5.3	0.9
	50	16.0	5.6	11.6	3.1
	250	16.9	6.5	12.3	2.9
Onion	0	3.4	0.5	0.1	0.7
	50	6.2	1.2	2.3	1.6
	250	9.1	0.8	5.4	3.2

1st sampling: 4 (onions)/ 7 (garlic); 2nd sampling: 9 (onions)/ 14 (garlic) weeks after planting

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The (iso)alliin content of garlic and onions changed significantly during the vegetation period (Table 1) with decreasing concentrations in the leaves. S fertilization increased the (iso)alliin content in bulbs of garlic and onions about 3 - 4.5 times (2nd sampling). The absolute increase of the (iso)alliin content in the leaves was even higher than in the bulbs: S fertilization increased the alliin content in leaves of garlic around 4 - 7 mg g^{-1} and in leaves of onions around 5.3 - 5.7 mg g^{-1} (Table 1). The isoalliin content correlated significantly with the total S content in bulbs of onions (Fig. 1). The relationship between both parameters was very similar at the two sampling dates (16 and 23 weeks after sowing). In leaves, 38 % of the total S and in the bulbs, more than 50 % of the total S was metabolized to isoalliin. Thus the higher the S supply of onions and garlic, the more alliin is metabolized in leaves and bulbs and consequently, the higher is the quality of the product.

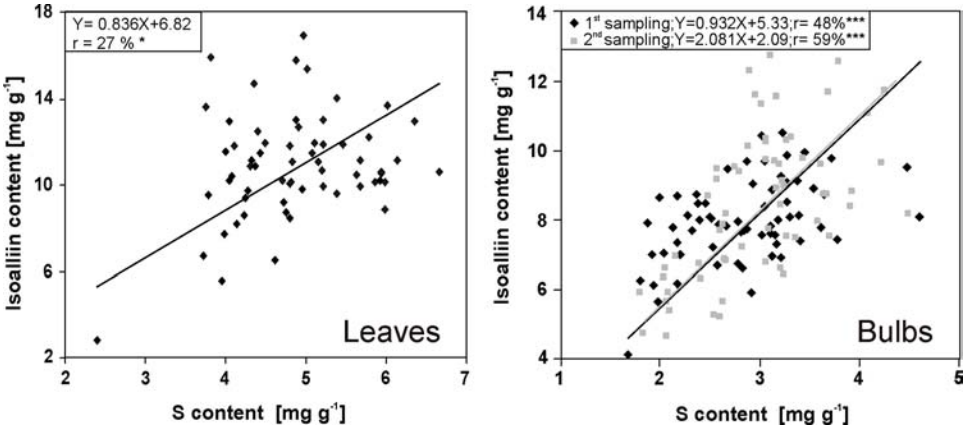

Fig. 1. Relationship between isoalliin content and total sulfur content in bulbs and leaves of *Allium cepa* L (field experiment). (1st sampling 16 weeks after sowing, 2nd sampling at harvest).

The customer decides whether he wants to use fresh products or phytopharmaceuticals. (Iso)alliin decomposes extremely fast after peeling and cutting of the onion or garlic bulb. It can be assumed that only 7 % of intact (iso)alliin is consumed when the fresh product is used. This means that the daily intake of only 1 onion which was fertilized with S would be sufficient to cover the demand of 12 mg alliin. In contrast, 1.75 bulbs from fresh onions grown at a low S supply need to be consumed for a similar dose.

The results imply for the phytopharmaceutical use that the maximum dose of (iso)alliin in onions can be increased by S fertilization from 5.8 to 9.6 mg g^{-1}. For the customer this means that the amount of onions in the diet can be almost halved. Garlic may contain even higher alliin contents compared to onions. The results of the presented experiments reveal that S fertilization significantly enhances the phytopharmaceutical quality of onions and garlic.

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MODEL BASED PROGNOSIS OF SULFUR DEFICIENCY

Elke Bloem, Silvia Haneklaus and Ewald Schnug
Institute of Plant Nutrition and Soil Science, Federal Agricultural Research Centre (FAL), Bundesallee 50, 38116 Braunschweig,Germany

Plant available soil sulfate is highly mobile in agricultural soils with a pH > 5 and follows the water movement in the soil. Therefore, the analysis of soil sulfate delivers only a momentary value which can change quickly. The result is a very high temporal and spatial variability of sulfate in soils (Bloem *et al.* 2001). The assessment of the plant sulfur (S) status requires a method which predicts the supply reliably. Only then, the S fertilizer demand can be addressed site-specifically.

It is the aim of the newly developed model MOPS to produce S risk maps for agricultural sites. The model is based on site-specific parameters and processes which are used to calculate the S supply of any agricultural site. In table 1 the main processes and components are listed which characterize the S supply of a site as well as the required input parameters. Site-specific stable and variable factors can be used to calculate parameters, which characterize the water flow in the soil. Factors which affect the soil water regime such as soil texture, groundwater level and climatic conditions are the most important parameters of the model, because of the high mobility of plant available sulfate in agricultural soils.

Groundwater contains about 5 - 100 mg l^{-1} sulfate-S and is therefore a huge storage pool for S. When plants have access to groundwater, either directly, or *via* capillary rise they are regularly sufficiently supplied with S. Investigations also revealed a close relationship between soil texture and S nutritional status of oilseed rape (Bloem 1998). The higher the clay content was, the better was the S nutrition of the crop. With increasing clay contents soils store more water and thus also plant available sulfate. Sulfate leaching decreases while capillary rise and field capacity are higher. The climatic conditions, especially over winter, are also important as precipitation and evapo-transpiration together with soil texture influence the extent of leaching and capillary rise. The individual crop and the crop rotation are important for calculating the S demand (oilseed rape > cereals > sugar beet), the biodegradability of the residues (sugar beets > cereals) and the time of the highest S demand: oilseed rape and cereals have the highest demand in spring while that of sugar beet and corn is during summer. This means that these crops can use S inputs through capillary rise or by irrigation.

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Table 1. Main processes of the model for the prediction of sulfur deficiency (MOPS) and relevant input parameters.

Main processes	Input parameters
Leaching of S	*Soil texture, field capacity, precipitation, temperature, groundwater level, organic matter content*
Capillary rise of S-rich soil water	*Groundwater level, soil texture, precipitation, temperature, evapo-transpiration*
Lateral S-fluxes	*Geomorphology*[1)]
Mineralization/immobilization	*Organic matter content, soil water content, temperature, soil structure*
Weathering of parent material	*Geology (important for S-rich parent material)*
Main components	**Derived parameters**
Constant:	
Groundwater level	*Capillary rise, soil water content, redox-conditions*
Groundwater S concentration	*Amount of S input*
Soil texture	*Leaching, capillary rise, storage of sulfate, field capacity*
Stable:	
Precipitation	*Leaching, mineralization, evapo-transpiration*
Temperature	*Capillary rise, leaching*
Crop type	*S demand, residual S*
Cultivation	*S supply by irrigation, fertilization, crop rotation*
Rooted soil depth	*Storage of sulfate*
Other available data:	
Nitrate leaching model	*Sulfate leaching*

[1]Calculated from DEM (Digital Elevation Models); *italic* are parameters which are available in digital form for Northern Germany

Various digital data are available for different soil features and weather conditions in a scale of 1 : 5000 to 1: 500,000 for Northern Germany. All important processes (Table 1) can be calculated from available digital data. Most of the parameters are inter-correlated such as leaching, texture and precipitation. Algorithms have been developed to combine the different parameters in such way that the risk of S deficiency can be calculated for different sites and crops. At the moment the model is validated for different training areas in Northern Germany. First results confirm earlier findings that S deficiency is not probable if the groundwater level is near the surface. Loamy soils are regularly better supplied with S than sandy soils and heavy rainfall especially during winter increases the risk of S deficiency.

A model based prognosis of S deficiency on the basis of digital data is a promising way to deliver S risk maps for farmers. The method is easy and the results are reliable as the calculations are based on stable field parameters such as groundwater and soil texture, and easy to assess variable factors such as climate and crop rota-

tion. MOPS is designed for agricultural sites in Lower Saxony, but a simplified questionnaire may be used to obtain estimates for any other areas (Bloem 1998).

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GLUTATHIONE TRANSPORTER HOMOLOGUES FROM *ARABIDOPSIS THALIANA*

Olivier Cagnac, Andree Bourbouloux and Serge Delrot
UMR CNRS 6161,Transport des assimilats, Physiologie, Biochimie et Biologie Moleculaire Végétales. Université de Poitiers, 40 avenue du Recteur Pineau 86022 cedex Poitiers, France

GSH plays a key role in plant defense against biotic and abiotic stresses. The most efficient detoxifying compounds derived from GSH are *S*-conjugates and phytochelatins; they take part in the inactivation and compartmentation of organic xenobiotics including heavy metals. The transport of Cd-phytochelatins and *S*-conjugates through the tonoplast is catalyzed by ABC transporters (Martinoia *et al*. 1993), which have been cloned in plants and yeast (Li *et al*. 1996).

Plasma membrane transport was studied in our lab (Jamai *et al*. 1996) and allowed for the physiological characterization of a proton/GSH co/transporter in *Vicia faba* cells. This transporter can also transport GSSG and *S*-conjugates. It has been cloned in yeast for the first time in our lab (Bourbouloux *et al*. 2000). This *HGT1* gene encodes for a high affinity GSH transporter in *S. cerevisiae*. Protein sequence data show that several *HGT1* homologues are present in yeast and plant genomes but not in *E. coli* and animal cells. Nine homologues were found in *Arabidopsis* genome. These nine transporters contain several highly conserved domains. Three cDNA corresponding to AtOPT3, 6 and 7 were amplified by PCR, and sub-cloned in the pDR shuttle vector under control of PMA1 promoter. These constructions were used to transform the yeast strain ABC 822 which is disrupted on the *hgt1* gene. A growth test assay was performed in a sulfur-free liquid medium, all salts containing sulfur being replaced by chloride salts. This medium was complemented with 500 μM GSH as the sole source of sulfur. On this medium, the wild type at first was able to grow exponentially before leveling-off after 40 h. The disrupted strain was unable to grow in such conditions. ABC 822 transformed with the *Arabidopsis* cDNA was also able to grow, but more slowly than the wild type. The same experiment was performed with 500 μM of GSSG instead of GSH; similar results were obtained. The three type of yeasts had similar growth kinetics on SC-medium. We thus concluded that AtOPT6 partially restored the wild phenotype. GSH uptake was then measured with radio-labelled GSH. These reactions were performed in MES/KOH buffer at pH 5.0. The uptake was measured at 1, 3 and 5 min and contrary to the *AtOPT6* clone, yeast-822 transformed with the empty vector does not show any increase of GSH absorption over time. These results confirm those previously obtained. To determine the specificity of the GSH transporter, we perfomed

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competition experiments with unlabelled amino acids, dipeptides and tripeptides, the competitors being 10 times more concentrated than labelled GSH. Methionine, glutamine, some dipeptides, as well as GSSG and GS-NEM were all more competitive than GSH. In parallel, the amount of mRNA level in the leaves, stems and roots of 6 week-old *Arabidopsis* plants stressed by 20 μM Cd or 80 nM Primisulfuron during 24 h was monitored. We used the Quantitative RT-PCR technique to detect *AtOPT6* expression. Cd^{2+} treatments had no effect on AtOPT6 expression, whereas Primisulfuron increases transcript level significantly in roots and shoots, but not in leaves.

It may be concluded that *AtOPT6* expression partially restored the wild phenotype. This result was confirmed by kinetics assay. Even though AtOPT6 mediates the transport of GSH GSSG and *S*-conjugate, it is not strictly specific for GSH. Results obtained on a rice HGT1 homologue confirm some of this results.

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SULFUR STATUS AND ACQUISITION IN RESPONSE TO DIFFERENT SULFATE CONCENTRATIONS AND TIME EXPOSURE IN MAIZE

David Carden, Silvia Quaggiotti and Mario Malagoli
Dipartimento di Biotecnologie Agrarie, Università degli Studi di Padova, Agripolis, Via dell'Università 16, I-35020 Legnaro, Italy

For sulfate acquisition, plants possess a variety of identified transporters which differ in their expression, location, affinity, and regulation. Their regulation, however, is not fully understood. To complement these molecular data, whole-plant physiology is also needed to identify physiological traits important for agricultural use. With environmental considerations arguing against high-input agriculture, there is also an emphasis on sufficient productivity under conditions of low input.

Previously two hybrids of maize (*Zea mays* L.), which both performed well under conditions of low-input, were shown to differ in their SO_4^{2-} uptake characteristics (Quaggiotti *et al.* 2000). To investigate further, the constitutive and induced uptake systems of both hybrids were compared by measuring the short-term uptake of $^{35}SO_4^{2-}$ at different concentrations. Fig. 1A shows that both cultivars had similar SO_4^{2-} uptake rates of approximately 135 nmol g^{-1} h^{-1} for the high-affinity (100 μM) constitutive uptake system, and this similarity may be related to their similar low-input agricultural yield (KWS Italia, personal communication). At higher concentrations of 200 μM and 1 mM both hybrids recorded higher uptake rates, but hybrid KW7 was more efficient, suggesting differences between their constitutive systems in this concentration range. This matched the greater productivity of KW7 under high-input conditions.

Additionally, the characteristics of the de-repression of the sulfate uptake systems were investigated by starving both hybrids of sulfate after previously being grown with sulfate. A typical de-repression of uptake was measured for both cultivars already 4 h after the onset of starvation, although the hybrid KW2 had higher uptake rates than those of KW7 throughout the experiment (Fig. 1B). The rates for KW2 after 72 h of starvation reached over 900 and 2000 nmol g^{-1} h^{-1} when measured at an external concentration of 100 and 200 μM respectively, while KW7 reached almost 700 and 1300 nmol g^{-1} h^{-1} (Fig. 1B). The fact that KW2 recorded higher induced uptake rates than those of KW7 is in contrast to their constitutive uptake rates. These results indicate that the efficiency of the inducible high-affinity uptake systems is independent to that of the constitutive high-affinity systems, suggesting that the suitability of physiological markers for agriculture would vary ac-

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cording to the criteria examined. Strongly buffering the pH (10 mM MES/Tris, pH 5.7) of the 100 µM uptake solution, preventing differences in external pH altering the thermodynamics of H^+-coupled co-transport, did not change the induced uptake differences between the cultivars when measured at 48 h (718 and 461 nmol g^{-1} h^{-1} for KW2 and KW7 respectively). This indicates that the observed uptake rate differences between the cultivars were not due to differences in the root surface pH which might arise from altered pump activity.

In addition to uptake differences, the proportion of sulfate translocated to the shoot can also differ between hybrids (Quaggiotti *et al.* 2000). This was further investigated using the time course experiment at 100 µM SO_4^{2-} in Fig. 1B. The proportion of sulfate translocated to the shoot in this short-term experiment increased from approximately 10 % at 0 h to over 30 % after 96 h, with that of KW7 consistently 2 to 5 percentage points higher than that of KW2. The greater SO_4^{2-} translocation of KW7 is in contrast to the differences in efficiency of induced uptake, suggesting the possible importance of translocation efficiency for crop productivity.

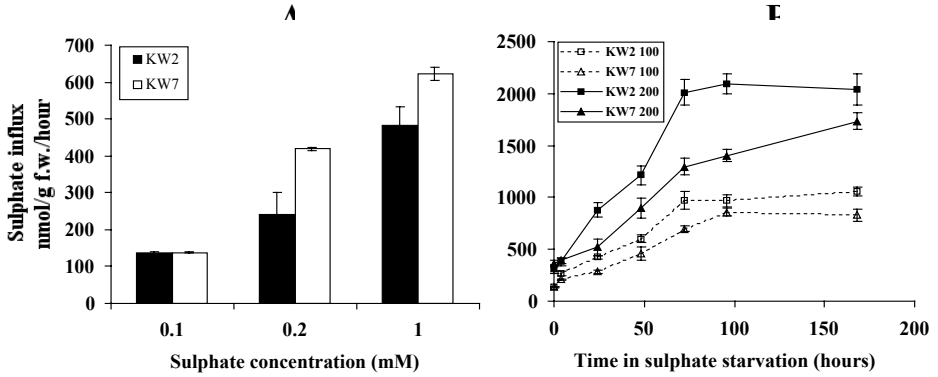

Fig. 1. Total plant uptake of sulfate by two hybrids of *Zea mays*. A) The constitutive uptake at three different SO_4^{2-} concentrations of 0.1, 0.2 and 1 mM. The hybrids were grown previously for seven days in SO_4^{2-}- containing solutions. B) The de-repression of uptake by sulfate starvation for 7 days, during which uptake was measured at two different sulfate concentrations. The plants were previously grown in the presence of SO_4^{2-}. All plants were grown hydroponically in a complete nutrient solution, and sulfate uptake was determined by measuring the short-term (10 min) uptake of $^{35}SO_4^{2-}$. Data presented are means of 3 to 4 bunches of 4 to 5 plants each. Error bars represent SE.

These results together indicate the complexity of SO_4^{2-} uptake and translocation mechanisms. Furthermore, the results suggest that similar transport mechanisms can be differently regulated even between two such closely related genotypes as hybrids of the same species. The fact that the constitutive uptake rates of both cultivars also correlates with their field performance under both high- and low-input conditions (KWS Italia, personal communication) supports the linking of efficiencies of field productivity of selected populations with physiological characteristics such as

whole-plant V_{max} and K_m measured using short-term uptake at different concentrations (Malagoli *et al.* 1993).

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NITROGEN AND SULFUR REQUIREMENT OF *BRASSICA OLERACEA* L. CULTIVARS

Ana Castro, Ineke Stulen and Luit J. De Kok
Laboratory of Plant Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Proteins contain both sulfur and non-sulfur amino acids and for this reason the availability of nitrogen and sulfur interacts with the utilization of nitrogen and sulfur for proteins and plant growth. Cysteine plays a key role in the synthesis of organic sulfur compounds and its synthesis is a major reaction in the direct coupling between sulfur and nitrogen metabolism in the plant (Brunold 1993). In general, research on uptake and metabolism of nitrogen and sulfur by plants has mainly been focussed on elucidation and characterization of the pathways and of the transporters and the enzymes involved (Stulen and De Kok 1993). Conclusions on the possible mutual regulation of nitrogen and sulfur metabolism have often been based on changes in activity and/or expression of the transporters and enzymes by imposing pedospheric sulfur and nitrogen deprivation. The main goal of our current research is to obtain more insight into the interrelated regulatory aspects of uptake and assimilation of nitrogen and sulfur, at the whole plant level under steady state conditions. In order to search for plants differing in nitrogen and sulfur requirement for growth, a preliminary comparative study was carried out with five cultivars of *Brassica oleracea* L.

There were only slight differences in relative growth rate (RGR), shoot/root ratio, dry matter content, and S and N content between the tested cultivars of *Brassica oleracea* L. (Table 1). Cultivar Tarvoy had the highest total N and the lowest total S content, while cultivar Arsis had the highest total S and the lowest total N content. Apparently in all five *B. oleracea* cultivars sulfur is mainly present as sulfate, *viz.* between 70 - 88 % of the total sulfur content.

Calculation of the nitrogen and sulfur requirement for growth from the data given in Table 1 shows that cultivar Tarvoy has the highest nitrogen requirement, while cultivar Arsis has the highest sulfur requirement (Fig. 1). However, the main fraction of the total sulfur content is in the non-reduced, inorganic form. The concept of sulfur requirement for "growth", therefore, has to be re-defined. For "structural growth" organic sulfur is the better parameter in the calculation of sulfur requirement.

The observed differences in nitrogen and sulfur requirement were not very substantial, and the pre-dominant proportion of the sulfur was present as sulfate. Therefore, we conclude that the *B. oleracea* cultivars used in this study are not suitable

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for comparative experiments on the regulatory control of nitrate and sulfate assimilatory pathways.

Table 1. Growth parameters and inorganic and organic sulfur and nitrogen compounds in seedlings of five *B. oleracea* cultivars; curly kale cv. Arsis, red cabbage cv. Rodon, pointed cabbage cv. Duchy, white cabbage cv. Castello and savoy cabbage cv. Tarvoy. For growth conditions, growth analysis and analysis of inorganic and organic N and S compounds see Westerman *et al.* (2000, 2001a,b). Relative growth rate (RGR) on a fresh weight (FW) basis is expressed as g g^{-1} day^{-1}, shoot to root ratio (S/R) is given on a FW basis, dry matter content (DMC) is given as % of FW, nitrogen and sulfur compounds are expressed as μmol g^{-1} FW. Data represent the mean of 3 experiments, with 12 plants in each (± SD).

	Growth parameters			N and S compounds				
	RGR	S/R	DMC	Total N	NO_3^-	Amino acids	Total S	SO_4^{2-}
Arsis	0.37±0.04	4.0±1.3	11.6±0.4	416±54	100±20	15±1.8	50±5.0	44±1.7
Rodon	0.33±0.03	5.0±1.0	11.2±0.9	432±15	97± 9	13±0.8	47±4.8	42±3.6
Duchy	0.32±0.09	5.1±0.6	12.7±0.8	434± 6	99±20	12±4.9	46±9.6	36±3.8
Castello	0.35±0.02	4.7±0.4	11.9±1.4	465±34	131±20	18±1.1	47±8.0	40±4.0
Tarvoy	0.30±0.04	3.5±0.5	13.6±1.9	546±11	99±20	19±3.4	44±8.0	32±7.2

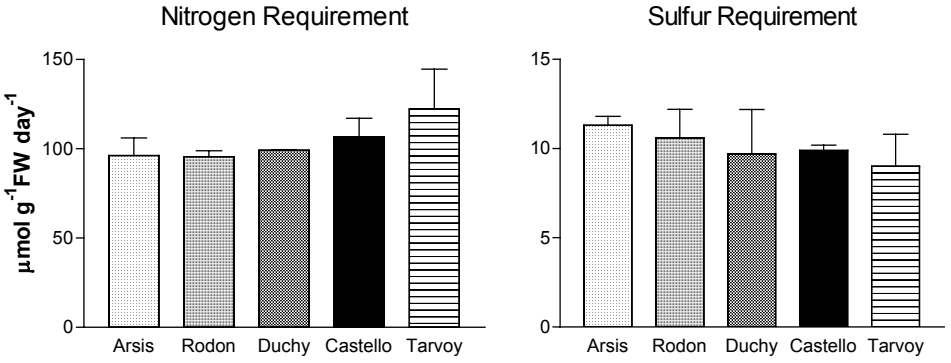

Fig. 1. Nitrogen and sulfur requirement of five cultivars of *B. oleracea*, calculated on the basis of the data on RGR and total nitrogen and sulfur content given in Table 1, after De Kok *et al.* (2000).

H_2S impact studies showed that sulfate uptake by the roots of seedlings of *B. oleracea* is well regulated and adjusted to the organic sulfur requirement for growth (De Kok *et al.* 2000; Westerman *et al.* 2000, 2001a,b). The occurrence and nature of the extremely high ratio of sulfate to organic sulfur in shoots of seedlings of *B. oleracea* needs further evaluation.

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GLUTATHIONE CONTENT AND STATUS OF *IN VITRO* PLANTLETS TRANSFERRED TO *EX VITRO*: DEMAND FOR GROWTH OR RESPONSE TO OXIDATIVE STRESS?

Luísa De Carvalho and Sara Amâncio
DBEB/CBAA, Instituto Superior de Agronomia, 1349-017 Lisboa, Portugal

In plants glutathione is the main storage and transport form of organic sulfur, its allocation regulating plant growth and the defense to oxidative stress. GSH accounts for *ca.* 90 % of the total glutathione (Noctor and Foyer 1998), however, the levels of GSSG may increase under severe stress. After transfer to *ex vitro* under higher irradiances, *in vitro* plantlets can show photoinhibition and oxidative stress. The aim of this research was to relate the high GSH in *in vitro* and *ex vitro* plants to the fast growth during acclimatization or to oxidative stress. High CO_2 was applied to evaluate the reversion of oxidative stress.

Grapevine and chestnut microplantlets were acclimatized until full development of two (in some cases three) new leaves (Table 1), maintaining some of the *in vitro* leaves (persistent). GSH and GSSG content, and GR activity were determined as described respectively by Anderson *et al*. (1992) and Schaedle and Bassham (1977).

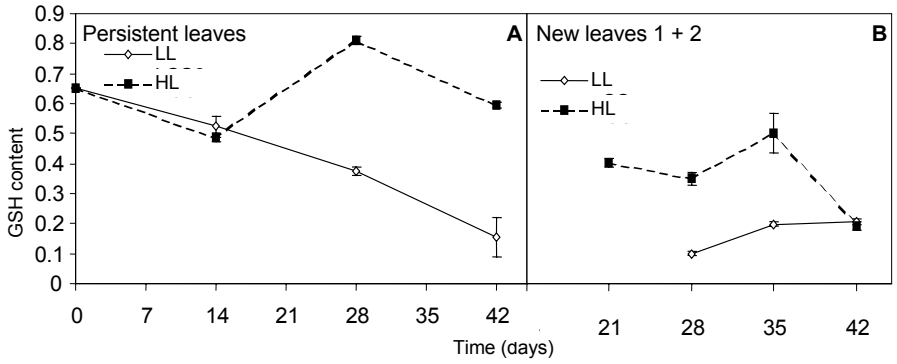

Fig. 1. GSH content in persistent (A) and first and second new leaves (B) of chestnut (μmol g^{-1} fresh weight) under LL, 150 μmol m^{-2} s^{-1} LCO_2, 350 μl l^{-1} and HL, 300 μmol m^{-2} s^{-1} HCO_2, 700 μl l^{-1} .

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Table 1. Number of new leaves (NL) and GR activity in new leaves of grapevine and chestnut (μmol NADPH oxidized mg^{-1} protein min^{-1}). Values followed by letters (a, b, c) are significantly different, $p < 0.001$. For abbreviations see Fig. 1.

	Grapevine				Chestnut			
Time	NL	LL LCO_2	NL	HL HCO_2	NL	LL LCO_2	NL	HL HCO_2
21	2	942a	2	861b	-		1	644a
28	2	966a	3	343a	2	590a	2	761b
35	-		-		2	712b	2	912c
42	-		-		2	524a	3	900c

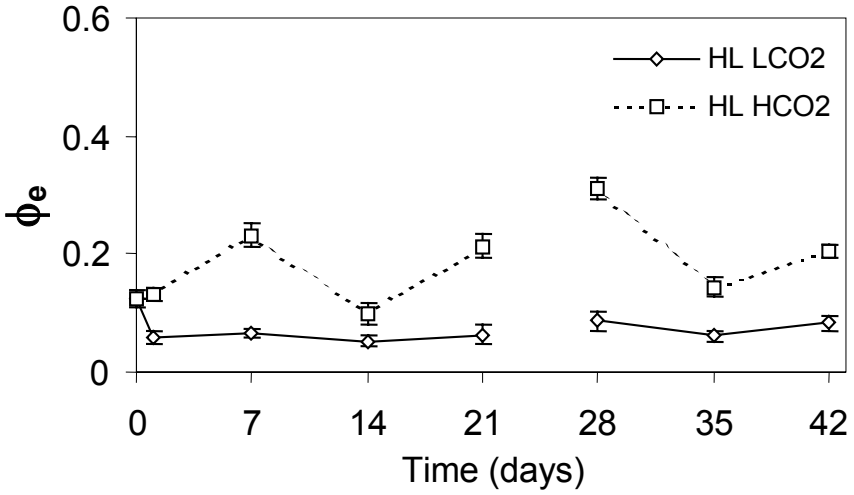

Fig. 2. ϕ_e in persistent leaves (left of the gap) and new leaves (right of the gap) of chestnut during acclimatization under the same conditions as in Fig. 1; ϕ_e in field leaves 0.511 ± 0.036.

In mature leaves the level of GSH decreased as it is allocated to developing structures. In chestnut persistent leaves (Fig. 1A) and the first two new leaves (Fig. 1B) behaved as mature leaves. The export of GSH was evident after day 35 under $HLHCO_2$ (Fig. 1), when a third leaf was expanding (Table 1). Under $LLLCO_2$ only two new leaves were formed and the mobilization of GSH was not observed. In grapevine, persistent leaves accumulated GSH during the whole period of acclimatization. In both species GSH/GSSG ratio increased in persistent and new leaves under $HLHCO_2$ (Carvalho and Amâncio 2002). Comparing to *in vivo* plants (Navari-Izzo *et al.* 1997), the new leaves of both species showed low GR activities (Table 1), even decreasing under $HLHCO_2$. Apparently, the ascorbate-glutathione cycle was not activated.

The quantum yield of electron transport (ϕ_e) can indicate photoinhibition symptoms. Unlike grapevine (not shown), in chestnut HL caused photoinhibition, re-

versed by HCO_2 to FL values (Fig. 2). $HLHCO_2$ also reversed oxidative stress (low GR activity and high GSH/GSSG). In conclusion, in the present system, high GSH is more related to fast growth than to oxidative stress.

Acknowledgements

PhD grant, BD/3097/96 from FCT, L.C.; CBAA funded by FCT Plurianual.

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SULFUR DYNAMICS IN FALLOW SOIL AND IN THE RHIZOSPHERE OF FIELD-GROWN RAPE AND BARLEY

Odile Dedourge, Phuy-Chhoy Vong, Françoise Lasserre-Joulin, Emile Benizri and Armand Guckert
UMR Agronomie et Environnement ENSAIA-INRA-INPL, 2 avenue de la Forêt de Haye, B.P. 172 – 54505 Vandoeuvre-lès-Nancy, France

By their arylsulfatase activity, some microorganisms, stimulated by the rhizodeposits, may contribute to the release of sulfates available for plants. This study aimed to compare the evolution of sulfur immobilization in relation to microbial biomass S and soil arylsulfatase activity in fallow and rhizosphere soils of rape and barley.

The three types of soil were collected from April to June 2001, with five replicates each. The rhizosphere soils were extracted with 0.01 M $CaCl_2$ for SO_4^{2-}-S analysis. The soil samples (50 g pot^{-1}), mixed with a ^{35}S carrier-free solution ($Na_2{}^{35}SO_4$, 88 kBq pot^{-1}) to 80 % of soil WHC, were incubated at 25°C for 7 days. The microbial biomass ^{35}S (^{35}S-MB) was then measured by fumigation with chloroform, and the non-immobilized ^{35}S was extracted with 0.01 M $CaCl_2$. The soil arylsulfatase activity (ARS) was determined on air-dried soil (Tabatabai and Bremner 1970).

The ^{35}S immobilized (Fig. 1) was significantly higher under fallow (72.8 to 84.5 %), than in barley (43.3 to 76.5 %) and in rape rhizosphere soils (27.6 to 53.9 %).

Due to the negligible contents of S-SO_4^{2-} (< 1 mg S kg^{-1} soil) (results not shown), the dilutions with non-labelled S were negligible. The higher percentage of ^{35}S immobilized in fallow than in rape and barley rhizosphere soils was explained by the higher percentage of ^{35}S-MB which followed the similar order: 31.6 to 48.5 %, 14.0 to 28.9 % and 8.8 to 18.1 %, for fallow, barley and rape rhizosphere soils respectively (Fig. 2). Rape and barley rhizosphere soils showed significant seasonal variations of ^{35}S immobilization when compared with fallow. Without cultivation, the accumulation of soil organic matter enhanced by the presence of $CaCO_3$ (pH water = 8) would be important under fallow and may explain the constant and larger ^{35}S immobilized in this soil. In contrast, the release of biocidal compounds from glucosinolate decomposition would lower the biological immobilization of ^{35}S (Brown and Morra 1997) as showed the results in rape rhizosphere soil. Fig. 2 shows significant correlation between immobilized ^{35}S and ^{35}S-MB. This confirms a central role of microbial biomass in the SO_4^{2-}-S incorporation (O'Donnell *et al*.

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1994). Concerning ARS, significant correlation ($r = 0.79$, $p < 0.01$) was found with immobilized ^{35}S. This underlines the implication of microbial ARS in sulfur cycle and confirms that sulfates were limiting in the studied rhizosphere soils. Quantitatively, the ARS (results not shown) expressed in mg *p*-nitrophenol kg^{-1} soil, were higher under fallow (82.2 to 108.2), than in barley (69.3 to 74.2) and in rape rhizosphere soils (59.5 to 71.5). However, the mean ratio values of ARS to ^{35}S-MB were significantly higher in rape than in barley rhizosphere soil and fallow (Table 1). Therefore, rape rhizosphere microbial biomass is the most active in producing ARS.

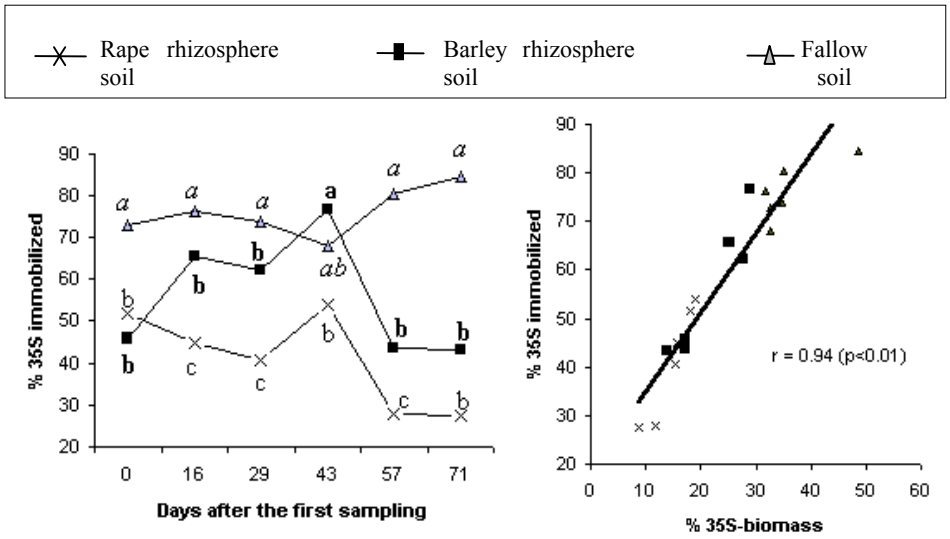

Fig. 1. Percentage of ^{35}S immobilized in the course of time (Newman-Keuls test, 5 %).

Fig. 2. Correlation between % of ^{35}S immobilized and % of ^{35}S-biomass.

Table 1. Average ratio values of arylsulfatase activity (mg *p*-nitrophenol kg^{-1} soil) to ^{35}S-biomass (kBq kg^{-1} soil) in the rape and barley rhizosphere soils and under fallow. Different letters mean significant different values (Newman-Keuls test, 5 %), $LSD_{p=0.05} = 0.07$.

	Rape rhizosphere soil	Barley rhizosphere soil	Fallow soil
Ratio values	0.58a	0.46b	0.34c

The overall results showed a strong relationship between immobilized ^{35}S, ^{35}S-MB and ARS. The specificity of rape plant, which needs high S and also releases glucosinolates into soil suggests that its rhizosphere microorganisms are genetically more specific in sulfur cycling when compared with barley. In fact, rape rhizosphere microorganisms immobilized less S but were most efficient in producing arylsulfatase in order to face the sulfate S limiting conditions.

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O-ACETYLSERINE (THIOL)LYASE CONFERS TOLERANCE TO HEAVY METALS

José R. Domínguez-Solís[1], M. Carmen López-Martín[1], M. Dolores Ynsa[2], Francisco J. Ager[2], Luis C. Romero[1] and Cecilia Gotor[1]
[1]Instituto de Bioquímica Vegetal y Fotosíntesis,CSIC-Univ. de Sevilla. Av Américo Vespucio s/n, E-41092 Sevilla, Spain; [2]Centro Nacional de Aceleradores. Av. Thomas A. Edison s/n, E-41092 Sevilla, Spain

Significant inductions of the *O*-acetylserine (thiol)lyase (OAS-TL) activity and transcript accumulation of *Atcys-3A,* gene coding for the cytosolic OAS-TL, is observed in *Arabidopsis* leaves in response to cadmium stress. These data suggest that high rate cysteine biosynthesis is required in *Arabidopsis* under heavy metal stress. This hypothesis is supported by the observation that intracellular levels of cysteine and glutathione decreased after treatment with $CdCl_2$. These decreases can be explained on the basis that either cysteine or glutathione is required for the synthesis of phytochelatins, the cysteine-rich polypeptides that complex heavy metals as a plant defense mechanism (Cobbett 2000).

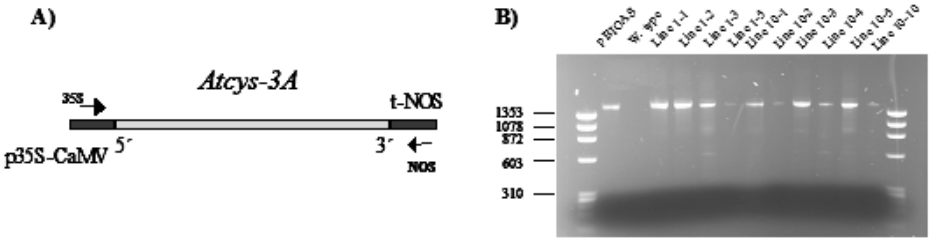

Fig. 1. Arabidopsis plant transformation. A) Integration of *Atcys-3A* into the plant nuclear genome was analyzed by PCR, using the transformation plasmid pBIOAS as a positive control of the PCR, and wild type DNA as a negative control. B) Two synthetic primers, 35S and NOS, localized in the pBIOAS plasmid were used for the PCR reactions.

To address whether *Atcys-3A* plays a role in plant responses to heavy metals, we have produced transgenic *Arabidopsis* plants overexpressing the *Atcys-3A* gene. Ten independent transformed lines were used for further analysis. A PCR approach was used to check the integration of the *Atcys-3A* gene in the nuclear genome of the

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different transgenic lines. In all ten lines a DNA bond of expected size of 1400 pb was obtained (Fig. 1).

Northern blot analysis shows divergent transcript abundance levels between the transformed lines compared to wild type. Some lines such as 1-3, 10-2 and 10-10, accumulate more *Atcys-3A* mRNA while other, lines 1-2 and 10-3, accumulate less than wild type plants (Domínguez-Solís *et al*. 2001). These differences in transcript levels, correlate with different responds to cadmium stress. When seeds are germinated on solid MS medium contaninig 250 μM $CdCl_2$, both wild type and transformed lines with low transcript levels barely germinate while transformed lines with high transcript levels are able to germinate and produce green leaves (Domínguez-Solís *et al*. 2001).

All results suggest that increased cysteine availability is responsible for cadmium tolerance, as transformed plants are able to supply the required thiol precursor for PC synthesis (Cobbett 2000). Similar results have been obtained in *B. juncea*, where the limiting step for cadmium tolerance seems to be GSH biosynthesis and overexpression of γ-EC synthetase enhances the tolerance to similar level we observe in *A. thaliana* (Zhu *et al*. 1999).

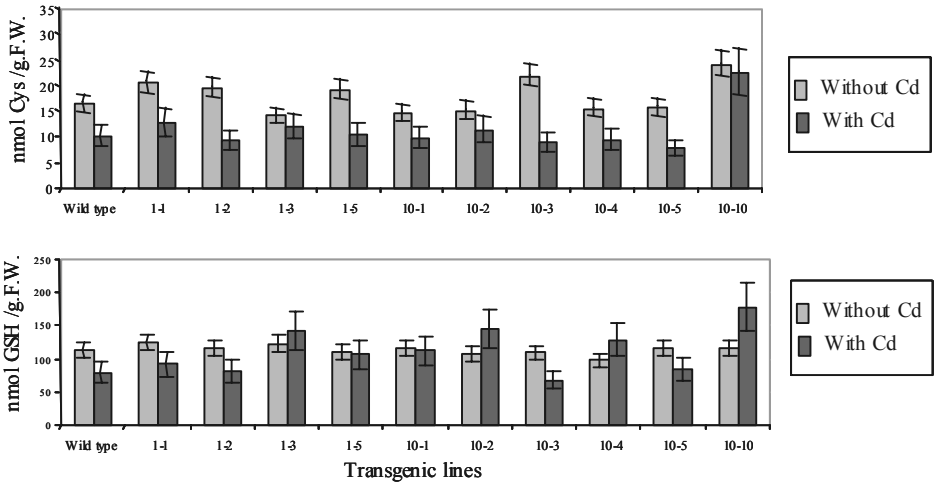

Fig. 2. Cysteine and glutathione contents upon cadmium treatment. *Arabidopsis* transgenic plants were incubated with 250 μM $CdCl_2$ for 18 h, and the leaves were collected for cysteine and glutathione determinations. Values are means and standard deviations of at least five experiments.

Cysteine and glutathione contents were also determined in leaves of transformed plants treated with 250 μM $CdCl_2$ for 18 h and compared to the contents in the absence of the metal. Contents of different transformed plants are in general very similar to the ones observed in wild type under physiological conditions. However under cadmium treatment although cysteine contents are again very similar among different lines, we observed, an increase in GSH contents in lines with high transcript level such as 1-3, 10-2 and 10-10 (Fig. 2).

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IMPACT OF ATMOSPHERIC H_2S ON SULFUR AND NITROGEN METABOLISM IN *ALLIUM* SPECIES AND CULTIVARS

Mark Durenkamp and Luit J. De Kok
Laboratory of Plant Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Allium species contain alliins, secondary sulfur compounds, which consist of stereoisomers of *S*-alk(en)ylcysteine sulfoxides and which are synthesized with cysteine as precursor (Block 1992). Atmospheric H_2S is taken up *via* the stomates, directly metabolized with high affinity into cysteine and it can be utilized as sulfur source for growth (De Kok *et al*. 1998, 2000; Westerman *et al*. 2000). Durenkamp and De Kok (2002) observed a substantial increase in the total sulfur content in the shoot of onion (*Allium cepa* L.) upon H_2S exposure. This increase was in the greater part probably due to an increase in the content of alliins.

The aim of the current study was to investigate the impact of atmospheric H_2S on growth and sulfur and nitrogen metabolism in different cultivars of *A. cepa* L. (onion), *A. fistulosum* L. (bunching onion) and *A. porrum* L. (leek). Twenty four day-old seedlings were grown in vermiculite trays for 18 days, exposed continuously to 0 and 0.15 µl l^{-1} H_2S for 18 days and watered with 25 % Hoagland nutrient solution daily. Growth and metabolite contents of shoots were determined according to Durenkamp and De Kok (2002).

A. cepa had a higher biomass production and a lower dry matter content than *A. fistulosum* and *A. porrum* (Fig. 1). Prolonged H_2S exposure (0.15 µl l^{-1}) resulted in a reduced shoot growth of *A. cepa* cv. Wolf F1 and Bravo F1 and *A. porrum* cv. NiZ 33-2015 F1, whereas the dry matter content (DMC) was hardly affected in all species and cultivars.

Atmospheric H_2S exposure resulted in an accumulation of inorganic, organic and total sulfur compounds in all *Allium* species and cultivars studied (Fig. 2). The accumulation in organic sulfur compounds was most likely due to an increase in the content of alliins and its precursors and not in the content of proteins. Since, H_2S exposure resulted in a more than two-fold decrease in the organic N/S ratio. This can likely can be attributed to an increase in non-protein organic sulfur compounds (the molar ratio of N/S in alliins and its precursors is ≤ 2, compared to approx. 20 in organic sulfur; Durenkamp and De Kok 2002). The inorganic, organic and total nitrogen content was hardly affected by H_2S exposure and differences between cultivars could largely be explained by variations in dry matter content. The impact of

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H_2S exposure on the content and composition of the different alliins and their precursors will further be investigated.

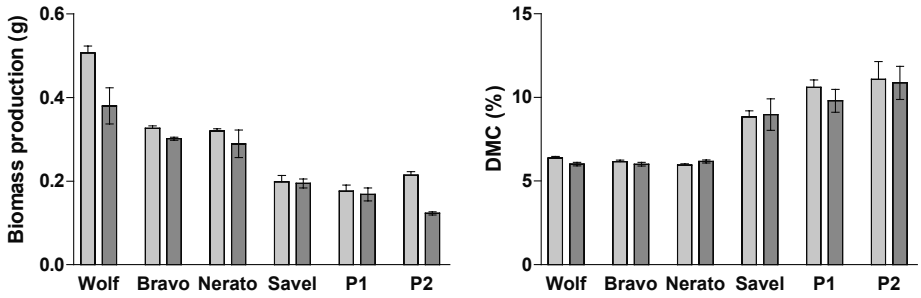

Fig. 1. Impact of atmospheric H_2S on growth (biomass production over a 18-day period, g) and dry matter content (DMC, %) of shoots of *Allium*. The cultivars are *A. cepa* L. cv. Wolf F1, Bravo F1 and Nerato F1, *A. fistulosum* L. cv. Savel and *A. porrum* L. cv. Bluetan F1 (P1) and NiZ 33-2015 F1 (P2). Plants were exposed to 0 and 0.15 µl l^{-1} H_2S (light and dark bars, respectively). Data on growth and DMC represent the mean of 3 measurements with 80 plants in each (± SD).

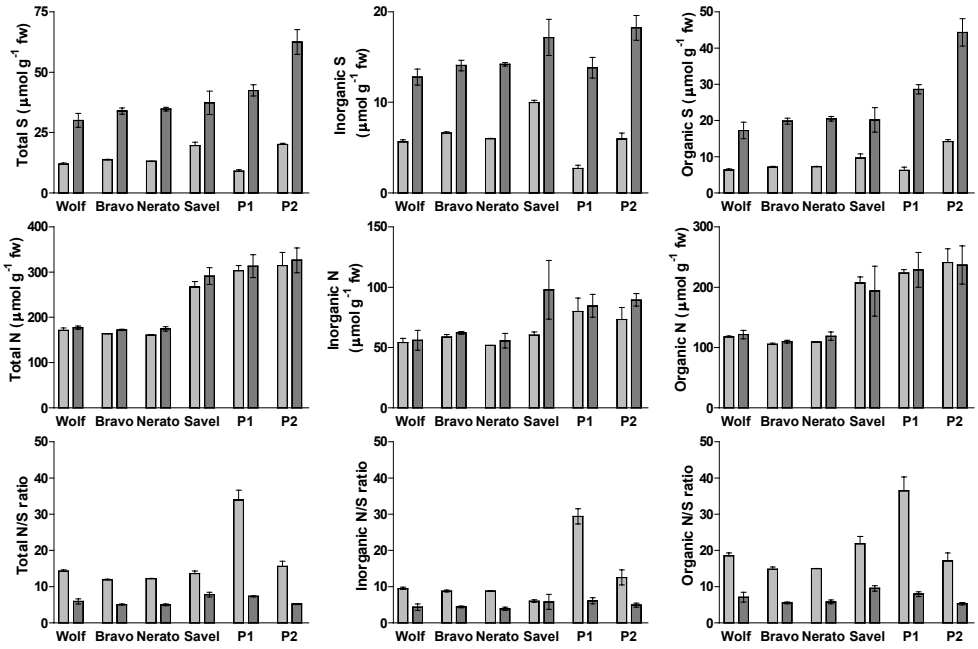

Fig. 2. Impact of atmospheric H_2S on the inorganic, organic and total sulfur and nitrogen content (µmol g^{-1} fresh weight) and N/S ratio of shoots of *Allium*. For material and methods, see Fig. 1.

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IDENTIFICATION AND CHARACTERIZATION OF *ARABIDOPSIS THALIANA* MUTANTS RESISTANT TO SELENATE

Elie El Kassis[1], Nicole Cathala[1], Pierre Fourcroy[1], Pierre Berthomieu[1], Abderrahmane Tagmount[2], Norman Terry[2] and Jean-Claude Davidian[1]
[1]Biochimie et Physiologie Moléculaire des Plantes (UMR 5004), ENSA-M - INRA, Place Viala, 34060 Montpellier cedex 1, France; [2]Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102, USA

The *Arabidopsis* Genome Initiative (2000) enabled the identification of a multigenic family, containing 14 members which encode putative sulfate transporters (*sultr)*. Expression of many of these genes in response to sulfur availability has been studied (Vidmar *et al.* 2000); however, their respective functions *in planta* need to be further characterized. Our aim was to isolate and to characterize *Arabidopsis* mutant lines resistant to selenate, a mutation which could affect sulfate transport and/or the sulfate metabolism pathway.

We have screened 20.000 T-DNA insertion *A. thaliana* lines, ecotype Wassilevskija (Ws-0), by growing them on a selenate containing medium. Selenate is a sulfate toxic analogue thought to adopt the same membrane transport systems and assimilation pathway as sulfate (Cherest *et al*. 1997; Terry *et al*. 2000). Lines were grown for two weeks on a low sulfate agarose medium containing 10 µM selenate in presence of djenkolate, an organic sulfur source. Several lines resistant to selenate were identified by a normal root-growth phenotype compared to the selenate sensitive lines. One of the mutants called B78 was further characterized at a genetic, physiological and molecular level.

We found that the B78 mutant was not resistant to kanamycine, a feature normally conferred by the kanamycin resistance gene held by the T-DNA insert. This indicated that the mutation was not T-DNA tagged. Positional cloning indicated that the mutation was located at the bottom arm of the chromosome 1. Two candidate genes encoding sulfate transporters, *sultr*1;2 and *sultr*2;2 were identified in this region and located on the BAC F28K19 (accession AC009243).

RT-PCR analysis could not identify any transcripts of either the *sultr1;2* or *sultr2*;2 genes in the B78 line, indicating that the mutation affected at least these two genes. We investigated by PCR the size of the deletion on both sides of the *sultr* genes. We found it to concern a total of 16 different genes representing a total of 65 kilobases (Fig. 1).

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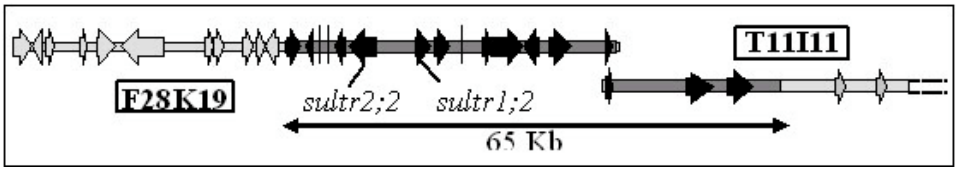

Fig. 1. Description of the deletion in mutant B78. The mutation covers a 65 kilobase region which contains *sultr*1;2 and *sultr2*;2 and 14 other genes. F28K19 and T11I11 are the BACs containing the deletion.

Measurements of short term ^{35}S-sulfate uptake by roots detected no differences between the mutant and the wild type (not shown). This result, in addition to the fact that the B78 mutant was able to grow normally in the presence of sulfate as the sole sulfur source, suggested that sulfate could be taken up and reduced normally in the mutant. We found that sulfate and selenium content values of both roots and shoots of the mutant were comparable to the values of the control line Ws-0 (Fig. 2) indicating that accumulation of both sulfate and selenium in the roots and their transport to the shoots were not affected by the deletion.

We identified by X-ray absorption spectroscopy that the chemical form under which selenium was accumulated in roots and shoots in both Ws and B78 lines, was selenate. No significant amount of selenium-organic forms were detected.

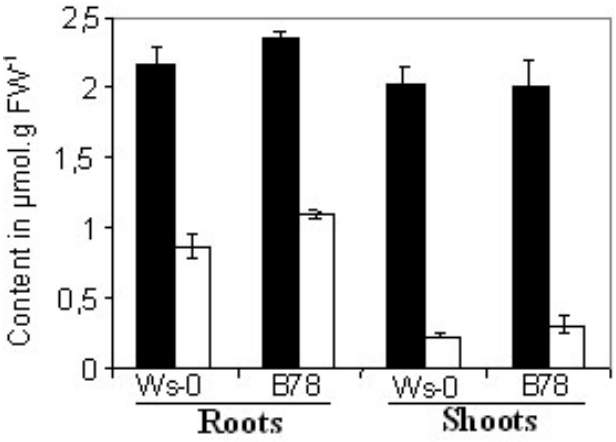

Fig. 2. Sulfate and selenium contents of roots and shootss of Wild type (Ws-0) and mutant (B78). Plants were grown in hydroponic conditions for 2 months. Sulfate (■) and selenium (□) contents of roots and shoots were determined 48 h after addition of selenate (0.5 mM) to the growing solution.

We suggest that the mutation conferring a resistance to toxic selenate treatment could be the result of a sequestration of selenate in the vacuole, away from the sul-

fate reduction sites. We propose a model in which the mutation would affect the functioning of a transporter located at the tonoplast and involved in sulfate efflux from the vacuole. This mutation could then limit sulfate and selenate remobilization from the vacuole. We propose that the *sultr*2;2 gene encodes a tonoplastic sulfate efflux transport system.

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THE EFFECT OF CATCH CROPS ON SULFATE LEACHING AND AVAILABILITY OF SULFUR IN THE SUCCEEDING CROP

Jørgen Eriksen[1] and Kristian Thorup-Kristensen[2]

[1]Department of Crop Physiology and Soil Science, Danish Institute of Agricultural Sciences, P.O. Box 50, 8830 Tjele, Denmark
[2]Department of Horticulture, Danish Institute of Agricultural Sciences P.O. Box 102, 5792 Aarslev, Denmark

Sulfate leaching losses may reduce the long-term possibility of maintaining the S supply of crops in low input farming systems. In order to maintain a sufficient S supply in the future when further reductions in the atmospheric deposition are expected, it is important to reduce leaching losses of sulfate. As leaching primarily occurs during autumn and winter, a plant cover in this period must be expected to affect the quantity of S leached. It has been demonstrated that a catch crop succeeding the main crop can absorb nitrate from the root zone during autumn and winter and thereby reduce nitrate leaching (Thorup-Kristensen and Nielsen 1998). Similar beneficial effects of catch crops on sulfate leaching may be expected.

The ability of catch crops (Italian ryegrass [*Lolium multiflorum* Lam], winter rape [*Brassica napus* L.] and fodder radish [*Raphanus sativus* L.]) to reduce soil sulfate concentrations in autumn and make it available to a succeeding crop was investigated in a field experiment on sandy loam soil, as described by Eriksen and Thorup-Kristensen (2002). All catch crops reduced soil sulfate concentrations in the autumn compared to bare soil (Fig. 1). Especially the cruciferous catch crops had the ability to deplete efficiently soil sulfate levels and thus, reduce the sulfate leaching potential. The S uptake in aboveground catch crop was 8, 22 and 36 kg S ha^{-1} for ryegrass, winter rape and fodder radish, respectively. In the following spring, sulfate levels of the autumn bare soil were low in the top 0.5 m and a peak of sulfate was found at 0.75 - 1 m depth (Fig. 1). In contrast, where a fodder radish catch crop had been grown, high sulfate levels were present in the top 0.5 m but only small amounts of sulfate were found at 0.5 - 1.5 m depth.

The release of S to barley (*Hordeum vulgare* L.) were investigated in pot experiments (setup described by Eriksen *et al.* 1995) after incorporation of the catch crops *Cichorium intybus* L., *Medicago lupulina* L., *Anthyllis vulneraria* L., *Trifolium repens* L., *Pastinaca sativa* L., *Sanguisorba minor* L., *Lupinus polyphyllos* L., *Lolium perenne* L. and *Raphanus sativus* L. The S mineralization rates were highest

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for cruciferous crops and lowest for legumes and differences were partly explained by the C/S ratio ($r^2 = 0.49$) that varied from 48 to 265 (Fig. 2).

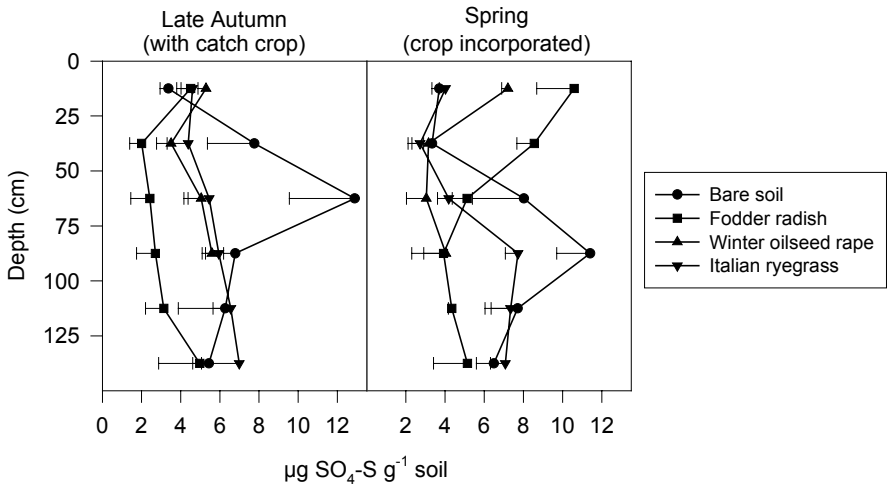

Fig. 1. Soil sulfate concentrations under different catch crops in Autumn and after incorporation the following spring. Error bars: SE.

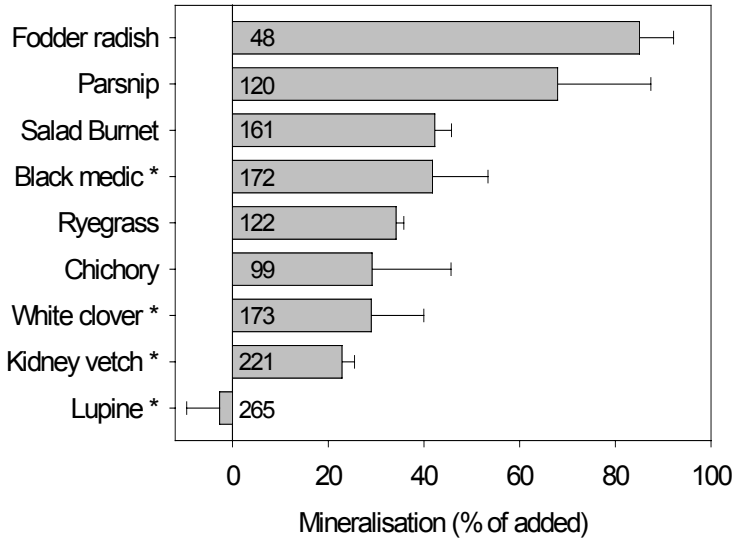

Fig. 2. Mineralization of catch crop S during the growing season of spring barley. Legumes are marked '*'. Inserted in bars are catch crop C/S ratios. Error bars: SE.

In the field, Eriksen and Askegaard (2000) found that sulfate leaching from an organic dairy crop rotation, on sandy soil was 20 kg S ha^{-1} as average of 4 years or equivalent to 60 % of the total input. Sulfate leaching was very variable and ranged from 4 to 45 kg S ha^{-1} for the same crop in different years.

The catch crops showed a great potential for reducing sulfate leaching. Especially cruciferous crops, having a high S demand and vigorous root growth efficiently depleted the soil sulfate pool. In a crop rotation, including both low S-demanding cereals and high S-demanding main crops a suitable catch crop strategy may prevent excess sulfate from leaching in years with low S-demanding crops and instead transfer S to the following high S-demanding crop. This is most important in low input systems, *e.g.* organic farming, but also of relevance to other farming systems.

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CHARACTERIZATION AND DIFFERENTIAL EXPRESSION OF TWO SULFATE TRANSPORTERS IN *ARABIDOPSIS THALIANA*

Pierre Fourcroy, Elie El Kassis, Elisa Dalla Pria, Nicole Cathala and Jean-Claude Davidian
Biochimie et Physiologie Moléculaire des Plantes (UMR 5004), ENSA-M - INRA, 2 Place Viala, 34060 Montpellier cedex 1, France

In *Arabidopsis* 13 genes can be considered as putative sulfate transporters on the basis of a consensus sequence (Prosite motif PDOC00870) located in the second transmembrane region and found in all sulfate transporters from prokaryotes or eukaryotes. *Sultr1.2* (At1g78000) shares a high homology with *Sultr1.1* (At4g08620), which is a high affinity sulfate transporter previously isolated and characterized (Vidmar *et al.* 2000). Our aim was to clone and characterize a cDNA coding *Sultr1*.2 from *Arabidopsis thaliana*.

The *A. thaliana* ecotype WS was cultivated hydroponically in a modified Hoagland medium; a sulfur-free medium was prepared using magnesium nitrate instead of magnesium sulfate. RNA was extracted and reverse-transcribed using M-MLV (Promega).

Cloning of the 3'end of *Sultr1.2* was obtained by PCR from a cDNA bank constructed in the λ Zap II vector using an oligo specific of *Sultr1.2* and the T3 promotor sequence from the λ vector). The sequence of the 3' UTR allowed us to design a second *Sultr1.2* specific oligo for the RT-PCR analysis.

For yeast expression, the complete coding sequence was introduced into the pYES 2 vector (In Vitrogen) and the resulting plasmids were used to transform the YSD1 mutant deficient in sulfate transport (Smith *et al.* 1995).

As previously described for *Sultr1.1* (Vidmar *et al.* 2000) the expression of *Sultr1.2* is restricted to roots (data not shown). *Sultr1.2* expression is inducible by sulfate starvation and exposure to selenate (Fig. 1). As a control, RT-PCR of actin was carried out leading to equal amplification of products regardless of the organs or treatments.

The YSD1 yeast mutant is not able to grow on media containing sulfate as the sole source of sulfur. Expressing SULTR1.2 under the GAL promoter of pYES2 complement the mutant and reestablishes normal growth in presence of 0.2 mM sulfate (Fig. 2A) demonstrating that SULTR1.2 is involved in sulfate transport. We did not recorded significant differences in terms of sulfate affinity or selenate sensitivity in yeast complemented by either SULTR1.1 or SULTR1.2 (Fig. 2B).

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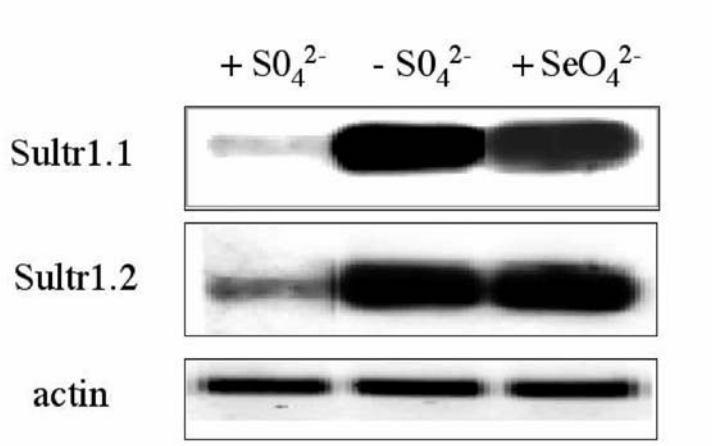

Fig. 1. Differential expression of two genes encoding sulfate transporters in response to either sulfate or selenate treatments. RNA were extracted from roots of 3 weeks old *Arabidopsis* plants grown in hydroponic conditions on sulfate at 1.5 mM ($+SO_4^{2-}$) , then either sulfate-starved for 2 days ($-SO_4^{2-}$) or treated with 0.5 mM selenate ($+SeO_4^{2-}$) for 2 days. RT-PCR analysis were conducted with the appropriate oligos for 25 cycles. Actin was used as a control.

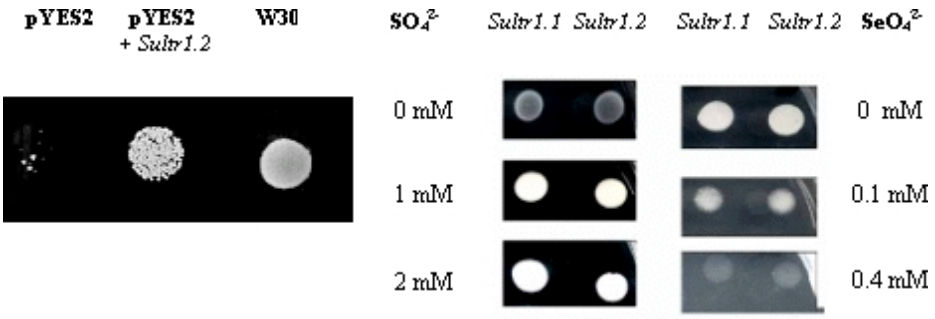

Fig. 2. Drop tests for yeast mutant YSD1 functional complementation. Left panel: the mutant YSD1 was transformed with either the vector pYES2 alone as a negative control (pYES2) or containing the cDNA sequence (+Sultr1.2). The yeast strain W303-1A was used as a positive control. Yeast were grown at 30°C for 48 h on an agarose medium supplemented with 0.2 mM sulfate as a sole sulfur source. Right panel: the YSD1 mutant was complemented by the pYES2 vector containing either the Sultr1.1 or Sultr1.2 coding sequences; yeast were grown as for (A) except that sulfate and selenate concentrations were as indicated. For selenate treatments, sulfate concentration was 0.2 mM.

More recently, a third gene called *Sultr1.3* (At1g22150) has been identified. Attempts to detect an expression of this gene in *A. thaliana* failed (data not shown).

Taken together the results indicate that like SULTR1.1, the protein SULTR1.2 is most likely involved in the uptake of sulfate from the soil solution in cases of sulfur

limitation. Our results are in agreement with recent data obtained by Yoshimoto *et al*. (2002). Interestingly, an *A. thaliana* selenate resistant mutant lacking *the sultr1.2* gene is able to grow normally (Elkassis *et al*. this volume).

Up to now, regulation of sulfate transporter expression in plants has been studied at the transcriptional level. Recently, a conserved C-terminal domain has been identified in many putative sulfate transporters. This domain has been called STAS for "Sulfate Transporter and Anti-Sigma factor" and shares homology with the bacterial anti-Sigma factor (Aravind and Koonin 2000). Investigating the role of this possible regulator domain could provide clue to decipher the post-transcriptional mechanisms controlling sulfate trafficking in plants.

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THIOLS IN ACORNS AND FEEDING MITES COLLECTED AT SITES WITH NATURALLY ELEVATED ATMOSPHERIC SULFUR CONCENTRATIONS

Dieter Grill[1], Michael Tausz[1], B. Strnad[1], Astrid Wonisch[1], Maria Müller[1] and Antonio Raschi[2]

[1]Institute of Plant Physiology, University of Graz, Schubertstraße 51, A-8010 Graz, Austria; [2]National Research Council, Institute of Environmental Analysis and Remote Sensing for Agriculture (IAT-CNR), P. le Cascine 18, I-50144 Firenze, Italy

Mites of *Balaninus glandium* Mrsh. (Coleoptera, *Curculionidae*) feed on acorns, hazelnuts, and chestnuts causing considerable loss of seeds (Amann 1965). In Tuscany, Italy, these mites are found in acorns of holm oak (*Quercus ilex* L.) and downy oak (*Quercus pubescens* Willd.) with naturally different levels of sulfur supply. Some oak populations grow near CO_2 springs, which may also emit H_2S. At these sites, H_2S can reach concentrations affecting plant sulfur metabolism albeit without inducing visible damage symptoms (Schulte *et al.* 1997; De Kok *et al.* 2002). The questions addressed in the present paper are (1) whether such natural low-level life-time exposure to H_2S leads to changes in sulfur metabolism in acorns and (2) whether such possible changes also affect sulfur compounds in feeding mites.

Acorns of holm oak (*Quercus ilex* L.) were sampled at the CO_2 spring in Bossoleto near Siena, Italy. Control seeds were taken from comparable plots in a distance of some km (details in Schulte *et al.* 1999). Acorns were collected in bales extended under the tree canopies in autumn and brought to the laboratory in Graz. They were stored at sufficient humidity for recalcitrant seeds at 8 °C. After some weeks *Balaninus glandium* Mrsh. mites left the seeds and were collected and frozen in liquid N_2. The testa was removed from intact acorns and cotyledons were frozen in liquid N_2. Plant material was ground in a dismembrator, lyophilized, and analyses were done on resulting dry powder. Because of small amounts of material mites were extracted directly with mortar and pestle on ice. Low molecular weight thiols were determined on a gradient HPLC after labelling with monobromobimane (Kranner and Grill 1993) and total sulfur was determined after combustion in O_2 over H_2O_2 by ion-exchange HPLC (Pilch and Grill 1995).

As in most plant tissues (*c.f.* Grill *et al.* 2001) the most abundant low-molecular weight thiol in acorn cotyledons was glutathione (GSH), whereas cysteine concentrations amount to 2 - 4 % of GSH (Table 1). γ-Glutamyl-cysteine was below detec-

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tion limits. In mites, cysteine concentrations were pronouncedly higher (up to 20-fold) than GSH concentrations. Comparable results were also found on acorns of *Q. pubescens* at another CO_2 spring (Solfatara/Viterbo, data not shown). Cotyledons of acorns collected in the CO_2 spring (exposed to elevated H_2S) contained significantly more total GSH and total S than those from control plot. As reviewed by De Kok *et al.* (2002), H_2S is known to increase thiol concentrations in affected plants. H_2S effects on seeds are largely unknown. Feeding mites supplied with more GSH in their diet contain more cysteine. Of course, not only GSH is responsible for changes in the sulfur metabolism in mites, but predominantly the increased total sulfur in acorns, which comprises organic (protein) sulfur, the main cysteine source for the mites.

Table 1. Thiol- (nmol g^{-1} dry weight) and total sulfur concentrations (mg g^{-1} dry weight) in acorns (cotyledons) of *Quercus ilex* and in feeding mites of *Balaninus glandinum.* Medians ± median deviations of n > 20 replicates for acorns and n = 5 replicates for mites. Asterisks indicate significant differences between samples from CO_2 spring and controls (*p < 0.05,***p < 0.001, Mann-Whitney U-test).

		GSH	GSSG	Cysteine	Cystine	Total S
Control	Cotyledons	1393±169	267±74	27±5	12±2	0.47±0.10
	Mites	37±8	n.d.	238±208	53±26	-
CO_2 spring	Cotyledons	1634±235***	406±95***	27±11	12±4	0.67±0.10***
	Mites	36±5	n.d.	701±130*	86±26	-

Possible physiological consequences of increased cysteine contents in mites are still unknown. It remains to be elucidated whether increased GSH concentrations in acorns increase their attraction for feeding animals.

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ROLE OF ATP SULFURYLASE IN THE REGULATION OF THE SULFATE ASSIMILATION PATHWAY IN PLANTS

Yves Hatzfeld[1], Nicole Cathala[3], Dao-Yao He[2] and Jean-Claude Davidian[3]
[1]CropDesign N.V, Technologie Park 3, B-9052 Gent, Belgium; [2]Ernest Gallo Research and Clinic Center, University of California at San Francisco, 5858 Horton Street, Emeryville, CA 94608, USA; [3]Biochimie et Physiologie Moléculaire des Plantes (UMR 5004), ENSA-M - INRA, Place Viala, 34060 Montpellier cedex 1, France

ATP sulfurylase, the first enzyme of the sulfate assimilation pathway, has long been considered as a likely regulatory node in sulfur assimilation (Leustek 1996). To investigate this hypothesis, we constitutively overexpressed an *Arabidopsis thaliana* ATP sulfurylase cDNA (*APS2*) (Logan *et al*. 1996) in transgenic tobacco plants.

Five transgenic lines were studied in detail: PBI19 contained a 35S-GUS expression cassette, while ASA11, ASA34, ASA47 and ASA70 contained a 35S-APS2 expression cassette.

Table 1. Biochemical characterization of transgenic tobacco plant. Tobacco plants (*Nicotiana tabacum* var. SR1 "Petit Havana") were germinated and grown for 40 days in pots containing peat maintained in a cabinet under controlled conditions, with a 16-hour light and 8-hour dark period at 24 °C / 20 °C. Plants were watered every other day alternately with tap water or with a 10 % Hoagland solution. The control line PBI19 contained a GUS expression cassette under the control of a CaMV-35S-promotor, while the lines ASA11, ASA34, ASA47 and ASA70 contained an ATP sulfurylase (APS2) expression cassette under the control of a CaMV-35S-promotor.

Genotypes	PBI19	ASA34	ASA70	ASA11	ASA47
ATP sulfurylase activity (milli-U mg^{-1} proteins)	71 ± 17	25 ± 9	74 ± 9	2283 ± 326	1530 ± 119
Sulfate (µmol g^{-1} FW)	3.5 ± 1.2	6.5 ± 3.2	3.7 ± 0.7	1.8 ± 0.7	1.5 ± 0.4
Proteins (mg g^{-1} FW)	2.0 ± 0.4	2.1 ± 0.4	2 ± 0.5	2.1 ± 0.2	2.0 ± 0.2
Total glutathione (µM)	63 ± 19	63 ± 19	54 ± 21	53 ± 17	52 ± 19
Cysteine, cystine (µM)	3.1 ± 0.9	2.7 ± 0.9	1.5 ± 1	1.9 ± 0.8	1.4 ± 0.7
Methionine (µM)	1.8 ± 0.4	1.5 ± 0.5	0.9 ± 0.4	1.6 ± 0.3	1.0 ± 0.4
Free amino acids (µM)	487 ± 140	576 ± 286	315 ± 108	473 ± 141	339 ± 66

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No phenotypic differences were observed between the 5 genotypes. The ATP sulfurylase activity measured in the leaves of the 5 transgenic lines ranged between 0.3 to 32 times that measured in control lines (Table 1). The ATP sulfurylase activity was located in chloroplasts and was correlated with the abundance of a polypeptide the size of which was similar to that of the APS2 deduced mature protein (not shown). The leaf contents in free amino acids, total proteins, cysteine, methionine or glutathione, showed no significant variation whatever the level of ATP sulfurylase activity. The sulfate contents of the leaves were negatively correlated to the constitutive ATP sulfurylase activities. Thus, modifications in ATP sulfurylase abundance do not have an effect on the steady-state equilibrium of the sulfate metabolism in transgenic tobacco.

Table 2. Incorporation of ^{35}S into protein in transgenic tobacco plants. Tobacco plants were grown for 4 weeks in peat, then transferred into hydroponics on a 10 % Hoagland nutrient solution (+S) which contained 2 mM $MgSO_4$, with a 16-hour light and 8-hour dark period at 20 °C and 70 % humidity. Forty-five-day old tobacco plants were incubated for a 3 h labelling period in a nutrient solution containing 0.4 mM $MgSO_4$ and ^{35}S-sulfate (74 kBq ml^{-1}), after which the radioactivity of the TCA precipitable fraction was quantified. The control line PBI19 contained a GUS expression cassette under the control of a CaMV-35S-promotor, while the lines ASA11, ASA34, ASA47 and ASA70 contained an ATP sulfurylase (APS2) expression cassette under the control of a CaMV-35S-promotor. Data represents the mean of 5 independent analyses.

Genotypes	PBI19	ASA34	ASA70	ASA11	ASA47
Roots (nmol S mg^{-1} proteins h^{-1})	0.5 ± 0.6	0.5 ± 0.6	1.0 ± 1.1	0.3 ± 0.3	0.7 ± 0.8
Shoots (nmol S mg^{-1} proteins h^{-1})	0.2 ± 0.2	0.1 ± 0.2	0.2 ± 0.2	0.3 ± 0.3	0.6 ± 0.7

ATP sulfurylase overexpression did not change the sensitivity of the plant to selenate, a toxic analogue of sulfate (not shown), nor the incorporation rate of ^{35}S into proteins (Table 2). Thus, the rate of sulfate metabolism was likely not affected by the ATP sulfurylase over-expression. These results agree which those obtained previously with transgenic isolated BY2 tobacco cells (Hatzfeld *et al*. 1998).

Our results do not agree with those obtained by others (Pilon-Smiths *et al.* 1999), who showed that a 2-fold over-expression of the *Arabidopsis* APS1 ATP sulfurylase isoform in Indian mustard was sufficient to lead to a slight but significant increase in glutathione and thiol contents, and to an increased resistance to selenate. The discrepancies between the 2 studies could reflect the occurrence of some important differences in sulfur metabolism between *Solanaceae* (tobacco) and *Brassicaceae* (Indian mustard).

We propose that in tobacco plants, variation in the efficiency of sulfate assimilation pathway, due to changes in constitutive ATP sulfurylase abundance, could be balanced by the sulfate uptake by roots. The ATP sulfurylase protein could be present mainly in an APS-mediated inhibited state, particularly in the ATP sulfurylase over-expressing genotypes. The enzyme could buffer the changes in cell sulfate content to allow a tight control of the APS concentration and of the sulfur flux in the

assimilation pathway. We suggest that the sulfur assimilation pathway in tobacco could be limited by the abundance of the APS-consuming enzyme APS reductase.

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INVESTIGATION OF THE FE-S CLUSTER BIOSYNTHESIS IN HIGHER PLANTS

Nicole Hausmann[1], Holger Eubel[2] and Jutta Papenbrock[1]
[1]Institut für Botanik, Universität Hannover, Herrenhäuserstr. 2, 30419 Hannover, Germany; [2]Institut für Angewandte Genetik, Universität Hannover, Herrenhäuserstr. 2, 30419 Hannover, Germany

Iron-sulfur (Fe-S) proteins play an important role in electron transfer processes and in various enzymatic reactions. In eukaryotic cells Fe-S proteins are localized in every cell compartment. In yeast, mitochondria are the major sites of Fe-S cluster biosynthesis. However, little is known about the biosynthetic machinery of Fe-S clusters in plants. Biochemical analysis demonstrated the formation of the Fe-S cluster of ferredoxin in isolated spinach chloroplasts (Takahashi *et al*. 1986) whereas genetic evidence so far indicated the localization of *Arabidopsis* proteins homologous to the yeast proteins involved in Fe-S cluster biosynthesis exclusively in plant mitochondria (Mühlenhoff and Lill 2000).

A mutation in the *Arabidopsis* gene *STARIK* leads to dwarfism and chlorosis of plants with an altered morphology of leaf and cell nuclei. The *STARIK* gene encodes the mitochondrial ABC transporter Sta1 that belongs to a subfamily of *Arabidopsis* half-ABC transporters. Sta1 can substitute for the Atm1p protein in yeast that is involved in the transport of Fe-S clusters from mitochondria into the cytosol (Kushnir *et al*. 2001). Therefore this mutant is a very good model system to investigate the consequences of impairments in Fe-S cluster biosynthesis and transport. We are currently analyzing the proteoms of mitochondria from wild type and mutant plants.

Suspension cell cultures of *Arabidopsis* C24 wild type and the *starik* mutant were established from callus cultures (Fig. 1). By means of centrifugation techniques mitochondria were isolated from both cell cultures and checked for their purity. The protein composition was analyzed by two-dimensional gel electrophoresis (2D-PAGE).

We could not detect any major differences in the proteoms of mitochondria isolated from cell cultures established from wild type and the *starik* mutant plants (Fig. 2). The results could be explained by several reasons: The physical properties of the proteins which might be differentially expressed may be unsuitable for isoelectric focussing or silver staining. Respective proteins may have isoelectric points not in the range of the pH gradients used here (pH 3 to 10). Involved proteins may be low abundant proteins which will not be visible on the 2D-PAGE. Differences in the protein patterns are not expressed in dark-grown etiolated cell cultures.

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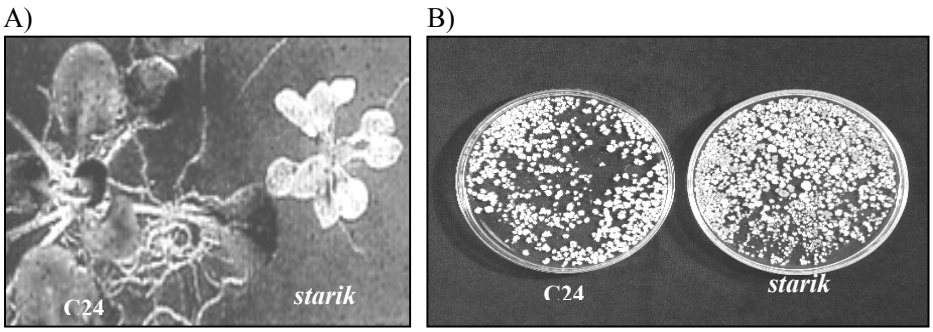

Fig. 1. A) *Arabidopsis* wild type C24 and *starik* plants. B) Suspension cell cultures of C24 wild type and the s*tarik* mutant.

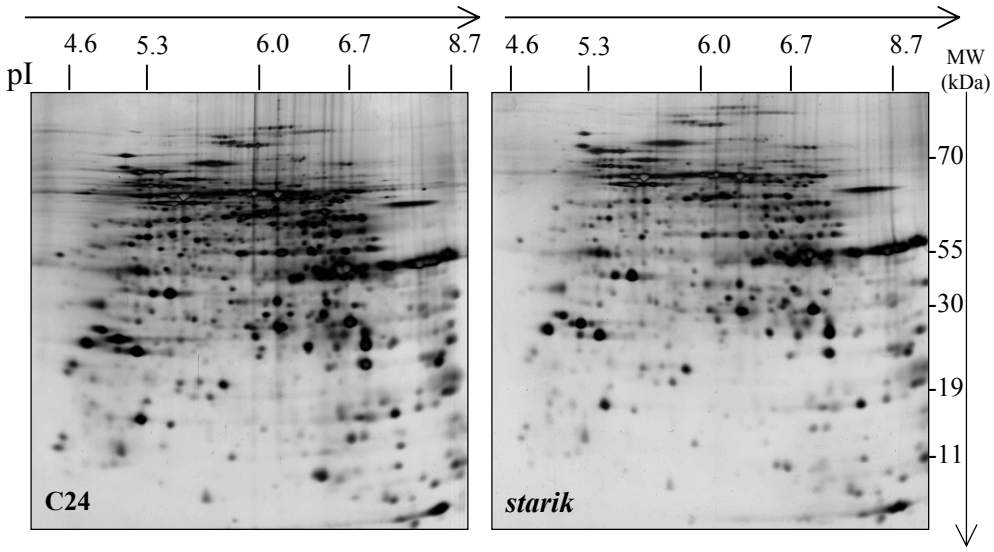

Fig. 2. The proteins of mitochondria isolated from of wild type and C24 suspension cultures were separated in the first dimension according to their pI and in the second dimension according to their molecular mass by SDS-PAGE. The gels were silver-stained.

Currently, we are isolating both mitochondria and chloroplasts from green tissue of wild type and *starik* plants and are analyzing their proteoms. First results showed differences in the protein pattern. Differentially expressed proteins are being analyzed by mass spectrometry.

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TRANSCRIPTOME ANALYSIS OF SULFUR-STARVED *ARABIDOPSIS* BY DNA ARRAY: OAS IS A POSITIVE REGULATOR OF GENE EXPRESSION UNDER SULFUR DEFICIENCY

Masami Yokota Hirai[1,2], Toru Fujiwara[3], Motoko Awazuhara[1], Tomoko Kimura[3], Masaaki Noji[1] and Kazuki Saito[1,2]

[1]Department of Molecular Biology and Biotechnology, Graduate School of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1-33, Inage-ku, Chiba 263–8522, Japan; *[2]CREST, JST (Japan Science and Technology Corporation), Japan; [3]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan*

Sulfur assimilation and sulfur amino acid biosynthesis are controlled both by feedback regulation of enzyme activities and regulation of gene expression involved in the sulfur assimilation pathway. When plants are exposed to sulfur deficiency, the expression of the genes encoding sulfate transporters and sulfur assimilatory enzymes is up-regulated. In sulfur-starved *Arabidopsis*, the concentration of *O*-acetyl-L-serine (OAS), a direct precursor of cysteine biosynthesis, increases (Kim *et al*. 1999). Exogenous application of OAS to plants led to an increase of mRNA levels of sulfate transporters and sulfur assimilatory enzymes, suggesting that OAS is a positive regulator of sulfur assimilatory genes in response to sulfur nutrition in plant as previously found in bacteria.

To investigate the changes in profiles of mRNA accumulation in response to sulfur deficiency, approx. 13,000 non-redundant *Arabidopsis* ESTs corresponding to approx. 8,000 genes were analyzed by DNA macro-array. Three-week-old *Arabidopsis* plants grown on an agarose-solidified sulfur-sufficient medium (1.5 mM sulfate) were transferred to a sulfate-free (0 mM sulfate) or OAS-supplemented (1.0 mM OAS / 1.5 mM sulfate) media and grown for 48 h. Plants transferred to sulfate-free media had reduced concentrations of sulfate. OAS concentrations increased by both treatments (Table 1).

Transcript profiles in response to sulfur deficiency and OAS treatments were compared. The changes in expression levels by sulfur deficiency, that is, ratios of the signal intensities of sulfur-starved sample to those of control sample, were plotted against those by OAS treatment (Fig. 1). There was a positive correlation between the changes in expression levels by sulfur deficiency and those by OAS

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treatment. This suggests that mRNA accumulation of a number of genes under sulfur deficiency is mainly controlled by OAS concentrations in tissues.

Table 1. Sulfate and OAS contents in roots of sulfur-starved and OAS-treated *Arabidopsis*. Average and SD of five replicates are shown.

	SO_4^{2-} (μmol g FW^{-1})	OAS (nmol g FW^{-1})
Control	5.43 ± 0.94	13.1 ± 3.8
S deficiency	2.74 ± 1.02	62 ± 21
OAS treatment	4.95 ± 1.34	830 ± 306

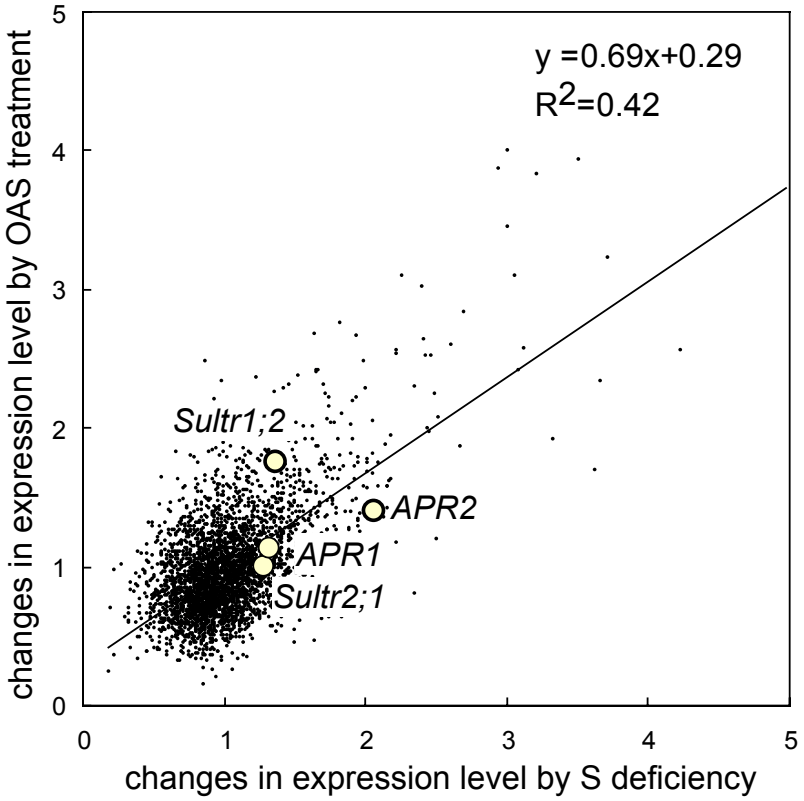

Fig. 1. Scatter plot of changes in expression levels by sulfur deficiency versus by OAS treatment. The horizontal axis shows ratio of the signal intensities of sulfur-starved root sample to those of control root sample. The vertical axis shows ratio of the signal intensities of OAS-treated root sample to those of control root sample. The dots represent the ESTs. The line represents a linear regression of the plot. The equation and the correlation coefficient are shown in the figure. Sulfate transporter genes (*Sultr1;2* and *Sultr2;1*) and APS reductase genes (*APR1* and *APR2*) that are known to be up-regulated by sulfur deficiency are highlighted.

Acknowledgments

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TRANSCRIPT PROFILING OF SULFUR DEPLETION IN *ARABIDOPSIS THALIANA*

Rainer Höfgen[1], Victoria Nikiforova[1], Jens Freitag[1], Stefan Kempa[1], Kerstin Riedel[2] and Holger Hesse[2]

[1]Max-Planck-Institut für Molekulare Pflanzenphysiologie, Dept. of Prof. Lothar Willmitzer, Am Mühlenberg 1, 14476 Golm, Germany; [2]Freie Universität Berlin, Institut für Biologie, Angewandte Genetik, Albrecht-Thaer-Weg 6, 14195 Berlin, Germany

Due to their immobility plants' adaptation to sulfur depletion is confined to a high physiological flexibility demanding the precise temporal and spatial adjustment of different complex tasks, *e.g.* signal perception and transduction, and gene responses. Metabolic engineering of amino acid biosynthesis in plants has largely unraveled the biosynthetic pathways of sulfur metabolism (Galili and Höfgen 2002; Nikiforova *et al.* 2002a). To analyze in an unbiased way the complex network of simultaneous adaptive gene regulations under conditions of limited sulfur a major focus is now put on the multiparallel analysis of plants' responses to sulfur deprivation using the transcriptomics approach. Analysis of regulatory elements was started to establish a catalogue of gene responses to sulfur depletion and to describe and understand the complete network of sulfur metabolism and its correlation to carbon and nitrogen metabolism (Nikiforova *et al.* 2002b; 2003).

Arabidopsis thaliana seedlings were grown between 6 and 13 days on sulfur depleted media. Seeds were either sown on normal medium and transferred to –S medium after eight days or they were sown directly on sulfur-depleted medium. In both set-ups sampling was done at time point A with no visible changes in phenotype and at time point B when first phenotypical changes were observed. Special efforts have been taken to design the experiment in a way to obtain statistically secure data, *i.e.* seedlings were germinated on agarose in five lines per petri-dish, and one sample was comprised of one row of seedlings per dish with a minimum of five petri-dishes per sample. In the sulfur depletion experiment eight samples were included resulting in 40 complex hybridizations. Analysis of the RNA expression profiles including the normalization procedure was performed with the mathematical tools incorporated in the *Haruspex* database (http://www.mpimp-golm.mpg.de/haruspex).

The isolated RNA was used to challenge the MSU collection of 16,000 ESTs spotted on nylon filters. This low redundant collection represents about 7200 genes of the *Arabidopsis* genome. The identification of genes known to be expressed under sulfur deficient conditions, *e.g.* sulfate transporters, proved the validity of the

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approach. Thus, we were able to identify a wide variety of genes overexpressed or reduced under the experimental conditions of sulfur deprivation.

Evaluation of the profiling results was done exemplarily by Northern blot analysis of an isoflavonoid reductase homologue (data not shown) which was overexpressed with a mean of 20 fold under sulfur deprivation at all experimental time points. Plant material was harvested from *Arabidopsis* grown in hydroponic culture and the up-regulation of expression under sulfur depletion was clearly demonstrated both in leaves and in roots in a time dependent manner.

Altogether, in this profiling study overexpression under sulfur deprivation was demonstrated for 3378 clones at least in one of four experimental points, and 2170 clones were down-regulated (Table 1). The majority of differentially expressed clones were related to energy balance and general metabolism followed by protein synthesis and transcription. Extended data-mining is currently performed to reveal correlations within and between the diverse pathways in response to sulfur deprivation. *Arabidopsis* will be used as a model system to draw a detailed map of the direct sulfur metabolism pathway and its interplay with other plant pathways.

Table 1. Clones differentially expressed under sulfur depletion sorted by functional categories.

Category	Up-regulated	Down-regulated
Cellular biogenesis, cell growth, cell division, DNA synthesis	189	94
Cell rescue, defense, cell death and aging	105	45
Cellular organisation and communication, signal transduction	151	81
Cellular transport, transport mechanisms and facilitation	151	90
Energy and metabolism	847	596
Protein destination and synthesis	370	140
Transcription	225	123
Not identified	1317	992

Acknowledgements

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TISSUE AND CELL SPECIFIC LOCALISATION OF A SULFATE TRANSPORTER IN MAIZE

Laura Hopkins and Malcolm J. Hawkesford
Rothamsted Research, Agriculture and Environment Division, Harpenden, Hertfordshire, AL5 2JQ, U.K.

The plant sulfate transporter gene family may be divided into four distinct groups (Hawkesford *et al.*, this volume). There is increasing evidence to suggest that the different groups may have specific/distinct roles in S assimilation and transport within the plant. The high affinity S transporters (Group 1) being responsible for primarily but not exclusively the transport of sulfate from the external environment and the root apoplast, and the lower affinity S transporters (Group 2, 3 and 4) involved in the movement of sulfate around the plant towards and between sink tissues (Hawkesford and Wray 2000). The availability of gene probes specific for individual isoforms enables the localization of the expression of the sulfate transporters at the tissue and cellular level in response to S nutrition.

A high-affinity sulfate-transporter (Group 1: ZmST1, Accession No. AF355602) has been cloned from maize by RT-PCR. Tissue and cell specific localization of this sulfate transporter has been determined along the developmental gradient of the root and in leaves of different age. In S-sufficient conditions there was uniform low expression of ZmST1 in the root and very low expression in the leaves (Fig. 1). S starvation increased ZmST1 expression in roots, especially at the top of the root (just behind the seed, the area possessing most laterals and root hairs) compared to the root tip. This result supports the idea that root hairs and the root tip are important sites of initial S uptake. Increased expression of sulfate transporters in root tissue in response to S starvation is part of the classic de-repression model of regulation of S uptake and assimilation (Hawkesford and Wray 2000). There is also an increase in ZmST1 expression in younger leaves under S starvation, probably as they are growing fastest, have an increased S requirement and are first to become S deficient (Fig. 1).

In situ hybridization using antisense and sense (negative control) riboprobes has been used to analyze spatial patterns of RNA accumulation at the cellular level. In S-starved maize roots there is localized high expression of ZmST1 in the endodermis and pericycle (Fig. 2), indicating a specific role in transport from the cortex to the xylem. This suggests that after sulfate has been transported apoplastically through the cortex to the endodermis, transport beyond the Casparian strip requires an active uptake step.

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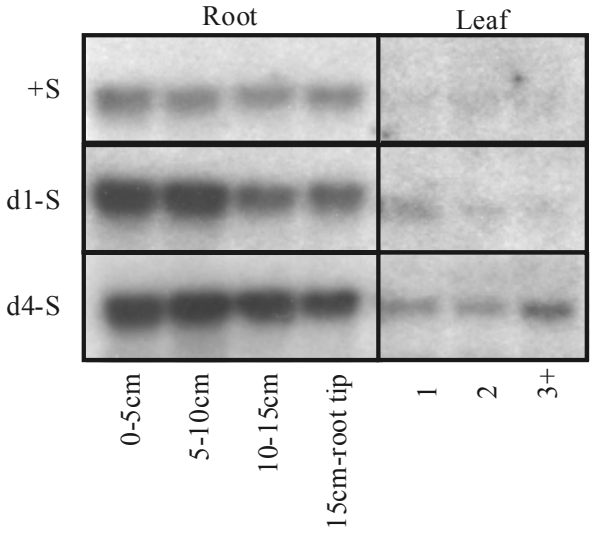

Fig. 1. Effect of S starvation on the expression of the high-affinity sulfate transporter (ZmST1) along the developmental gradient of the root and in different aged leaves of maize. Plants were grown hydroponically for 7 days in 1 mM +S before transfer to –S. Distances are in cm from the seed. Leaves are numbered from the oldest to the youngest.

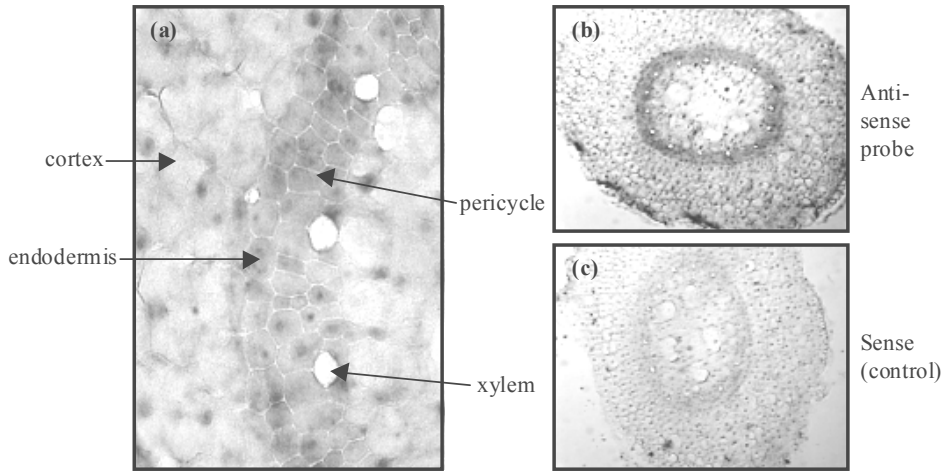

Fig. 2. In situ localization of ZmST1 in S-starved maize roots. Plants were grown hydroponically for 7 days in 1 mM +S then S-starved for 7 days. Transverse section through maize root (a), (b) hybridized with digoxigenin-labelled antisense riboprobe generated from ZmST1 (c) hybridized with digoxigenin-labelled sense riboprobe (control) generated from ZmST1.

Acknowledgements

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THE ROLE OF ELEMENTAL SULFUR IN TOMATO AS A PHYTOALEXIN RESPONSE TO *VERTICILLIUM* INFECTION

Jonathan R. Howarth[1], Jane S. Williams[2], Richard M. Cooper[2] and Malcolm J. Hawkesford[1]
[1]Rothamsted Research, Agriculture and Environment Division, Harpenden, Hertfordshire, AL5 2JQ, U.K.; [2]Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, U.K.

Recently the deposition of elemental sulfur in response to plant pathogen infection has been described and is suggested to function as an inorganic phytoalexin (Cooper *et al.* 1996). This sulfur production is *via* an as yet uncharacterized pathway. In this project, tomato (*Lycopersicon esculentum*) infected with the soil-borne vascular pathogen *Verticillium dahliae* has been developed as a tractable model system to investigate this unique aspect of plant S metabolism at the biochemical and genetic level.

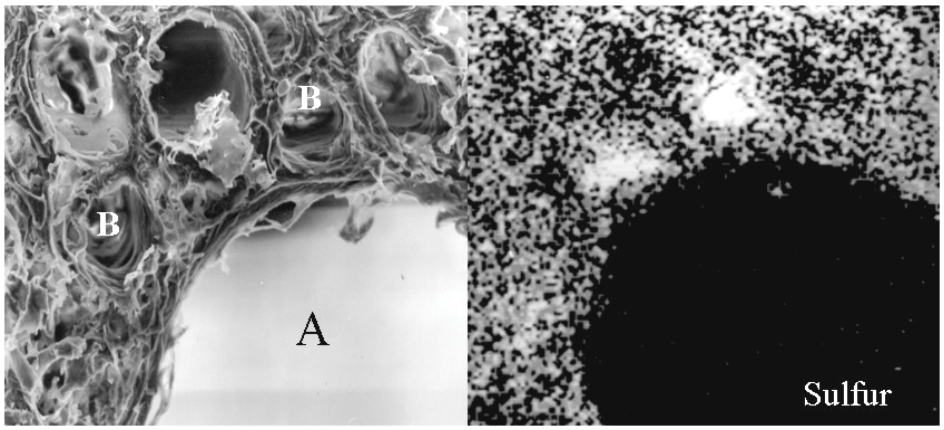

Fig. 1. Distribution of S in a transverse section of tomato stem. The SEM image (left) shows the xylem vessel (A) and xylem parenchyma (B). The EDX sulfur analysis (right) locates the accumulation of S mainly to the xylem parenchyma cells at 28 dpi, as shown by the intense regions of the X-ray dot map.

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Eight-week-old pot-grown plants of a disease resistant (GCR218) and a susceptible (GCR26) tomato line were infected with spore solution. Infection status, S accumulation and gene expression were then analyzed at 7, 14 and 21 days post-infection (dpi).

In infected susceptible plants, rapid acropetal hyphal colonization was observed whereas in resistant plants colonization by *V. dahliae* was limited to the roots throughout the time course. Scanning Electron Microscopy-Energy Dispersive X-ray Microanalysis (SEM-EDX) revealed a greater accumulation of sulfur in vascular cells of the resistant tomato line than the susceptible line. Accumulations of S were detected in xylem parenchyma, cell walls and vascular gels. These structures would be in potential contact with the xylem invading fungus, implicating sulfur in plant defense (Fig. 1). The sulfur produced is suggested to be in the form of S_8 as shown by Gas Chromatography–Mass Spectrometry (GC-MS) of vascular extracts. GC-MS analysis revealed more rapid and 5 times greater accumulation of potentially fungitoxic levels of S_8 in the resistant line than that in the xylem of the susceptible plants at 21 dpi. No S_8 accumulation occurred in uninfected plants. HPLC analysis of reduced S revealed transient increases in glutathione and cysteine levels in the infected xylem tissues of resistant but not susceptible line, reaching the highest levels at 14 dpi (Williams *et al.* 2002).

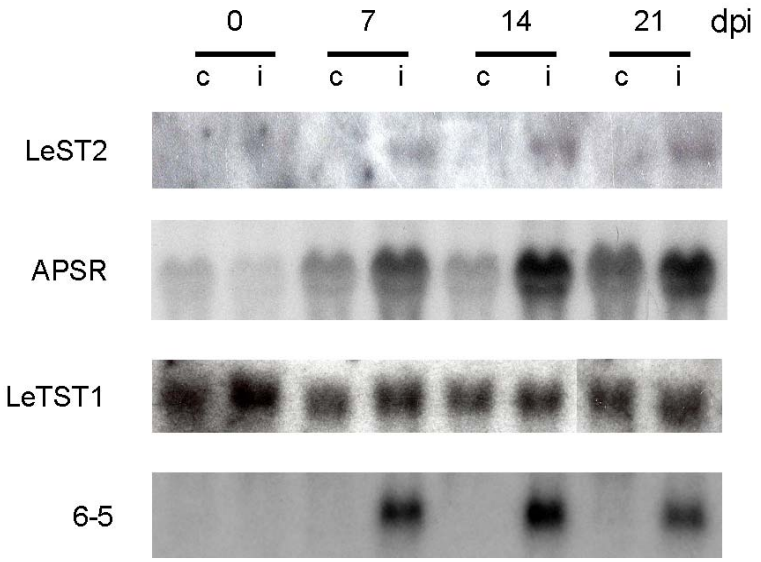

Fig. 2. Northern analysis of genes potentially involved in the production of elemental S in tomato (GCR218) in response to *Verticillium dahliae* infection. RNA was extracted from uninfected control © and infected (i) vascular tissues at 0, 7, 14 and 21 days post infection (dpi). Blots were hybridized with probes encoding LeST2 (sulfate transporter), APSR (adenosine 5'-phosphosulfate reductase), LeTST1 (vascular sulfur transferase) and gene fragment 6 - 5 of unknown function cloned by cDNA-AFLP.

To determine the genetic basis for the deposition of elemental S, two approaches were used. A targeted approach studying the expression of genes known to be involved in sulfate uptake and the metabolism of reduced S compounds, and a non-targeted cDNA-AFLP approach to clone genes differentially expressed in response to infection. In the targeted approach, a sulfate transporter (LeST2) and an APS reductase gene were induced in the vascular tissue of resistant plants in response to infection. No induction of expression of a vascular sulfur transferase (LeTST1) was observed (Fig. 2).

The cDNA-AFLP approach screened the relative gene expression of 15,000 gene fragments. 35 putatively up-regulated gene fragments were isolated and subjected to a secondary reverse Northern screen to remove false positive clones. One clone of unknown function (6-5) was induced in the vascular system of resistant plants on infection by *Verticillium* (Fig. 2).

Acknowledgements

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SELECTION OF METHIONINE-ENRICHED SOYBEAN SEEDS

John Imsande[1] and M. Paul Scott[2]
[1]Plant Genetics Group, Department of Agronomy, Iowa State University, Ames Iowa 50011, U.S.A.; [2]USDA-ARS, Ames Iowa 50011, U.S.A.

Protein accounts for approximately 40 % of the dry weight of a soybean seed; however, soy protein is deficient in both cysteine and methionine. To overcome this sulfur deficiency, animal feeders supplement soy protein rations with costly methionine, which is more stable than cysteine. To alleviate the need for methionine supplementation, we mutagenized seeds and selected genetic soybean lines, the protein of which is enriched approximately 20 % for both cysteine and methionine. Animal rations prepared from these genetic lines should not require methionine supplementation.

Soy protein is used world-wide as a major nutrient in the diets of humans and other animals. The main limitation of soy protein as an animal food, however, is its deficiency in the sulfur amino acids, especially methionine. A methionine deficiency is deleterious to animals for two reasons. First, animals can not synthesize cysteine *de novo* and second, dietary methionine is converted to cysteine more readily than is cysteine to methionine (Finkelstein *et al.* 1988). Also, because of the abundance of transmethylation reactions that rely upon *S*-adenosylmethionine, methionine utilization by animals exceeds that of cysteine. Hence, a primary concern of this research was to increase the mole percent of the sulfur amino acids in soybean seeds.

Two procedures were developed for the isolation of methionine over-producing soybean plants produced by chemically mutagenized seeds (Imsande 2001). Plants producing seeds with an elevated sulfur concentration were selected either by an ethionine resistance assay that relied upon hydroponic growth, for example mutants 20a2 and 86a1, or by a field phenotype of methionine over-producing plants, for example plants H52 and H82. Finally, genetic crosses were constructed using plants selected by the field-phenotype assay as recipients and the ethionine-resistant plants as pollen donors (Imsande 2001).

Pods containing the F1 seeds were collected and the seeds were planted in the greenhouse, producing F2 seeds. F2 seeds were field planted and F3 seeds from selected plants were collected. Seeds highest in percent seed sulfur (*i.e.*, S/N ratio) were selected and field grown. F3 seeds were field-planted yielding F4 seeds, which were analyzed for seed sulfur and S/N ratio (Imsande 2001). Plants selected by these procedures included H52 x 86a1 and H82 x 20a2. Seeds from these plants were

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assayed, and remnant seeds were planted and selected for elevated seed methionine, producing F5 seeds. Subsequently, protein from H82 x 20a2 seeds was analyzed for mole percent methionine and cysteine. These data show that the methionine content of line H82 x 20a2 is 31 % greater than that of typical soy protein whereas the cysteine content is 27 % greater (Imsande 2001).

Subsequently, M8 seeds were obtained from H52 #5 HF in 2000 and M9 seeds in 2001. Also, F5 seeds were obtained from crosses H52 x86a1 and H82 x20a2 in 2000 and F6 seeds from these two lines in 2001. The seeds produced by these lines were analyzed repeatedly for methionine concentration by growth support of an *E. coli* methionine auxotroph (Sambrook *et al.* 1989; Wright and Orman 1995). These analyses demonstrated a statistically significant increase in the methionine concentration of these lines (Table 1). A typical one-kg feed ration for animals contains approximately 150 g of soy protein. The mean mole percentage value for L-methionine of soybean protein is 1.40. Also, a typical one-kg ration is supplemented with 1 g of DL-methionine. Hence, the methionine concentration of soy protein must be increased approximately 21 % to provide as much L-methionine as is present in the supplemented ration, or 42 % to provide as much total methionine. The methionine mole percentage of our lines is approximately 1.84, which is approximately 31 % greater than the 1.40 mole percentage methionine found in a random sample of soybean meal. Because the mole percentage of cysteine in our lines also is increased approximately 27 %, rations prepared using these newly developed lines should not require methionine supplementation. Also, the increased cysteine content may enhance the firmness of tofu.

Table 1. Performance summary: 2000, without seed coat; 2001, with seed coat.

	Methionine content	CV	Increase in methionine (%)	Seed yield kg/10 foot row
2000 Samples				
H52 #5HF	0.308	5.6	9.2	ND
H52 x 86a1	0.317	6.7	12.4	ND
H82 x 20a2	0.312	5.5	10.6	ND
Kenwood 94	0.282	3.9	0 (control)	ND
2001 Samples				
H52 #5HF	0.340	29	25	0.760
H52 x 86a1	0.301	14	10	0.731
H82 x 20a2	0.312	15	14	0.675
Kenwood 94	0.273	14	0 (control)	0.753

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COR - AN *ARABIDOPSIS THALIANA* PROTEIN WITH CYSTINE LYASE ACTIVITY

Patrik Jones and Kazuki Saito
Department of Molecular Biology and Biochemistry, Graduate school of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan

Cystine lyases catalyze the breakdown of L-cystine to thiocysteine, pyruvate and ammonia (Ramirez and Whitaker 1998). Until now there are no reports of the identification of a cystine lyase at a molecular level and it is not clear what functional role this class of enzymes have. We isolated a cystine lyase from broccoli and the cDNA clone corresponding to the amino acid sequence of the purified protein showed homology to a group of plant genes annotated as tyrosine aminotransferases (unpublished). The strongest homologue in *Arabidopsis* (COR - 78.8 % identity, 85.1 % similarity) was recently described by Loupukhina *et al.* (2001) who showed that recombinant COR is capable of tyrosine aminotransferase activity. The COR transcript is up-regulated by coronatine (Loupukhina *et al.* 2001), jasmonic acid (Loupukhina *et al.* 2001) and salt stress (Gong *et al.* 2001).

A full-length COR cDNA was obtained from a public EST library (acc. nr. AV442456; Kazusa DNA institute). Recombinant COR was synthesized in *E. coli* using the pETBlue2 (Novagen) bacterial expression vector according to manufacturer's protocols. Recombinant protein preparations were prepared as described in Jones *et al.* (1999). Assays for tyrosine aminotransferase activity were conducted according to Loupukhina *et al.* (2001) and assays for cystine lyase activity were conducted according to Anderson and Thompson (1979). Both assays were calibrated with standards of the respective end-products. Protein preparations from induced *E. coli* harboring empty pETBlue2 were employed as negative controls.

Active recombinant COR was synthesized in *E. coli* and partially purified by affinity chromatography (Fig. 1). Assays for cystine lyase and tyrosine aminotransferase activity were conducted and crude bacterial protein preparations were capable of both cystine lyase and tyrosine aminotransferase activity (Fig. 2). Isolated recombinant COR displayed only cystine lyase activity and protein preparations from bacteria harboring the empty vector were also capable of tyrosine aminotransferase activity.

Thus, our preliminary results suggest that COR encodes a cystine lyase (*COR*) with no tyrosine aminotransferase activity in contrast to the results of Lopukhina *et al*. (2001). However, it is possible that COR also has additional activities that we have not assayed for yet. Interestingly, both aspartate and alanine aminotransferases

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from porcine heart tissue have previously been shown to catalyze cystine lyase reactions *in vitro* (Adcock *et al*. 1996). To achieve an insight into the true role of COR *in planta* our primary strategy is to create transgenic *Arabidopsis* with reduced or abolished expression of *COR*.

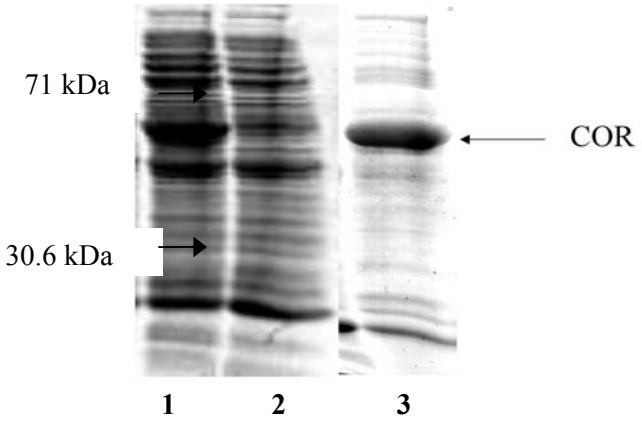

Fig. 1. Synthesis and partial purification of recombinant COR (rCOR). SDS-PAGE of crude protein preparations obtained from *E. coli* harboring (Lane 1) pETBlue2-*COR* and (Lane 2) pETBlue2. Lane 3 is SDS-PAGE of recombinant COR following hist-tag affinity chromatography.

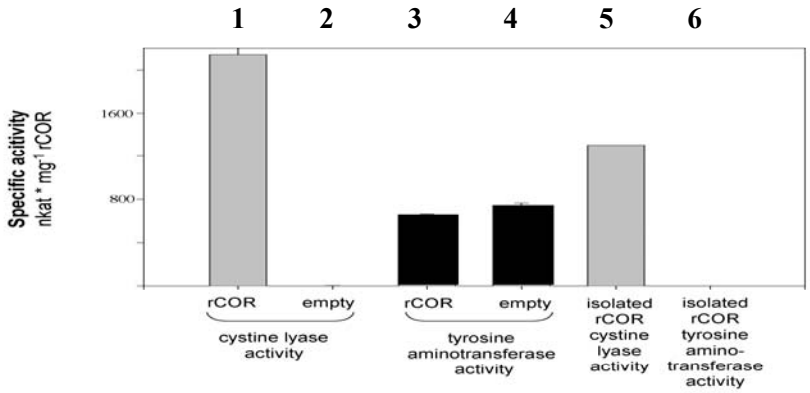

Fig. 2. Enzyme activity assays using crude and partially purified preparations of recombinant COR. Activity-values of protein preparations shown by bars 1, 2 and 5 were assayed for cystine lyase activity whilst 3, 4 and 6 were assayed for tyrosine aminotransferase activity. Protein preparations used in the assays were crude recombinant COR (bars 1 and 3), crude empty vector (bars 2 and 4) and partially purified recombinant COR (bars 5 and 6). The protein preparations are shown in Fig. 1.

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NEW APPROACHES TO STUDY "SULFUR-INDUCED RESISTANCE" AGAINST FUNGAL PATHOGENS IN *ARABIDOPSIS THALIANA*

Ricarda Jost[1], Paul Scholze[2] and Rüdiger Hell[1]
[1]Institute of Plant Genetics and Crop Plant Research, D-06466 Gatersleben, Germany; [2]Federal Centre for Breeding Research on Cultivated Plants, D-06484 Quedlinburg, Germany

Over the last decade signal transduction pathways of plant-pathogen interaction have been studied extensively. Interestingly, many products of the plants' response turned out to be S-containing compounds, *i.e*. the phytoalexin camalexin, isothiocyanates, or sulfur-rich peptides (Tierens *et al*. 2001; Vignutelli *et al*. 1998). Field experiments pointed at a positive correlation between S fertilization and resistance of oilseed rape against fungal pathogens, leading to the question, whether sulfate assimilation rate could be a limiting factor for the ability of plants to fight against fungal infection (Schnug *et al*. 1995). To address this question, the model plant-pathogen system *Arabidopsis thaliana* / *Alternaria brassicicola* was cultivated in a newly developed sterile aeroponical system.

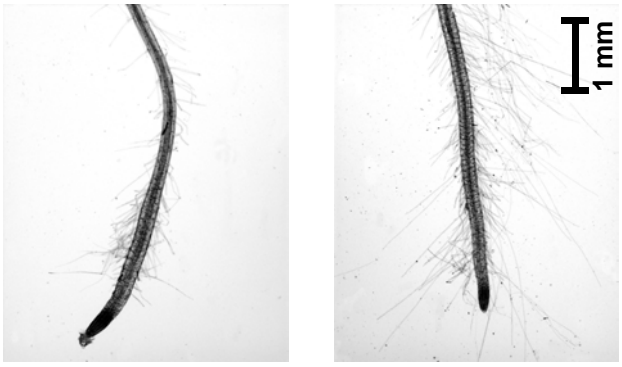

Fig. 1. Phenotypical changes in root architecture observed after 7 weeks of aeroponical culture with 50 % Hoagland solution. Plants with 10 % of the regular sulfate supply show increased root hair formation as well as a dramatic elongation of individual root hairs.

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Arabidopsis thaliana L. Col-0 was grown on a metal grid covered with a thin layer of rockwool in sterile culture vessels. Roots were imbibed twice a week in 50 % Hoagland solution with 40 µM Fe-EDTA. Sulfate concentrations varied from 0.5 mM (control) to 0.05 mM (minimal). Plants were continuously grown under these different sulfate regimes for 6 weeks under short day conditions before inoculation with fungal spore solution was carried out.

Even with minimal sulfate supply the shoot displayed no visible deficiency symptoms, while the root architecture was dramatically altered (Fig. 1). Depending on the sulfate concentration of the nutrient solution the plants displayed typical changes in the levels of sulfate, nitrate, L-cysteine and glutathione (Jost and Hell 1998). While in infected plants sulfate and thiol contents of control leaves rather increased, they were further decreased under limiting sulfate supply (Table 1). This might be a first indication for the high demand of sulfur containing compounds under fungal attack that can hardly be satisfied by the sulfate-deprived cultures.

As a positive control for fungal elicitation *Thi2.1* promotor GUS plants grown on control solution were tested for GUS activity (Fig. 2). GUS activity was comparable between plants treated with 200 µM methyljasmonate for 6 h and those infected with *Alternaria* spores. In the corresponding control plants GUS activity could hardly be detected indicating that the aeroponical culture led to no pre-elicitation *i.e.* by wounding or bacterial infection.

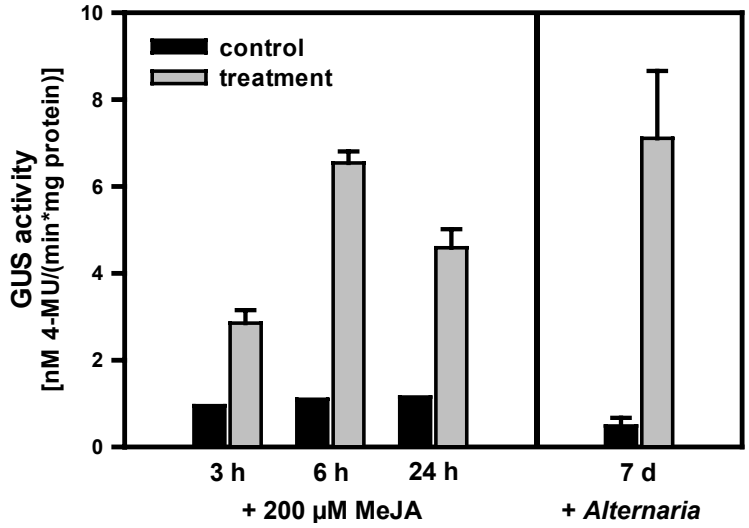

Fig. 2. Induction of reporter gene activity of *Thi2.1* promoter GUS plants (Vignutelli *et al*. 1998) by treatment of 4 week-old plants with methyljasmonate (left panel) or inoculation of 6-week-old aeroponically grown plants with *Alternaria brassicicola* (10^6 spores ml^{-1}, right panel).

The data presented here demonstrate that the aeroponical culture system is well suited to follow the concept of a "sulfur-induced resistance" in *Arabidopsis* by allowing both, the application of defined nutrient solutions and high reproducibility of fungal infections. cDNA arrays can now be used to identify regulatory steps for the production of S-containing defense compounds. Transgenic lines altered in their sulfur status will be analyzed with respect to their altered susceptibility towards fungal pathogens.

Table 1. Fungal infection leads to a rapid depletion of internal sulfate pools of plants cultured on minimal solution. Plants were grown as described in the text and harvested 7 days after inoculation (5×10^5 spores ml^{-1}). Data represent mean values obtained from leaf / root material from three independent experiments.

	Mock-treated with 0.1 % Tween		After *Alternaria* infection	
	0.5 mM Sulfate	0.05 mM Sulfate	0.5 mM Sulfate	0.05 mM Sulfate
Shoot-FW (mg culture $vessel^{-1}$)	304 ± 64	272 ± 23	256 ± 6	269 ± 40
Root-FW (mg culture $vessel^{-1}$)	58 ± 17	65 ± 5	48 ± 18	82 ± 14
Sulfate (µmol g^{-1}FW)	43 ± 8	7 ± 1	46 ± 8	1.4 ± 0.3
Nitrate (mmol g^{-1} FW)	2.3 ± 0.1	3.2 ± 0.6	2.0 ± 0.2	2.8 ± 0.3
L-Cysteine (nmol g^{-1} FW)	21 ± 3	13 ± 1	26 ± 4	11 ± 3
GSH (nmol g^{-1} FW)	272 ± 48	139 ± 12	286 ± 40	105 ± 26

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TEMPORAL AND SPATIAL EXPRESSION ANALYSIS OF SERINE ACETYLTRANSFERASE ISOFORMS IN *ARABIDOPSIS THALIANA*

Cintia G. Kawashima, Masaaki Noji and Kazuki Saito
Department of Molecular Biology and Biotechnology, Graduate School of Pharmaceutical Sciences, Chiba University, CREST, JST, Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan

Sulfur is an essential macronutrient required for plant growth. It is primarily used to synthesize cysteine, methionine, and numerous essential and secondary metabolites derived from these amino acids. In plants, sulfur is assimilated into cysteine through the cysteine biosynthetic pathway (Saito 1998). Cysteine is synthetized by condensation of *O*-acetylserine (OAS) and sulfide catalyzed by *O*-acetylserine (thiol)lyase. SATase (EC 2.3.1.30) has an important function in this pathway, by catalyzing the formation of *O*-acetyl-L-serine (OAS) from L-serine and acetyl-CoA catalyzed by, connecting serine metabolism to cysteine biosynthesis (Noji *et al.* 1998). SATase and OAS are proposed to be regulatory factors in the biosynthesis of cysteine in plants (Brunold 1993; Saito *et al.* 1994; Smith *et al.* 1997). In the genome of *Arabidopsis thaliana*, there are five predicted SATase-like genes. Therefore, it is important to clarify the temporal and spatial expression levels of the different putative SATase isoforms (SAT-p, -c, -m, -#5, and -106) during the development of *Arabidopsis thaliana*.

Arabidopsis thaliana (ecotype Columbia) seeds were sterilized, and sowed on GM medium with 1 % sucrose and 0.2 % gelrite. Tissue samples were harvested every week for 5 weeks. Total RNA was isolated using 100 mg of plant material from stored and stratified seeds, cotyledon, leaf, stem, root, silique, and flower tissues. 5 ng of total RNA was used for Real-time Quantitative PCR analysis, and the N-terminal region of all the SATases cDNA sequence was chosen for amplification. The Real-time Quantitative PCR analysis was normalized using 18S ribosomal RNA as an internal control together with an equal quantity of sample RNA, and a respective plasmid DNA of each sample.

During plant development SAT-p, -c, -m, -#5, and -106 mRNAs showed similar expression patterns, revealing distinct expression levels between aerial and root regions (Table 1). SAT-p and SAT-c showed an increased expression levels in root at the vegetative stage (2 and 3 weeks). The expression was apparent reduced after the plants entered the reproductive stage accompanied by a relatively lower apparent expression in aerial tissue samples. Unlike SAT-p and SAT-c, SAT-m, SAT-#5, and

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SAT-106 had no significantly increased expression levels in aerial and root regions at the vegetative stage. Notably, SAT-p mRNA expression levels were high compared with the other known SATases (Table 1).

The expression level in stored seed and silique tissues was distinct among the different SAT isoforms. SAT-m and SAT-106 expression was detected in relatively low levels. In contrast, there was no detectable expression of SAT-p, SAT-c, or SAT-#5 in these tissues. Accordingly, we propose a division of the known SAT isoforms into two distinct groups: SAT-m/-106 and SAT-p/-c/-#5 (Table 1).

OAS and thiol contents were analyzed by HPLC using the same tissues as in the real-time quantitative PCR analysis. The levels of cysteine, glutathione, and OAS in seedling, leaf, root, silique, and flower showed a similar pattern during development. The amounts of cysteine, glutathione, and OAS in aerial region were higher than in root, in concordance with the localization of sulfur reduction in chloroplasts.

The different expression levels among the known and putative SATase isoforms suggest that they may have distinct roles in different cells in the cysteine biosynthetic pathway.

Table. 1. Real-time quantitative PCR analysis of SAT-p, SAT-c, SAT-m, SAT-#5, and SAT-106 from wild type *Arabidopsis thaliana* during its development from seed to flower stage. The amounts of mRNA are given in pmol ng^{-1} total RNA.

Analyzed Tissues	SATases mRNA Expression by the Real-time Quantitative PCR Average values of three samples [pmol ng^{-1} total RNA]				
	SAT-p	SAT-c	SAT-m	SAT-#5	SAT-106
Stored seed	-	-	0.000032	-	0.00000844
Stratified seed	0.041	0.0026	0.05	0.0000549	0.000158
1 week cotyledon	0.336	0.092	0.0039	0.000172	0.00000553
2 weeks shoot	1.687	0.399	0.035	0.000416	0.00054
2 weeks root	0.916	0.4	0.0047	0.0000429	$9.08.10^{-17}$
3 weeks rosette leaf	23.982	8.983	0.169	0.012	0.00086
3 weeks root	2.69	0.897	0.021	0.0000104	0.000162
5 weeks rosette leaf	0.118	0.016	0.00173	0.0022	0.000126
5 weeks stem	0.133	0.014	0.00755	0.000592	0.0000728
5 weeks root	0.158	0.013	0.00254	0.00015	0.000141
Silique	-	-	0.0000214	-	0.00000433
Flower	0.000005	0.0069	0.0076	0.00257	0.344

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THE EFFECT OF LEAD ON SULFOQUINOVOSYL DIACYLGLYCEROL CONTENT IN LEAVES AND ROOTS OF WHEAT SEEDLINGS

Oksana Kosyk, Alexander Okanenko and Nataliya Taran
National Taras Shevchenko University, Plant Physiology & Ecology Department, Volodymyrska, 64, UA 01033, Kyiv, Ukraine

The quantity of available heavy metals in soil is increased greatly in industrial areas and if plants take them up they may induce toxic and adaptive responses. Heavy metals may affect lipid composition of plants. It has been demonstrated that Cd induced a decrease in the monogalactosyl diacylglycerol (MGDG) and sulfoquinovosyl diacylglycerol (SQDG) in *Brassica napus* leaves (Youssef *et al.* 1998).

In our experiments plants were grown as hydroponic cultures at various levels (0.0048, 0.48 and 48 μM) of $Pb(NO_3)_2$. Plants were harvested after 14 days and glycolipids (MGDG, digalactosyl diacylglycerol (DGDG)) and SQDG content of shoot and roots were separated chromatographically and the content of galactolipids were determined according to Rougham and Batt (1968), and SQDG according to Kean (1968).

The pigment content and composition of leaves of wheat seedlings was not affected upon exposure to Pb in the nutrient solution (data not shown), however, the protein content significantly increased, both in leaves and roots (Fig. 1A). Galactolipid content was not significantly affected by Pb, except at 48 μM. On contrary, there was a Pb concentration dependent decrease in SQDG content, especially in the roots (Fig. 1B,C,D). Similar results were obtained when the data were expressed on a chlorophyll basis (data not shown). Expressed on a protein basis, all lipids decreased drastically in leaves and especially SQDG in roots.

We assume, that SQDG as a strong anion can create complexes with the Pb atom. It is possible competitive usage of sulfur for sulfur containing cysteine-rich peptides (phytochelatins) and protein synthesis (judging on increasing protein content). This according to the suggestion that the cells utilizes sulfur preferentially for the synthesis of essential metabolites, such as proteins, rather than for SQDG synthesis (Sato et al. 2000). But information presented allows also to suggest that metabolism changes induced by Pb depend upon plant species and conditions of metal action. For example, while acute exposure of the moss, *Rhytidiadelphus squarrosus*, to low levels (1 - 10 μM) of $Pb(NO_3)_2$ did not change the radio-labelling of SQDG significantly, populations gathered from Pb-polluted soils showed more labelling of chloroplast lipids than moss from unpolluted areas. It was considered the increased

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metabolism of chloroplast lipids in moss from Pb-polluted regions might represent an adaptive response (Guschina and Harwood 2002).

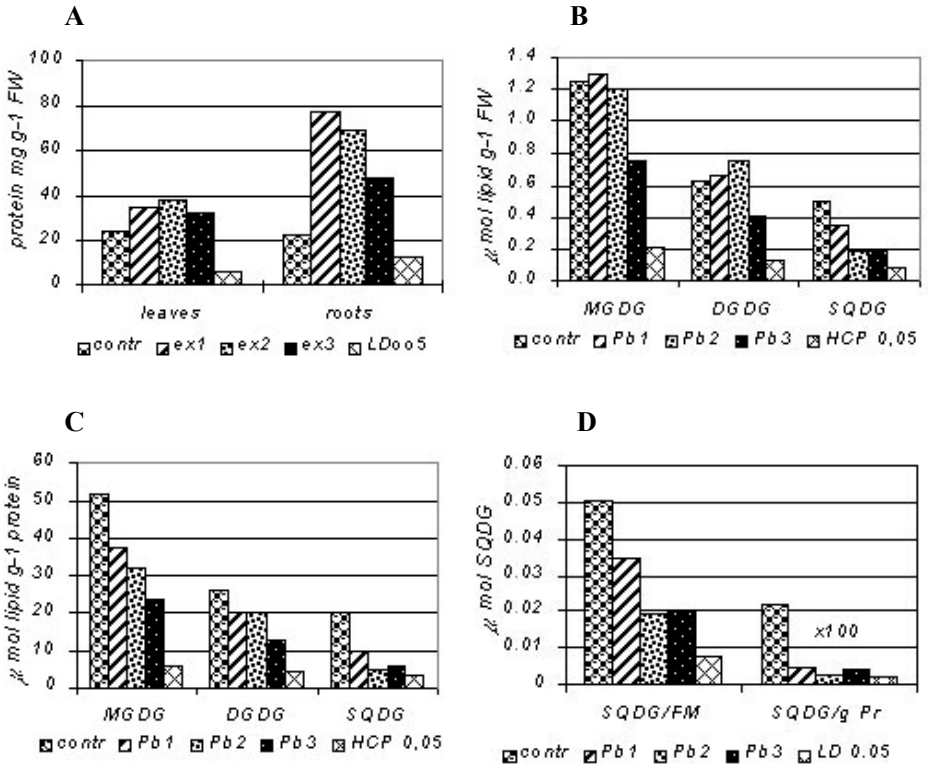

Fig. 1. Lead effect upon protein (A) and glycolipid content in wheat seedling leaves (B, C) and roots (D): Pb 1, 0.0048 μM $Pb(NO_3)_2$; Pb 2, 0.48 μM $Pb(NO_3)_2$; Pb 3, 48 μM $Pb(NO_3)_2$.

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GRASS SULFUR STATUS AND NON-PROTEIN NITROGEN ACCUMULATION: A PREVIEW IN THE SOUTHEASTERN PART OF BELGIUM

Richard Lambert, Michaël Mathot, Bernard Toussaint and Alain Peeters
Laboratoire d'Ecologie des Prairies - Université catholique de Louvain, place Croix du Sud n°5 bte 1, B-1348 Louvain-la-Neuve, Belgium

Sulfur deficiency may become more widespread as atmospheric sulfur depositions is reduced. A shortage of sulfur may limit plant growth. It may also limit the synthesis of proteins; leading to the accumulation of non-protein nitrogen in the plant *e.g.* nitrates (Schnug 1990) and amides (Freney *et al.* 1978; Millard *et al.* 1985). Once ingested these compounds may be dangerous for animal health and also affect the metabolic utilization of nitrogen. In previous studies, the N/S ratio was considered as a good estimator of the risk of sulfur deficiency (Metson 1973; Dijkshoorn *et al.* 1960). For grass growth, there is a risk of S shortage when the N/S ratio is higher than 16:1. In the ruminant diet, the optimum N/S ratio is considered to be between 10 and 15 (Metson 1973; Whitehead 1995).

The first aim of our work was to make a preview of the sulfur status of the grass crop in the South-East part of Belgium to assess the need for further studies and secondly, to study the relationship between non-protein nitrogen content in function of sulfur content in order to evaluate the quality.

Fifteen grass forage samples were taken from farms in the southeastern part of Belgium (Ardenne, Famenne and Jurassic area) in 1993 and twenty-seven other samples in Belgian Ardennes in 1995 and 1996. The southeastern part of Belgium is a rural area with a high rainfall (800 - 1010 mm $year^{-1}$) and soils with a high organic content (3.5 % humus). The N and S content of the 42 samples were determined and the N/S ratio was calculated. For the calculation of the N/S ratio, nitrogen is measured using the Kjeldahl method.

Protein nitrogen was determined using the Studzer method (Kjeldahl analysis of proteins precipitated with $CuSO_4$) on the 27 forage samples taken from farms in 1995 and 1996 (13 of fresh grass, 5 of hay and 9 of grass silage). Non-protein nitrogen (NPN) was calculated as the difference between the $N_{Kjeldahl}$ and the $N_{Studzer}$. However, the Kjeldahl method does not take into account the nitrate.

Ten out of the 42 samples (24 %) presented a risk of S deficiency for optimum grass growth (N/S values above 16:1, with a maximum of 21:1) and 23 samples (55 %) were in the range $10 < N/S < 16$. S concentration was in the range 0.14 - 0.35 % dry matter (DM) with a mean of 0.19 % DM.

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A significant positive correlation (r = 0.835) was found for the linear regression between NPN content of forage and the N/S ratio (Fig. 1). When considering this correlation for the different forage categories separately, only the relation for grass silage did not appear significant probably due to degradation of proteins occurring during silage. The NPN concentration (% DM) is also higher in grass silage than in fresh grass or hay.

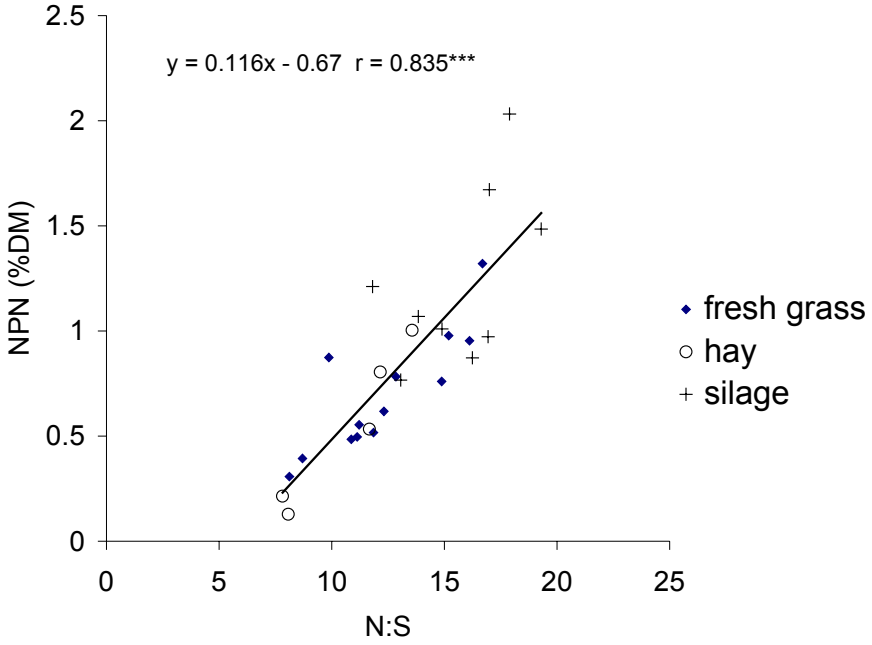

Fig. 1. Relation between non-protein nitrogen content of forage samples and N/S ratio

The correlation coefficient for the linear regression was less or not significant when confronting non-protein nitrogen with $N_{Kjeldahl}$ content (r = 0.573) or with S content (r = 0.179), separately. The weak correlation between NPN and $N_{Kjeldahl}$ content indicates that in our sample, nitrogen excess is not the main factor that explains non-protein nitrogen accumulation. The N/S ratio explains 70 % of the variance.

The results showed that S might be a limiting factor for grass growth and for ruminant nutrition in the southeastern part of Belgium. Approximately one-fourth part of the samples had a N/S ratio above 16. The limiting effect of S in the diet may be increased by the fact that the grass forage is not usually fed alone. Maize silage or cereals that are poor in S are also included in the diet. They also confirm that there is an accumulation of NPN in plants when N/S ratio increases and thus that the S shortage may limit protein synthesis.

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COMPARISON OF OILSEED RAPE AND BARLEY RHIZOSPHERE MICROBIAL COMMUNITIES INVOLVED IN SULFUR IMMOBILIZATION - PRELIMINARY RESULTS

Françoise Lasserre-Joulin, Romain Boulan, Emile Benizri, Phuy-Chhoy Vong and Armand Guckert
UMR Agronomie et Environnement ENSAIA-INRA, 54505 Vandoeuvre-lès-Nancy, France

Plant rhizodeposition plays a main role in biogeochemical cycles, and rhizosphere corresponds to a bioreactor where microbial communities are directly involved in sulfur immobilization/mineralization processes. The quantity of carbon annually released by plants towards soil corresponds to about 2.4 to 3 t ha^{-1} yr^{-1} (Kuzyakov and Domanski 2000). This carbon may increase rhizosphere microbial biomass by factor 10 to 100, comparing to bulk soil. But knowledge on the nature and the activities of these rhizosphere microbial communities is very poor.

This study aimed at comparing rhizosphere microbial communities of two plant species differing by their needs in sulfur: oilseed rape (30 kg S ha^{-1} yr^{-1}) and barley (15 - 20 kg S ha^{-1} yr^{-1}). From field-grown plants, rhizosphere (RH) and corresponding non-rhizosphere soils (NRH) were sampled. Microorganisms (bacteria) were extracted and cultured in M56 media (Na_2HPO_4 5.4 g l^{-1}, K_2HPO_4 3.55 g l^{-1}, $Mg(NO_3)_2$ 0.1 g l^{-1}, $(NH_4)_2HPO_4$ 1 g l^{-1}, $Ca(NO_3)_2$ 3.5 g l^{-1}, $FeCl_3$ 0.25 g l^{-1}), containing or not glucose (G), and mineral (K_2SO_4 5 g l^{-1}) or organic sulfur compounds: methionine (Met) 0.2 g l^{-1} or laurylsulfate (LS) 0.2 g l^{-1}.

Bacterial communities, initially contained in oilseed rape or barley (RH) and (NRH) soil samples, and able to grow on different M56 media, were estimated using the Most Probable Number method (De Man 1975) (Fig. 1).

For all soil inocula, all sulfur sources were used by bacteria, except methionine alone. Laurylsulfate without glucose seemed to be a better organic-S compound for bacterial growth than methionine (Fig. 1). Methionine could not be used as the carbon source alone, and it allowed microbial growth only in association with glucose. Nevertheless, the association methionine plus glucose was more efficient than glucose alone. Concerning laurylsulfate, it was not better than K_2SO_4 to favor microbial growth, which supports the generally use of estersulfates as sulfur source rather than as carbon. Estersulfates are known to be easily used by microorganisms, and to be the most important (Eriksen *et al*. 1998) and the most active soil organic S (Bettany and Stewart 1983). Nevertheless, oilseed rape rhizosphere bacteria were most nu-

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merous to develop with laurylsulfate, in comparison with barley. This result suggests that oilseed rape rhizosphere would be able to select a specific and therefore homogeneous microbial communities able to mobilize greater quantities of estersulfate, compared with barley. This result also supports the observations of Grayston and Germida (1991), who suggested that oilseed rape taproot system would be poorer in microsites for microorganisms and so, would harbor a more homogeneous microbial biomass than barley, which possesses a dense fibrous root system. Moreover, the glucosinolate presence in crucifers contributes to isothiocyanate production in the vicinity of roots, which would inhibit the development of numerous microorganisms, particularly mycorhizae (Brown and Morra 1996).

This study emphasized the importance of the rhizosphere effect on microbial communities involved in sulfur cycle, particularly the efficiency of sulfur source in rhizosphere bacteria development. The results showed that oilseed rape and barley rhizodeposition could favor microbial communities able to use a wide spectrum of sulfur compounds: K_2SO_4, laurylsulfate and methionine (in association with glucose). But they also revealed a positive selection of estersulfate, above all by oilseed rape rhizosphere microbial populations. Nevertheless, further study is needed to improve knowledge on these rhizosphere microbial communities involved in sulfur cycles.

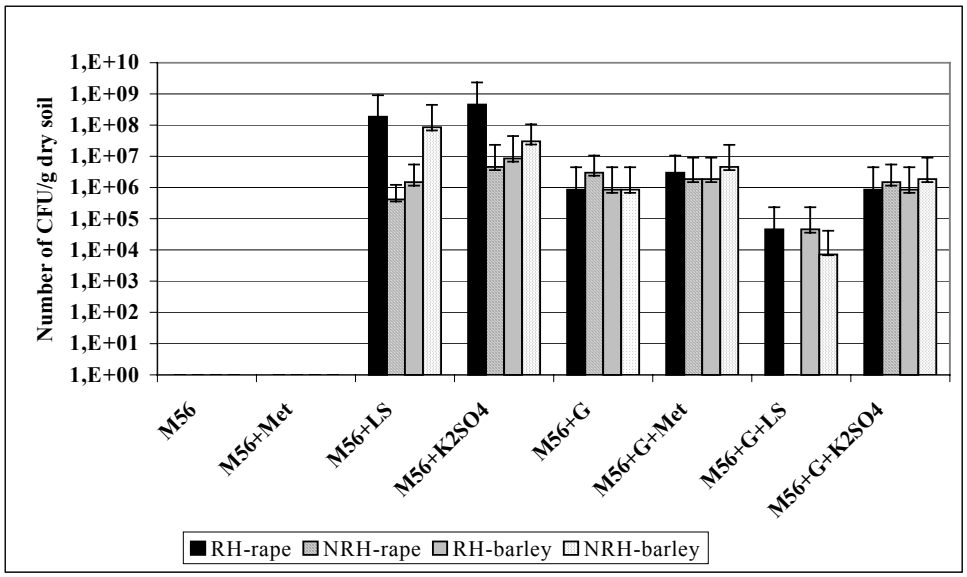

Fig. 1. Oilseed rape and barley rhizosphere (RH) or non-rhizosphere (NRH) bacterial initial soil densities in different media, containing or not sulfur in mineral (K_2SO_4) or organic forms (methionine or estersulfate)

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IDENTIFICATION AND CHARACTERIZATION OF THE *ATNFS2* GENE FROM *ARABIDOPSIS THALIANA* ENCODING A NIFS-LIKE PLASTIDIAL CYSTEINE DESULFURASE

Sebastien Leon, Brigitte Touraine, Jean-François Briat and Stephane Lobreaux
Biochimie & Physiologie Moléculaire des Plantes, UMR 5004
CNRS/INRA/Agro.M/UM2, 2-Place Viala, F-34060 Montpellier Cedex 1, France

NifS-like proteins are ubiquitous proteins that are involved in many roles, such as sulfur-containing compounds as well as iron-sulfur (Fe/S) cluster biosynthesis. *Azotobacter vinelandii* NifS protein is an essential component for nitrogen fixation in these bacteria, involved in the biogenesis of the Fe/S centers of nitrogenase (Zheng *et al.* 1993). NifS is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes the breakdown of cysteine to yield alanine and elemental sulfur, which then binds on a catalytic cysteine residue of the NifS protein (Zheng *et al.* 1993). A NifS homologue has been found in *A. vinelandii* (Zheng *et al.* 1998) but also in non-nitrogen fixing bacteria such as *Escherichia coli*, referred to as IscS, where it is involved in the biogenesis of cellular Fe/S clusters (Schwartz *et al.* 2000). The *Saccharomyces cerevisiae* homologue of IscS, NFS1p, is indeed involved in Fe/S cluster biogenesis (Kispal *et al.* 1999; Li *et al.* 1999; Strain *et al.* 1998). This protein is mainly targeted to mitochondria, which is the essential site for Fe-S cluster biogenesis in eukaryotic cells (Mühlenhoff and Lill 2000).

Fe/S cluster assembly in plants has not been studied at the molecular level. A NifS protein of *Arabidopsis thaliana* (AtNFS1) has been identified and is targeted to the mitochondria (Kushnir *et al.* 2001), although functional data are still not available. This subcellular localization is in agreement with an Fe/S cluster assembly in plant mitochondria, as well as the characterization of the yeast Atm1p homologue from *Arabidopsis* (AtSta1) which is an ABC-transporter from the mitochondria essential for the maturation of cytosolic Fe/S proteins (Kushnir *et al.* 2001).

We present here data concerning a second *NifS* gene detected by a BLAST search on *A. thaliana* genome, that we named *AtNFS2* (GenBank AY078068) (Léon *et al.* 2002). This protein shows strong homology to other NifS-like enzymes (Fig. 1). Particularly, AtNFS2 shares high homology to *Synechocystis* PCC6803 Csd3 protein, a putative cysteine desulfurase essential for cyanobacterial growth (Seidler *et al.* 2001).

Since cyanobacteria and plastids share a common ancestor, we were interested in the subcellular localization of AtNFS2. By transient expression in *A. thaliana* pro-

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toplasts, we observed that the N-terminal third of AtNFS2 addresses the chimeric protein within plastids (Léon *et al.* 2002). We overexpressed AtNFS2 as a His-tagged protein into *E. coli* cells and purified it by nickel resin-based affinity chromatography. Its yellowish color is typical of PLP-containing enzymes, and visible absorption spectra showed a major peak at 418 nm, consistent with this hypothesis. Moreover, this spectrum is modified in presence of cysteine (Léon *et al.* 2002), a feature that was already observed for *A. vinelandii* NifS protein (Zheng *et al.* 1993). We assayed the ability of AtNFS2 to use cysteine as a substrate; upon addition of 0.5 mM cysteine in the medium, we were able to detect alanine, by mean of HPLC analysis (Léon *et al.* 2002). This shows that AtNFS2-His6 bears a cysteine desulfurase activity *in vitro*.

In summary, we have characterized for the first time a NifS enzyme from *Arabidopsis thaliana*, and showed that this protein is targeted to plastids (Léon *et al.* 2002). The physiological roles of AtNFS2 are still unclear. Some sulfur-containing metabolites are synthesized in this compartment and could require a cysteine desulfurase for their biosynthesis, for instance thiamine of which the thiazole moiety requires cysteine as the sulfur donor (Julliard and Douce 1991). Another attractive hypothesis would be that Fe/S clusters might be synthesized in the plastids, as many of these cofactors are required to support photosynthesis. Further experiments will test these hypotheses.

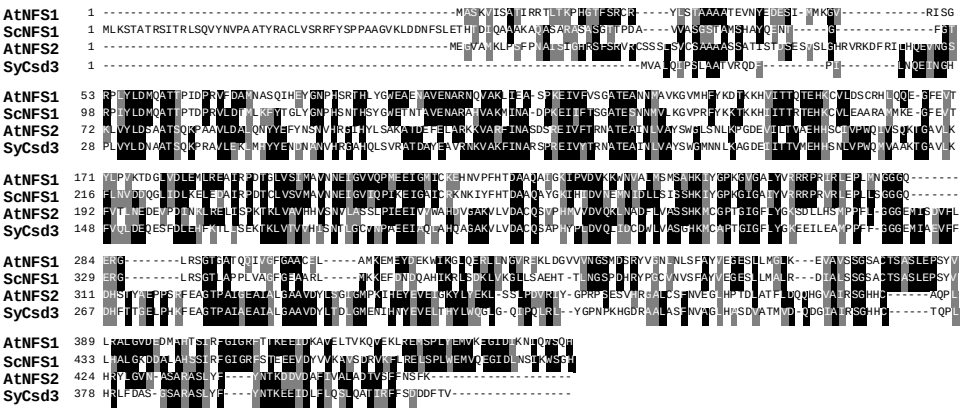

Fig. 1. Primary sequence alignment of AtNFS2 with other proteins. The deduced amino acid sequence of AtNFS2 is compared to those of AtNFS1 (Genbank Accession number O49543), yeast NFS1p (P25374), and *Synechocystis* Csd3 (Q55793). Amino acid residues conserved in most sequences are boxed in black, and those that are partially conserved (identical or similar) are boxed in grey.

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ANALYSIS OF TRANSGENIC TOBACCO LINES EXPRESSING BACTERIAL *CYSK* GENE ENCODING *O*-ACETYLSERINE (THIOL)LYASE A

Frantz Liszewska and Agnieszka Sirko
Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawinskiego 5A, 02-106 Warsaw, Poland

A significant enhancement of serine acetyltransferase (SAT) activity, due to heterologous expression of *Escherichia coli cysE* gene, has been previously reported by us for transgenic tobacco (Blaszczyk *et al.* 1999) and by others for transgenic potato (Harms *et al.* 2000). In tobacco, overproduction of bacterial SAT resulted not only in the elevated levels of cysteine and glutathione but it ameliorated also resistance of transgenic plants against oxidative stress generated by hydrogen peroxide. The two final enzymes of cysteine biosynthesis pathway, SAT and *O*-acetylserine (thiol)lyase (OAS-TL), form a bi-enzymatic complex in both, *E. coli* (Mino *et al.* 2000) and plants (Droux *et al.* 1998). Molecular interactions between these enzymes are responsible for the regulation of their respective activities. The aim of this study was to investigate the influence of simultaneous overproduction of bacterial SAT and OAS-TL in the cytosol of transgenic tobacco on the level of non-protein thiol-containing compounds and plant resistance to the oxidative stress.

Three groups of transformants, CK, CEK and CEMK, were obtained upon transformation of the wild type tobacco and previously described (Blaszczyk *et al.* 1999) transgenic lines CE-20 (overproducing wt SAT of *E. coli*) and CEM-12 (overproducing mutated SAT of *E. coli*, insensitive to the feedback inhibition by cysteine) with plant expression cassettes containing *E. coli cysK* gene encoding OAS-TL A. In the regenerated plants the presence of OAS-TL A has been verified by immunodetection. The CK group appeared to be the most numerous since in eight plants the bacterial protein has been detected. Much less numerous were the groups obtained by super-transformation of transgenic plants overproducing bacterial SAT. Only in one CEK and in four CEMK transformants OAS-TL A protein has been detected (Liszewska *et al.* 2001).

The CEK-2, CEMK-4, CEMK-9 and CK-5 lines have been selected for the subsequent experiments. Both, OAS-TL and SAT have been assayed in the leaves of primary transformants (T_0) and their self-pollinated progeny (T_1). OAS-TL activity was increased 5 to 12-fold in the CEK plants, 30 to 200-fold in the CEMK, and 10 to 100-fold in CK transformants in comparison to the controls. The enzymatic activities were dependent on the leaf position from the apex, the plant age and the

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transformant group. Consistently, the highest OAS-TL activities were measured in the youngest leaves of all transgenics.

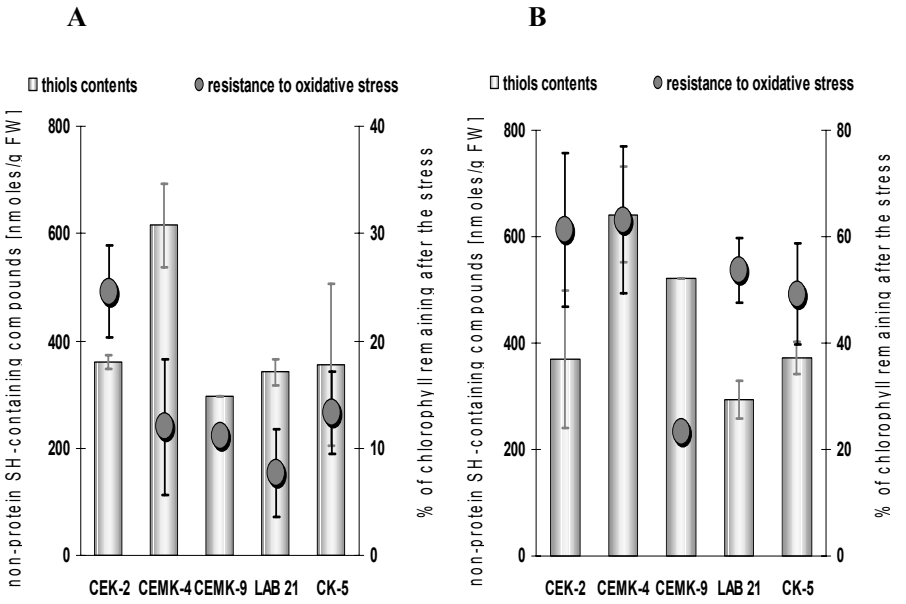

Fig. 1. Comparison of the level of non-protein SH-containing compounds (columns) and the oxidative stress resistance (points). The results are averages of the assays in 3 - 4 independent individuals of T_1 generation of the respective lines with SD indicated. Resistance against H_2O_2-generated stress was assayed only in 11-week-old plants (panel A), and resistance against MV-generated stress was assayed only in 14-week-old plants (panel B). The stress resistance was calculated as a percentage of chlorophyll remaining in the leaf discs after incubation in the oxidant solution versus control incubation in H_2O. The leaf discs were incubated in the constant light (medium light intensity).

The contents of low-molecular-weight SH-containing compounds have been measured in the leaf tissue of analyzed plants. The most significant elevation was found in CEMK-4 line. In parallel to the thiols assays, the resistance of the leaf tissue to the oxidative stress generated by floating of the leaf discs on the solutions of either 0.5 M H_2O_2 or 5 μM methyl viologen (MV) has been checked. Results of thiols assessment and the resistance to the applied oxidative agent are shown in Fig. 1. The assays were done with 11-week-old plants (panel A) and 14-week-old plants (panel B) grown in soil in a greenhouse conditions. The material has been collected from the most upper fully developed leaves (fourth-sixth from the apex).

In the conditions of plant growth and oxidative stress treatments used in this experiment some differences between the transgenic and control plants in the resistance to either H_2O_2 or MV have been detected. The most striking observation is the

low resistance of CEMK-4 transformants against H_2O_2 despite the relatively high level of SH-containing compounds. The number of analyzed transgenic lines was small; therefore it is impossible to conclude whether any relationships exist between the type of allele (wt or mutated) of bacterial SAT and the resistance of plant tissues to oxidative stress. Our results indicate simply that the level of SH-containing compounds is not the only factor determining oxidative stress resistance in the analyzed transgenic plants.

Acknowledgements

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EFFECTS OF NITROGEN AVAILABILITY ON SULFATE UPTAKE IN COMMON REED

Mario Malagoli, Claudia Bragato and Anna-Rita Trentin
Dipartimento di Biotecnologie Agrarie, Università degli studi di Padova, Agripolis, I-35020 Legnaro PD, Italy

Common reed (*Phragmites australis* (Cav.) Trin. Ex Steudel) is a perennial aquatic plant with a wide geographical distribution. It shows a high level of adaptation, growing in different habitats, from salt marshes, littoral zone of lakes, fens, to relatively dry areas (Haslam 1970). In the last years, an extensive die-back of *P. australis* has been recorded in many European wetlands (Ostendorp 1989; Armstrong *et al*. 1996a), and eutrophication has been suggested as the possible primary cause. Several studies have reported on the effects of high level of nutrients on root structure (Votrubová and Pecháckova 1966), internal aeration (Armstrong *et al*. 1996a), carbohydrate storage (Woitke *et al*. 1997), and on accumulation of toxic reduced compounds (Armstrong *et al*. 1996b). However, the knowledge on the nutrient uptake physiology of *P. australis* is limited to ammonium (Romero *et al*. 1999) and sulfide (Fürtig *et al*. 1996). Although the below ground apparatus of *P. australis* experiences anoxic conditions, the internal gas transport system provides roots and rhizomes with sufficient oxygen and facilitates a partial oxidation of the rhizosphere. Therefore, oxidized inorganic forms of N and S could be available for the plants. In the present work we wanted to study the effect of the availability of ammonium or nitrate on the uptake capacity of sulfate.

Reed plants were obtained from rhizomes and grown for 20 days in sand, supplied with modified Hoagland solution containing 1.6 mM SO_4^{2-} and either 5 mM NH_4^+ or 5 mM NO_3^-. After rhizome excision, plants were transferred to hydroponics containing the same nutrient solutions as in sand and left for 7 days to adapt to the new conditions. Before the experiments plants were conditioned for 5 days, in the presence of either ammonium (NH_4^+) or nitrate (NO_3^-), in the absence of N (-N) or S (-S). Ammonium or nitrate net uptake experiments were carried out as described in Malagoli *et al*. (2000). Sulfate influx was measured as reported in Malagoli *et al*. (1993).

The known adaptation of common reed plants to reducing soil conditions was confirmed by the net uptake experiments: ammonium uptake was significantly higher (5.1 μmol NH_4^+ g^{-1} fresh weight h^{-1}) than nitrate (3.7 μmol NO_3^- g^{-1} fresh weight h^{-1}) (Fig. 1A). Five days of N deprivation affected net uptake rates, with a reduction of 20 % for the plants grown with ammonium as N source and approximately 50 % for the plants grown in nitrate (Fig. 1A). In common reed plants, grown

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in presence of S, sulfate influx rates were affected by the nitrogen form supplied. In the presence of ammonium the influx rate was significantly lower than the rate measured in presence of nitrate (Fig. 1B), while 5 d of N deficiency did not affect the sulfate influx. Five days of sulfur starvation resulted in an increase of about 40 % in the sulfate influx rates in both of the nitrogen conditions (Fig. 1B).

The results presented indicate that in *P. australis* the ammonium transport system is more efficient than nitrate; ammonium net uptake rates were higher both with N availability and after 5 d of deprivation. However, common reed plants are able to take up nitrate when this is the only form of N available, without any evident effect on growth (data not shown). Sulfate influx is affected negatively by the presence of ammonium, but the transport system is equally de-repressed after 5 d of S starvation in presence of either ammonium or nitrate. The adaptability of common reed is confirmed by its capacity to take up nutrients in oxidized as well as reduced forms.

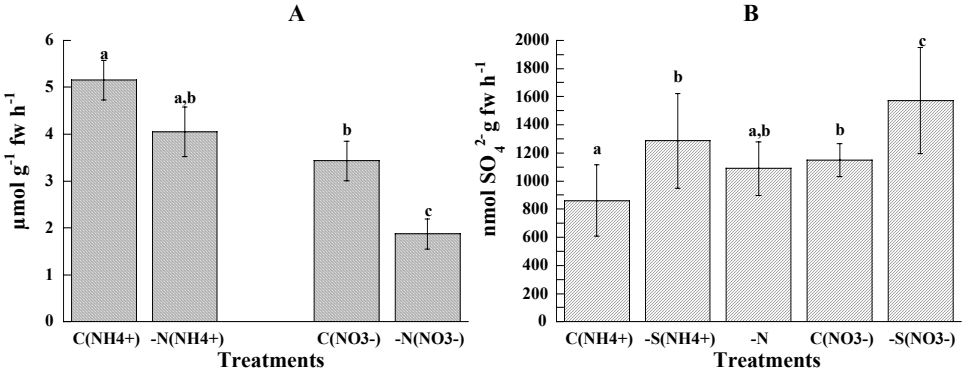

Fig. 1. (A.) Ammonium (NH_4^+) and nitrate (NO_3^-) net uptake rates (µmol g^{-1} fresh weight h^{-1}) measured in plants grown in nutrient solutions and conditioned for 5 d in presence (C) or absence (-N) of N. (B) Sulfate influx rates (nmol SO_4^{2-} g^{-1} fresh weight h^{-1}) measured in plants grown in nutrient solutions containing either (NH_4^+) or nitrate (NO_3^-) and conditioned for 5 d in presence (C) or absence (-S) of sulfur, or in absence of N (-N). Data presented are means of 4 replicates of 5 plants each. Error bars indicate SE; different letters indicate significant differences at $p < 0.05$.

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LOCALIZATION OF γ-GLUTAMYL-TRANSFERASE ACTIVITY IN PLANT TISSUE

Antonio Masi, Tiziana Destro and Massimo Ferretti
Department of Agricultural Biotechnology, Strada Romea 16 – Agripolis, I-35020 Legnaro (PD), Italy

Glutathione synthesis has been thoroughly studied and its regulation elucidated using biochemical and molecular approaches, but the sequence of reactions leading to cysteine release from glutathione remains to be clarified. Two pathways have been proposed for glutathione degradation in plants. In one pathway, the glycine residue is first removed by a carboxypeptidase, then cysteine is hydrolized by a γ-glutamyl-cyclo-transferase. These activities have been demonstrated in tobacco suspension cell cultures (Steinkamp and Rennenberg 1984). In another pathway, similar to the one already known in animal tissues, the glutamyl residue is removed by γ-glutamyl-transferase (GGT) activity to yield cysteinyl-glycine, which is further hydrolized by a dipeptidase.

GGTs are widely found in monocots and dicots and both soluble and membrane-associated forms have been found. Activities have been reported to be constitutive or to increase throughout fruit development and ripening in tomato (Martin and Slovin 2000). In *A. thaliana*, GGT is encoded by a small gene family with at least four isoforms that might serve different functions and might have different cellular localizations. The *Arabidopsis* GGT is very similar to animal enzymes, and all amino acid residues necessary for the catalytic activity of the mammalian GGT are conserved in the plant enzyme. A putative hydrophobic transmembrane domain was also found at the N-terminus of the plant protein. In transgenic tobacco plants over-expressing AtGGT, activity was localized on the outer surface of the plasma membrane. In animals and in plants, GGT is composed of two subunits derived from post-translational modification of a single polypeptide chain precursor, which is cleaved and assembled to its final heterodimeric form. As predicted by sequence analysis, GGTs are often heavily glycosilated. Biochemical evidences confirm that *Arabidopsis* GGTs have catalytic characteristics very similar to those of yeast, mammalian and other plant GGTs (Storozhenko *et al.* 2002), but the *in vivo* role for this enzyme has not been ascertained.

Since localization may help elucidation of enzyme functions, we developed a method for the demonstration of GGT histochemically in plants, based on the original technique of Rutenberg *et al.* (1969), where 4-methoxy-2-naphtylamine, liberated enzymatically by GGT activity, reacts with the diazonium salt Fast Garnet

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GBC to form a reddish to purplish-brown insoluble azo-dye. Enzyme-histochemical analysis revealed that GGT activity is not uniformly distributed among the examined tissues. In maize and barley, it is specifically localized in the parenchyma cells of conductive tissues that are closely associated with vascular bundles (Fig. 1a). This is evident both in leaves and in stems, and suggests that GGT may be involved in the regulation of glutathione distribution in the plant. This physiological role, might be crucial when plants have to cope with environmental oxidative challenges. An attempt to isolate genes involved in plant protection toward oxidative damage led to the isolation of a cDNA from *A. thaliana* encoding a putative GGT, whose expression in yeast conferred the highest tolerance to diamide, a thiol-oxidizing drug (Kushnir *et al*. 1995). In another study, when maize plants were treated with UV-B radiation, an increase in cysteinyl-glycine content was observed in shoots (Masi *et al*. 2002), possibly reflecting an enhanced degradation of glutathione conjugates with dangerous metabolites, as lipid peroxides. In the same experiment the root γ-glutamyl-cysteine content also increased, indicating enhanced GSH synthesis. Since glutathione is the transport metabolite, the increase in cysteinyl-glycine could also reflect a process of delivery of glutathione to sink tissues, involving glutathione degradation by GGT. This possibility is compatible with GGT localization in conductive tissues as shown in this work.

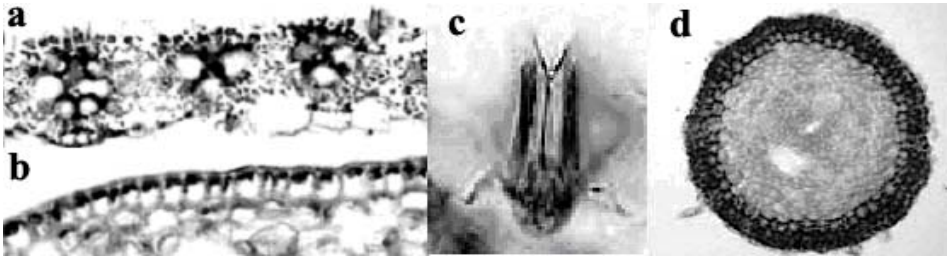

Fig. 1. Enzyme-histochemical visualisation of GGT activity in (a) maize leaf section; (b) barley root; (c) maize stomata; (d) epidermal cell layer from barley epicotyl. Dark grey indicates enzyme activity.

Interestingly, activity was also found in the epidermal layer in the barley epicotyl and in maize stomata (Fig. 1b,c). This distribution may signify the salvage of glutathione involved in antioxidative events at the interface with the external environment. In barley, but less so in maize, it is possible to observe an intensive staining of the root epidermis and cortex cells (Fig. 1d), which may signify retrieval of external glutathione. In pea and broad bean plants, very limited staining was observed in corresponding tissues (not shown); this finding might reflect interspecific differences. Although the enzyme promotes degradation of glutathione, other major functions different from glutathione catabolism may be envisaged for GGT, pointing to its involvement in a γ-glutamyl cycle in plants too.

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EFFECTS OF NITROGEN AND SULFUR FERTILIZATION ON GRASS YIELD AND QUALITY IN BELGIUM

M. Mathot[1], R. Lambert[1], J. Mertens[2], B. Toussaint[1] and A. Peeters[1]
[1]Laboratoire d'Ecologie des Prairies, Université catholique de Louvain, place Croix du Sud N°5 bte 1, B-1348 Louvain-la-Neuve, Belgium; [2]Soil Service of Belgium, W. de Croylaan 48, B-3001 Heverlee, Belgium

Sulfur deficiencies have been reported in grasslands in most European countries. Field trials were set up on Belgian soils to investigate the effects of sulfur and sulfur-nitrogen interaction on dry matter yield and chemical composition of herbage.

Five trials were set up in 2001 in different sites throughout Belgium (Table 1). The results of two previous experiments carried out in 1993 (Lambert *et al*. 2000) and 1995 at site 3 are also presented. The experimental sites were located on potentially sulfur deficient soils. Increasing N and S fertilization levels were applied (Table 1). All the experiments were designed as randomized blocks with 4 replicates.

Table 1. Sites characteristics, number of cuts, total N (kg N ha^{-1}) and total S (kg SO_3 ha^{-1})* fertilization levels. Species studied were *Lolium multiflorum* (Lm) and *Lolium perenne* (Lp).

	Soil			Species	Year	Cuts	N			S
Sites	Texture	% C	pH KCl				N1	N2	N3	
1. Bocholt	Sand	2.8	5.3	Lp	2001	5	207	414	538	0-60-150
2. Heure	Silt loam	3.1	5.7	Lp	2001	2	80	160	208	0-20-40-60-100
3. Michamps	Silt loam	1.9	5.6	Lp	1993	2	-	160	240	0-40-60
				Lp	1995	3	-	240	480	0-30
				Lp	2001	3	120	240	312	0-90-150
4. Nethen	Silt	1.7	7.5	Lm	2001	5	90	180	234	0-20-40-60-100
5. Tielen	Sand	2.4	4.9	Lp	2001	5	211	422	549	0-60-150

*(kg SO_3 ha^{-1}), European fertilization standard!

Dry matter yields were recorded. All samples were analyzed for N by NIRS. Total sulfur content was determined by Leco Fp2000, in samples where a sulfur effect on yield or on N content was detected.

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A significant effect of the S fertilization on the yield was observed on the first cut on site 1 (+ 50 %, 500 kg DM ha^{-1}). For this cut, without S fertilization, the N : S ratio was 15.2 and the S content was 0.18, indicating a possible S deficiency for plant growth. The critical values are 15 for N : S and 2 g S kg^{-1} DM for the S content (Whitehead 2000). Total yield in site 3 in 1993, in site 1 and in site 5 in 2001 were increased, but not significantly, by the S fertilization (Table 2).

A 50 kg SO_3 supply increased the grass N content significantly (+10 %) at the third cut in site 5. However, the S content of the forage reached the toxicity level for cattle (0.4 S % DM).

In all grass samples analyzed for sulfur the S fertilization increased significantly the S content and decreased the N/S ratio. Forty-five percent of the samples of the zero SO_3 treatments had a S content lower than 0.2 % DM and a N/S ratio higher than 15 indicating that they were S deficient. Among these, the highest N fertilization showed the highest rate of S-deficient samples (62.5 %). At the lowest N fertilization only 12.5 % of the samples was S deficient. These results show that supplying N strongly influences plant S content, by increasing the yields and diluting S in the biomass.

Table 2. Total yield of the different sites in relation with total SO_3 (kg ha^{-1})* and nitrogen level applied (N1, N2 or N3). Results are given as relative yield (%) to the corresponding value between brackets (ton dry matter ha^{-1}).

		Site 1			Site 2					Site 4				
S		0	60	150	0	20	40	60	100	0	20	40	60	100
N	N1	(9.4)	94	99	(4.2)	102	89	85	106	(4.6)	98	97	96	99
	N2	113	122	117	137	117	107	132	124	114	111	117	109	111
	N3	122	131	130	120	123	104	121	127	121	125	119	123	119

		Site 3 (1993)			Site 3 (1995)		Site 3 (2001)			Site 5		
S		0	40	60	0	30	0	90	150	0	60	150
N	N1				(2.9)	106	(5.0)	97	101	(10.1)	103	100
	N2	(6.6)	103		290	292	172	179	175	113	116	121
	N3	109		125	427	396	201	193	188	120	122	123

*(kg SO_3 ha^{-1}), European fertilization standard!

Finally, it appears that occasional S deficiency may occur whatever the year or the site and may affect as well herbage quality as dry matter yield. Such deficiencies were avoided by supplying sulfur at a rate of about 20 kg SO_3 ha^{-1} cut^{-1}. Further investigations have to be carried on to determine the main factors influencing the occurrence of S deficiency and to establish a S fertilization advice system.

Acknowledgements

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GENOMIC AND BIOCHEMICAL STUDIES OF SULFUR ASSIMILATION IN ONION

John McCallum[1], Meeghan Pither-Joyce[1], Martin Shaw[1], Anya Lambert[2] and Michael T. McManus[2]
[1]Crop and Food Research, Private Bag 4704, Lincoln, Christchurch, New Zealand; [2]Institute of Molecular BioSciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

Allium vegetables vary widely in their content of alkyl cysteine sulfoxides (ACSOs), the sulfur amino acid derivatives that determine their aroma and health qualities (Lancaster and Bolland 1990). It is known that ACSO content is influenced by S and N nutrition but the molecular basis for this genetic and environmental variation is not known. We have now isolated cDNAs for all steps leading from S assimilation to glutathione formation and are using these for genetic and metabolic analysis of onion S metabolism. Our strategy is to compare genotypes that are documented high accumulators of ACSOs (pungent) with milder lines and in the present study, we have observed significant differences in the expression of some S-assimilation genes. These observations have been extended by analyzing *O*-acetylserine (thiol)lyase activity in these same lines.

Seedlings of mild (cv. 'Houston Grano') and pungent ('CLK' or 'P12' selections of cv. PLK) onions were grown hydroponically in Hoaglands media. We isolated cDNAs either by RT-PCR of root and leaf cDNAs and/or screening a root cDNA library, or by sequencing clones from a differential cDNA library enriched for transcripts up-regulated in onion (*cv* 'PLK') root by 48 h of sulfate starvation. Gene expression studies were performed either using Northern analysis or quantitative dot-blot analysis as described in MaCallum *et al.* (2002). The determination of *O*-acetylserine (thiol)lyase activity in leaf extracts was performed as described in Rolland *et al.* (1992).

Gene expression in roots was compared first by Northern analysis of the pungent CLK line in response to 48 h of S starvation (Fig. 1A), and an up-regulation of a high-affinity sulfate transporter (HAST), an ATP sulfurylase (ATPS), an APS reductase (APSR), and a sulfite reductase all of plastid origin was observed. When these plants were grown in steady-state conditions [S-sufficient (2 mM S), S-deprived media (0.05 mM S)], expression of the HAST and ATPS genes were up-regulated significantly in the low S treatment, the expression of the sulfite reductase gene remained constant but the expression of ATP sulfurylase decreased. Dot-blots were used to compare the mild and pungent genotypes grown under high and low sulfur conditions (Fig. 1B), and in common with the pungent CLK line, an up-

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regulation of HAST and APSR gene expression was observed in the Houston Grano line. Again, a significant decrease in the expression of the chloroplastic ATPS gene was observed suggesting that ATPS expression is a key candidate for analysis of genotype variation in onion S assimilation. Comparison of *O*-acetylserine (thiol)lyase activity in chloroplasts of leaves from the pungent line (P12) with the mild line (Houston) grown in S-sufficient and S-starved conditions (Table 1) reveals that enzyme activity was highest in the pungent onion line (P12), with greatest induction of activity in response to low S observed in the mild line, suggesting differences in the biochemical regulation of S assimilation in onion.

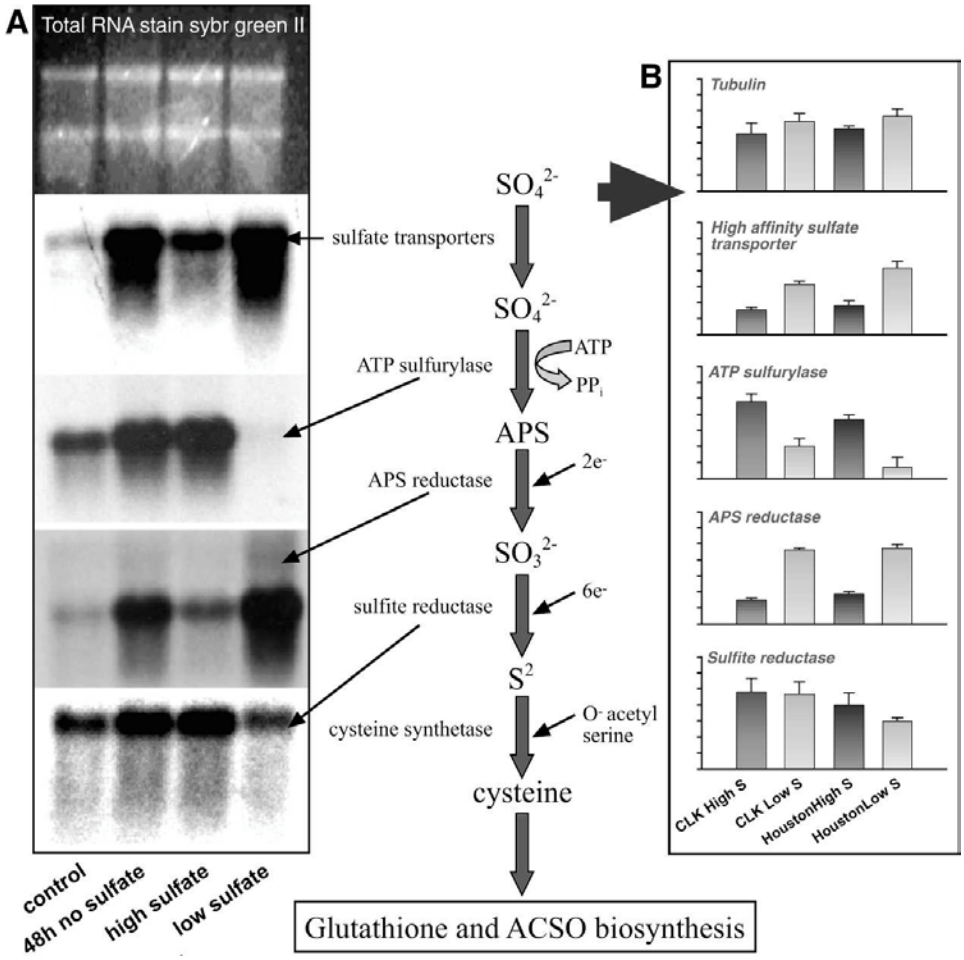

Fig. 1. Changes in the expression of S-assimilation genes in response to S supply.

Table 1. Cultivar comparison of *O*-acetylserine (thiol)lyase activity in chloroplasts.

Onion Cultivar	Treatment	*O*-Acetylserine (thiol)lyase activity (nmol mg^{-1} protein min^{-1})
Houston Grano	High (2 mM) S	33
	Low (0.5 mM) S	105
P12	High S	80
	Low S	120

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EFFECT OF MINERAL SULFUR AND ORGANIC FERTILIZERS ON YIELD AND SULFUR UPTAKE OF GRASS ON BELGIAN SANDY SOILS

J. Mertens, G. Verlinden and M. Geypens
Soil Service of Belgium, W. de Croylaan 48, B-3001 Heverlee, Belgium

Sulfur is an essential nutrient for optimal plant growth. Because of the decreased use of S-containing fertilizers and the decreasing atmospheric sulfur deposition, sulfur deficiency can be expected for different crops. Because of the importance of grassland in Belgian agriculture and the occurrence of sulfur deficiency in the neighboring countries, field trials and pot experiments were set up to study the effect of sulfur fertilization on grass yield on Belgian sandy soils.

Two field experiments were set up in the northern part of Belgium on sandy soils with a low organic matter content. A characterization of the soils is given in Table 1.

Table 1. Location and characteristics of experimental soils.

Location	Soil texture	pH KCl	% C	Total N (mg/100g)	Total S (mg/100g)
Tielen	Sand	4.9	2.4	157	21
Bocholt	Sand	5.3	2.8	119	15

The experiment consisted of 9 treatments with 4 replicates each, distributed in a randomized block design. Three N application rates (recommended rate ®, R-50 % and R+30 %), were combined with three S application rates (0, 20 and 50 kg SO_3 ha^{-1} for the first three cuts). N fertilization was applied as NH_4NO_3, S fertilization was applied as K_2SO_4. The grass (*Lolium perenne*) was harvested 5 times. The dry matter yield at each cut is presented in Table 2.

Generally, S fertilization increased dry matter yield, but N and S had a clear interactive effect: there was no, or even a negative effect of sulfur fertilization on dry matter yield at low N application rates. However, at the recommended and high N application rates, a positive effect of S fertilization on dry matter yield was observed: yield increase varied from 230 to 890 kg ha^{-1}. Especially at the first grass cut in Bocholt, dry matter yield was increased significantly by sulfur fertilization. This

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research will continue to evaluate these first indications of sulfur deficiency on Belgian sandy soils.

The effect of mineral S and organic fertilizers on yield and sulfur uptake was also investigated in a pot experiment. Pots were filled with a sandy soil and cropped with Italian rye grass (*Lolium multiflorum* L.). The sandy soil (top 20-cm) was collected from the field in Tielen (Table 1). Five different treatments were set up. The five treatments consisted of two mineral fertilizer applications (NPK fertilization without S and NPK fertilization with S, applied at the beginning of the experiment and after each cut) and three organic material (cattle slurry, poultry manure and compost) applications. Cattle slurry (0.67 % N, 0.05 % S), poultry manure (2.64 % N, 0.34 % S) and compost (1.60 % N, 0.63 % S) were applied at a rate of 125, 25 and 100 g pot^{-1} respectively and were mixed with the soil at the beginning of the experiment. The grass was harvested 4 times.

At each grass cut, a positive effect on yield and S uptake of both mineral and organic fertilization was observed. The grass took up most S in the mineral fertilized treatment. When the three organic fertilizers are compared, the grass fertilized with poultry manure took up most S. The availability of sulfur in the organic fertilizers was sufficient for an optimal grass growth during the first weeks of the experiment. However, after the first cut, S mineralization from the organic S pool in the cattle slurry and poultry manure could not fulfill the S demand of the grass, so grass yield and S uptake decreased. From the second to the fourth cut, only 4.2 to 0.9 % (cattle slurry) and 5.1 to 3.1 % (poultry manure) of the total applied sulfur were taken up. For the treatment with compost, yield and S uptake was optimal for the first two cuts: only a small part of the applied sulfur was taken up (1.1 %), but because of the high concentration of S in the compost, growth was optimal. The next two cuts, S recovery decreased to 0.56 and 0.28 %.

Table 2. Dry matter yield (ton ha^{-1}) on a sandy soil in (A) Tielen and (B) Bocholt (2001). R, recommended rate.

	N	R	R	R	R-50%	R-50%	R-50%	R+30%	R+30%	R+30%
	S	0	20	50	0	20	50	0	20	50
A	cut 1	2.50	2.36	2.47	2.12	2.26	2.00	3.16	2.75	2.99
Cut 2		3.28	3.23	3.47	2.77	2.67	2.83	3.13	3.34	3.29
Cut 3		1.53	1.63	1.59	1.27	1.33	1.31	1.53	1.77	1.71
Cut 4		1.75	1.99	2.23	1.83	1.76	1.77	1.86	2.20	2.16
Cut 5		2.36	2.43	2.43	2.10	2.32	2.18	2.39	2.26	2.27
Total		11.4a	11.6a	12.2a	10.1b	10.3b	10.1b	12.1a	12.3a	12.4a
B	cut 1	1.16	1.70	1.73	1.20	0.94	0.98	1.66	2.09	2.26
Cut 2		3.02	3.19	3.07	2.58	2.36	2.54	3.26	3.35	3.33
Cut 3		2.27	2.48	2.33	1.84	1.76	1.99	2.44	2.76	2.62
Cut 4		1.71	1.75	1.63	1.40	1.32	1.41	1.66	1.67	1.66
Cut 5		2.49	2.42	2.26	2.41	2.48	2.38	2.49	2.53	2.42
Total		10.7	11.5	11.0	9.4	8.8	9.3	11.5	12.4	12.3
		abc	ab	abc	bc	c	bc	ab	a	a

The mineral S fertilization significantly increased the S content of the grass at each grass cut. The organic fertilizers increased the S content of grass at the first cut. The S content varied from 0.08 to 0.11 % for the no-fertilized treatment, from 0.12 to 0.16 % for the mineral fertilized treatment and from 0.07 to 0.18 % for the organic fertilized treatments.

Acknowledgements

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ARABIDOPSIS SULFUR TRANSFERASES: INVESTIGATION ON THEIR ROLE IN THE ORGANISM

Tanja Meyer, Meike Burow, Michael Bauer and Jutta Papenbrock
Institut für Botanik, Universität Hannover, Herrenhäuserstr. 2, D-30419 Hannover, Germany

Sulfur transferases (ST)/rhodaneses are a group of enzymes widely distributed in plants, animals, and bacteria that catalyze the transfer of a sulfur atom from a donor molecule to a thiophilic acceptor substrate. The exact function of ST in the plant organism is not known so far. Several functions were suggested: such as a role in cyanide detoxification in analogy to mammalian ST (Westley 1973), the detoxification of free oxygen radicals in a thioredoxin reductase reaction (Nandi *et al*. 2000), providing sulfur for biosynthetic reactions, *e.g.* for Fe-S cluster (Bonomi *et al*. 1977), mobilization and transport of reduced sulfur, especially during aging of plants (Papenbrock and Schmidt 2000). Here, we would like to investigate the putative role of ST1 in cyanide detoxification in the non-cyanogenic plant *Arabidopsis thaliana*.

Experimental design: 4-week-old *Arabidopsis* plants grown on soil were sprayed with a 20 mM cyanide solution and compared with control plants. At the time points indicated in Fig. 1 the parts above ground were cut and frozen in liquid nitrogen. The expression on the RNA and protein levels, and the respective enzyme activities were determined as described (Papenbrock and Schmidt 2000) for ST and for *β*-cyano-L-alanine synthase (CAS), another enzyme supposed to be involved in cyanide detoxification in higher plants. The cyanide contents were determined according to Guilbault and Kramer (1966).

In comparison to the expression in control plants the levels of *ST* and *CAS* RNA were unimpaired (Fig. 1). It was shown by Western blot analysis using monospecific antibodies that the levels of ST and CAS did not change after the plants were treated with cyanide (data not shown). The enzyme activities remained almost constant in control and cyanide treated plants or even decreased after cyanide treatment (Table 1). However, it was shown that the cyanide contents in the plants were raised at least 5 times after being sprayed with cyanide.

Increasing of the cyanide contents by a factor of 5 in *Arabidopsis* plants has neither an effect on the expression of *ST* and *CAS* mRNA and the respective protein contents nor on the ST and CAS enzyme activities. Constitutive expression levels might be sufficient for the detoxification of cyanide. In non-cyanogenic plants cya-

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nide is mainly formed in the last step of ethylene biosynthesis in the cytosol. However, both enzymes, ST and CAS, are localized in the mitochondria. Therefore one has to postulate a transport of cyanide into the mitochondria. In summary, the evidence for an involvement of ST and also CAS in cyanide detoxification as their only role in the metabolism is very low.

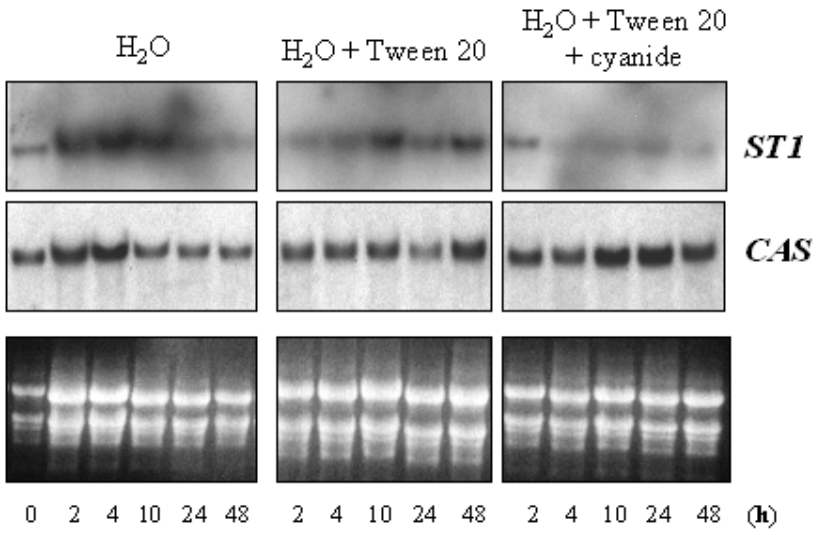

Fig. 1. Northern blot analysis. 4-week-old *Arabidopsis* plants were grown on soil and sprayed with 10 ml of H_2O (left), H_2O and 0.1 % (v/v) Tween 20 (middle), and H_2O, 0.1 % (v/v) Tween 20 and 20 mM cyanide (right). Total RNA was extracted, separated and blotted. The membranes were hybridized with *ST1* and *CAS* DNA probes. The boxes at the bottom show the respective ethidium bromide-stained gels to demonstrate equal loading.

Table 1. Cyanide contents and determination of enzyme activities in plants treated with 20 mM cyanide solution in comparison to untreated plants. The ST activity was determined by using 3-mercaptopyruvate as sulfur donor and cyanide as sulfur acceptor. The CAS activity was determined according to Blumenthal *et al*. (1968). The values for the second control, H_2O and 0.1 % Tween 20, were very similar to the H_2O control. The standard error was always below 5 %. FW, fresh weight.

	Cyanide ($\mu g\ g^{-1}$ FW)		Thiocyanate formed (nmol mg^{-1} protein min^{-1})		Sulfide formed (nmol mg^{-1} protein min^{-1})	
	H_2O	20 mM KCN	H_2O	20 mM KCN	H_2O	20 mM KCN
0 h	0.11	-	48.6	-	67.6	-
2 h	0.05	0.81	60.5	37.8	50.5	40.7
4 h	0.13	0.51	52.3	44.5	42.9	38.2
10 h	0.05	0.41	50.9	41.7	49.1	36.9
24 h	0.16	0.36	59.9	50.3	57.1	47.9
48 h	0.14	0.25	54.0	44.5	50.4	62.8

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SUBCELLULAR DISTRIBUTION OF GLUTATHIONE – A HIGH RESOLUTION IMMUNOGOLD ANALYSIS IN LEAVES OF PUMPKIN (*CUCURBITA PEPO* L.)

Maria Müller, Bernd Zechmann, Michael Tausz and Günther Zellnig
Institute of Plant Physiology, University of Graz, Schubertstraße 51, A-8010 Graz, Austria

Glutathione has numerous roles in cellular defense mechanisms and in sulfur metabolism. The functions depend on the concentration and the redox state of glutathione pools. The concentrations in different cells and tissues are an equilibrium between synthesis, degradation, use, and short and long-distance transport (Foyer *et al.* 2001).

As a first step some workgroups have examined the cellular and roughly the subcellular distribution of glutathione in different plant tissues and single cells by using monochlorobimane and fluorescence microscopy (*e.g.* Fricker *et al*. 2000; Meyer *et al*. 2001; Müller *et al*. 1999, 2002). With this background, it is of interest to collect more precise data on GSH localization and GSH concentrations in cell organelles by using ultrastructural investigations. In this work, an electron microscopic immunogold labelling was applied for the first time to investigate the subcellular localization of glutathione in plant cells.

Small sections of pumpkin leaves (*Cucurbita pepo* L.) were fixed according to a standard fixation protocol (*cf.* Zellnig *et al*. 2000) or in 0.5 % glutaraldehyde / 2.5 % paraformaldehyde, dehydrated in a graded series of ethanol and embedded in LR-white resin (Figs. 1-3). Ultrathin sections were incubated with an antiglutathione (GSH) rabbit polyclonal antiserum (Signature Immunologics, Inc.) followed by a 10 nm gold-labelled secondary antibody (British Biocell Int.). The GSH antiserum was shown to react selectively with GSH, although it does not differentiate between the reduced and oxidized forms of glutathione. To confirm the selectivity of the immunolabelling in the present set of experiments, the tissue sections were either incubated with a primary antibody, which was exposed to 5 mM GSH prior to the application, followed by the secondary antibody or treated with the secondary antibody alone. Both methods resulted in a negative immunolabelling (Fig. 2).

All investigated cells showed gold particles after the immunoreaction but the labelling intensity varied over a wide range between the compartments from very low intensities in peroxisomes and ER to high intensities in mitochondria (Figs. 3, 3A). The density of gold particles in chloroplasts and especially in the nuclei was lower than expected from either biochemical data (Noctor *et al.* 2002) or from staining

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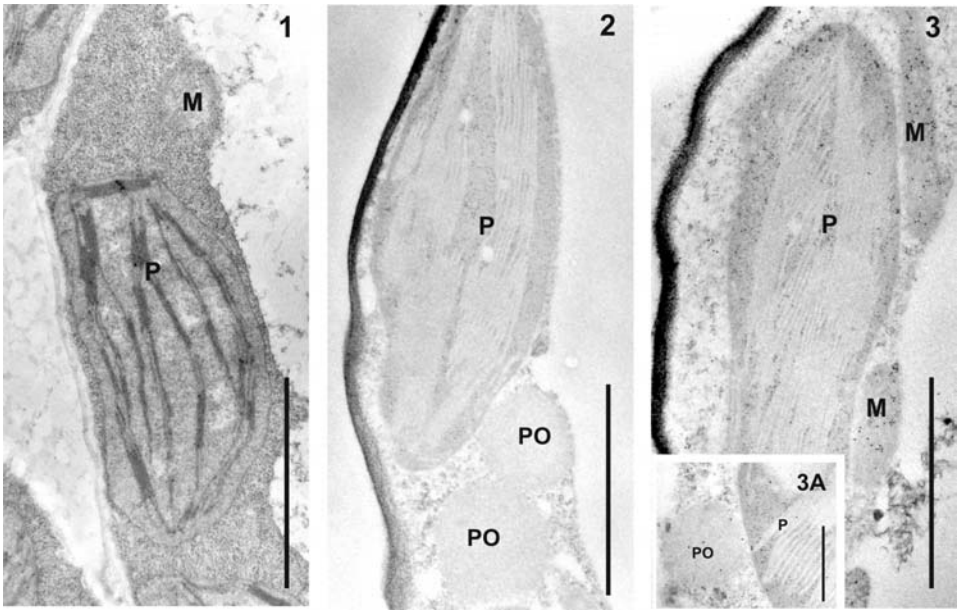
with bimane derivates. Gold particles were never found in vacuoles and the apoplast.



Figures 1-3. Transmission electron microscopy of mesophyll leaf cells of *Cucurbita pepo* L.
Fig. 1. Part of a cell after standard fixation (sections stained with lead citrate and uranyl acetate) showing a chloroplast (P) and a mitochondrion (M). Bar = 2 μm.
Fig. 2. Part of a cell after GSH addition to the primary antibody prior to the section labelling, showing a chloroplast (P) and peroxisomes (PO) without gold particles. Bar = 2 μm.
Fig. 3. Electron micrograph showing the distribution of GSH in various compartments after immunolabelling; gold particles are present in the chloroplast (P) and in an increased number in the mitochondria (M). Bar = 2 μm.
Fig. 3A. Electron micrograph of an immunolabelled section showing gold particles in the chloroplast and a peroxisome. Ba r = 0.5 μm.

The presented results clearly show different labelling patterns of glutathione in the cell compartments. Further work would be necessary including quantitative analysis of the total pool of glutathione, the subcellular compartmentation of glutathione precursors, and related enzymes. Another important question, which can be addressed using this method, is the origin of mitochondrial glutathione and the possibility of a transporter located on the inner mitochondrial membrane (*cf.* Noctor *et al.* 2002).

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CLUSTER ANALYSIS OF GENE RESPONSES TO SULFUR DEPLETION IN *ARABIDOPSIS THALIANA*

Victoria Nikiforova[1], Carsten Daub[1], Jens Freitag[1], Stefan Kempa[1], Kerstin Riedel[2], Holger Hesse[2] and Rainer Höfgen[1]

[1]Max-Planck-Institut für Molekulare Pflanzenphysiologie, Dept. of Prof. Lothar Willmitzer, Am Mühlenberg 1, 14476 Golm, Germany; [2]Freie Universität Berlin, Institut für Biologie, Angewandte Genetik, Albrech Thaer-Weg 6, 14195 Berlin, Germany

In higher plants inorganic sulfate is assimilated into a variety of sulfur-containing organic compounds with numerous biological functions (Leustek & Saito 1999). To elucidate the interactions in this network of different pathways and their regulating cascades, we performed an array expression analysis of *Arabidopsis* seedlings subjected to different conditions of sulfur depletion. The temporal expression behavior of ~7200 non-redundant genes was analyzed simultaneously.

To obtain differential expression of genes interconnected with the sulfur-assimilatory pathway, *Arabidopsis* seedlings were subjected to sulfur depletion for 6 and 10 days, and for 10 and 13 days in two experimental sets. In the first experimental set the seedlings were pre-grown on sulfur-sufficient medium for 8 days. The respective transcript levels of approximately 16,000 EST clones represented in the MSU collection were determined *via* array hybridizations with five repetitions of independently sampled material (Hoefgen *et al.* 2003, this volume).

The correlated expressional behavior of different ESTs representing the same gene nicely proved the reproducibility of the temporal expression patterns (Fig. 1A and B). However, the complexity of the expressional behavior of randomized ESTs representing different genes (Fig. 1C) required special mathematical tools to identify gene clusters. Thus, the analytical challenge posed by the coordinated and complex response of several hundred genes in this study, was assessed with bioinformatic tools which, for this purpose, had to be partially newly developed or adapted.

By applying a modified informational entropy measurement (Butte and Kohane 2000) to the gene expression profiles obtained in response to sulfur depletion the amount of mutual information for each gene pair was calculated. By data set permutation and re-calculation of the mutual information content of different randomized data sets a significance threshold was defined to distinguish between significant and non-significant correlation. By single linkage clustering based on the significant mutual information gene clusters with similar expressional behavior were identified and a network of non-linear correlations was set up. Within this network of correlated gene expression connections of different orders can be extracted to display for

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example the immediate correlation partners of a selected gene of interest. Reducing the complexity to the nearest neighbors produces distinct sets of genes correlated in their expressional behavior based on their amount of mutual information. For example, cysteine synthase is directly correlated with 13 genes which are not only members of the downstream sulfur metabolism (*e.g. S*-adenosyl methionine synthetase, *S*-adenosyl homocysteine hydrolase) but also potential regulatory genes (*e.g.* two splicing factors, one transcription factor, one phosphatase). Whether these *in silico* generated relations also correspond to physiological relations is currently verified.

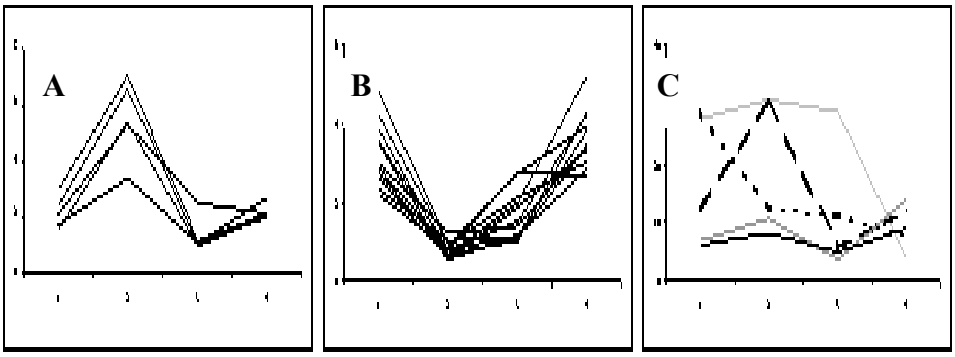

Fig. 1. Patterns of expressional behavior of different ESTs as revealed by calculating the amount of mutual information. (A) and (B) the expression of different ESTs of a given gene is correlated proving the reproducibility of the approach. (C) expression patterns of randomized ESTs of different genes demonstrating the necessity for mathematical tools to assess the amount of data.

By applying bioinformatic tools to gene expression data sets it is thus possible to provide tractable hypotheses about complex expression patterns of pathways *e.g.* under sulfur depletion. This approach holds the potential to exceed classical, pathway driven studies as the data set is analyzed in an unbiased way. Confirmation of the inter-regulation of sulfur-responding pathways will be performed by comparing the expression profile data with metabolic networks obtained by metabolite profiling as well as by detailed analyses of respective knock-out mutants.

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ALTERED EXPRESSION OF SERINE ACETYLTRANSFERASE GENE IN TRANSGENIC *ARABIDOPSIS* RESULTED IN MODULATED PRODUCTION OF CYSTEINE AND GLUTATHIONE

Masaaki Noji[1], Fumiko Saito[1], Tomoko Ochiai[1], Yumiko Shirano[2,3], Hiroaki Hayashi[4], Daisuke Shibata[2,5], Tomohiko Kato[5], Satoshi Tabata[5] and Kazuki Saito[1,6]

[1]Department of Molecular Biology and Biotechnology, Graduate School of Pharmaceutical Sciences,Chiba University, Chiba 263-8522, Japan; [2]Mitsui Plant Biotechnology Research Institute (disbanded in March 1999), Ibaraki 305-0047, Japan; [3]Boyce Thompson Institute, Cornell University, NY 14853-1901, U.S.A.; [4]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan; [5]Kazusa DNA Research Institute, Chiba 292-0812, Japan; [6]CREST, JST, Japan

Serine acetyltransferase (SATase) catalyzes the formation of *O*-acetylserine (OAS) that is the key intermediate of cysteine biosynthesis. OAS is not only a dominant limiting intermediate for cysteine formation but was recently suggested to be a possible signal molecule for gene expression in cysteine biosynthesis. From these indispensable roles of OAS, one can expect the presence of regulatory mechanism of SATase for cysteine biosynthesis (Saito 2000). In *Arabidopsis*, it has been shown that the activity of the cytosolic SATase (SAT-c) was feedback inhibited by cysteine at physiological concentration, however, the activities of the plastidic and the mitochondrial SATases (SAT-p and SAT-m) were not subjected to this feedback regulation (Noji *et al.* 1998). This finding suggests that some SATase isoforms could play a regulatory role in cysteine biosynthesis through the feedback inhibition. To reveal the role of SATase in the regulation of cysteine biosynthesis, and to modulate the sulfur metabolite in plants, we characterized transgenic *Arabidopsis* plants in which SATase activities in cytosol or in chloroplasts were varied by overexpression of heterologous SATase gene, or by suppression of the endogenous SATase gene.

Watermelon SATase is localized in cytosol and its activity is inhibited by low concentration of cysteine (Saito *et al.* 1995). We previously made a point-mutated watermelon SATase, SATG277C, which is not subjected to the feedback regulation by cysteine (Inoue *et al.* 1999). We constructed four different transgenic *Arabidopsis*, SATW, RuSATW, SATM and RuSATM, overexpressing watermelon SATase or mutant SATase gene, SATG277C. In SATW and SATM, produced SATase was localized in cytosol; whereas in RuSATW and RuSATM produced SATase was

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localized in chloroplasts by the function of the fused transit peptide sequence Rubisco small subunit. In SATW and RuSATW, watermelon SATase produced was highly sensitive to the feedback inhibition by L-cysteine. However, the mutated SATase produced in SATM and RuSATM was insensitive to this feedback regulation because of the point mutation of Gly-277 to Cys. In the transgenic *Arabidopsis*, SATase activity was enhanced 25 - 80 folds as compared to that of non-transgenic *Arabidopsis*. The contents of OAS, cysteine, and glutathione in transgenic *Arabidopsis* overexpressing the mutant SATG277C were significantly increased as compared to wild type plants and to the transgenic plants overexpressing the wild type SATase (Fig. 1). In cell-free extracts of SATW and RuSATW, we detected highly enhanced SATase activity by *in vitro* assay, however, the enzymatic activity of SATase is presumably inhibited by cysteine that was synthesized in plant cells. Therefore, overaccumulation of OAS and cysteine did not remarkably occur in the transgenic plants, SATW and RuSATW, overexpressing wild type SATase gene compared to SATM and RuSATM overexpressing mutated SATase gene. Interestingly, GSH contents were increased not only in SATM and RuSATM plants but also in SATW and RuSATW plants. These results suggest that the metabolic flux through cysteine to GSH is likely enhanced in all transgenic plants, although the steady-state concentration of cysteine is not increased in SATW and RuSATW plants. These *in vivo* results clearly indicate that the feedback inhibition by cysteine to the activity of SATase plays an important role for OAS and cysteine synthesis in plant cells.

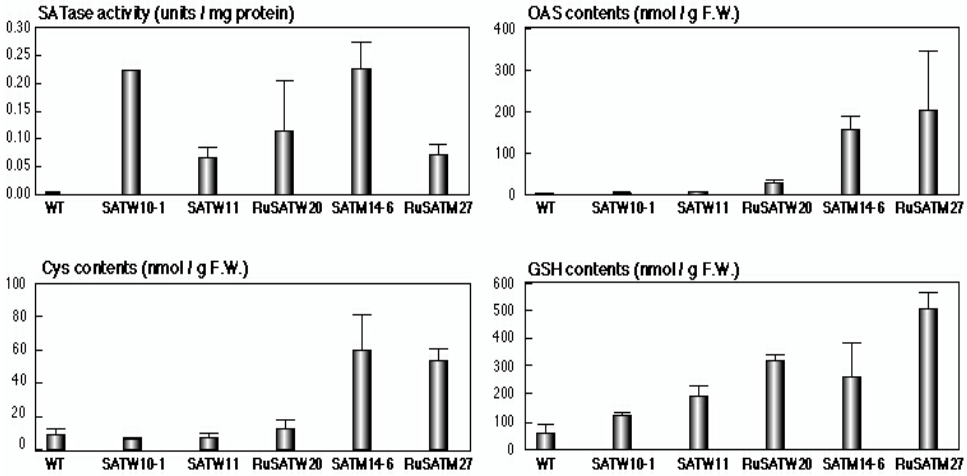

Fig. 1. SATase activity, and the contents of OAS, Cys and GSH in 3-week-old transgenic plants. WT, wild type *Arabidopsis* plants.

Antisense transgenic *Arabidopsis* repressing the expression of *SAT-p* gene, ANSP, and T-DNA insertion knock-out mutant in *SAT-c* gene, KOC, were also analyzed. The expression level of SAT-p mRNA in ANSP was decreased to 30 % of

that of wild type *Arabidopsis* plants. In KOC, the expression of SAT-c was completely repressed. The contents of OAS, cysteine and glutathione were decreased both in ANSP and in KOC, again suggesting the important role of SATase for the regulation of OAS and cysteine synthesis in plants.

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TRANSGENIC *ARABIDOPSIS THALIANA* EXPRESSING GFP IN RESPONSE TO SULFUR NUTRITION

Naoko Ohkama[1], Yoshitaka Sogawa[1], Derek B. Goto[2], Kentaro Takei[3], Hitoshi Sakakibara[3], Nakako Shibagaki[1], Hiroaki Hayashi[1], Tadakatsu Yoneyama[1], Satoshi Naito[2] and Toru Fujiwara[1,4]

[1]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences,The University of Tokyo, Tokyo 113-8657, Japan; [2]Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan; [3]Riken (The Institute of Physical and Chemical Research) Plant Science Center, Hirosawa, Wako,Saitama, 351-0198 Japan; [4]Present address; Biotechnology Research Center, The University of Tokyo, Tokyo 113-8657, Japan

Transgenic *Arabidospsis thaliana*, NOB lines (Naoko Ohkama's ß lines), were generated which express green fluorescence protein (GFP) under control of a chimeric sulfur responsive promoter. The plants have been used for physiological and genetic study of sulfur nutrition-regulated gene expression. Here we summarize our studies using NOB transgenic plants. Details of the analysis have been described elsewhere.

The chimeric promoter used for expression of GFP in the NOB transgenic lines are comprised of the cauliflower mosaic virus (CaMV) 35S RNA promoter and the gene promoter for the ß subunit of ß-conglycinin, one of the seed storage proteins of soybean. The ß subunit gene is up-regulated by sulfur deficiency in transgenic plants including *A. thaliana* at the level of transcription (Naito *et al.* 1994; Hirai *et al.* 1995). Deletion analysis of the ß subunit gene promoter identified a 235bp-region responsible for –S induction (Awazuhara *et al.* 2002). Three copies of this sulfur-responsive region of the ß subunit gene promoter ($ß_{SR}$) was inserted tandemly into CaNV 35S promoter at the position -90. This chimeric promoter-ß glucuronidase fusion gene conferred a S-response also to non-seed tissues including rosette leaves in transgenic *A. thaliana* plants (Awazuhara *et al.* 2002).

Using the chimeric promoter, we generated transgenic *A. thaliana* plants named as NOB carrying GFP gene under this chimeric promoter (Ohkama *et al.* 2002a). In NOB plants, GFP expression was induced by sulfur deficiency in all tissues observed including roots, leaves, stems, flowers, siliques and seeds. After exposure to sulfur deficient conditions, increase in GFP fluorescence was observed prior to the appearance of chlorosis. Increase in GFP accumulation was associated with reduction of sulfate and thiol content, and with increase of *O*-acetyl-L-serine in leaves

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(Ohkama *et al.* 2002a). These results establish that the level of GFP accumulation in transgenic NOB plants is a good indicator of S status.

Using NOB as a reporter, we examined several features of sulfur deficiency-induction of the chimeric promoter. We first examined the effect of methionine, which downregulates the ß promoter (Hirai *et al.* 1994). The NOB plants were crossed with *A. thaliana mto1-1* mutant plants which overaccumulate soluble methionine (Inaba *et al.* 1994). In seeds of NOB plants carrying the *mto1-1*mutation, GFP accumulation was lower than the control NOB plants without the mutation. But, repression by the *mto1-1* mutation of the NOB transgene was not evident in rosette leaves, suggesting tissue-specificity of methionine-regulation of gene expression (Ohkama *et al.* 2002a). Interestingly, the full length (1kb) promoter of the ß subunit gene was downregulated in the presence of the *mto1-1* mutation in rosette leaves when fused to the CaMV 35S RNA promoter.These results suggests presence of a mechanism of gene regulation in rosette leaves responsible for downreglation of gene expression in response to elevated levels of methionine.

NOB plants also respond to exogenous application of OAS and glutathione (Sogawa *et al.* 2002). We also examined the effects of plant hormones (Ohkama *et al.* 2002b). Cytokinins were found to upregulate GFP accumulation in NOB plants. Accumulations of the 5'-adenosine phosphoreductase 1 (*APR1*) and *Sultr2;2* transcripts were also increased by cytokinins, suggesting roles of cytokinin in upregulation of sulfur responsive genes and the sulfur assimilation pathway.

We recently carried out experiments suggesting that disruption of *Sultr1;2* (Shibagaki *et al.* 2002) increase GFP accumulation in NOB. It is likely that a number of genes are responsible for –S response of NOB. To identify such genes, mutants were screened from M2 generation of ethyl methanesulfonate-mutagenized NOB plants. Currently several lines were identified that accumulate GFP at a high level under control conditions. Genetic and physiological characterizations of these newly identified mutants are under way.

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HEALTHINESS OF WINTER OILSEED RAPE FERTILIZED WITH NITROGEN AND SULFUR

Anna Podlesna[1], Malgorzata Jedryczka[2] and Elzbieta Lewartowska[2]
[1]Institute of Soil Science and Plant Cultivation, Czartoryskich 8, 24-100 Pulawy, Poland; [2]Institute of Plant Genetics, Polish Academy of Sciences, Strzeszynska 34, 60-479 Poznan, Poland

Oilseed rape is under field conditions subjected to various diseases and pests. Seed yield losses caused by diseases are estimated at 15-20 %, but in the extreme cases it may be as high as 80 % (Mrowczynski *et al.* 2000). Today economical and ecological aspects are taken into consideration in modern plant protection. Pesticides are recommended in the case of strong infection and other actions ought to results in an increase of natural plant resistance. Previous studies have indicated that adequate sulfur fertilization may have beneficial effect on oilseed rape resistance to pests (Sadowski *et al.* 2000; Schnug and Ceynowa 1990).

In current study, long-term field experiments were carried out at two replicate sites at the Experimental Station of Institute of Soil Science and Plant Cultivation (central Poland). Winter oilseed rape, Kana variety, was cultivated at various levels of nitrogen fertilization: N1, without nitrogen; N2, 120 and N3, 200 kg N per hectare. In addition, plants were subjected to two levels sulfur fertilization: without sulfur and with 94 kg S per hectare. Nitrogen and sulfur were applied as ammonium sulfate, single superphosphate and potassium sulfate. At the site without sulfur, equivalent amounts of NPK were applied as ammonium nitrate, triple superphosphate and potassium chloride. The main observations of plant healthiness were conducted 2 weeks before harvest. At statistical analysis was used Turkey confidence interval at $\alpha = 0.05$. Analysis of variance was done with Statgraphics Plus, v. 4.0.

It was observed that oilseed rape showed infection symptoms caused by following fungal pathogens: *Leptosphaeria maculans* (*Phoma lingam*), *Alternaria* spp., *Verticilium* spp. and *Erysiphe cruciferarum*. High levels of nitrogen fertilization caused greater infection of oilseed rape by fungal diseases (Table 1). Greater tillering and foliage of plants fertilized with nitrogen resulted in a greater plant density, which created good conditions to intensification of diseases (Lipa 1992). Statistically significant influence of applied sulfur was found in relation to black spot caused by *Alternaria* spp. and powdery mildew as a result of *Erysiphe cruciferarum* infection. According to Schnug and Ceynowa (1990), this protective role of sulfur results from its presence at glucosinolates and at products their degradation. *In vivo* studies conducted with oilseed rape showed temporary accumulation of glucosinolates in its leaves after infection by *Alternaria brassicea* (Doughty *et al.* 1991).

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Gullner and Kömives (2001) refer to studies, which showed that also GSH plays important regulatory roles in defense after recognition of the attacking pathogen. On this base we can suppose that this kind of resistance is connected with adequate supply of plants at sulfur. The date obtained in presented experiment can be confirmation of hypothesis mentioned above because higher concentration of sulfur was found at leaves of plants fertilized with this element (Fig. 1).

Table 1. The percent of winter rape plants infected with fungal pathogens.

Description		*Leptosphaeria maculans*	*Alternaria* spp.	*Verticillium* spp.	*Erysiphe Cruciferarum*
N doses:	0	6.0a*	19.5a	3.0a	63.0a
	120	12.0b	38.0b	5.0b	69.0b
	200	23.5c	51.0c	6.0b	69.5b
S fertilization:	-S	11.8a	42.7a	3.7a	72.3a
	+S	15.3b	29.7b	5.7b	62.0b

*Numbers in columns marked with the same letters do not differ significantly

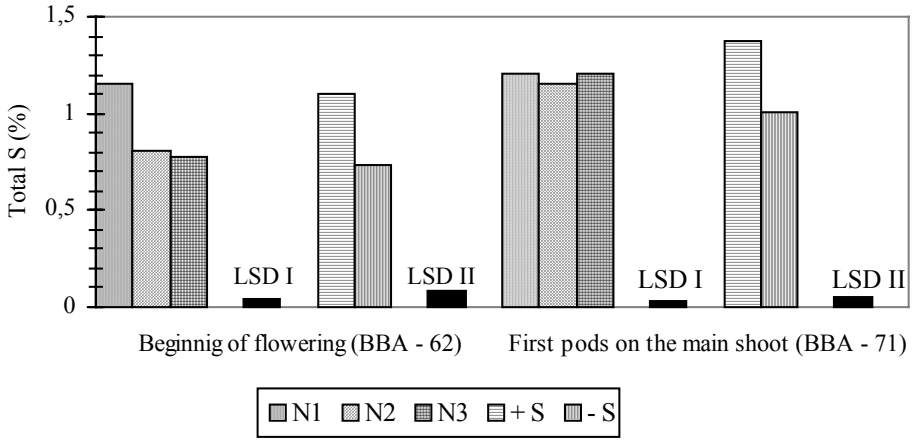

Fig. 1. Total sulfur content in leaves of winter oilseed rape (% of dry weight).

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GLUTATHIONE TRANSPORT IN PEDUNCULATE OAK ROOTS AND ITS COMPARISON WITH SULFATE AND GLUTAMINE TRANSPORT

Heinz Rennenberg and Stefan Seegmüller
Chair of Tree Physiology, Institute of Forest Botany and Tree Physiology, Albert-Ludwigs-University Freiburg, Georges-Köhler-Allee 053/054, D-79110 Freiburg i.B., Germany

Sulfur is available to plants not only as sulfate, but also as organic sulfur to the roots. Organic sulfur may be of particular significance in natural environments, where decaying plant material rather than sulfate-containing fertilizer is the predominant sulfur source. Since glutathione (GSH) is present in plant cells in high amounts (Bergmann and Rennenberg 1993) and may readily be liberated during mineralization, we studied whether mycorrhizal pedunculate oak (*Quercus robur* L.) roots are able to take up this sulfur containing tripeptide and to allocate it to the shoot by xylem transport. The kinetic parameters of glutathione uptake and xylem loading were compared with those of sulfate and glutamine (Gln).

The experiments showed that GSH was taken up by excised mycorrhizal oak roots (Table 1). In the concentration range studied (5 - 500 μM) uptake of GSH exhibited biphasic Michaelis-Menten kinetics suggesting the presence of a high (HAUS) and a low affinity uptake system (LAUS). Similar kinetics were found for the uptake of Gln, a dominant amino compound in various tissues of pedunculate oak (Seegmüller, Schulte and Rennenberg, unpublished results). Uptake of sulfate also showed biphasic kinetics, but with significantly lower V_{max} and higher K_m compared to GSH and Gln uptake, indicating superior performance of the organic sulfur and nitrogen uptake systems than sulfate uptake systems.

Xylem loading of GSH showed monophasic kinetics in the concentration range between 5 and 500 μM (Table 2), again with similar V_{max} and K_m values than xylem loading of Gln. The V_{max} of xylem loading of both GSH and Gln was low compared to the HAUS and LAUS of these compounds, the K_m between that of HAUS and LAUS (compare Table 1 and 2). Xylem loading of sulfate proceeded at lower V_{max} and higher K_m than xylem loading of GSH or Gln (Table 2). Apparently, also the transport system(s) responsible for xylem loading of GSH and Gln exhibit superior performance than the transport system mediating xylem loading of sulfate.

From the similar kinetic properties of the uptake and the xylem loading systems of GSH and Gln it may be concluded that GSH and Gln transport are mediated by general amino acid permeases. Such a general amino acid permease has recently

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been identified from the ectomycorrhizal fungus *Amanita muscuria* (Nehls *et al*. 1999), but the specificity for GSH has not been tested. Whether GSH and Gln uptake in the present study were mediated by fungal or/and plant root tissues will be analyzed in future studies.

Table 1. Catalytic properties of GSH, sulfate and Gln uptake. Pedunculate oak (*Quercus robur* L.) trees were grown from seeds in the presence of the mycorrhizal fungus *Laccaria laccata* as previously described (Seegmüller and Rennenberg 1994) and kinetic parameters of GSH, sulfate and Gln uptake were determined with detached mycorrhizal roots as reported by Herschbach and Rennenberg (1991).

	V_{max} (± % SE) (nmol g fresh weight^{-1} h^{-1})		K_m (±% SE) (µM)	
	HAUS*	LAUS*	HAUS*	LAUS*
GSH uptake	87 (± 18)$^{a,B\#}$	359 (± 16)^{b,C}	5.6 (± 71)^{a,A}	140 (± 33)^{b,A}
Sulfate uptake	16 (± 31)^{a,A}	132 (± 37)^{b,A}	10.0 (± 56)^{a,A}	3180 (± 22)^{b,B}
Gln uptake	73 (± 21)^{a,B}	201 (± 20)^{b,B}	9.1 (± 57)^{a,A}	115 (± 52)^{b,A}

*HAUS, high affinity uptake system; LAUS, low affinity uptake system

a,b: significant differences between HAUS and LAUS at $p < 0.05$; A,B,C: significant differences between compounds at $p < 0.05$; n = 9 to 30

Table 2. Catalytic properties of xylem loading of GSH, sulfate and Gln. Pedunculate oak (*Quercus robur* L.) trees were grown from seeds in the presence of the mycorrhizal fungus *Laccaria laccata* as previously described (Seegmüller and Rennenberg 1994) and kinetic parameters of xylem loading of GSH, sulfate and Gln were determined with detached mycorrhizal roots as reported by Herschbach and Rennenberg (1991).

	V_{max} (± % SE) (nmol g fresh weight^{-1} h^{-1})	K_m (± % SE) (µM)
Xylem loading of GSH	5.0 (± 33)$^{b;\#}$	47 (± 43)^{a}
Xylem loading of sulfate	1.4 (± 31)^{a}	900 (± 28)^{b}
Xylem loading of Gln	3.6 (± 37)^{b}	48 (± 43)^{a}

a,b: significant differences between compounds at $p < 0.05$; n = 9 to 30

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SUCCESSFUL ENGINEERING OF METHIONINE METABOLISM IN POTATO

Kerstin Riedel[1], Michaela Zeh[2], Anna Paola Casazza[2,3], Rainer Höfgen[2] and Holger Hesse[1]

[1]Freie Universität Berlin, Institut für Biologie, Angewandte Genetik, Albrecht-Thaer-Weg 6,14195 Berlin, Germany; [2]Max-Planck-Institut für Molekulare Pflanzenphysiologie, Dept. of Prof. Lothar Willmitzer, Am Mühlenberg 1, 14476 Golm, Germany; [3]present address: Università degli Studi di Milano, Dipartimento di Biologia, Via Celoria 26, 20133 Milan, Italy

To analyze the physiological impact of threonine synthase (TS) activity on the control of the branched pathway of the aspartate family members threonine (Thr) and methionine (Met), we engineered TS antisense potato plants (*Solanum tuberosum* cv. Désirée) using the constitutive cauliflower mosaic virus 35S promoter. Transgenic lines with dramatically reduced TS activity accumulated significantly higher levels of free Met in both leaves and tubers. At least in tubers this increase was not on the expense of Thr. These plants offer a major biotechnological advance toward the development of crop plants with improved nutritional quality.

Met and Thr are essential for mammals, and must be derived entirely from the diet. However, in most crop plants they are limited and improving their content would lead to an improved nutritional quality. In plants, their biosynthetic pathways diverge at the level of *O*-phosphohomoserine (OPHS). Cystathionine γ-synthase (CgS) and TS compete for this common substrate demanding an effective regulation of their enzyme activities. There is no evidence for feedback inhibition of CgS but in contrast, plant TS is activated by low concentrations of SAM and inhibited by cysteine (Galili and Höfgen 2002). Neither precursors, nor reaction products, nor nitrogenous compounds have any effect on plant TS expression, essentially excluding metabolic regulation (Casazza *et al.* 2000). To determine *in vivo* the physiological relevance of TS and its role in controlling the competing pathways of Met and Thr transgenic potato plants with gradually reduced TS expression at both transcription and enzymatic activity level were generated *via* antisense inhibition (Zeh *et al.* 2001).

The reduced TS steady-state mRNA levels in transgenic potato leaves correlated with a reduction in TS enzyme activity down to 16 to 6 % of wild type level and was reflected in growth retardation and chlorosis. In the most strongly inhibited line also an acute reduction in tuber yield was observed.

The amount of free Thr was reduced by 2 to 7 fold in leaves but not changed in tubers (Table 1). A significant increase in free Met was observed by up to 44 fold in

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leaves and up to 30 fold in tubers. A significant increase of aspartate in leaves highlights the importance of TS for carbon allocation into the aspartate pathway, and an increase in the Met intermediate homocysteine further indicates a redirection of the carbon flow from Thr to Met. No other amino acids were changed neither in leaves nor in tubers nor were major changes in mRNA or protein levels of Met biosynthetic genes like CgS, CbL, and MS detected. And also the activity of the competing enzyme, CgS, was not significantly changed (data not shown).

Our observations indicate that in contrast to *Arabidopsis* there is a different mode of regulation of Met synthesis in potato. In *Arabidopsis* the levels of mRNA and activity of CgS are reduced in the presence of excess Met (Galili and Höfgen 2002) whereas the accompanying increase of SAM activates TS (Giovanelli *et al.* 1984) and thereby diverts OPHS from the equilibrium towards synthesis of Thr at the expense of Met and SAM synthesis. Our findings, however, indicate that Met accumulation does not affect expression or activity of CgS nor of any other corresponding biosynthetic gene. In potato it seems that regulation occurs at the level of the ratio of TS to CgS activity rather than at the level of CgS mRNA stability. It seems likely that the TS to CgS ratio of activity acts as the sole switch, directing the carbon backbone towards Thr synthesis as soon as sufficient Met or Met-derived products like SAM are available.

From an applied perspective, our result is a major achievement for plant breeding to improve the nutritional quality of food or feed as not only Met is increased in an agronomically important crop plant but also another essential amino acid, Thr, is not reduced.

Table 1. Analysis of source leaf and tuber metabolites of 8-week-old TS antisense lines. Metabolites were determined as described in Zeh *et al.* (2001). Values are given as μmol g^{-1} fresh weight.

	Leaves					Tubers				
	Wt	# 16	# 45	# 35	# 61	Wt	# 16	# 45	# 35	# 61
Threonine	0.05	0.04*	0.03*	0.03*	0.03*	0.49	0.42	0.47	0.43	0.46
Methionine	0.01	0.02	0.13*	0.14*	0.22*	1.21	9.73*	9.81*	20.2*	35.4*
Aspartic acid	1.02	1.09	1.56*	2.57*	2.62*	4.03	2.06*	3.15	3.13	3.05
Homocysteine	0.00	0.01	0.04*	0.04*	0.08*	--	--	--	--	--
Isoleucine	0.03	0.01*	0.02	0.01	0.02*	1.73	4.56*	6.23*	8.26*	6.27*

*Statistically significant changes ($P < 0.05$)

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EFFECTS OF SULFUR NUTRITION ON CARBON METABOLISM AND NITROGEN ASSIMILATION IN *CHLORELLA SOROKINIANA*

Carmelo Rigano[1], Simona Carfagna[1], Vittoria Di Martino Rigano[2], Vincenza Vona[1], Sergio Esposito[1] and Graziella Massaro[1]
[1]Dipartimento di Biologia Vegetale, Università di Napoli Federico II, Via Foria 223, I-80139 Napoli, Italy; [2]Dipartimento di Farmacologia Sperimentale, Università di Napoli Federico II, Via Montesano 49, I-80131 Napoli, Italy

Sulfur removal from the growth medium of the unicellular alga *Chlorella sorokiniana* resulted in a decrease of the cellular content of cysteine, in a decline of growth and in a dramatic reduction of NH_4^+ uptake. Over 24 h of S starvation, the photosynthetic capacity of the alga was reduced by 74 %. The cellular content of starch, however, increased by 240 %, and there also occurred a net increase in the pools of free glutamate, asparagine and serine (Di Martino Rigano *et al.* 2000). Sulfur starvation caused also an increase in the concentration of *O*-acetyl-L-serine (OAS) (unpublished results), the metabolic precursor of cysteine, which agrees with the statement that a high carbon and nitrogen status of the cell favors synthesis of OAS (Hawkesford 2000).

Sulfate re-supply to cells starved of S for 24 h, resulted in an immediate formation of cysteine, in a rapid mobilization of starch and in a prompt increase of the rate of respiratory oxygen consumption, and promoted a prompt resumption of growth and a time dependent recovery of photosynthetic capacity. This suggests that S resupply promoted immediate synthesis of cellular components necessary to support resumption of growth, or *de novo* synthesis of those proteins which were dramatically broken down during S starvation. Here is shown that for the biosynthesis of N compounds stimulated by S addition, the cells used amino acids accumulated during the period of S starvation as an immediate preferred N source, and that an use of the exogenous NH_4^+ was either delayed, or significantly limited.

Chlorella sorokiniana was grown in a continuous light. Nutrient medium, culture conditions, free amino acids and NH_4^+ determination as reported before (Di Martino Rigano *et al.* 2000).

When sulfate was added to S-starved cells, the concentration of OAS immediately decreased (not shown) and that of cysteine increased (Di Martino Rigano *et al.* 2000). This demonstrated the importance that an accumulation of OAS plays for a prompt assimilation of sulfur by S-starved cells when these cells are supplied with sulfate, which is in agreement with observations in other organisms (Neuenschwan-

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der *et al.* 1991; Clarkson *et al.* 1999; Hawkesford 2000). The rapid synthesis of cysteine also suggests that sulfate added to S-starved cells was almost immediately absorbed, reduced and subsequently incorporated into cysteine. Sulfate re-supply to cells starved of S for 24 h, resulted also in an immediate decrease in the intracellular content of glutamate, asparagine and serine.

The increase in cysteine concentration suggests that after S re-supply, the S-starved cells of *C. sorokiniana* rapidly shifted to conditions of a surplus of internal S. Furthermore, the decrease in concentration of glutamate and other amino acids accumulated during S starvation, suggests that there was an increase in the nitrogen demand of the cell, due to an intense biosynthetic activity stimulated by the sudden sulfur availability. Despite this increased N demand, NH_4^+ uptake occurred at very slow rates over the first hour, after which it exhibited a rate which was still as low as 65 % that of S-sufficient cells over the successive hours (Fig. 1A,B).

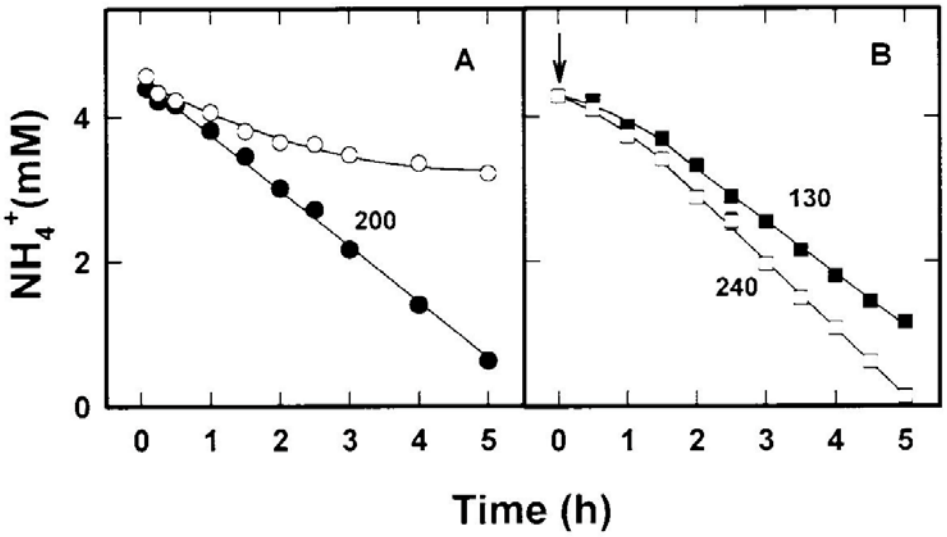

Fig. 1. NH_4^+ uptake by illuminated cell suspensions of *C. sorokiniana*. The suspensions, kept at 35°C, were flushed with 5 % CO_2 air. (A) S-sufficient cells: NH_4^+ uptake in a complete medium (●-●); NH_4^+ uptake in a medium without S (○-○). (B) Cells starved of S for 24 h: NH_4^+ uptake in a medium added with 1.2 mM sulfate (■-■); NH_4^+ uptake in a medium added with 1.2 mM sulfate plus 10 g l^{-1} glucose (□-□). Number in parenthesis indicates uptake rates expressed as µmol NH_4^+ absorbed ml^{-1} PCV (packed cell volume) h^{-1}. Bars show ± SE (when larger than the symbol); n = 3.

The slow NH_4^+ uptake by S-starved cells over the first hour was certainly due to the fact that during this period the accumulated amino acids acted as a preferred N source. The fact, however, that after this initial period NH_4^+ uptake by S-starved cells supplied with 10 g l^{-1} glucose occurred at a higher rate than that exhibited by

S-sufficient cells, supports that nitrogen assimilation might be limited by carbon availability.

Acknowledgments

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EXPLAINING SULFUR AND NITROGEN INTERACTIONS IN THE GROWTH OF CROPPED HYBRID RYEGRASS

Maria Da Graça Serrão[1], Michael Tausz[2], Maria João Neves[1], John Keith Syers[3] and Fernando Pires[1]

[1]Soil Science Department, Estação Agronómica Nacional, Quinta do Marquês, 2784-505 Oeiras,Portugal; [2]Institute of Plant Physiology, University of Graz, Schubertstraße 51, 8010 Graz, Austria; [3]Department of Natural Resources and Environmental Science, Naresuan University, Phitsanulok 65000, Thailand

A deficient nitrogen supply may depress plant response to sulfur application, with subsequent reflexes on growth and quality, even in relatively low demanding S crops such as grass (Syers *et al.* 1987). In fact, both nutrients are essential for protein synthesis and the N/S ratio of proteins varies only slightly (Mengel and Kirkby 1987). This work aims to clarify previously reported significant interactions between N and S on dry matter yield of hybrid ryegrass (*Lolium multiflorum* x *Lolium perenne*) cv. Ariki cropped in a Haplic Arenosol.

Three S rates (S_0-0, S_1-15, and S_2-30 kg S ha^{-1}) were applied as gypsum and two N rates (N_1-140 and N_2-280 kg N ha^{-1}) as ammonium nitrate (20.5 % N), in a factorial block design with three replicates of each treatment. At the N_1 rate, the S_2 application rate substantially depressed yield at the first cut and the yield over two harvests. In contrast, when N was added at the N_2 rate, the S_2 application rate gave significant positive responses in yield. Distribution of plant total N and S concentrations (Nt, St) in inorganic (NO_3-N, SO_4-S) and organic (No, So) forms, at both harvests, as affected by the six combinations of N and S rates was examined, as well as Nt/St ratio, total N and S uptake by ryegrass tops, residual absorbed NO_3-N and SO_4-S, and "metabolized" No (the difference between total N uptake and residual absorbed NO_3-N) and "metabolized" So (calculated as for "metabolized" No).

Nt and St contents in ryegrass tops (<0,5 mm) were determined by the Kjeldahl method and by digestion with HNO_3, $HClO_4$, and HCl, followed by SO_4^{2-} measurement by turbidimetry (Blanchar *et al*. 1965). NO_3-N and SO_4-S contents were determined in water extracts by anion-exchange HPLC with conductivity detector (Pilch and Grill 1995). No and So were estimated as the differences between Nt and NO_3-N, and St and SO_4-S, respectively.

At the 2nd harvest, Nt contents in the forage tops significantly increased with the N_2 application rate (Table 1), in which they approach the minimum level considered as adequate for the growth of perennial and annual ryegrass (3.5 - 4.0 g 100 g^{-1} ac-

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cording to Kelling and Matocha 1990). Nt : St ratio in plant material, ranging from 6.9 to 7.7 and from 5.7 to 9.5 at the 1st and 2nd harvests, respectively, was much lower than the critical value (12 - 14) for optimum graminae yield, which may indicate either S deficiency when there is not other nutrient deprivation (Bergmann 1992) or N supply limitation. In contrast, St concentrations in ryegrass tops were relatively adequate, near the range 0.27- 0.32 g 100 g^{-1} referred as adequate for perennial ryegrass (Smith 1986).

Because residual NO_3-N concentrations were negligible (Table 1), total N uptake (over two harvests) was almost identical to the "metabolized" No values. Both parameters tended to decrease (- 21.6 kg ha^{-1}) with the N_1S_2 treatment and to increase (14 kg ha^{-1}) with the N_2S_2 treatment when compared to the respective "nil" S treatments.

Table 1. Total nitrogen, nitrate, organic nitrogen, total sulfur, sulfate, and organic sulfur contents in hybrid ryegrass tops at two harvests. Means of three replicates.

Treatments	1st harvest						2nd harvest					
	Nt	NO_3-N	No	St	SO_4-S	So	Nt	NO_3-N	No	St	SO_4-S	So
	(g N 100 g^{-1})			(g S 100 g^{-1})			(g N 100 g^{-1})			(g S 100 g^{-1})		
N_1S_0	1.82	0.00	1.82	0.26	0.10	0.16	2.39	0.00	2.39	0.29	0.14	0.15
N_1S_1	2.03	0.00	2.03	0.28	0.09	0.19	2.36	0.00	2.36	0.32	0.17	0.15
N_1S_2	1.88	0.00	1.88	0.25	0.11	0.14	2.07	0.00	2.07	0.37	0.21	0.16
N_2S_0	2.35	0.02	2.33	0.33	0.15	0.18	3.05	0.09	2.96	0.33	0.17	0.16
N_2S_1	2.13	0.01	2.12	0.28	0.10	0.17	3.21	0.11	3.10	0.34	0.18	0.16
N_2S_2	2.24	0.01	2.23	0.29	0.15	0.15	3.06	0.09	2.97	0.38	0.25	0.13

"Metabolized" So was lower at the N_1S_2 treatment (6.9 kg ha^{-1}) than at the N_1S_0 treatment (9.5 kg ha^{-1}) whereas it did not differ between the N_2S_0 and N_2S_2 treatments. Because N is more limiting than S for ryegrass production in this soil, the variations in "metabolized" No in ryegrass tops can in part explain why ryegrass yields had the same behavior. The results will be complemented by soil data (mineral N and available S).

Acknowledgements

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SELENATE RESISTANT MUTANTS OF *ARABIDOPSIS THALIANA* IDENTIFIES A REGION IN SULFATE TRANSPORTER GENE REQUIRED FOR EFFICIENT TRANSPORT OF SULFATE IN ROOTS

Nakako Shibagaki[1], Alan Rose[2], Toru Fujiwara[3], John P. Davies[4] and Arthur R. Grossman[1]

[1]Carnegie Institution of Washington, Department of Plant Biology, Stanford, CA 94305, U.S.A.; [2]Section of Molecular and Cellular Biology, Division of Biological Sciences, University of California, Davis, One Shields Avenue, Davis, CA 95616, U.S.A.; [3]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, the University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan; [4]Exelixis Plant Science, 16160 SW Upper Boones Ferry Rd, Portland, OR 97224, U.S.A.

We identified mutants resistant to selenate, a toxic analog of sulfate, in *Arabidopsis thaliana* (Rose 1997; Shibagaki *et al.* 2002). Almost all of the selenate resistant mutants that we identified are allelic and have lesions in the sulfate transporter gene, *Sultr1;2*. We named the locus *sel1*. The amino acid residues altered in the mutant strains are listed in Table 1.

To prove sulfate transport function of the Sultr1;2 protein, we expressed the *Sultr1;2* gene in the budding yeast mutant, CP154-1B, which is null for two major sulfate transporters, SUL1 and SUL2 (Cherest *et al*. 1997). CP154-7B cells can not grow in medium containing 500 μM sulfate as a sole sulfur source; they require methionine supplement for growth. The wild type *Arabidopsis Sultr1;2* gene can complement the yeast mutant for growth on sulfate. However, the *Sultr1;2* genes that carry any of the missense mutations, *sel1-1*, *sel1-3*, *sel1-4*, *sel1-7* and *sel1-8*, are unable to complement the methionine auxotrophy of CP154-7B. Growth of the strains transformed with the wild type and mutant *Sultr1;2* genes were also assayed for growth in liquid cultures (Table 1).

In growth medium supplemented with 0.4 mM methionine, all of the transformants grew at approximately the same rate as wild type yeast cells. In contrast, only the strain harboring the wild type *Sultr1;2* gene grew at a rate similar to that of wild type cells in medium lacking methionine; the transformants with the mutant *Sultr1*:*2* genes grew very poorly in this unsupplemented medium. Two serine residues located in the 1st and 2nd membrane-spanning domain (MSD) are important for the

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activity of Sultr1;2 in both yeast and *Arabidopsis*. This is in agreement with the finding that the first three MSDs and serine residue 126 are highly conserved among sulfate transporters from most organisms.

Interestingly, we determined that three of the five *sel1* mutations were in the region between the 12th MSD and its carboxyl terminal extension. These results suggest that the region between the last MSD and the carboxyl terminal extension of Sultr1;2 is required for efficient sulfate transport or for proper targeting (or stability) of the polypeptide to its site of function. Furthermore, the carboxyl terminal extension on many sulfate transporters, including Sultr1;2, is designated STAS domain, based on similarity with a bacterial antisigma factor antagonist (Aravind and Koonin 2000). The STAS domain is likely to have a regulatory function relating to sulfate transport activity of Sultr1;2, although currently there is no experimental evidence to support this conjecture. To begin to probe the function of STAS domain, we attempted to complement the yeast CP154-1B mutant with a Sultr1;2 construct deleted for the STAS domain; the truncated transporter was unable to complement the sulfate transport-deficient phenotype of the mutant strain (Table 1). This result suggests that the STAS domain is required for the transport function of Sultr1;2, its integration into the cytoplasmic membranes or its stability within the membranes. A more detailed analysis of point mutations within the STAS domain may reveal the relationship of this putative regulatory sequence with sulfate transport function.

Table 1. Mutations in *Sultr1;2* and the growth of resulting yeast strains. The *Sultr1;2* cDNA derived from the *sel1* mutants of *Arabidopsis* were tested for their ability to complement CP154-7B on minimal glucose plates supplemented with 500 μM sulfate. 'YES' or 'NO' indicate growth or no growth observed visually after three days of incubation at 30 °C. Doubling times for growth at 30°C with shaking in liquid minimal medium supplemented with 500 μM sulfate and 400 μM methionine or 500 μM sulfate and no methionine. Values are means (± SD) from at least three independent experiments.

Yeast strains	Type of mutation	Complementation on plate	Doubling time in liquid culture (hours) + Methionine	- Methionine
W303-1A (*MATa, ade2, his3, leu2, trp1, ura3*)			2.1 ± 0.6	3.4 ± 0.5
CP154-1B (*MATa, ade2, his3, leu2, trp1, ura3, sul1::LEU2, sul2::URA3*)			2.6 ± 1.2	35 ± 18
Transformants with				
Sultr1;2-WT		YES	2.4 ± 0.2	4.1 ± 0.5
Sultr1;2-sel1-1	Ser96 → Phe (tC^{287}c→ tTc)	NO	2.6 ± 0.6	27 ± 13
Sultr1;2-sel1-3	Gly509 → Glu (gG^{1526}a→ gAa)	NO	2.0 ± 0.1	22 ± 14
Sultr1;2-sel1-4	Ser126 → Asn (aG^{377}t→ aAt)	NO	3.0 ± 1.2	16 ± 6
Sultr1;2-sel1-7	Pro503 → Leu (cC^{1508}t→cTt)	NO	2.6 ± 1.0	34 ± 12
Sultr1;2-sel1-8	Ile511 → Thr (aT^{1532}t → aCt)	NO	2.6 ± 0.3	31 ± 2
Sultr1;2-noSTAS-1	deletion of C-terminal after F493	NO	2.5 ± 0.3	25 ± 13
Sultr1;2-noSTAS-2	deletion of C-terminal after R518	NO	2.5 ± 0.1	37 ± 16

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SULFUR UPTAKE BY RYE GRASS AS AFFECTED BY DIFFERENT SULFUR SOURCES CONTAINED IN CALCIUM NITRATE

Bal Ram Singh[1] and Leif Ruud[2]
[1]Department of Soil and Water Sciences, Agricultural University of Norway, Box 5028, N-1432 Aas, Norway; [2]Hydro Agri Norway, 0240 Oslo, Norway

Sulfur requirement of agricultural crops in Norway has increased in recent years (Singh 1994). Since calcium nitrate is extensively used as N fertilizer for topdressing, it was of interest to produce S-containing calcium nitrate and to test its agronomic importance. Greenhouse experiment was conducted for 3 years to investigate the effect of different sources of S contained in calcium nitrate on the yield and S uptake by rye grass.

Two soils, a sandy soil mixed with peat material (20 % w/w) and a clay soil (clay and silt contents of 20 and 37 %, respectively) were used. Both soils were limed to adjust pH at 6.0. Rye grass was used as test plant. A basal dose of NPK at the rate equivalent to 120, 60 and 120 kg ha^{-1} was applied to all pots. The treatments consisted of two S sources (kieserite and elemental S) and three rates of S (0, 1.5 and 2.5 %) as shown below.

Treatments: calcium nitrate; calcium nitrate with 1.5 % S incorporated through kieserite; calcium nitrate with 1.5 % S coated with elemental S; calcium nitrate with 2.5 % S incorporated through kieserite; calcium nitrate with 2.5 % S coated with elemental S; calcium nitrate with 2.5 % S as elemental S applied separately; calcium nitrate with 1.5 % S incorporated through kieserite + 1 % coated with elemental S.

Kieserite was mixed but elemental S was either mixed or coated with calcium nitrate. Rye grass was harvested 3 to 4 times in different years and the dry matter yield was recorded. The total dry matter yield for each year was calculated. Plant samples collected after each harvest were analyzed for S, N, K, Ca, and Mg.

The dry matter yield increased significantly with S application, irrespective of the source of S, in both soils but the yield levels were higher in the clay soil than in the sandy soil. The effect of S application on rye grass yield was more pronounced from 2nd to 4th harvests and it was also higher for kieserite than for elemental S.

Elemental S applied separately gave higher yields than with S coated calcium nitrate. The effect of S coated fertilizer was higher in the 3rd and 4th harvests than the earlier harvests. Rate of S application beyond 1.5 % had only marginal effects on rye grass yield. All treatments with kieserite gave significantly higher yields than those with elemental S (Fig. 1). In the sandy soil the yields increased consistently

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with increased rate of kieserite including the treatments where kieserite was combined with elemental S.

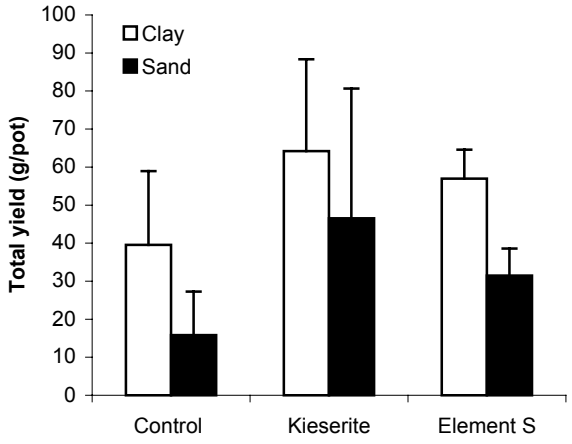

Fig. 1. Effect of sources on dry matter yield of rye grass.

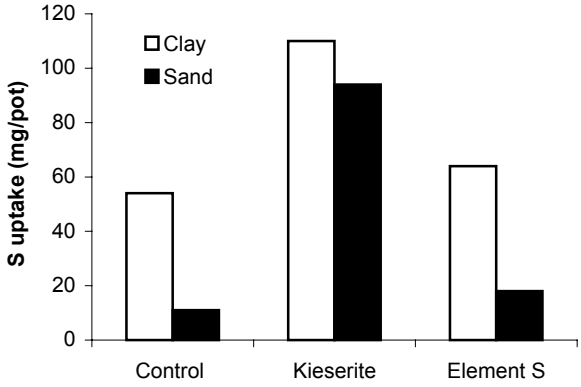

Fig. 2. Effect of S sources on S uptake by rye grass.

The concentration and uptake of S in rye grass was little affected by elemental S application except when it was applied separately, but the effects varied among years. In contrast to S uptake, the concentration of N in the elemental S-treated pots was higher than in those with kieserite in both soils (Results not shown). This implies that lower concentration of S in rye grass resulted in increased uptake of N. Kieserite application increased the concentration in plants at all rates of its application. The total uptake of S in kieserite treated pots was many folds higher than the control or those treated with elemental S (Fig. 2). These effects were more pro-

nounced in the sandy soil. Kieserite application also gave a very positive effect on the uptake of other nutrients (N, K, Ca and Mg).

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JUNGERMANNIIDAE SPECIES RESPOND TO CADMIUM IN A DIFFERENT MANNER TO OTHER BRYOPHYTES

Kristin Sutter, Sieglinde Menge, Herbert Tintemann, Angelika Schierhorn and Gerd-Joachim Krauss
Martin Luther University, Dept. Biochemistry/Biotechnology, Kurt-Mothes-Str. 3, 06099 Halle,Germany

Plants evolved strategies at the cellular level to respond to heavy metal toxicity (Hall 2002). Phytochelatins (PCs) derived from glutathione (GSH) can act as chelators for heavy metals in higher plants, algae and fungi. The drop in GSH reflects the consumption of this tripeptide during the synthesis of PCs (Cobbett and Goldsbrough 2002). We observed, that in a number of aquatic and terrestrial mosses cadmium (Cd) lets increase the GSH pool without any detectable PC synthesis (Bruns *et al.* 2001).

In contrast to higher plants, the aquatic biomonitor moss *Fontinalis antipyretica* Hedw. shows that specific effect to Cd in the range of 100 - 500 µM (Bruns *et al.* 2000). The cytoplasmic S content increased during Cd exposure. EEL-spectra (electron energy loss spectroscopy) indicate that in the cytoplasm Cd is chelated by SH groups (Bruns *et al.* 2000, 2001). All findings support the idea, that GSH seems to play an essential role in heavy metal detoxification in the investigated moss species, representing 11 orders of the divisions Marchantiophyta and Bryophyta (Bruns *et al.* 2001). Interestingly, under Cd exposure species of the order Polytrichales (division Bryophyta) show a slow increase in Ellman positive coumarins, but not phytochelatins (Berlich *et al.* 2002). Coumarins give misleading absorbance with Ellman's reagent suggestive of thiol conjugates.

Further experiments were performed with species of the subclass *Jungermanniidae* or leafy liverworts (class Jungermanniopsida, division Marchantiophyta; Crandall-Stotler and Stotler 2000). Plant material from three *Jungermanniidae* species (*Trichocolea tomentella* (Ehrh.) Dumort., *Scapania nemorea* (L.) Grolle*., Calypogeia arguta* Nees & Mont. ex Nees) was harvested from aseptic cultures and shaken in a reduced Knop medium (Bruns *et al.* 2001).

Unlike other bryophytes these species showed a remarkable induction of phytochelatins (PC2, PC3, PC4) during 10 days exposure with 100 µM Cd (Fig. 1). The increase of the PC pool is accompanied with a decrease in glutathione content. The content of PC3 and PC4 was significantly higher than that of PC2. In S*phagnum fallax* Klinggr., which represents a moss species without any detectable PC induc-

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tion Cd lets increase the glutathione pool (Fig. 1). GSH and PCs were identified by Electrospray-Ionization Mass Spectrometry (ESI-MS). A significant increase of γ-glutamyl-cysteine, the precursor of GSH occurred in *Jungermanniidae* species and S*phagnum fallax* Klinggr. Like in higher plants phytochelatins seem to play an important role in Cd detoxification in *Jungermanniidae* species. These findings could support the idea derived from morphological data that leafy liverworts are resolved a monophyletic group of the *Jungermanniopsidae* (Crandall-Stotler and Stotler 2000).

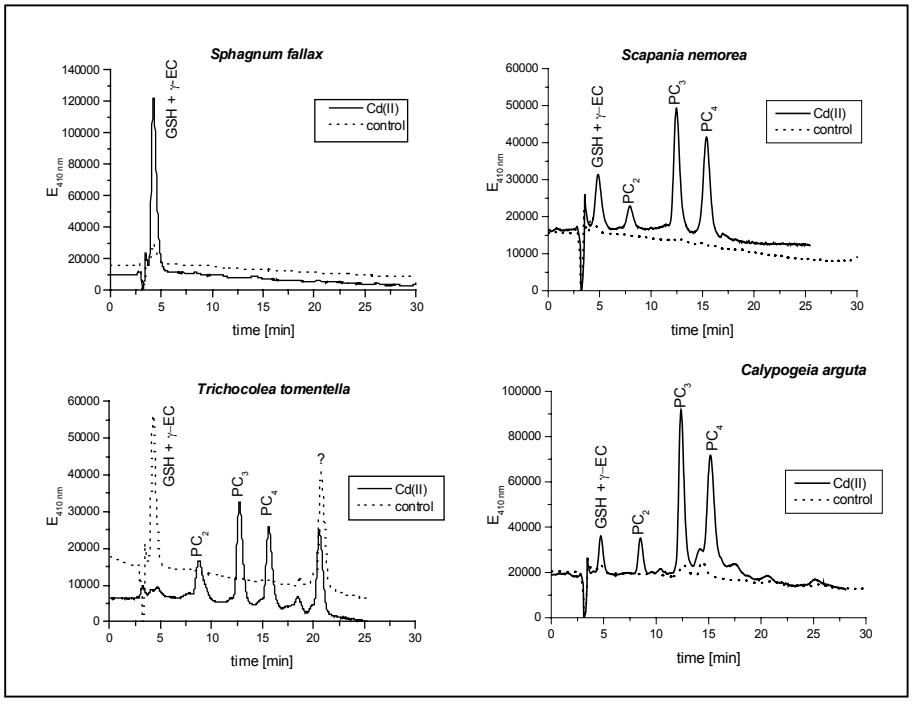

Fig. 1. PC and GSH contents of *Jungermanniidae* species during 10 days of exposure to 100 µM Cd. The content of the thiols was determined by RP-HPLC after online post-column derivatization with Ellmans's reagent according to Grill *et al.* (1987). UV/Vis absorbance was measured at 410 nm.

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SULFUR TRANSPORT AND ASSIMILATION IN DEVELOPING EMBRYOS OF CHICKPEA (*CICER ARIETINUM*)

Linda M. Tabe, Ingrid Venables, Anita Grootemaat and David Lewis
CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia

Because parts of the pathway of sulfur reduction and assimilation in plants are thought to be exclusively localised in the plastids, there has been a tendency to assume that mature, photosynthetic leaves are the major sites of sulfur assimilation in plants. However, there is increasing evidence that photosynthetic sink organs such as roots, developing leaves and developing seeds are active in sulfur reduction and sulfur amino acid biosynthesis (Brunold and Suter 1989; Sexton and Shibles 1999; Hartmann *et al.* 2000; Tabe and Droux 2001). We have previously demonstrated that it is predominantly oxidized sulfur that is delivered to developing lupin pods in the phloem (Tabe and Droux 2001). Here, we investigate the capacity of developing chickpea embryos for active uptake of sulfate.

Cotyledons were dissected from developing chickpea seeds at mid-maturation (approximately 30 days after flowering). The cotyledons were separated into matched pairs, and one cotyledon of each pair was incubated at 24 ^{o}C in the light, in an isotonic uptake solution containing ^{35}S-sulfate (2 % w/v sucrose, 20 mM 2[N-morpholino]ethanesulfonic acid, pH 4.5, 5.0 or 6.0 (or 20 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, pH 7.0), 1 mM $CaCl_2$, 50 μM Na_2SO_4 plus 10 μCi ml^{-1} $^{35}SO_4$). The other cotyledon was incubated in the same solution in the dark, on ice, to act as a control for surface contamination of the tissue with ^{35}S. Cotyledons incubated at 24 ^{o}C showed characteristics of active sulfate uptake. Sulfate accumulation was pH dependent (Fig 1) and saturable with respect to sulfate concentration (results not shown).

An RT-PCR approach was used to investigate the sulfate transporters that may be involved in sulfate uptake by developing chickpea embryos. Consensus oligonucleotides were designed to highly conserved regions in genes encoding plant sulfate transporters, and RT-PCR was used to isolate partial cDNA clones encoding three putative sulfate transporters from chickpea organs (Fig 2). Sequence comparisons showed up to 78 % identity between the predicted amino acid sequences (which represented approximately one third of the full length protein) and sequences of known plant sulfate transporters. The three partial cDNAs were designated CaSultr2-1, 3-1 and 3-3 in accordance with the nomenclature of the *Arabidopsis*

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thaliana sulfate transporter genes to which they were most similar (Takahashi *et al.* 2000).

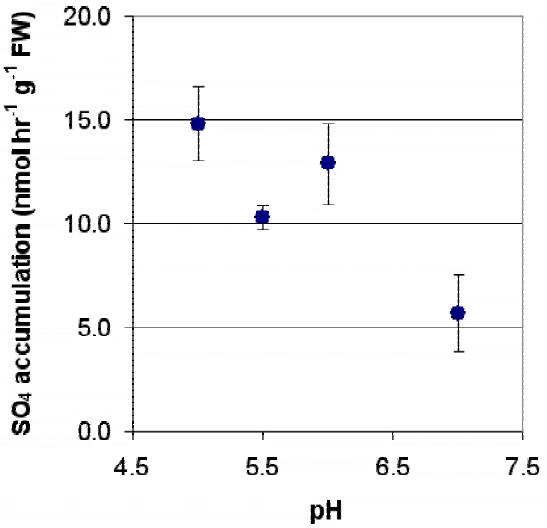

Fig 1. Accumulation of sulfate by mid-maturation chickpea cotyledons *in vitro*. Sulfate uptake was calculated from the quantity of [^{35}S] in cotyledons incubated at 24 ^{o}C, minus the ^{35}S in matched cotyledons incubated on ice. The tissues were rinsed briefly in excess, unlabelled uptake solution at 0 ^{o}C before extraction into 25 mM HCl and counting in scintillation fluid, values are mean +/- standard error, n = between 2 and 8 for each value.

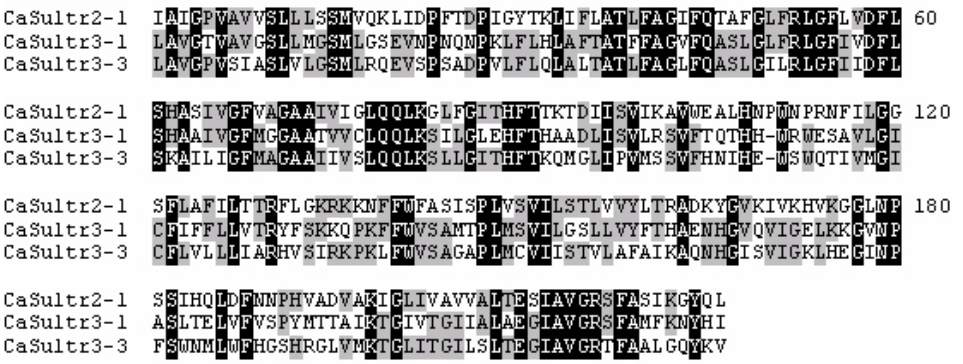

Fig. 2. Predicted amino acid sequences of putative sulfate transporters from *Cicer arietinum*. RT-PCR was performed (using a One-step RT-PCR kit from Qiagen) on total RNA isolated from either roots, leaves, or developing pods or embryos of chickpea. The RT-PCR products were cloned and sequenced.

Gene-specific oligonucleotides were designed, and RT-PCR was used to investigate the expression of the three putative transporters in chickpea. CaSultr2-1 and CaSultr3-1 transcripts were detected in RNA from roots, leaves, stems and developing chickpea pods, and the testa of developing seeds. In developing embryo, CaSultr2-1 transcripts were undetectable, while a very low amount of CaSultr3-1 transcript was detected. CaSultr3-3 transcripts were detected in the above organs (except developing testa) and were abundant in developing chickpea embryos. The results presented here suggest that developing chickpea embryos are capable of active sulfate uptake and that this process may involve a transporter with similarity to the AtSultr3-3 gene of *Arabidopsis*. The expression of the CaSultr3-3 gene in almost all chickpea organs investigated suggests that it is involved in sulfate uptake by many cell types, or that it is involved in a sub-cellular transport step.

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T-DNA INSERTION MUTAGENESIS OF SULFATE TRANSPORTERS IN *ARABIDOPSIS*

Hideki Takahashi, Akiko Watanabe-Takahashi and Tomoyuki Yamaya
RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, 230-0045, Japan

Membrane-bound transporters facilitate absorption and distribution of nutrients in plants. Sulfate transporter gene family in *Arabidopsis* consists of 14 members with different kinetic properties and cell-type specific expression. The initial uptake of sulfate in *Arabidopsis* root involves Sultr1;1 and Sultr1;2 high-affinity transporters expressed in the epidermis and cortex (Takahashi *et al.* 2000; Yoshimoto *et al.* 2002; Shibagaki *et al.* 2002). Sulfate is subsequently transported through the endodermis to reach the vascular bundle. Several vascular localizing transporters are suggested to participate in inter-organ movement of sulfate through the vascular tissue (Takahashi *et al.* 1997; 2000). Within the individual cell, sulfate anion mostly accumulates in the vacuole as inert sulfur pool. When sulfur is limiting, plants activate high-affinity sulfate transporters in roots to facilitate the uptake of sulfate from the soil. Alternatively, they utilize vacuolar sulfate as sulfur source for synthesis of cysteine and methionine.

We have isolated the knockout mutants for 12 isoforms of sulfate transporters in *Arabidopsis* (Fig. 1). PCR screening of pooled DNA of T-DNA insertion lines was carried out using gene specific primers. The use of this reverse genetic approach enabled us to identify *in planta* functions of vacuolar sulfate transporters, Sultr4;1 and Sultr4;2 in *Arabidopsis*. Localization of Sultr4;1 and Sultr4;2 was investigated using GFP. The coding sequence of GFP was fused to the C-terminal ends of Sultr4;1 and Sultr4;2, and expressed with their own promoters in transgenic plants. Sultr4;1-GFP and Sultr4;2-GFP were localized in the tonoplast membranes. Based on prediction with the computer programs and bialistic transformation (Takahashi *et al.* 1999), the N-terminal region of *Arabidopsis* Sultr4;1 was suggested to encode a chloroplastic transit peptide. Discrepancies of the results may indicate that the truncated cDNAs fused to GFP in the previous experiments were not sufficient to locate the fusion proteins to the correct subcellular compartments. Insertion of T-DNA in *Sultr4;1* and *Sultr4;2* caused increased accumulation of sulfate and decrease of cysteine and glutathione contents when plants were grown under low sulfate conditions. These results indicate that dysfunction of Sultr4;1 and Sultr4;2 transporters at the tonoplast membranes resulted in restricting supply of sulfate to cysteine biosynthesis. It was suggested that these transporters are responsible for the efflux of sulfate from the vacuole.

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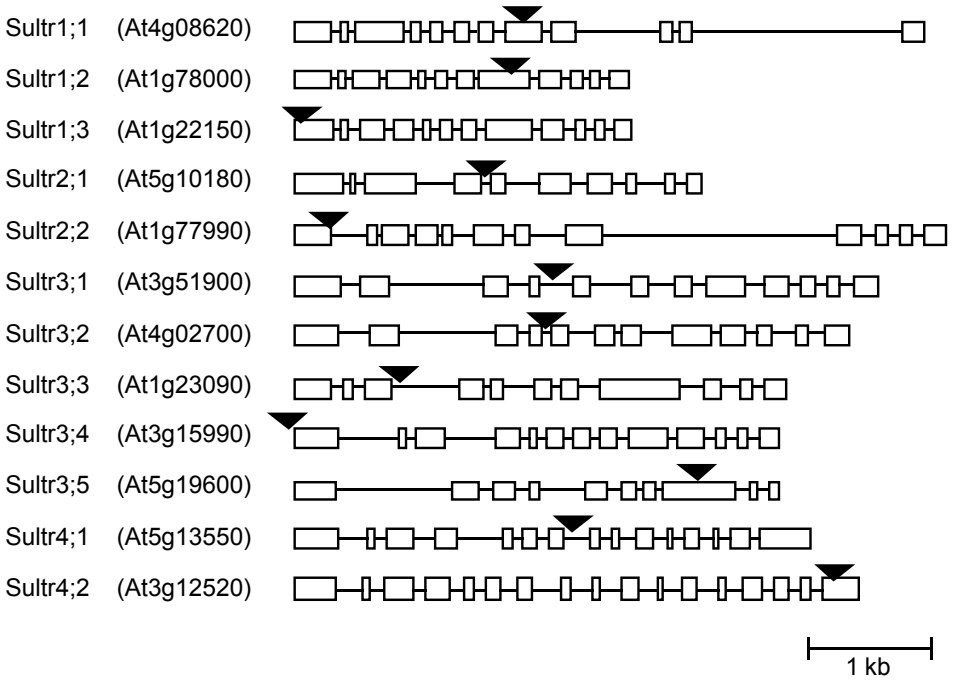

Fig. 1. T-DNA insertion mutants of sulfate transporters in *Arabidopsis*. Open bars and lines indicate exons and introns, respectively. Arrow heads indicate insertion sites of T-DNAs.

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MODIFICATION OF SULFUR METABOLISM IN SPRUCE TREES BY H_2S STUDIED BY RADIOLABELLED SULFATE UPTAKE

Michael Tausz[1], Astrid Wonisch[1], Wilfried Weidner[1,2], Luit J. De Kok[2] and Dieter Grill[1]

[1]Institute of Plant Physiology, University of Graz, Schubertstraße 51, A-8010 Graz, Austria; [2]Laboratory of Plant Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Hydrogen sulfide is a phytotoxic gas locally important as air pollutant, but widely used as a tool to study S metabolism in plants (De Kok *et al*. 2002). It enters plant sulfur assimilation pathway at the reduction state of sulfide and is easily incorporated into cysteine without energy consuming reduction steps. Herbaceous plants, such as *Brassica*, can grow with H_2S as exclusive S source (De Kok *et al.* 1997). *Brassica* plants switch only partly from sulfate uptake to H_2S if sulfate supply to roots is sufficient, though H_2S appears to be the preferred source for organic S (Westerman *et al.* 2000). Knowledge on responses of S metabolism in trees to H_2S is scarce. In the present paper we investigate the effect of H_2S on sulfur utilization in spruce roots and needles.

Two-year-old *Picea abies* (L.) Karst. (Norway spruce) seedlings were grown in 25 % Hoagland nutrient solution containing 0.5 mmol l^{-1} SO_4^{2-}. Before the start of the experiment, the nutrient solution was changed to one containing $^{35}SO_4^{2-}$ (9.4 Mbq $mmol^{-1}$). Six randomized trees were exposed to 250 nl l^{-1} H_2S and corresponding control (0 nl l^{-1} H_2S) in stainless steel cabinets with polycarbonate top for 6 days. Day/night T were 23/18 °C, relative humidity 65 %, PPFD 250 - 270 µmol m^{-2} s^{-1} PAR for 12 h day^{-1}. Needles of the youngest fully developed age class were sampled in liquid N_2. After removing nutrient solution roots were washed three times in 250 ml of demineralized water and fine roots (< 2 mm diameter) were sampled. Material was lyophilized and ground under liquid nitrogen in a dismembrator. Total S was determined after combustion in O_2 over H_2O_2 and SO_4^{2-} in water extracts on an anion exchange HPLC. Organic S was calculated as the difference between total S and SO_4^{2-}. Fractions were collected and radioactivity was measured in a liquid scintillation counter.

After 6 days of exposure to atmospheric H_2S pools of organic S and SO_4^{2-} increased in spruce needles. In fine roots, only SO_4^{2-} accumulated, whereas the organic S pool was hardly affected (Fig. 1).

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In needles, the amount of labelled S, which must originate from SO_4^{2-} taken up from the nutrient solution, decreased in the (larger) organic S pool. In coincidence with results on other plants (De Kok *et al.* 2002), this indicates that H_2S is predominantly used for the synthesis of organic S in the needles. Use of reduced S from H_2S enables the plant to circumvent energy consuming S reduction (De Kok and Tausz 2001). On the other hand, the amount of S originating from the nutrient solution increased in the needle sulfate pool. Apparently, sulfate import into the foliage continued in spite of the atmospheric S supply, but mainly the flux (sulfur reduction pathway) from the foliar sulfate pool into foliar organic S was inhibited upon H_2S supply.

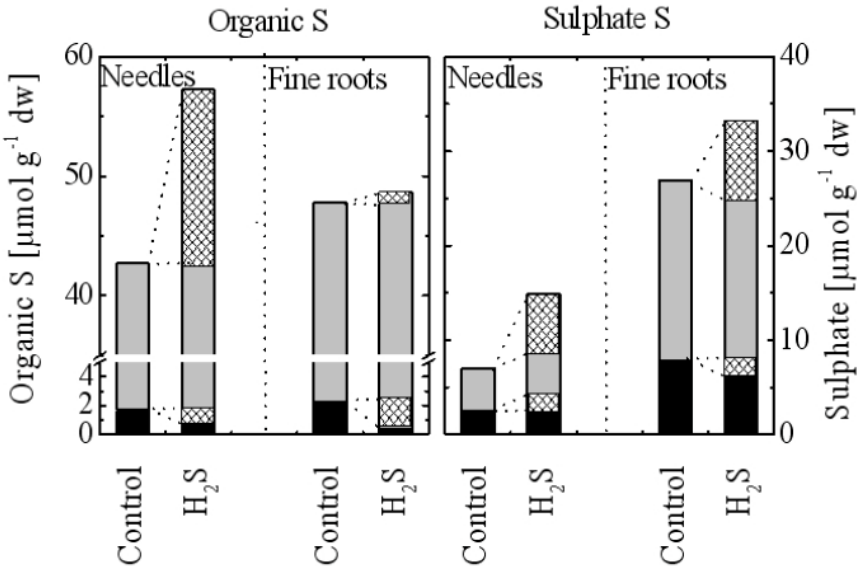

Fig. 1. Sulfur fractions in needles and roots of *Picea abies* exposed to 0 ("control") and 250 nl l^{-1} H_2S ("H_2S"). Sulfur originating from the nutrient solution (■) and sulfur from non-labelled sources, *i.e.* from H_2S or internal S cycling (▨). Crosshatched areas indicate changes compared to the control (without H_2S). dw, dry weight.

In the organic S fraction of roots, sulfur from the nutrient solution was replaced by non-labelled S without change in the size of the organic S pool. Increase in the root SO_4^{2-} pool was due to unlabelled S, while the amount of S coming from the nutrient solution even decreased. These data point towards a reduced uptake of SO_4^{2-} from the nutrient solution and, most probably, a net import of organic S from the needles into the roots. In spruce trees a basipetal (phloem) transport of glutathione, the most common transport form of reduced S, could not be demonstrated (Herschbach and Rennenberg 2001). Increased proportion of non-labelled S in the

root organic S fraction may still be caused by internal cycling and limited export, but since sulfate import into needles seems to continue in spite of H_2S exposure (see above), this explanation is less probable.

Acknowledgement

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BIOSYNTHESIS OF CYSTEINE AND GLUTAMATE IN *CHLAMYDOMONAS REINHARDTII*: EFFECT OF NITRATE OR SULFATE STARVATION AND CADMIUM STRESS

José M. Vega[1], Antonio Benitez-Burraco[1], Javier Vigara[2] and Carlos Vilchez[2]
[1]Dpto. Bioquímica Vegetal y Biología Molecular, Universidad Sevilla. Sevilla. Spain; [2]Dpto. Química y Ciencia de Materiales. Universidad de Huelva. Huelva. Spain

Assimilation of sulfate in *Chlamydomonas reinhardtii* requires its transport inside the cell and into the chloroplast, where it is activated and reduced to sulfide. Cysteine biosynthesis occurs, in the cytosol, chloroplast and mitochondria of the alga, by sulfhydrylation of the carbon skeleton of serine, catalyzed by cysteine synthase complex, which involves two different enzymes; serine acetyltransferase (SAT; EC 2.3.1.30) which catalyzes the synthesis of *O*-acetylserine (OAS) from L-serine and acetyl-CoA; and the *O*-acetylserine (thiol)lyase (OAS-TL; EC 4.2.99.8), which catalyzes the production of cysteine from OAS and sulfide. Three OAS-TL isoenzymes have been separated from *C. reinhardtii* crude extract and its physicochemical properties characterized (Prieto *et al.* 1998). Otherwise, glutamate synthesis is produced in the *C. reinhardtii* chloroplast and cytosol by the reductive amination of 2-oxoglutarate, catalyzed by the glutamine synthetase (GS, EC 6.3.2.1), glutamate synthase (Fd-GOGAT, EC 1.4.7.1) cycle. The cysteine biosynthesis is the key point in which CO_2 fixation, nitrate, and sulfate assimilation are connected.

C. reinhardtii cells (wild type, strain 21 gr) were grown at 25 ºC in Sueoka liquid medium, with 10 mM nitrate and 0.3 mM sulfate. The cultures were flushed with air supplemented with 5 % CO_2, and continuously illuminated with white light, 50 W m^{-2}. Crude extract were prepared by freezing the cells in liquid nitrogen and the soluble proteins were separated by centrifugation (16,000g, 20 min). More information about methods see Ravina *et al*. (1999).

Sulfur-starved cells induce gene expression of both, SAT and OAS-TL (3.5 fold its original value in S-repleted cells, after 36 h of treatment), and activities (6- and 7-fold, respectively after 24 h). In addition, S-starvation promotes OAS-TL protein synthesis, quantified by specific antiserum, and the time course shown is similar to that observed with the activity (Fig. 1). These data reveal transcriptional and post-transcriptional mechanisms regulate the cysteine synthase activities in *C. reinhardtii*. We study the postranscriptional regulation of the OAS-TL activity and our data indicate that in order to get maximal expression of activity, light, nitrate and CO_2

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are simultaneously required in the cells culture (Fig. 2). The three OAS-TL isoenzymes are inhibited by L-methionine, but the mitochondrial one is not sensible to the feedback inhibition by cysteine. Moreover, S-starvation decreases, after 9 hours, 60 % the alga photosynthetic activity and also down 50 % the consumption rate of nitrate and nitrite.

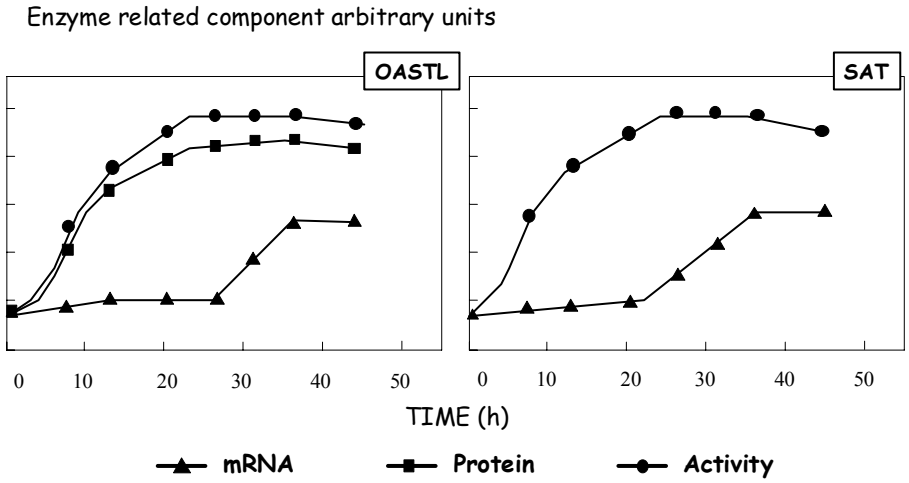

Fig.1. Effect of S-starvation on the cysteine synthetase activities of *C. reinhardtii*. At zero time, the cells were re-suspended (20 μg Chl ml^{-1}) and grown in a S-free culture medium. At the indicated times, the activities were measured and the Northern and Western blot analysis performed.

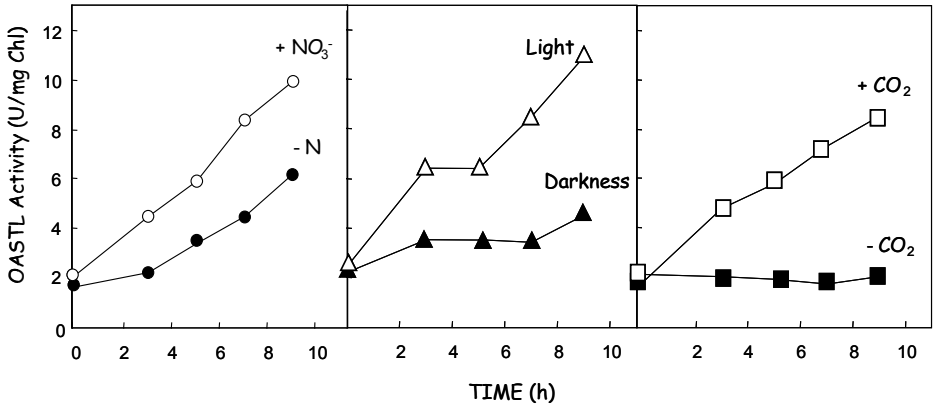

Fig. 2. Effect of light, nitrate and CO_2 on the OAS-TL activity induction in S-starved *C. reinhardtii* cells. At zero time, the cells were resuspended (20 μg Chl ml^{-1}) in S-free culture medium, and grown under the indicated conditions. OAS-TL activity was determined when indicated.

Sulfate is required with nitrate in the medium, for full expression of nitrate reductase (NR), nitrite reductase (NiR) and GOGAT activities, but does not affect GS activity. Otherwise, nitrate is specifically required in the culture medium for full induction of NR and NiR, while GS and GOGAT remain high in N-starved cells.

The presence of high concentration (300 μM) of cadmium in the culture medium inhibits the growth rate of the algae and limits in 20 % the productivity, while no inhibition was observed at 150 μM Cd^{2+}. The nitrate consumption rate was 118.7 nmol mg^{-1} Chl min^{-1} under normal growing conditions, and increases to 127.3 in the presence of 150 μM Cd^{2+}, but go down to 96.7 nmol mg^{-1} Chl min^{-1}, in the presence of 300 μM of Cd^{2+}.

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MOBILIZATION OF ^{35}S IN RHIZOSPHERE SOIL OF RAPE AND BARLEY: RELATIONSHIP BETWEEN ROOT-^{35}S UPTAKE AND SOIL ARYLSULFATASE ACTIVITY

Phuy-Chhoy Vong, Françoise Lasserre-Joulin and Armand Guckert
UMR Agronomie et Environnement ESAIA-INRA, 54505 Vandoeuvre lès Nancy, France

Rape is known to require more S for its secondary metabolite synthesis. Its rhizosphere harbors predominantly sulfur-oxidizing bacteria (Grayston and Germida 1991). In addition, rape roots, which are not mycorrhized, are able to exude large amounts of organic acids (Jones 1998). Information about P acquisition by plant rhizosphere is abundant, but that about S in relation to arylsulfatase activity is still limited.

After labelling the organic S of a calcareous soil with $Na_2{}^{35}SO_4$ for three months, an equivalent of 608 g dry soil was placed into PVC pots (13 cm height x 13 cm diameter). In the center of each pot, a rhizobag (10 cm height x 8 cm diameter), filled with the same ^{35}S-labelled soil was inserted (Lasserre *et al*. 2000). Before sowing, the pots received a single dose of 76.9 mg N kg^{-1} soil as ammonium nitrate and three doses of S as $MgSO_4$: S1 (20.4), S2 (30.8) and S3 (61.5 mg S kg^{-1} soil). Four seeds of "Tanto" rape and "Scarlett" barley were sown in the rhizobag and then thinned to one plant 8 days after sowing (DAS). In total we had 72 pots with 4 replicates for each treatment. The plants were in a growth chamber under controlled conditions. Destructive pot samplings were made on 20, 42 and 56 DAS before the flowering stage. The rhizosphere soil in the rhizobag was manually separated from the roots. Shoots and roots were digested with HNO_3-$HClO_4$ and then ^{35}S analyzed. Soil S-SO_4^{2-} was extracted by 0.01M $CaCl_2$ and the residue by hot water at 70°C for 18 h (Sparling *et al*. 1998). The soil arylsulfatase was analyzed according to the method of Tabatabai and Bremner (1970).

As a whole, rape took up significantly more ^{35}S than barley (Fig. 1), especially at the high level of S (S3). For roots, similar patterns of evolution were noted for both plants with a large ^{35}S uptake by rape roots. As expected, significantly large decrease of ^{35}S-SO_4^{2-} (Fig. 2A,B) and to a lesser extent ^{35}S-hot water (Fig. 2C,D) was observed for rape rhizosphere but not for barley. Significant correlations of ^{35}S uptake with ^{35}S-SO_4^{2-} ($r^2 = 0.78$, $p < 0.01$) and ^{35}S-hot water ($r^2 = 0.75$, $p < 0.05$) found in rape rhizosphere soil (Fig. 2A,C) but not in barley (Fig. 2B,D) indicated the capability of rape to regulate the absorption of ^{35}S in both mineral and organic forms.

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In addition, significant correlation between soil arylsulfatase activity and ^{35}S uptake by roots of rape (Fig. 3A) and not of barley (Fig. 3B) explained the ability of rape roots to regulate ^{35}S uptake *via* the soil arylsulfatase activity. Rape took up more ^{35}S than barley, but the soil arylsulfatase activity values [mg *p*-nitrophenol (PN) kg soil^{-1}] found in both plants were similar. This explains that most of ester sulfate synthesized by rape rhizosphere microorganisms would not be mainly in the form of aryl-esters. In summary, rape requires most S. Among all strategies adopted the regular release of arylsulfatases by both roots and rhizosphere microorganisms would be the ones used by rape.

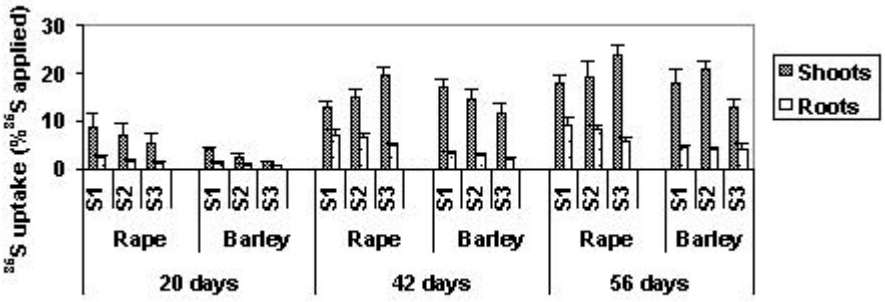

Fig. 1. ^{35}S uptake by shoots and roots of rape and barley. Vertical bars: (± SE).

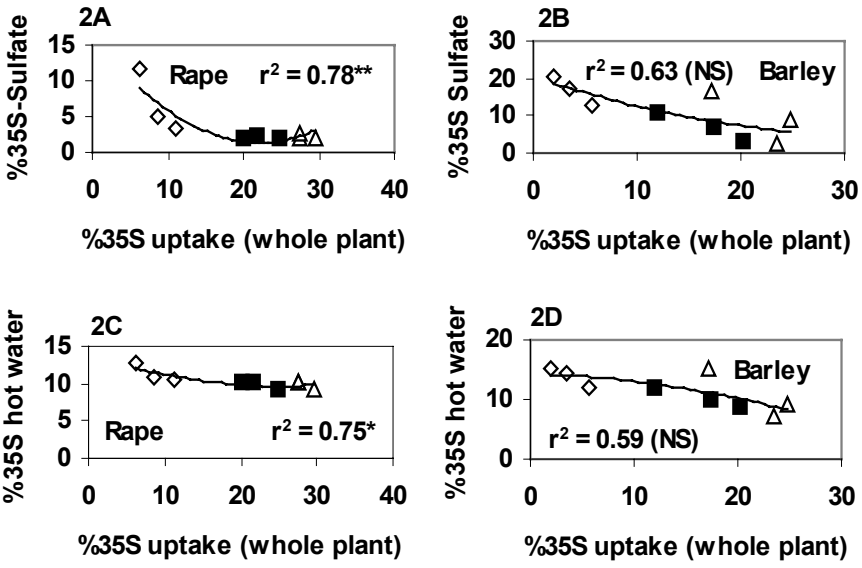

Fig. 2. Correlations between ^{35}S-sulfate, ^{35}S-hot water and ^{35}S uptake. ◊: 20 days, ■: 42 days, Δ: 56 days.

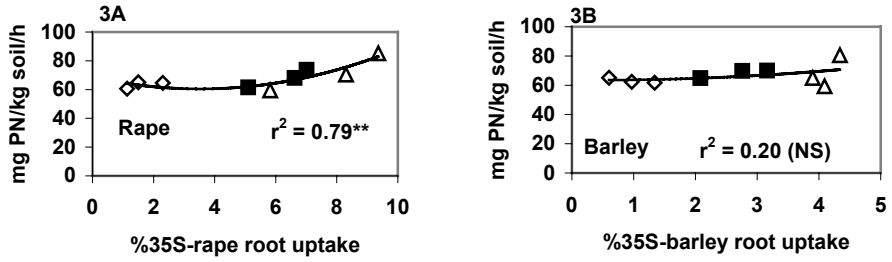

Fig. 3. Correlation between ^{35}S uptake by roots and soil arylsulfatase activity.

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WOOL QUALITY AND SULFUR SUPPLY

Shiping Wang[1], Yanfen Wang[1], Zuozhong Chen[1], Ewald Schnug[2] and Silvia Haneklaus[2]

[1]The Laboratory of Vegetation Quantitative Ecology, Institute of Botany, CAS, Beijing 100093,China; [2]Institute of Plant Nutrition and Soil Science of FAL, Bundesallee 50, D-38116 Braunschweig,Germany

Sulfur fertilization increased yield of gramineous crops in S-deficient areas of the Inner Mongolia Steppe and other parts of China (Fan and Messick 1997; Puoli *et al.* 1991; Wang *et al.* 2001, 2002). S fertilization improves forage quality too and corrected a microbial S deficiency in the rumen of sheep (Murphy *et al.* 1983). Feeding experiments with lamb, sheep and goats confirmed the favorable effect of S-fertilized forage on animal production (Weston *et al*. 1988; Puoli *et al*. 1991; Qi *et al*. 1992). Sheep is more sensitive to S deficiency than cattle, because of its particular S demands for wool production (Murphy *et al*. 1983). Wool fiber production is characterized by a high demand for S-containing amino acids as epidermal tissues of all animals are rich in S (Tisdale 1977; Qi *et al.* 1994). The keratin fiber that grows from epidermal tissue is particularly rich in S amino acids. Therefore fiber-producing animals such as sheep express S deficiency more pronounced (Qi *et al.* 1994). The results of the presented field experiment revealed that S fertilization significantly increased not only the S content of the forage crop, but also relevant wool parameters.

The experiment was conducted at the Inner Mongolia Grassland Ecosystem Research Station of the Chinese Academy of Sciences (43°26' N; 116°04′ E). The soil type is a chestnut soil and the vegetation is dominated by *Leymus chinense*, *Cleistogenes squarrosa* and *Artemisia frigida*. Three-hectare paddocks were fenced and fertilized with 0, 30 and 60 kg S ha^{-1} as gypsum. The stocking rate was 4 sheep ha^{-1} and the sheep bred was the Inner Mongolia fine wool sheep (1.5 year-old wethers with about 36 kg live weight). The sheep grazed continuously from June 1 to October 5. The aboveground biomass was determined by cutting five one-square meter plots at the soil surface; then the material was dried at 65 °C until constancy of weight. The S, N and P content were determined in all plant samples. The experimental sheep were sheared on 15 June. Wool samples were collected after the grazing period from the mid-side of four sheep. The wool was cleaned by light petroleum and air-dried. The absolute length, diameter, strength, elongation of wool was determined.

The aboveground biomass, total S and P concentration increased significantly by S fertilization while the total N concentrations remained unaffected (Table 1). The

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natural S supply of the forage crop of 1.8 mg S g^{-1} was not sufficient to meet the requirements of growing sheep which range from 1.8 to 3.1 mg S g^{-1} (NRC 1985; Qi *et al.* 1994), because wool quality was significantly improved by S fertilization (Table 2).

Table 1. Effect of S fertilization on the aboveground biomass of the natural plant community and total N, S and P concentrations.

Parameter	Sulfur fertilization (kg S ha^{-1})		
	0	30	60
Aboveground biomass (g DM m^{-2})	117.4a	137.1b	143.4b
Total N (g kg^{-1})	18.2a	18.3c	19.6a
Total S (g kg^{-1})	1.8a	2.5c	2.6c
Total P (g kg^{-1})	1.6a	2.3c	2.7c

a and b or b and c ($p < 0.05$), a and c ($p < 0.01$)

Table 2. Effect of S fertilization on wool yield and quality.

S fertilization (kg S ha^{-1})	Absolute length (cm)	Diameter (mm)	Strength (g)	Elongation (%)	Cleaned wool weight (kg)
0	3.6a	21.2a	8.1a	41.4a	0.5a
30	4.0b	23.3b	9.7b	51.6c	0.7b
60	4.1b	24.9b	10.9b	51.5c	0.7b

a and b or b and c ($p < 0.05$), a and c ($p < 0.01$)

S fertilization significantly increased the apparent digestibility of the forage by 5 %, whereas voluntary intake and average daily gain were not affected by S fertilization (data not shown). In comparison to the control, S fertilization increased the absolute length, diameter, strength, elongation and cleaned wool weight by 13.7 %, 17.8 %, 35.8 %, 24.5 % and 26.0 %, respectively.

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COMPARATIVE BIOCHEMICAL CHARACTERIZATION OF OAS-TL ISOFORMS FROM *ARABIDOPSIS THALIANA*

Markus Wirtz and Rüdiger Hell
Institute of Plant Genetics and Crop Plant Research, Corrensstrasse 3, 06466 Gatersleben, Germany

The exclusively entry for sulfur into plant metabolism is formation of cysteine. It is catalyzed by *O*-acetylserine (thiol)lyase (OAS-TL) using *O*-acetylserine (OAS) as the acceptor for reduced sulfur. In *Arabidopsis thaliana* three major OAS-TL isoforms are present (Jost *et al.* 2000), which have been located in the cytosol, plastids and the mitochondria. The need for cysteine synthesis in all these compartments is still controversially discussed. Biochemical investigations of OAS-TL from various sources led to different results with respect to kinetic mechanism and K_M affinities (Roland *et al.* 1996; Warrilow and Hawkesford 2000). Recently, OAS-TL has been proposed to function in cysteine degradation at high pH values (Burandt *et al.* 2002). The aim of this study was to clarify the biochemical characteristics and mechanisms of the individual OAS-TL isoforms form *Arabidopsis thaliana* to understand the compartment specific function of OAS-TL in cysteine metabolism.

Since recombinant OAS-TL activity proved to be sensitive to fusion tags, the recombinant proteins were expressed without tags in *E. coli* cells using the pET-vector system and purified by conventional column chromatography. The K_M values for OAS and sulfide were measured varying the substrate concentration up to 10 mM for OAS and 2 mM for sulfide, respectively. Other conditions were as described in (Jost *et al.* 2000). The produced cysteine was determined in parallel with two assays: (1) the Gaitonde test (Gaitonde 1967), (2) derivatization of cysteine with monobromobimane followed by HPLC analysis. The latter assay allows more sensitive detection of cysteine and therefore the use of less enzyme to ensure a high substrate to activity ratio.

Table 1. Kinetic features of the mitochondrial *Arabidopsis thaliana* OAS-TL differ with respect to the assay method used for quantification of produced cysteine.

Assay	Specific activity	K_M (OAS)	K_M (sulfide)
Gaitonde	351 ± 32	480 ± 62 µM	380 ± 50 µM
Monobromobimane	382 ± 24	434 ± 24 µM	3.1 ± 0.5 µM

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The purified OAS-TL isoforms have about 100 times higher maximal velocity rates than reported earlier (Burandt *et al.* 2002). The specific activities of the isoforms differ between 400 to 600 µmol min^{-1} mg^{-1} for organellar isoforms and about 900 µmol min^{-1} mg^{-1} for the cytosolic isoform. Gaitonde and monobromobimane test result in comparable maximal velocity rates for all analysed OAS-TL isoforms. Substrate K_M values obtained with both assays are similar for OAS but differ about 100 times for sulfide as shown for the mitochondrial isoform in Table 1.

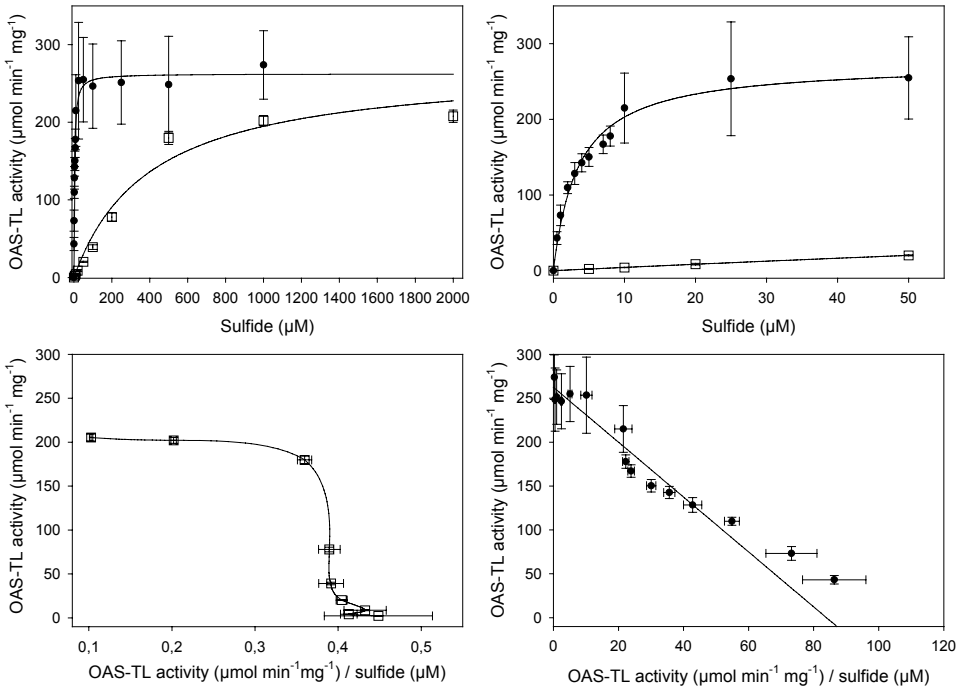

Fig. 1. Velocity against substrate plots (upper panels) and Eady-Hofstee plots (lower panels) for mitochondrial OAS-TL from *A. thaliana*. Data were obtained with the Gaitonde (□) or the monobromobimane (●) assay. The right side of the upper part presents the same dataset as the left side, but only up to 50 µM sulfide and at a larger scale. The biphasic behaviour of the Gaitonde data in the Eadie-Hofstee plot demonstrates that one substrate limits the reaction.

A typical sulfide saturation experiment with the Gaitonde and the monobromobimane assay for mitochondrial OAS-TL as an example is shown in Fig. 1 as a velocity against substrate plot (upper part) and an Eadie-Hofstee plot (lower part). The data set obtained with the Gaitonde assay shows a biphasic behaviour. This indicates that sulfide is completely consumed during the incubation period due to excess enzyme activity in the assay. However, at low sulfide concentrations the use of less enzyme results in production of undetectable amounts of cysteine with the Gaitonde test. Using less enzyme and the monobromobimane assay it was demonstrated that

the enzyme was active even at very low sulfide concentrations, yielding an apparent K_M value for sulfide of about 3 μM. The linearity of the Eadie-Hofstee plot of these data confirms that the OAS-TL reaction follows a typical Michaelis-Menten kinetics without any cooperativity as reported for bacterial OAS-TL (Becker *et al.* 1969).

Our results were obtained by careful comparison of the assay methods and added a note of caution for the interpretation of kinetic data of sulfide fixation by OAS-TL in plants.

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EFFECTS OF SO_2 EXPOSURE ON SULFUR DISTRIBUTION IN CURLY KALE (*BRASSICA OLERACEA* L.) INVESTIGATED BY ^{35}S-LABELLED NUTRIENT SOLUTION

Astrid Wonisch[1], Wilfried Weidner[1], Michael Tausz[1], Sue Westerman[2], Luit J. De Kok[2] and Dieter Grill[1]

[1]Institute of Plant Physiology, University of Graz, Schubertstrasse 51, A-8010 Graz, Austria; [2]Laboratory of Plant Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Several studies showed that curly kale is able to use both pedospheric sulfate and atmospheric H_2S as a sulfur source for growth (De Kok *et al.* 1997, 1998), whereas the utilization of the different sulfur sources is regulated and in tune the sulfur requirement for growth (Westerman *et al.* 2000). Plant shoots form a sink for SO_2, which has to be reduced prior to its metabolism into organic compounds. However, it may also be oxidized to sulfate and subsequently transferred to the vacuole where it is poorly accessible for metabolism (De Kok *et al.* 1997; Tausz *et al.* 1998).

This study was intended to investigate the interactions between metabolism of atmospheric SO_2 in the shoot and sulfate uptake by the roots of curly kale (*Brassica oleracea* L.). Sulfur distribution within the different sulfur pools was determined by using ^{35}S-sulfate in the nutrient solution while simultaneously fumigating with SO_2. Curly kale (*Brassica oleracea* L., cv. Bornick F1, Nickerson-Zwaan, The Netherlands) was grown on a 25 % Hoagland nutrient solution for three weeks (see for details Westerman *et al.* 2000). At the start of the experiment plants were transferred to a ^{35}S-sulfate labelled 25 % Hoagland nutrition solution with specific activity of 4.86 MBq $mmol^{-1}$ sulfate. Plants were subsequently exposed to 400 nl l^{-1} SO_2 for 24 h in cylindrical stainless steel fumigation cabinets with a polycarbonate top (as described by Stuiver *et al.* 1992). Day/night temperature were 23/18°C, relative humidity 65 % and the photon fluence rate 250 - 270 µmol m^{-2} s^{-1} (within the 400 - 700 nm range) for 12 h day^{-1}. After removing the labelled nutrient solution roots were washed three times in 250 ml of demineralized water. Nine plants for each treatment (control, SO_2) were sampled and divided into shoots and roots (3 plants for one sample mixture, respectively). The lyophilized material was pulverized under liquid nitrogen in a dismembrator. Sulfate content was measured by an isocratic HPLC-method described in Tausz *et al.* (1996). The determination of the total sulfur content was carried out on plant dry matter, which was combusted in oxygen atmosphere over hydrogen peroxide. The formed sulfate was measured by HPLC analysis

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as described above. For estimation of the radioactivity of the organic and inorganic sulfur fractions, the injected samples were collected with a fraction collector and measured in a liquid scintillation counter.

SO_2 exposure resulted in increased organic and inorganic sulfur (sulfate) contents of both shoots and roots (Fig. 1). Sulfur uptake by roots was hardly affected by SO_2 exposure, since the proportion of ^{35}S in the plant originating from sulfate taken up from the nutrition solution was hardly affected. The increase in the sulfur fraction in both shoots and roots could be attributed to deposition and metabolism of SO_2. Enhanced levels of sulfur fractions in the roots indicate a transport of sulfur compounds from the shoots to the roots in the presence of excess sulfur in the shoots.

In contrast to studies with H_2S as atmospheric sulfur source (De Kok *et al.* 1997; Westerman *et al.* 2000), there is no strong interaction between atmospheric SO_2 and pedospheric sulfate nutrition resulting in increased levels of organic and inorganic sulfur pools.

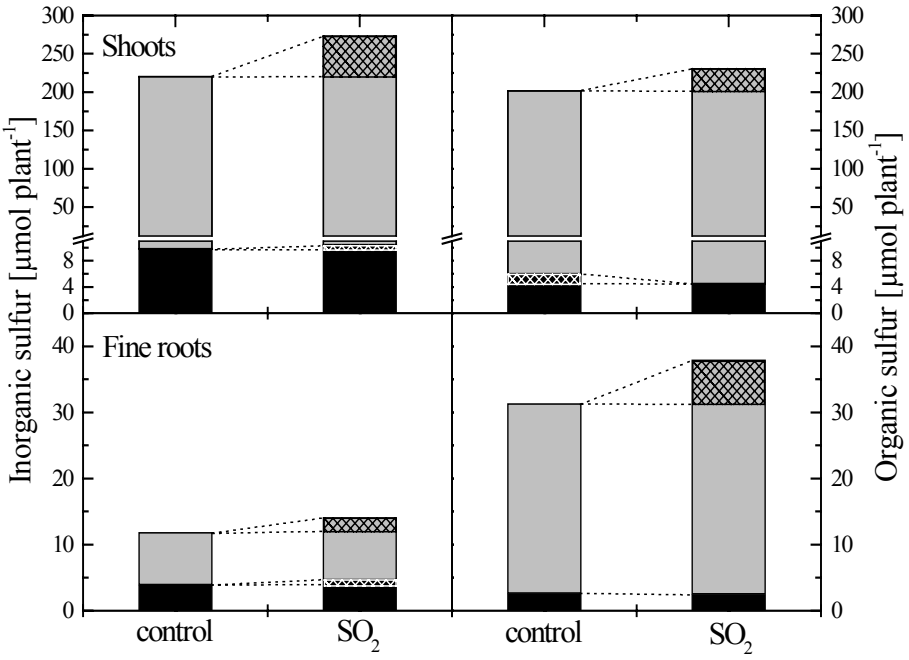

Fig.1. Sulfur pools in curly kale shoots and roots after fumigation with 400 nl l^{-1} SO_2 for 24 h. ■ = ^{35}S-sulfur originating from the nutrient solution, ▭ = sulfur from non-labelled sources, *i.e.* from SO_2 or internal S cycling. Cross-hatched signatures indicate changes relative to control (without SO_2).

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INTERACTION BETWEEN ATMOSPHERIC SULFUR DIOXIDE DEPOSITION AND PEDOSPHERIC SULFATE NUTRITION IN CHINESE CABBAGE

Liping Yang, Ineke Stulen and Luit J. De Kok
Laboratory of Plant Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

At present, sulfur deficiency appears to be one of the most frequent nutrient deficiencies occurring in high yielding arable crops. Sulfur is usually available to plants as sulfate from the pedosphere. Despite improved air pollution control legislation, SO_2 pollution is still rather serious in the vicinity of populated areas in China. Although atmospheric sulfur gases, *viz.* SO_2, H_2S, may be phytotoxic (Okpodu *et al*. 1996; De Kok 1990), they can also be utilized as a sulfur source for plant growth and may even be beneficial when the pedospheric sulfate supply is limited (De Kok 1990; De Kok and Stulen 1998; De Kok *et al.* 2000; Westerman *et al.* 2000). The present study was conducted to evaluate the possible significance of SO_2 as nutrient for Chinese cabbage, an important vegetable crop in Northern China.

Seedlings of Chinese cabbage (*Brassica oleracea* L., cv. Kasumi F1, Nickerson-Zwaan, The Netherlands) were grown on a 25 % Hoagland nutrient solution in a climate-controlled room for one week and subsequently transferred to a 25 % Hoagland nutrient solution with or without 0.5 mM sulfate. Plants were acclimated for one day in the fumigation cabinets and subsequently exposed to various levels of SO_2 (0.06 - 0.18 $\mu l\ l^{-1}$; see for experimental setup De Kok *et al.* 1997). The relative growth rate (RGR) of the plants, sulfate and total S were determined after 5 days of exposure (see for methods De Kok *et al.* 1997; Westerman *et al.* 2000).

Table 1. Impact of sulfate nutrition and SO_2 exposure on growth of Chinese cabbage. Plants were exposed for five days. Relative growth rate (RGR in % day^{-1}; determined over a five-day-time interval) and shoot/root ratio (S/R) are expressed on a fresh weight basis and represent the mean of 3 measurements with 9 plants in each (± SD).

	- Sulfate				+ Sulfate			
SO_2 ($\mu l\ l^{-1}$)	0	0.06	0.12	0.18	0	0.06	0.12	0.18
RGR	24±2	30±1	30±2	31±1	31±1	30±2	30±1	30±2
S/R	4.9±0.3	4.7±0.3	5.6±0.3	5.7±0.3	7.5±0.2	7.8±0.2	7.1±0.2	7.9±0.5

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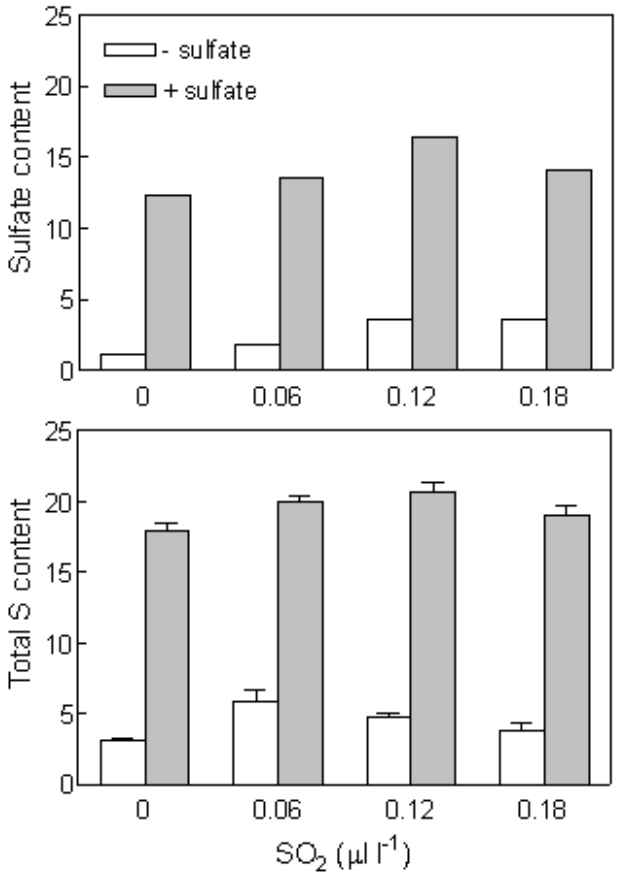

Fig. 1. Impact of sulfate nutrition and SO_2 exposure on total sulfur and sulfate content of shoots. Plants were exposure for five days. Data are expressed in µmol g^{-1} fresh weight and represent the mean of three measurements (± SD).

Chinese cabbage was not very susceptible to SO_2, since growth was not affected upon exposure to 0.06 - 0.18 µl l^{-1} SO_2 for 5 days (Table 1). When Chinese cabbage was grown without sulfate in the nutrient solution, it resulted in a significant reduction in growth, a decrease in shoot/root ratio and in rapid development of sulfur deficiency symptoms. The young developing leaves already started to yellow after two or three days of sulfate deprivation. Sulfate-deprived plants were characterized by a very low content of sulfate and total sulfur in both shoots and roots (Fig. 1). When sulfate-deprived plants were simultaneously exposed to levels of 0.06 - 0.18 µl l^{-1} SO_2, the development of sulfur deficiency symptoms was alleviated, growth was retained but shoot/root ratio remained lower (Table 1). The content of sulfate and total sulfur in plant tissue slightly increased upon exposure to the various levels

of atmospheric SO_2 both in presence and absence of sulfate in the pedosphere (Fig. 1).

The present results demonstrated that in the absence of sulfate in the pedosphere, Chinese cabbage was able to utilize foliarly absorbed atmospheric SO_2 as the sulfur source for growth. The interaction between atmospheric SO_2 deposition and pedospheric sulfur nutrition will further be studied under both laboratory and field conditions at polluted sites in China in the vicinity of Beijing.

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CHARACTERIZATION OF TWO FUNCTIONAL HIGH-AFFINITY SULFATE TRANSPORTERS FOR UPTAKE OF SULFATE IN *ARABIDOPSIS* ROOTS

Naoko Yoshimoto[1,2], Hideki Takahashi[2], Frank W. Smith[3], Tomoyuki Yamaya[2,4] and Kazuki Saito[1,5]

[1]Graduate School of Pharmaceutical Sciences, Chiba University; [2]RIKEN Plant Science Center, Suehiro 1-7-22, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan; [3]CSIRO Plant Industry, Long Pocket Laboratory, 120 Meiers Road, Indooroopilly, Queensland 4068, Australia; [4]Graduate School of Agricultural Sciences, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan; [5]CREST, JST, Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan

Uptake of sulfate from the soil is the initial and indispensable step for plants to acquire inorganic sulfur. When external sulfate is limited, plants increase sulfate uptake capacity in roots (Clarkson *et al.* 1983). Sulfate transporters localizing epidermis, cortex and endodermis of roots are suggested to mediate this step, since the Casparian strips restrict free movement of solute across endodermis. Completion of the genome sequencing indicates that *Arabidopsis* genome has at least 14 genes encoding sulfate transporter proteins (The *Arabidopsis* Genome Initiative 2000, Takahashi *et al.* 2000, Yoshimoto *et al.* 2002). The analysis of transgenic *Arabidopsis* transformed with the fusion gene constructs of their promoter regions and green fluorescent protein suggested that *Sultr1;1* and *Sultr1;2* expressed in the epidermal and cortical cells of roots. Sultr1;1 and Sultr1;2 were able to function as high-affinity sulfate transporters when expressed in yeast. K_m values for sulfate of Sultr1;1 and Sultr1;2 were 3.6 μM and 6.9 μM, respectively, suggesting their capacity to take up sulfate from soil solution with limited amount of sulfate (Table 1).

The major difference between *Sultr1;1* and *Sultr1;2* was the patterns of mRNA accumulation in response to sulfur deficiency. When grown with 50 μM sulfate, *Sultr1;1* mRNA in roots accumulated approximately 10 times higher than that of control plants grown with 1500 μM sulfate. On the other hand, the mRNA level of *Sultr1;2* under 50 μM sulfate was about twice the control condition, suggesting that this isoform is less-responsive to the change of sulfur conditions. Antisense plants of *Sultr1;1* were constructed to determine the *in planta* function of Sultr1;1 in *Arabidopsis*. Antisense plants could not accumulate *Sultr1;1* mRNA even under sulfur-deficient condition. Both the sulfate content and sulfate uptake rate in roots were reduced by antisense suppression of *Sultr1;1* under sulfur-deficient condition. Selenate, a toxic analogue of sulfate, is known to reduce sulfate content in roots and

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increase uptake rates of sulfate even the medium contains sufficient amount of sulfate (Takahashi *et al.* 2000). Twelve-day-old plants were cultivated for 2 days in the presence of 0.1 mM selenate and the mRNA levels of *Sultr1;1* and *Sultr1;2* were monitored. When treated with selenate, the mRNA level of *Sultr1;1* significantly increased synchronizing with the decrease in sulfate contents of roots, whereas, the mRNA level of *Sultr1;2* was not affected.

Table 1. Characteristics of *Sultr1;1* and *Sultr1;2*. Kinetic studies were performed using yeast mutant CP154-7A (*sul1, sul2*) expressing cDNA for *Sultr1;1* and *Sultr1;2*. K_m values were calculated by fitting first-order kinetic equation $Y = V_{max} \times X / (K_m + X)$ to sulfate uptake rates. Cell type-specificities were investigated in transgenic *Arabidopsis* expressing fusion gene constructs of promoter and green fluorescent protein. The effects of sulfur limitation on the mRNA levels were studied in plants grown continuously on media containing 50 μM sulfate. Plants grown with 1500 μM sulfate were used as control.

	K_m (μM)	Cell type-specificity in roots	mRNA expression under sulfur limitation
Sultr1;1	3.6	epidermis, cortex	≤ 10-fold
Sultr1;2	6.9	epidermis, cortex	≤2-fold

In conclusion, two high-affinity sulfate transporters Sultr1;1 and Sultr1;2 that co-localize at epidermis and cortex of roots are suggested to mediate sulfate uptake in *Arabidopsis* roots. According to their patterns of mRNA expression, Sultr1;1 is suggested to facilitate sulfate uptake under sulfur-deficient condition. Sultr1;2 may function for uptake of sulfate under a wide range of sulfur conditions. It is suggested that differential regulation of *Sultr1;1* and *Sultr1;2* expression can maintain sulfur acquisition under both sulfur-replete and deficient conditions.

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INFLUENCE OF SULFUR NUTRITION ON SUGAR BEET RESISTANCE TO APHIDS

Eva Zelená and František Zelený
Research Institute of Crop Production, Drnovská 507, 16106 Praha 6 - Ruzyně, Czech Republic

Nutrition of plants plays an important role in their resistance to pathogens and pests, especially in moderately sensitive species. It acts by effecting changes in growth pattern, in morphology and anatomy and, particularly, in chemical composition of the plants thus altering the quality and quantity of food available to pests (Marschner 1995). It has been well known that improved nitrogen nutrition and namely over-fertilization of plants with nitrogen increase infestation of plants with aphids. Aphids need in their food especially low molecular weight nitrogenous compounds, namely amino acids and amides (Klingauf 1987), the content of that is increased in S-deficient plants as a consequence of a disturbed proteosynthesis. The positive effect of S nutrition on resistance of plants to aphids should therefore manifest itself namely in plants sufficiently supplied with nitrogen. Moreover, S is involved in biosynthesis of phenolics and various plant glucosides that could act adversely on aphids. However, there is still a lack of information on the effects of S nutrition on plant resistance to this pest. During the last decades, sulfur was mainly considered as a pollutant, since there was an abundant supply to plants from industrial emissions. From this point of view also the effect of S on resistance of plants to aphids was studied (Koritsas and Garsed 1985, Kidd 1991).

Our experiments on the effect of S nutrition on infestation of sugar beet (*Beta vulgaris*) with *Aphis fabae* were carried out under constant conditions on soil taken up from sugar beet growing region well supplied with nutrients. Sulfur in the form of sodium sulfate and nitrogen, in the form of ammonium nitrate, were mixed into the soil before sowing at doses 0.1 g kg^{-1} (N, respectively S) and 0.2 g kg^{-1} (2N, respectively 2S) dry soil in different combinations at following variations: C – unfertilized, N, 2N, S, 2S, N+S, N+2S, 2N+S, 2N+2S. Aphids were pre-cultivated on *Vicia faba* plants and transferred on 3-week-old sugar beet plants, 5 adult aphids per plant.

Both nutrients applied separately stimulated plant growth but their effect was highly increased when applied simultaneously (Fig. 1). The effect of fertilization became apparent already on 2-week-old plants. At the beginning of growth, single S had even better effects than nitrogen.

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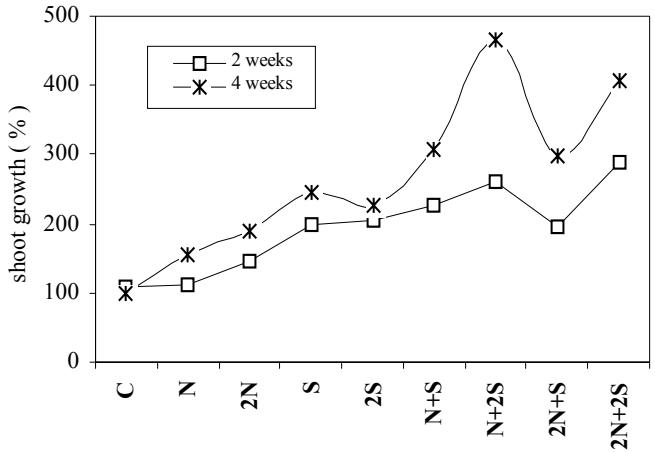

Fig. 1. Effect of S and N fertilization on growth of sugar beet cv. Granada. Fresh matter of shoots from 2 and 4 week-old plants expressed in percentage of the controls.

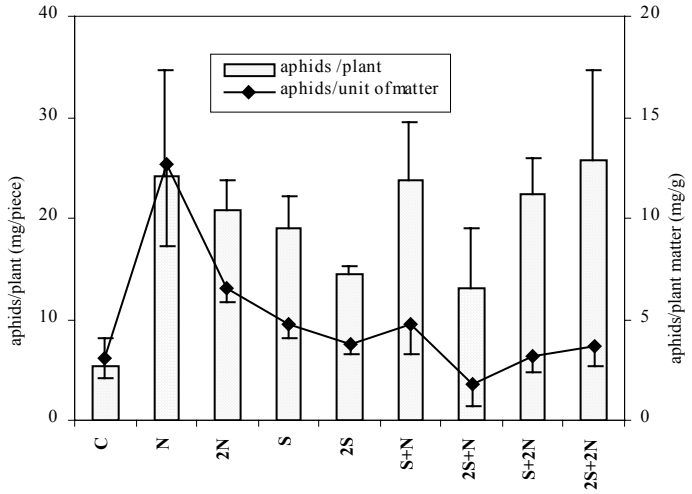

Fig. 2. The effects of S and N fertilization on infestation of sugar beet plants with *Aphis fabae* at 10 days after aphids treatment. The infestation expressed as a mass of aphids (mg) per one plant and per unit of plants matter, average of 4 replications ± SD.

The reproduction of aphids was significantly higher on all fertilized variants (Fig. 2). However, after the conversion of the amount of aphids per unit of produced matter, the infestation on all variants supplied with S significantly decreased. At the variant (N+2S) which was the best for plant growth, it dropped even under the con-

trol level. On contrary to findings published in literature, single N in the lower dose had bigger stimulatory effect on infestation than in higher dose. However, it can be explained just by different situation in S supply to soils in past, much higher than now. Finally such situation was simulated in our experiments in variants (N+2S) and (2N+2S).

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INFLUENCE OF SULFUR ON GROWTH OF RADISH

František Zelený and Eva Zelená
Research Institute of Crop Production, Drnovská 507, 16106 Praha 6 - Ruzyně, Czech Republic

Fertilization of crops with nitrogen, the most common nutrient limiting yields, utilized in high rates to ensure the crop yields, is often uneconomical and can have even the negative impacts on crops, quality of products and the environment. Nutrition of plants with sulfur, deficiency of that in agriculture lands has been increasing worldwide (Scherer 2001; Zelený and Zelená 1997, 2002), because of its important role in N metabolism should significantly increase the efficiency of N fertilizers. We investigated the effects of different N and S fertilizers on growth and the nutrients content in leaves and bulbs of radish, the crop that as a member of *Brassica* family should be especially sensitive to S shortage (Schnug and Haneklaus 1994; McGrath and Zhao 1996).

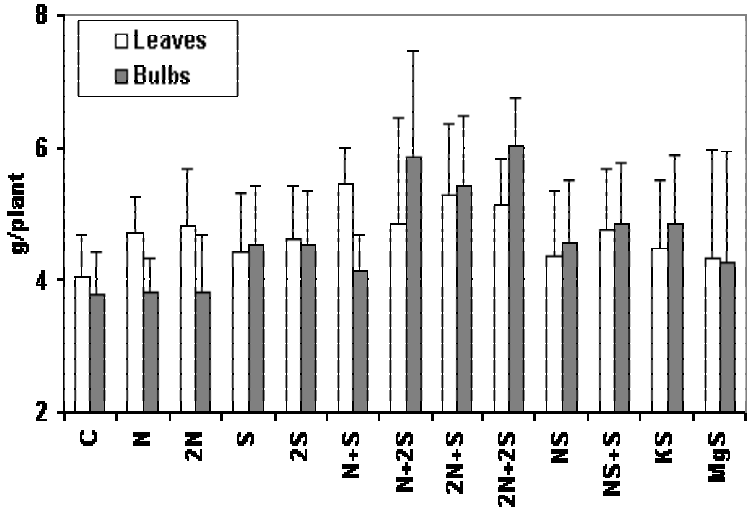

Fig. 1. Effect of nitrogen and sulfur application on the fresh matter of leaves and bulbs of radish. Data are expressed as an average (n = 8) ± SD.

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Radish (*Raphanus sativus* L.*, var. radicula)* cv. Duo was grown in constant conditions of light and temperature in growth chamber on a heavy soil with the total content of S = 98.5 mg kg^{-1}, N = 0.138 % and C = 0.98 %, water-soluble content of S 21.7 mg kg^{-1}, pH ($CaCl_2$) = 7.1, well supplied with macro and micronutrients. Nitrogen and sulfur in doses 0.1 g and 0.2 g kg^{-1} of dry soil were mixed into the soil before sowing in different combinations in the form of ammonium nitrate [N], sodium sulfate [S], ammonium sulfate [NS], potassium sulfate [KS] and magnesium sulfate [MgS]. Total contents of C, N and S in soil and plant material were determined by CNS 2000 analyzer LECO, water soluble S content in soil (extract 1:5) by ICP spectrophotometer TraceScan.

Nitrogen in the form of ammonium nitrate stimulated leaf growth of radish without any effect on fresh weight of bulbs. The higher nitrogen doses [2N] did not further increase the leaf biomass production. Sulfur on the contrary to nitrogen increased namely bulb growth. Simultaneous application of both nutrients had a clear interactive effect on growth intensity of plants, stimulated especially formation and growth of bulbs, producing significant increase in bulb yield, up to 60 %. Shoot/bulb growth ratio fluctuated from about 1.3 in var. 2N to 0.8 in var. N+2S and 2N+2S (Fig. 1).

N and S application increased concentration of the nutrients in tissues. N/S ratio was markedly higher in plants fertilized with nitrogen [N and 2N] but also in that grown in unfertilized control that demonstrates that radish plants were well supplied with nitrates. All S fertilizers significantly increased sulfur concentration in leaves as well in bulbs and sharply decreased N/S ratio in the tissues (Fig. 2).

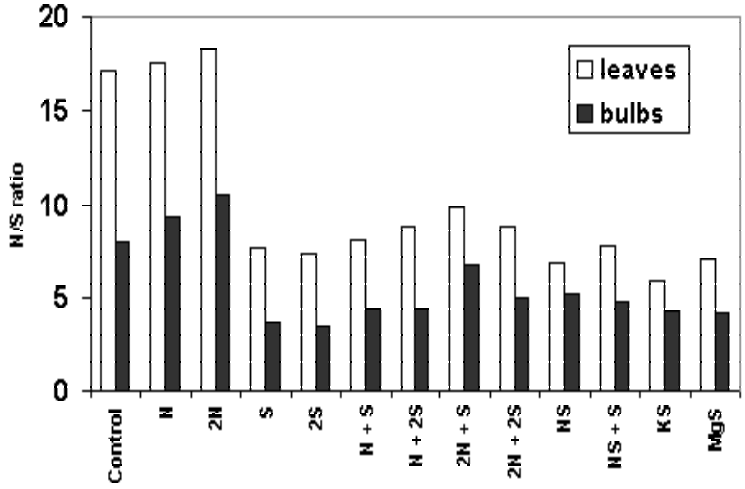

Fig. 2. The influence of N and S fertilization on N/S ratio in leaves and bulbs of radish.

Our results indicate that the level 20 mg water-soluble S kg^{-1} soil is not sufficient for intensive growth of radish bulbs. The demand of the crop for S is increasing with N supply. Higher sulfate supply is necessary to provide mainly the stimulation of bulb growth.

Acknowledgements

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