Proceedings of the International Plant Sulfur Workshop

Luit J. De Kok
Malcolm J. Hawkesford
Heinz Rennenberg
Kazuki Saito
Ewald Schnug *Editors*

Molecular Physiology and Ecophysiology of Sulfur

Springer

Proceedings of the International Plant Sulfur Workshop

More information about this series at http://www.springer.com/series/8885

Luit J. De Kok • Malcolm J. Hawkesford
Heinz Rennenberg • Kazuki Saito
Ewald Schnug
Editors

Molecular Physiology and Ecophysiology of Sulfur

Springer

Editors
Luit J. De Kok
Laboratory of Plant Physiology, Groningen
Institute for Evolutionary Life Sciences
University of Groningen
Groningen, The Netherlands

Heinz Rennenberg
Professur für Baumphysiologie
University of Freiburg
Freiburg, Germany

Ewald Schnug
Institute for Crop and Soil Science
Julius Kühn-Institut (JKI)
Braunschweig, Germany

Malcolm J. Hawkesford
Plant Biology and Crop Science
Rothamsted Research
Harpenden, Hertfordshire, UK

Kazuki Saito
Metabolomics Research Group
RIKEN Center for Sustainable Resource Science
Yokohama, Kanagawa, Japan

ISSN 2451-9073 ISSN 2451-9081 (electronic)
Proceedings of the International Plant Sulfur Workshop
ISBN 978-3-319-20136-8 ISBN 978-3-319-20137-5 (eBook)
DOI 10.1007/978-3-319-20137-5

Library of Congress Control Number: 2015953430

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

In Memory of Our Dear Colleague

Michael T. McManus
(3rd September, 1957–16th July, 2015)
Professor of Plant Physiology
Massey University, Palmerston North, New Zealand

Preface

This proceedings volume contains a selection of invited and contributed papers of the 9th International Workshop on Sulfur Metabolism in Plants, which was hosted by Heinz Rennenberg, Albert-Ludwigs-University Freiburg, and was held at Schloss Reinach, Freiburg-Munzigen, Germany from April 14–17, 2014. The focus of this workshop was on molecular physiology and ecophysiology of sulfur in plants, and the content of this volume presents an overview on the current research developments in this field.

We are delighted to dedicate this volume to Prof. Dr. Sara Amâncio, University of Lisbon, Portugal and Prof. Dr. Jean-Claude Davidian, SupAgro /INRA, Montpellier, France. Both of them have significantly contributed to the understanding of the regulation of uptake and assimilation of sulfur in plants and the success of the Plant Sulfur Workshops over more than two decades.

Groningen, The Netherlands	Luit J. De Kok
Harpenden, Hertfordshire, UK	Malcolm J. Hawkesford
Freiburg, Germany	Heinz Rennenberg
Chiba, Japan	Kazuki Saito
Braunschweig, Germany	Ewald Schnug

Acknowledgement

The editors thank Helen Jenkins for grammatical editing of the chapters.

Contents

Foreword: The Value of Sulfur for Grapevine

Sara Amâncio

Abstract The response to sulfate deficiency (−S) and sulfate resupply (+S) was analyzed in a cell system of *Vitis vinifera* cv. Touriga Nacional by measuring sulfate influx and the expression of sulfate transporter transcripts. After 24 h in −S medium, cells showed a significant increase in sulfate influx rate and the relative expression of sulfate transporters confirmed their strong de-repression in −S conditions. It was verified that in *V. vinifera* cell systems and leaves the sulfur-containing antioxidant metabolite glutathione (GSH), which participates in antioxidant homeostasis, is also a crucial player in the regulation of sulfur metabolism. Antioxidant phenylpropanoid compounds, namely flavonoids and stilbenes, are present in most grapevine tissues, accumulating in response to biotic and abiotic stress. Grapevine plantlets are a suitable model for studying the interplay between the phenylpropanoid pathway and nutrient deficiency. It was verified that *V. vinifera* under sulfur deficiency allocates resources to the phenylpropanoid pathway, probably consecutive to inhibition of protein synthesis, an eventually advantageous strategy to counteract oxidative stress symptoms evoked by −S conditions.

Introduction

Plants are able to reduce sulfate (SO_4^{2-}) to sulfide (S^{2-}), which is incorporated into cysteine; so the greater part of S from SO_4^{2-} absorbed by plants is ultimately used for protein synthesis. Organic sulfur is also found in the form of glutathione (GSH), the thiol-tripeptide that mediates redox reactions by the interchange of dithiol-disulfide.

Traditionally grapevine (*Vitis vinifera* L.) received large S inputs from copper sulfate and S° applied as fungicides. S° is probably the oldest pesticide unexpectedly produced as a component of plant defense system against vascular pathogens (Williams et al. 2002). In fact, sulfur applied to vine leaves and berries significantly

S. Amâncio (✉)
DRAT/LEAF, Instituto Superior de Agronomia, Universidade de Lisboa,
Lisboa 1349-017, Portugal
e-mail: samport@isa.ulisboa.pt

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_1

protected from powdery mildew infection. Treatments against this disease used copper sulfate as Bordeaux mixture or S° (Williams and Cooper 2004). S° was then identified as the only inorganic phytoalexin recorded to date.

Plant defensins are small peptides with a characteristic folding pattern stabilized by eight cysteines (Thomma et al. 2002). Grape genes encoding defensins are differentially expressed among cultivars, suggesting distinct patterns of gene expression between genotypes (Goes da Silva et al. 2005). Despite the advantages of elemental sulfur due to its target activity against powdery mildew, vineyard fungicides were substituted for chemicals without any sulfur. So grapevine sulfate assimilation and sulfur as biotic stress antagonist are important topics and advances on the expression and regulation of *V. vinifera* genes encoding for sulfur transporters and assimilating enzymes were obtained.

Sulfate Uptake and Assimilation

Grapevine Sulfate Transporters

Plants have evolved mechanisms to regulate sulfate uptake in response to sulfur availability. The primary response of numerous plant systems under sulfur depletion is an increase in uptake capacity due to the de-repression of sulfate transporter genes (Takahashi et al. 2011) and a rise in the expression of sulfate transporter proteins (Hawkesford 2000). The regulation by sulfur status at the molecular level is highly coordinated with physiological responses, either at the cellular or at whole plant level (Clarkson et al. 1999).

Sulfate transporter sequences from different plant species are organized into five groups based on the predicted protein sequences (Hawkesford 2003). The grapevine genome release (Jaillon et al. 2007; Velasco et al. 2007) made it possible to identify the protein sequences of 13 sulfate transporters assigned to the five sulfate transporter family groups (Tavares and Amâncio, unpublished results).

Group 1 comprises the genes for high-affinity sulfate transport regulated by external S conditions. In the *V. vinifera* cv. Pinot noir genome, two sequences were assigned to Group 1 and which had been previously amplified from *V. vinifera* cv. Touriga Nacional (Tavares et al. 2008; Amâncio et al. 2009a; Tavares 2009). Sulfate uptake by *V. vinifera* cells was significantly affected after sulfate removal (−S) in a time scale similar to that described for maize cells (Clarkson et al. 1999). The expression of Group 1 transcripts matched the de-repression of sulfate uptake, suggesting a transcriptional regulation of sulfate transport in response to S availability. A strong repression of sulfate influx as well as transcript abundance was observed after sulfate repletion in *V. vinifera* cells (Tavares et al. 2008). Thus, the regulation of *V. vinifera* Group 1 sulfate transporter by S starvation and S resupply occurs at the mRNA expression level and also at de novo protein synthesis, as reported for *Arabidopsis* (Maruyama-Nakashita et al. 2005).

The common characteristic among Group 2 sulfate transporters is a low affinity for sulfate. *V. vinifera* carries two isoforms that fit into this group, and *VvSultr2;1* mRNA depicted a mild up-regulation visible in cells after 7 days in −S conditions (Tavares 2009, Tavares and Amâncio, unpublished results). A large and diverse number of sulfate transporter isoforms have been assigned to Group 3 (Hawkesford and De Kok 2006). In the *V. vinifera* genome seven sequences were assigned to this group and six of them were expressed in cultured cells, in roots and in leaves of grapevine plants. After 4 days in −S conditions, one of *V. vinifera* Group 3 transcripts showed a moderate up-regulation (Tavares 2009). In *Arabidopsis thaliana* sulfate transporters from Group 4 have been associated with sulfate efflux from the vacuole (Kataoka et al. 2004). In contrast to *A. thaliana*, only one sulfate transporter from Group 4 was identified in the *V. vinifera* genome, which under sulfate deficiency conditions, showed a high up-regulation at the transcription level (Tavares and Amâncio, unpublished results). Sulfate transporters from Group 5 were also be identified in *V. vinifera* genome. However, in *A. thaliana*, Group 5 sulfate transporters do not show some of the characteristic sulfate transporter protein domains (Takahashi et al. 2011).

Grapevine Sulfur Assimilation: Genes and Enzymes

There is a high homology of *V. vinifera* ATP sulfurylase1 (VvATPS1) to AtATPS4, AtATPS3 and AtATPS1 and to isoforms from *Brassica spp*, *Camelia sinensis* and *Solanum tuberosum* and of VvATPS2 to *Populus* ATPS and AtATP-S2. The nucleotide sequences of *V. vinifera VvAPTS1* and *VvAPTS2*, reproduce the homology depicted at the amino acid level (Amâncio et al. 2009b). A partial sequence of *V. vinifera* genes encoding for adenosine phosphosulfate reductase (APSR) (EU275236) was cloned and deposited at Gene Bank (Amâncio et al. 2009b), Following grapevine genome sequencing (Jaillon et al. 2007; Velasco et al. 2007), the genes classified as putative isoforms of sulfate assimilation enzymes were confirmed. VvAPSR amino acid sequence confirms the main features of the plant type APSR structure: a C-terminal redox active TRX domain, a GSH-dependent TRX with glutaredoxin function, and an N-terminal reductase domain (Bick and Leustek 1998). The sole isoform of *V. vinifera* sulfite reductase, as in *A. thaliana*, contains two main domains: an iron-sulfur (4Fe-4S) cluster and siroheme domain and the ferredoxin – binding domain (Amâncio et al. 2009b).

Serine acetyltransferases (SERAT) are proteins containing hexapeptide repeats characteristic of transferase enzymes whose secondary structure formed by these repeats is involved in the interaction of SERAT with *O*-acetylserine (thiol)lyase (*O*ASTL) (Takahashi et al. 2011). Four *V. vinifera* SERAT sequences were identified in the grapevine genome (Tavares et al. 2015), and eight isoforms of *V. vinifera* *O*ASTL with the highly conserved pyridoxal-phosphate cofactor domain were obtained by homology analysis (Amâncio et al. 2009b). This compares to similar numbers in the *A. thaliana* genome: SERAT (5) (Kawashima et al. 2005) and *O*ASTL (9) (Watanabe et al. 2008). VvSERAT2;1 localized to the chloroplast of

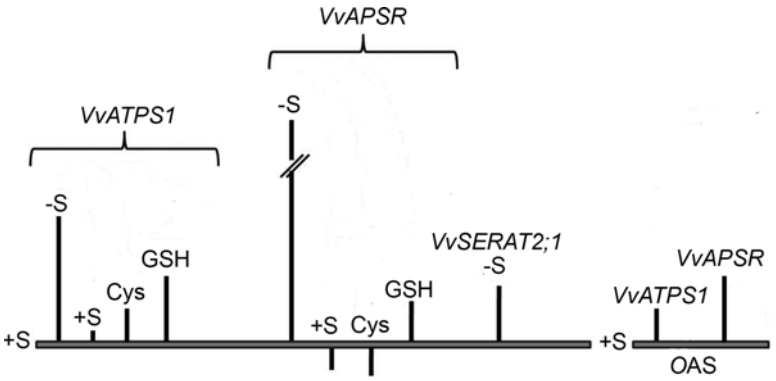


Fig. 1 Relative expression analyzed by RT- qPCR of *VvATPS1*, *VvAPSR* in *V. vinifera* S-depleted cells transferred to +S (1.5 mM) or receiving 1 mM GSH or 1 mM cysteine for 24 h; and in +S cells supplemented with 0.5 mM OAS. The relative expression of *VvSERAT2;1* in S-depleted cells is also shown (Tavares et al. unpublished results)

V. vinifera cells is the first plant SERAT identified so far that depicts a full serine acetyltransferase activity and does not interact with OASTL (Tavares et al. 2015).

A reverse correlation between sulfate availability and the expression of genes coding for sulfate assimilation enzymes is well documented for model plants and some other species. In grapevine cells under sulfate depletion, the expression of genes for sulfate metabolism enzymes showed that the relative abundance of *VvATPS1* and particularly *VvAPSR*, is up-regulated (Amâncio et al. 2009b), confirming the crucial role of APSR in sensing sulfur conditions and regulating the sulfate metabolism pathway. As in other species, the up-regulation of the above transcripts is significantly amplified in cell systems when compared with whole plant analysis (Amâncio et al. 2009a). Conversely to Arabidopsis, the *VvSERAT*2;1 transcript level was significantly de-repressed in cells after 5 days under sulfate deficiency conditions (Fig. 1). This late up-regulation seems more related to a long-term S-deficiency response (Tavares et al. 2015).

Regulation of Sulfate Assimilation

In many species sulfur uptake and assimilation are highly regulated processes. Control of gene expression limits excess uptake and activity of the assimilatory pathway. Reduced S-compounds, namely GSH, exert a negative regulatory effect while *O*-acetylserine (*O*AS), the carbon/nitrogen skeleton for cysteine, exerts a positive effect. Analysis of the transcript expression of *VvATPS1* and *VvAPSR* in *V. vinifera* cells as a response to sulfate deficiency, sulfate re-supply, GSH, cysteine or OAS addition (Fig. 1) confirmed that SO_4^{2-}, cysteine and GSH are strong negative regulators of *APSR*. In cells growing in +S medium the effect of *O*AS was responsible for four and threefold de-repression, respectively, of *VvAPSR* and *VvATPS* expression. Interestingly, the up-regulation of *VvATPS* is of the same order of magnitude as that of ATPS activity in maize cells treated with *O*AS (Clarkson et al. 1999).

Crosstalk Between Sulfur and the Antioxidant System in Grapevine

GSH as the major non-protein reduced sulfur compound plays important roles, from ROS processing to hampering protein denaturation, by assuring the reduction of Cys-thiol groups. In grapevine, the changes in GSH content upon abiotic stress are genotype-dependent. In fact, in cv. Touriga Nacional under environmental conditions that evoke oxidative stress, the GSH pool is sufficient to maintain the cell redox state, while in cv. Trincadeira the GSH pool is replenished de novo in a slower and eventually less efficient response (Carvalho et al 2015a, b).

Phenylpropanoids are phytocompounds not essential for plant survival, thus classified as plant secondary compounds. Grapevine bears a large variety of phenylpropanoid compounds, namely resveratrol (a stilbene) and anthocyanins (a flavonoid), which derive from two branches of the phenylpropanoid pathway. The synthesis of chalcone, the precursor of flavonoid compounds, depends on chalcone synthase (CHS), while stilbenes, such as resveratrol are produced by stilbene synthase (StSy), enzymes that define the first branching point of the phenypropanoid pathway. Besides the nutriceutical activity of anthocyanin and resveratrol, anthocyanins, present in all *V. vinifera* tissues, behave as powerful antioxidants while resveratrol acts as an antioxidant as well as phytoalexin. The anthocyanin pool increases upon abiotic and biotic stress (Winkel-Shirley 2002). Sulfur deficiency can bring about the accumulation of anthocyanins (Nikiforova et al. 2003). As reported in Tavares et al. (2013), *V. vinifera* cv. Touriga Nacional plantlets grown in −S conditions for 4 weeks significantly accumulated anthocyanins when compared to +S plantlets. In the same experimental system, the *trans*-resveratrol stilbene levels were raised by 1.5 and 2.5-fold in −S conditions after 2 and 4 weeks, respectively. In −S *V. vinifera* cv. Touriga Nacional cell cultures the *trans*-resveratrol glucoside increased by sevenfold as compared to +S cells after 4 days, a value that was maintained until the seventh day in −S cells. *CHS* and *StSy* transcription levels in −S plantlets increased 8.0 and 6.1 times, respectively, after 2 weeks, matching the increase in anthocyanins and stilbenes measured in equivalent plantlets. These results could be explained by a metabolic detour to secondary metabolism, namely to the phenylpropanoid pathway, as the outcome of an impairment in protein synthesis and the competition for phenylalanine.

Concluding Remarks

Very little research on sulfur uptake and assimilation has been reported for grapevine. The collaboration with Ineke Stulen and David Clarkson allowed me to approach sulfur uptake and assimilation and the interaction with nitrogen metabolism in maize leaves, roots, *callus* and cell suspensions. Encouraged by the results and by the successful collaborations with my European partners, together with the high socio-economic and cultural value of grapevine, it became a priority to open a line of research on sulfur in *V. vinifera* metabolism, the coordination between sulfur

primary metabolism and secondary metabolic pathways and the fine tuning of genomic regulation of the sulfur metabolic pathway. Successful collaborations with Jean-Claude Davidian on grapevine sulfate transporters, with Rüediger Hell and Markus Wirtz on the characterization of the SERAT gene family, have extended our perception of sulfur in grapevine.

References

Amâncio S, Tavares S, Fernandes JC (2009a) The up-regulation of *Vitis vinifera* sulfur assimilation by sulfate depletion decreases from cells to roots and to leaves. In: Sirko A, De Kok LJ, Haneklaus S, Hawkesford MJ, Rennenberg H, Saito K, Schnug E, Stulen I (eds) Sulfur metabolism in plants. Backhuys Publishers, Leiden, pp 49–53

Amâncio S, Tavares S, Fernandes JC, Sousa C (2009b) Grapevine and sulfur: old partners, new achievenments. In: Roubelakis-Angelakis KA (ed) Grapevine molecular physiology and biotechnology, 2nd edn. Springer Science+Business Media BV, The Netherlands, pp 31–52

Bick JA, Leustek T (1998) Plant sulfur metabolism – the reduction of sulfate to sulfite. Curr Opin Plant Biol 1:240–244

Carvalho C, Coito JL, Colaço S, Sangiogo M, Amâncio S (2015a) Heat stress in grapevine: the pros and cons of acclimation. Plant Cell Environ 38:777–789

Carvalho LC, Vidigal P, Amâncio S (2015b) Oxidative stress homeostasis in grapevine (*Vitis vinifera* L.). Front Environ Sci 3:20

Clarkson DT, Diogo E, Amâncio S (1999) Uptake and assimilation of sulfate by sulfur deficient *Zea mays* cells: the role of *O*-acetyl-L-serine in the interaction between nitrogen and sulfur assimilatory pathways. Plant Physiol Biochem 37:283–290

Goes da Silva F, Iandolino A, Al-Kayal F, Bohlmann MC, Cushman MA, Lim H, Ergul A, Figueroa R, Kabuloglu EK, Osborne C, Rowe J, Tattersall E, Leslie A, Xu J, Baek JM, Cramer GR, Cushman JC, Cook DR (2005) Characterizing the grape transcriptome. Analysis of expressed sequence tags from multiple *Vitis* species and development of a compendium of gene expression during berry development. Plant Physiol 139:574–597

Hawkesford MJ (2000) Plant responses to sulfur deficiency and the genetic manipulation of sulfate transporters to improve S-utilization efficiency. J Exp Bot 51:131–138

Hawkesford MJ (2003) Transporter gene families in plants: the sulfate transporter gene family: redundancy or specialization? Physiol Plant 117:155–163

Hawkesford MJ, De Kok LJ (2006) Managing sulfur metabolism in plants. Plant Cell Environ 29:382–395

Jaillon O, Aury J-M, Noel B, Policriti A, Clepet C et al (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449:463–467

Kataoka T, Watanabe-Takahashi A, Hayashi N, Ohnishi M, Mimura T, Buchner P, Hawkesford MJ, Tomoyuki Y, Takahashia H (2004) Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in Arabidopsis. Plant Cell 16:2693–2704

Kawashima CG, Berkowitz O, Hell R, Noji M, Saito K (2005) Characterization and expression analysis of a serine acetyltransferase gene family involved in a key step of the sulfur assimilation pathway in *Arabidopsis*. Plant Physiol 137:220–230

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel *cis*-acting element conferring sulfur deficiency response in Arabidopsis roots. Plant J 42:305–314

Nikiforova V, Freitag J, Kempa S, Adamik M, Hesse H, Hoefgen R (2003) Transcriptome analysis of sulfur depletion in Arabidopsis thaliana: interlacing of biosynthetic pathways provides response specificity. Plant J 33:633–650

Takahashi H, Kopriva S, Giordano M, Saito K, Rudiger H (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu Rev Plant Biol 62:157–184

Tavares S (2009) Uptake and assimilation of sulfate in *Vitis vinifera*: a molecular and physiological approach. PhD Thesis, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Lisboa, 172 pp

Tavares S, Sousa C, Carvalho LC, Amâncio S (2008) De-repressed transporters are strongly repressed after sulfate addition to sulfur depleted *Vitis* cells. Int J Plant Sci 169:987–997

Tavares S, Vesentini D, Fernandes JC, Ferreira RB, Laureano O, Ricardo da Silva JM, Amâncio S (2013) *Vitis vinifera* secondary metabolism as affected by sulfate depletion: diagnosis through phenylpropanoid pathway genes and metabolites. Plant Physiol Biochem 66:118–126

Tavares S, Wirtz M, Beier MP, Bogs J, Hell R, Amâncio S (2015) Characterization of the serine acetyltransferase gene family of *Vitis vinifera* uncovers differences in regulation of OAS synthesis in woody plants. Front Plant Sci 6:74

Thomma BPHJ, Cammue BPA, Thevissen K (2002) Plant defensins. Planta 216:193–202

Velasco R, Zharkikh A, Troggio M, Cartwright DA, Cestaro A et al (2007) A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS One 2(12):e1326

Watanabe M, Kusano M, Oikawa A, Fukushima A, Noji M, Saito K (2008) Physiological roles of β-substituted alanine synthase gene (Bsas) family in *Arabidopsis thaliana*. Plant Physiol 146:310–320

Williams JS, Hall AS, Hawkesford MJ, Beale MH, Cooper RM (2002) Elemental sulfur and thiol accumulation in tomato and defense against a fungal vascular pathogen. Plant Physiol 128:150–159

Williams JS, Cooper RM (2004) The oldest fungicide and newest phytoalexin – a re-appraisal of the fungitoxicity of elemental sulfur. Plant Pathol 53:263–279

Winkel-Shirley B (2002) Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5:218–223

Part I
Biochemistry and Physiology

Partitioning of Sulfur Between Primary and Secondary Metabolism

Stanislav Kopriva

Abstract Sulfur is an essential nutrient for all organisms. Plants are able to take up inorganic sulfate and assimilate it into a range of bioorganic molecules, either after reduction to sulfide, or activation to 3′-phosphoadenosine 5′-phosphosulfate. While the regulation of the reductive part of sulfate assimilation and the synthesis of cysteine has been studied extensively in the past three decades, much less attention has been paid to the control of synthesis of sulfated compounds. Only recently have the genes and enzymes activating sulfate and transferring it onto suitable acceptors been investigated in detail with the emphasis on understanding the control of partitioning of sulfur between the two branches of sulfate assimilation. These investigations brought a range of interesting new findings, such as a common regulatory network of sulfate assimilation and glucosinolate synthesis, and identified new components of the pathway, e.g. PAPS transporter or the 2′(3′),5′-diphosphoadenosine phosphatase. Here the new findings are reviewed and put into context of primary and secondary sulfur metabolism.

Introduction

Sulfur is an essential nutrient for all living organisms, but only plants, algae, fungi and some bacteria can use the major source of inorganic sulfur, sulfate, to meet their demands (Kopriva 2006; Takahashi et al. 2011). Except a few minor variations, the pathway of sulfate assimilation is conserved in all these organisms (Fig. 1; Patron et al. 2008). Sulfate is taken up into the cells by sulfate transporters and activated by adenylation catalyzed by ATP sulfurylase to adenosine 5′-phosphosulfate (APS). In plants, algae, and most bacteria APS is reduced to sulfite by APS reductase, whereas in fungi, cyanobacteria, and some proteobacteria a second

S. Kopriva (✉)
Botanical Institute, Cluster of Excellence on Plant Sciences (CEPLAS),
University of Cologne, Zülpicher Str. 47b, 50674 Cologne, Germany
e-mail: skopriva@uni-koeln.de

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_2

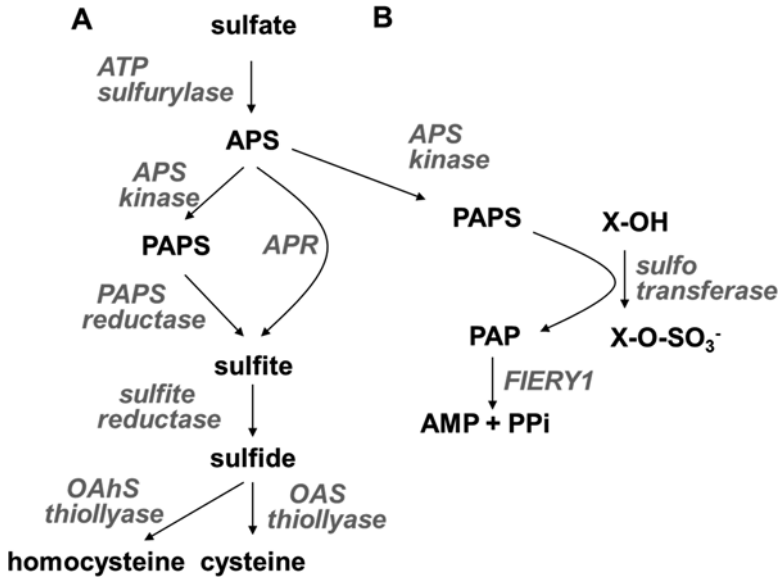


Fig. 1 Scheme of primary (**A**) and secondary (**B**) sulfate metabolism

activation step, phosphorylation of APS to 3′-phosphoadenosine 5′-phosphosulfate (PAPS), is necessary before the reduction to sulfite by PAPS reductase (Kopriva and Koprivova 2004). Sulfite is then reduced to sulfide by sulfite reductase and sulfide is incorporated into amino acid precursors to form cysteine, or homocysteine in the yeast and fungi (Takahashi et al. 2011). However, not all sulfur-containing metabolites are dependent on reduced sulfur, synthesis of a number of organic sulfo-compounds requires PAPS as a donor of activated sulfate or even partially reduced sulfur donors, such as sulfite for the synthesis of sulfolipids (Sanda et al. 2001). Since the majority of compounds containing sulfo- group are secondary metabolites, the reductive assimilation is often referred to as primary assimilation, whereas the branch leading to synthesis of sulfated products is called secondary assimilation (Kopriva et al. 2012). The two pathways of sulfate assimilation in plants are resolved and the genes have been identified in many species (Kopriva et al. 2009; Hell et al. 2002; Leustek et al. 2000; Ravilious and Jez 2012b; Takahashi et al. 2011). Sulfate assimilation is highly regulated both by sulfur demand and availability, probably because of the high reactivity and toxicity of the pathway intermediates. The focus of plant sulfur research has long been on the primary pathway and primarily the key enzyme, APS reductase, and secondary assimilation has been investigated in detail only relatively recently (Aubry et al. 2014; Kopriva et al. 2012; Mugford et al. 2009; Takahashi et al. 2011). Here, the efforts to characterize plant secondary sulfate assimilation and the regulation of sulfur partitioning between the primary and secondary pathways will be summarized.

APS Kinase, a Forgotten Enzyme

APS kinase is an essential enzyme for sulfate reduction in yeast and the PAPS reducing bacteria but in plants, which reduce APS directly, this enzyme is part of the secondary pathway. In plants, therefore, its significance was not considered as highly important, as the best known metabolites requiring sulfation, glucosinolates, are secondary products limited to *Brassicaceae* and not essential for plant survival (Halkier and Gershenzon 2006). This is in contrast with humans and animals, where defects in the production of PAPS result in serious developmental alterations and death (Dejima et al. 2006; Kurima et al. 1998). APS kinase has been identified in plants and shown to be well conserved in sequence to the proteins from other organisms (Jain and Leustek 1994; Lee and Leustek 1998; Mugford et al. 2009; Ravilious et al. 2012; Patron et al. 2008). Two isoforms have been initially described in Arabidopsis, but the genome sequence revealed that APS kinase is encoded by a small gene family of four members in this species (Jain and Leustek 1994; Lee and Leustek 1998; Mugford et al. 2009). Three of these isoforms contain a transit peptide, and one, APK3, appears to be cytosolic as the encoded protein is very similar in size to the bacterial enzymes. Indeed, this localization has been confirmed using GFP fusions, showing APK1, APK2, and APK4 were localized to the plastids, whereas APK3 is indeed cytosolic (Mugford et al. 2009). All four isoforms have been expressed in *E. coli* and the recombinant proteins shown to possess APS kinase activity. Only minor differences between the kinetic parameters of the individual isoforms have been observed, as well as in the transcript accumulation patterns, pointing to a possible functional redundancy (Mugford et al. 2009). However, at the transcript level *APK1* and *APK2* seem to be more highly expressed than *APK3* and *APK4*.

To dissect the biological function of the individual APS kinase isoforms, T-DNA lines disrupting the corresponding genes have been analyzed. Not surprisingly, the single mutants lacking one isoform did not show any phenotypes, at least at standard conditions (Mugford et al. 2009). Therefore, the mutants were crossed to obtain multiple knock-outs. Among the six possible combinations of double mutants, one pair, disrupted in *APK1* and *APK2*, showed a clear semi-dwarf phenotype (Mugford et al. 2009). To check whether the morphological phenotype is accompanied by disturbance in the secondary sulfur metabolites, glucosinolates as the best known sulfated metabolites, were analyzed and showed a remarkable reduction of around 85 % in the content of all individual glucosinolates. Accumulation of other sulfated metabolites, sulfo-jasmonic acid and phytosulfokines, was also reduced in the *apk1 apk2* mutants (Mugford et al. 2009). These experiments showed the importance of the donor of active sulfate for plant performance and so further crossing was carried out to test whether APS kinase is an essential enzyme in Arabidopsis. This question has already been answered at the stage of triple mutants (Mugford et al. 2010), because one combination, *apk1 apk3 apk4*, could not been obtained. This shows clearly that APS kinase is essential for Arabidopsis viability,

although the exact nature of the essential metabolites is not known. It cannot be the glucosinolates, as mutants in transcription factors controlling the pathway of glucosinolate synthesis, devoid of the metabolites, are viable (Frerigmann and Gigolashvili 2014; Sonderby et al. 2007). Phytosulfokines are also an unlikely candidate as the mutant in tyrosyl protein sulfotransferase, a single copy gene catalyzing the sulfation of phytosulfokines and other small peptides, is also viable (Komori et al. 2009). Recent analysis of the sulfur metabolome revealed a large number of unknown compounds containing sulfur (Glaser et al. 2014), among which the essential metabolite might be found in future. The analysis of the triple mutants revealed that loss of APK3 or APK4 in the *apk1 apk2* background further strengthens the dwarf phenotype. On the other hand, mutants with APK1 as the sole isoform of APS kinase are not distinguishable from WT plants showing that this is the major APS kinase isoform in Arabidopsis (Mugford et al. 2010). The structure of APK1 has been determined and the reaction mechanism, including the sequence of substrate binding (first ATP and second APS) has been solved (Ravilious and Jez 2012a; Ravilious et al. 2012). The analysis of APK1 structure revealed a novel redox regulation of plant APS kinase, in which the enzyme is activated in reduced environment (Ravilious et al. 2012).

Regulation of Primary and Secondary Sulfur Metabolism

As already mentioned, the reduced availability of PAPS in *apk1 apk2* mutants and two of the triple mutants resulted in a strong decrease in the sulfated secondary compounds, glucosinolates. Glucosinolates are a group of compounds important for plant pathogen defense but are also responsible for smell and taste of crucifers and with health protecting properties (Halkier and Gershenzon 2006; Sonderby et al. 2010). They are derived from the amino acids methionine and tryptophan or phenylalanine in a complex pathway, with sulfation of desulfo-precursors by sulfotransferase being the last step (Underhill et al. 1973). These precursors, which are almost undetectable in wild type plants, accumulate to very high levels in the *apk1 apk2* plants. This accumulation is several times higher than would account for unused substrates of the sulfotransferase (Mugford et al. 2009), pointing to an active process through increased synthesis rate. Indeed, the transcripts of genes involved in glucosinolate synthesis are coordinately induced in *apk1 apk2* plants (Mugford et al. 2009). This is true not only for the metabolic genes but also for genes encoding six MYB transcription factors controlling glucosinolate synthesis, *MYB28*, *MYB29*, and *MYB76* regulating the methionine-derived aliphatic glucosinolates and *MYB51*, *MYB34*, and *MYB122* of the indolic glucosinolate network (Gigolashvili et al. 2007, 2008; Sonderby et al. 2007). Given the importance of PAPS for glucosinolate synthesis this up-regulation posed the question of whether the genes involved in PAPS synthesis are also part of the regulatory network of these MYB factors. Indeed, transactivation assays, in which the individual MYB factors were co-expressed with constructs containing ß-glucurodinase under the control of the investigated

promoters, showed that *APK1*, *APK2*, and to some extent also *APK3*, are under the control of the MYB factors as well as *ATPS1* and *ATPS3* isoforms of ATP sulfurylase (Yatusevich et al. 2010). Interestingly, the genes for APS reductase and sulfite reductase of primary assimilation are also under the control of the MYB factors. The results of transactivation assays were corroborated by results of expression analysis of plants over-expressing the MYB factors. Thus, both primary and secondary sulfate assimilation is part of the same regulatory network controlled by the six "glucosinolate" MYB factors (Yatusevich et al. 2010). While the increased expression of MYB factors induced transcript levels for sulfate assimilation genes, it was not affected in mutants of the MYBs, showing that they do not contribute much to the constitutive regulation of sulfur metabolism.

The genes of primary and secondary sulfur metabolism, however, share other mechanisms of regulation. Both groups respond to sulfate deficiency where primary assimilation is up-regulated and the glucosinolate biosynthesis genes down-regulated (Hirai et al. 2005). For most of the genes in both pathways this regulation is controlled by *SULFATE LIMITATION1* (*SLIM1*) (Maruyama-Nakashita et al. 2006). When Arabidopsis plants are adapted to darkness for 36 h, sulfate assimilation and glutathione synthesis are very significantly reduced, but are rapidly induced by light (Kopriva et al. 1999). It has recently be shown that, for many genes, the early response to light is controlled by *LONG HYPOCOTYL5* (*HY5*) transcription factor (Huseby et al. 2013; Lee et al. 2011). Interestingly, primary and secondary sulfate assimilation and glucosinolate synthesis are preferentially expressed in a coordinated manner in bundle sheath cells of Arabidopsis (Aubry et al. 2014). Thus the pathways have to be precisely controlled to ascertain that sulfur is partitioned to the right metabolites according to the immediate demand. The redox regulation of APS kinase (Ravilious et al. 2012), which is complementary to regulation of APS reductase (Bick et al. 2001), might represent such a mechanism.

Partitioning of Sulfur

In the *apk1 apk2* plants the synthesis of PAPS was reduced, therefore we hypothesized that the flux through the primary assimilation might be increased. Indeed, the *apk1 apk2* plants accumulated several-fold higher glutathione than wild type controls (Mugford et al. 2009). The flux through primary assimilation has been higher, probably through up-regulation of APS reductase (Mugford et al. 2009, 2011). On the other hand, over-expression of APS kinase in plastids or cytosol did not affect glucosinolate synthesis, but surprisingly, slightly increased the flux through primary assimilation. APS kinase is thus the next enzyme regulating flux through primary sulfate assimilation after APR2, ATPS1 and sulfite reductase (Koprivova et al. 2013; Vauclare et al. 2002; Khan et al. 2010). APS reductase, particularly the APR2 isoform, was traditionally considered key for the control of the flux, based on several levels of evidence: a control flux analysis, a QTL analysis of sulfate content, and an analysis of natural variation in foliar sulfur (Loudet et al. 2007; Vauclare et al.

2002). Indeed, disruption of APR2 reduces the flux through the pathway, while over-expression of APS reductase increases the flux (Mugford et al. 2011; Tsakraklides et al. 2002). High levels of APS reductase, however, lead to accumulation of partially reduced sulfur compounds and toxicity (Martin et al. 2005; Tsakraklides et al. 2002). It is thus clear that the interplay between APS reductase and APS kinase controls the partitioning of sulfur between primary and secondary sulfur assimilation but other enzymes also participate at the fine tuning of the regulation, particularly of the primary pathway.

New Players in the Secondary Sulfur Metabolism

Recently, several new components of the secondary sulfur assimilation have been identified and/or linked to the network (Gigolashvili et al. 2012; Lee et al. 2012). The viability of *apk1 apk2 apk4* mutants, which possess only the cytosolic enzyme, and several mutants lacking *apk3* and so synthesizing PAPS only in the plastids is a clear evidence for an efficient exchange of PAPS between cytosol and plastids (Kopriva et al. 2012; Mugford et al. 2009, 2010). To identify the transporter, two strategies were employed, both utilizing the strong coregulation of the genes of the glucosinolate synthesis network. The microarray data of *apk1 apk2* plants were interrogated for up-regulated transporter genes and simultaneously, promoters of transporters of sugar nucleotide and mitochondrial carrier families that are co-regulated with glucosinolate synthesis genes were tested for activation by the MYB factors regulating glucosinolate synthesis. Both analyses resulted in identification of the same gene, previously described as encoding an ADP/ATP carrier in thylakoid membranes (Gigolashvili et al. 2012). The transporter has clearly a dual function, as besides ATP and ADP it exchanges PAPS with phosphoadenosine phosphate (PAP) in vitro. Loss-of-function mutants of the PAPS transporter (PAPST1) show a phenotype similar to *apk1 apk2* plants, exhibiting semi-dwarfism, lower glucosinolate content and accumulation of desulfo-precursors, all clearly indicative of the lower availability of PAPS in the cytosol. However, as the phenotypic alterations in *papst1* mutants are weaker than those of *apk1 apk2 apk4*, which has only the cytosolic APS kinase isoform, some transport from the plastids has to occur and a second transporter has been postulated (Gigolashvili et al. 2012) although the second plastidic PAPS transporter and a transporter responsible for delivery of PAPS to Golgi apparatus to support protein sulfation still await discovery.

The second new network component is linked to PAP. PAP is a co-product of sulfation and is transported back to the plastids to be dephosphorylated to AMP. The gene encoding the enzyme responsible for this reaction has been recovered in many different genetic screens, because the accumulation of PAP, or a second possible substrate inositol bisphosphate, is connected with multiple morphological phenotypes (Wilson et al. 2009; Gy et al. 2007; Kim and von Arnim 2009; Xiong et al. 2001; Robles et al. 2010). An additional phenotype connecting the SAL1/FRY1 to sulfur assimilation is a decrease in glucosinolates and accumulation of desulfo-glucosinolates (Lee et al.

2012). This phenotype can be caused either by blockage of PAPS export from the plastids due to accumulation of PAP, or a direct inhibition of the sulfotransferases involved in glucosinolate synthesis. This consequence of SAL1 loss is not surprising; the mutant has, however, further phenotypes connected with sulfur metabolism and homeostasis. Expression analysis of *sal1/fry1* mutants revealed that a set of genes including those of sulfur metabolism is regulated in the same way as during sulfate deficiency (Lee et al. 2012). However, this misregulation is not caused by alteration in signaling. Instead, the mutants possess significantly lower levels of sulfate and consequently the total sulfur content is lower (Lee et al. 2012). The mechanisms of the alteration of sulfur homeostasis are, however, completely unknown and may be a consequence of various other phenotypes of *fry1*, altered venation pattern or jasmonate synthesis (Rodriguez et al. 2010; Robles et al. 2010).

Conclusions and Outlook

Whereas traditionally primary sulfate assimilation was the center of attention in plant sulfur research, the last years have brought many new insights in the secondary pathway and its connection to metabolism of glucosinolates. APS kinase has been recognized as an essential enzyme and as an important player in regulation of sulfur metabolism. Through the investigations of secondary sulfur metabolism new transcription factors regulating primary assimilation have been discovered. PAPS transporter has been identified and another transporter postulated, increasing the knowledge of metabolite exchange between compartments.

These and other findings have, however, opened up a number of intriguing questions. What are the essential sulfated metabolites that make the APS kinase an essential enzyme? Is there a link between PAPS production and the number of phenotypes caused by disruption of SAL1/FRY1? This is particularly interesting given the possible function of PAP as a retrograde signal in drought stress signaling (Estavillo et al. 2011). What are the roles of cytosolic PAPS synthesis and generally the intracellular localization of sulfur metabolism? How important is the redox regulation of APS kinase for controlling the flux through sulfur metabolism? These questions are not trivial or easily answerable due to the complex nature of the pathways. However, many genetic tools are available and protein structures of many of the pathway components have been resolved so that the next few years should bring new breakthroughs in understanding the molecular nature of the control of sulfur partitioning between primary and secondary sulfate assimilation.

References

Aubry S, Smith-Unna RD, Boursnell CM, Kopriva S, Hibberd JM (2014) Transcript residency on ribosomes reveals a key role for the *Arabidopsis thaliana* bundle sheath in sulfur and glucosinolate metabolism. Plant J 78:659–674

Bick JA, Setterdahl AT, Knaff DB, Chen Y, Pitcher LH, Zilinskas BA, Leustek T (2001) Regulation of the plant-type 5′-adenylyl sulfate reductase by oxidative stress. Biochemistry 40:9040–9048

Dejima K, Seko A, Yamashita K, Gengyo-Ando K, Mitani S, Izumikawa T, Kitagawa H, Sugahara K, Mizuguchi S, Nomura K (2006) Essential roles of 3′-phosphoadenosine 5′-phosphosulfate synthase in embryonic and larval development of the nematode *Caenorhabditis elegans*. J Biol Chem 281:11431–11440

Estavillo GM, Crisp PA, Pornsiriwong W, Wirtz M, Collinge D, Carrie C, Giraud E, Whelan J, David P, Javot H, Brearley C, Hell R, Marin E, Pogson BJ (2011) Evidence for a SAL1-PAP chloroplast retrograde pathway that functions in drought and high light signaling in Arabidopsis. Plant Cell 23:3992–4012

Frerigmann H, Gigolashvili T (2014) MYB34, MYB51, and MYB122 distinctly regulate indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. Mol Plant 7:814–828

Gigolashvili T, Berger B, Mock HP, Muller C, Weisshaar B, Flugge UI (2007) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. Plant J 50:886–901

Gigolashvili T, Engqvist M, Yatusevich R, Muller C, Flugge UI (2008) HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana*. New Phytol 177:627–642

Gigolashvili T, Geier M, Ashykhmina N, Frerigmann H, Wulfert S, Krueger S, Mugford SG, Kopriva S, Haferkamp I, Flugge UI (2012) The Arabidopsis thylakoid ADP/ATP carrier TAAC has an additional role in supplying plastidic phosphoadenosine 5′-phosphosulfate to the cytosol. Plant Cell 24:4187–4204

Glaser K, Kanawati B, Kubo T, Schmitt-Kopplin P, Grill E (2014) Exploring the Arabidopsis sulfur metabolome. Plant J 77:31–45

Gy I, Gasciolli V, Lauressergues D, Morel JB, Gombert J, Proux F, Proux C, Vaucheret H, Mallory AC (2007) Arabidopsis FIERY1, XRN2, and XRN3 are endogenous RNA silencing suppressors. Plant Cell 19:3451–3461

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. Annu Rev Plant Biol 57:303–333

Hell R, Jost R, Berkowitz O, Wirtz M (2002) Molecular and biochemical analysis of the enzymes of cysteine biosynthesis in the plant *Arabidopsis thaliana*. Amino Acids 22:245–257

Hirai MY, Klein M, Fujikawa Y, Yano M, Goodenowe DB, Yamazaki Y, Kanaya S, Nakamura Y, Kitayama M, Suzuki H, Sakurai N, Shibata D, Tokuhisa J, Reichelt M, Gershenzon J, Papenbrock J, Saito K (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in Arabidopsis by integration of metabolomics and transcriptomics. J Biol Chem 280:25590–25595

Huseby S, Koprivova A, Lee BR, Saha S, Mithen R, Wold AB, Bengtsson GB, Kopriva S (2013) Diurnal and light regulation of sulphur assimilation and glucosinolate biosynthesis in Arabidopsis. J Exp Bot 64:1039–1048

Jain A, Leustek T (1994) A cDNA clone for 5′-adenylylphosphosulfate kinase from *Arabidopsis thaliana*. Plant Physiol 105:771–772

Khan MS, Haas FH, Samami AA, Gholami AM, Bauer A, Fellenberg K, Reichelt M, Hansch R, Mendel RR, Meyer AJ, Wirtz M, Hell R (2010) Sulfite reductase defines a newly discovered bottleneck for assimilatory sulfate reduction and is essential for growth and development in *Arabidopsis thaliana*. Plant Cell 22:1216–1231

Kim BH, von Arnim AG (2009) FIERY1 regulates light-mediated repression of cell elongation and flowering time via its 3′(2′),5′-bisphosphate nucleotidase activity. Plant J 58:208–219

Komori R, Amano Y, Ogawa-Ohnishi M, Matsubayashi Y (2009) Identification of tyrosylprotein sulfotransferase in Arabidopsis. Proc Natl Acad Sci U S A 106:15067–15072

Kopriva S (2006) Regulation of sulfate assimilation in Arabidopsis and beyond. Ann Bot-Lond 97:479–495

Kopriva S, Koprivova A (2004) Plant adenosine 5′-phosphosulphate reductase: the past, the present, and the future. J Exp Bot 55:1775–1783

Kopriva S, Muheim R, Koprivova A, Trachsel N, Catalano C, Suter M, Brunold C (1999) Light regulation of assimilatory sulphate reduction in *Arabidopsis thaliana*. Plant J 20:37–44

Kopriva S, Mugford SG, Matthewman C, Koprivova A (2009) Plant sulfate assimilation genes: redundancy versus specialization. Plant Cell Rep 28:1769–1780

Kopriva S, Mugford SG, Baraniecka P, Lee BR, Matthewman CA, Koprivova A (2012) Control of sulfur partitioning between primary and secondary metabolism in Arabidopsis. Front Plant Sci 3:163

Koprivova A, Giovannetti M, Baraniecka P, Lee BR, Grondin C, Loudet O, Kopriva S (2013) Natural variation in the ATPS1 isoform of ATP sulfurylase contributes to the control of sulfate levels in Arabidopsis. Plant Physiol 163:1133–1141

Kurima K, Warman ML, Krishnan S, Domowicz M, Krueger RC Jr, Deyrup A, Schwartz NB (1998) A member of a family of sulfate-activating enzymes causes murine brachymorphism. Proc Natl Acad Sci U S A 95:8681–8685

Lee S, Leustek T (1998) APS kinase from *Arabidopsis thaliana*: genomic organization, expression, and kinetic analysis of the recombinant enzyme. Biochem Biophys Res Commun 247:171–175

Lee BR, Koprivova A, Kopriva S (2011) The key enzyme of sulfate assimilation, adenosine 5′-phosphosulfate reductase, is regulated by HY5 in Arabidopsis. Plant J 67:1042–1054

Lee BR, Huseby S, Koprivova A, Chetelat A, Wirtz M, Mugford ST, Navid E, Brearley C, Saha S, Mithen R, Hell R, Farmer EE, Kopriva S (2012) Effects of fou8/fry1 mutation on sulfur metabolism: is decreased internal sulfate the trigger of sulfate starvation response? PLoS One 7:e39425

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol 51:141–165

Loudet O, Saliba-Colombani V, Camilleri C, Calenge F, Gaudon V, Koprivova A, North KA, Kopriva S, Daniel-Vedele F (2007) Natural variation for sulfate content in *Arabidopsis thaliana* is highly controlled by APR2. Nat Genet 39:896–900

Martin MN, Tarczynski MC, Shen B, Leustek T (2005) The role of 5′-adenylylsulfate reductase in controlling sulfate reduction in plants. Photosynth Res 86:309–323

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006) Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. Plant Cell 18:3235–3251

Mugford SG, Yoshimoto N, Reichelt M, Wirtz M, Hill L, Mugford ST, Nakazato Y, Noji M, Takahashi H, Kramell R, Gigolashvili T, Flugge UI, Wasternack C, Gershenzon J, Hell R, Saito K, Kopriva S (2009) Disruption of adenosine-5′-phosphosulfate kinase in Arabidopsis reduces levels of sulfated secondary metabolites. Plant Cell 21:910–927

Mugford SG, Matthewman CA, Hill L, Kopriva S (2010) Adenosine-5′-phosphosulfate kinase is essential for Arabidopsis viability. FEBS Lett 584:119–123

Mugford SG, Lee BR, Koprivova A, Matthewman C, Kopriva S (2011) Control of sulfur partitioning between primary and secondary metabolism. Plant J 65:96–105

Patron NJ, Durnford DG, Kopriva S (2008) Sulfate assimilation in eukaryotes: fusions, relocations and lateral transfers. BMC Evol Biol 8:39

Ravilious GE, Jez JM (2012a) Nucleotide binding site communication in *Arabidopsis thaliana* adenosine 5′-phosphosulfate kinase. J Biol Chem 287:30385–30394

Ravilious GE, Jez JM (2012b) Structural biology of plant sulfur metabolism: from assimilation to biosynthesis. Nat Prod Rep 29:1138–1152

Ravilious GE, Nguyen A, Francois JA, Jez JM (2012) Structural basis and evolution of redox regulation in plant adenosine-5′-phosphosulfate kinase. Proc Natl Acad Sci U S A 109:309–314

Robles P, Fleury D, Candela H, Cnops G, Alonso-Peral MM, Anami S, Falcone A, Caldana C, Willmitzer L, Ponce MR, Van Lijsebettens M, Micol JL (2010) The RON1/FRY1/SAL1 gene is required for leaf morphogenesis and venation patterning in Arabidopsis. Plant Physiol 152:1357–1372

Rodriguez VM, Chetelat A, Majcherczyk P, Farmer EE (2010) Chloroplastic phosphoadenosine phosphosulfate metabolism regulates basal levels of the prohormone jasmonic acid in Arabidopsis leaves. Plant Physiol 152:1335–1345

Sanda S, Leustek T, Theisen MJ, Garavito RM, Benning C (2001) Recombinant Arabidopsis SQD1 converts UDP-glucose and sulfite to the sulfolipid head group precursor UDP-sulfoquinovose i*n vitro*. J Biol Chem 276:3941–3946

Sonderby IE, Hansen BG, Bjarnholt N, Ticconi C, Halkier BA, Kliebenstein DJ (2007) A systems biology approach identifies a R2R3 MYB gene subfamily with distinct and overlapping functions in regulation of aliphatic glucosinolates. PLoS One 2(12):e1322

Sonderby IE, Geu-Flores F, Halkier BA (2010) Biosynthesis of glucosinolates-gene discovery and beyond. Trends Plant Sci 15:283–290

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu Rev Plant Biol 62:157–184

Tsakraklides G, Martin M, Chalam R, Tarczynski MC, Schmidt A, Leustek T (2002) Sulfate reduction is increased in transgenic *Arabidopsis thaliana* expressing 5′-adenylylsulfate reductase from Pseudomonas aeruginosa. Plant J 32:879–889

Underhill EW, Wetter LR, Chisholm MD (1973) Biosynthesis of glucosinolates. Biochem Soc Symp 38:303–326

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos P, Krahenbuhl U, den Camp RO, Brunold C (2002) Flux control of sulphate assimilation in Arabidopsis thaliana: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. Plant J 31:729–740

Wilson PB, Estavillo GM, Field KJ, Pornsiriwong W, Carroll AJ, Howell KA, Woo NS, Lake JA, Smith SM, Harvey Millar A, von Caemmerer S, Pogson BJ (2009) The nucleotidase/phosphatase SAL1 is a negative regulator of drought tolerance in Arabidopsis. Plant J 58:299–317

Xiong L, Lee B, Ishitani M, Lee H, Zhang C, Zhu JK (2001) FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in Arabidopsis. Genes Dev 15:1971–1984

Yatusevich R, Mugford SG, Matthewman C, Gigolashvili T, Frerigmann H, Delaney S, Koprivova A, Flugge UI, Kopriva S (2010) Genes of primary sulfate assimilation are part of the glucosinolate biosynthetic network in *Arabidopsis thaliana*. Plant J 62:1–11

Significance of Long-Distance Transport

Cornelia Herschbach

Abstract Sulfur cycling in plants is essential, not only to distribute this nutrient to the sites of its demand in growth and development, but also to signal the sulfur status of the plant and to control whole plant sulfur nutrition. Under most environmental conditions, uptake of sulfur compounds from the soil and their transport in the xylem to the shoot ensures adequate sulfur supply. However, metabolism of sulfur compounds in roots as well as in the shoot can result in both a surplus and a deficiency of individual sulfur compounds. Sinks and sources for individual sulfur compounds may change during the annual growth cycle, plant developmental stage and in response to environmental changes. In addition to the xylem, a second long-distance transport path, i.e. the phloem, plays an important role in whole plant sulfur cycling because it connects source and sink organs. However, a particular organ can change from source to sink and vice versa depending on environmental conditions as well as plant growth and developmental stage. Signaling of the sulfur demand is not only systemically, but also locally controlled. Still 'the systemic signal' does not appear to exist. Sulfate as a potential systemic signal communicating environmental stress from the roots to the shoot will be discussed.

Introduction

Long-distance transport in higher plants occurs in xylem and phloem, together constituting the plant vascular system. As the xylem consist of dead cells, the water flow in the xylem, i.e. the transpiration stream, is a physical process driven by a pressure gradient build up by water loss from leaves into the atmosphere and described by the cohesion-tension theory (CTT) (for a review see Stroock et al. 2014). Increasing water potential up to roots and between the root/soil interface is the driving force to suck water from the soil into plants. Plant nutrients in the soil water are taken up by the roots, transported into the xylem, and allocated to the

C. Herschbach (✉)
Fakultät für Umwelt und Natürliche Ressourcen, Institut für Forstwissenschaften, Albert-Ludwigs-Universität Freiburg, Georges-Köhler-Allee 053/054, 79110 Freiburg, Germany
e-mail: cornelia.herschbach@ctp.uni-freiburg.de

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_3

leaves within the bulk flow of water (Marschner 2012). The phloem is the long-distance transport path that connects sources and sinks of primary and secondary products of photosynthesis. The osmotically generated pressure gradient in the phloem, i.e. high osmotic pressure at the source and low osmotic pressure at sinks, mostly built by sucrose, conducts a mass flow from sources to sinks (De Scheeper et al. 2013). Within this mass flow other nutrients as well as inorganic cations and anions are co-transported. In annual plants usually mature leaves are sources for carbon but also for other nutrients supplied to sinks such as roots, flowers and seeds (Fig. 1). In perennial plants the source sink relationship depends on the season. During spring buds and developing leaves are sinks for carbon and other nutrients,

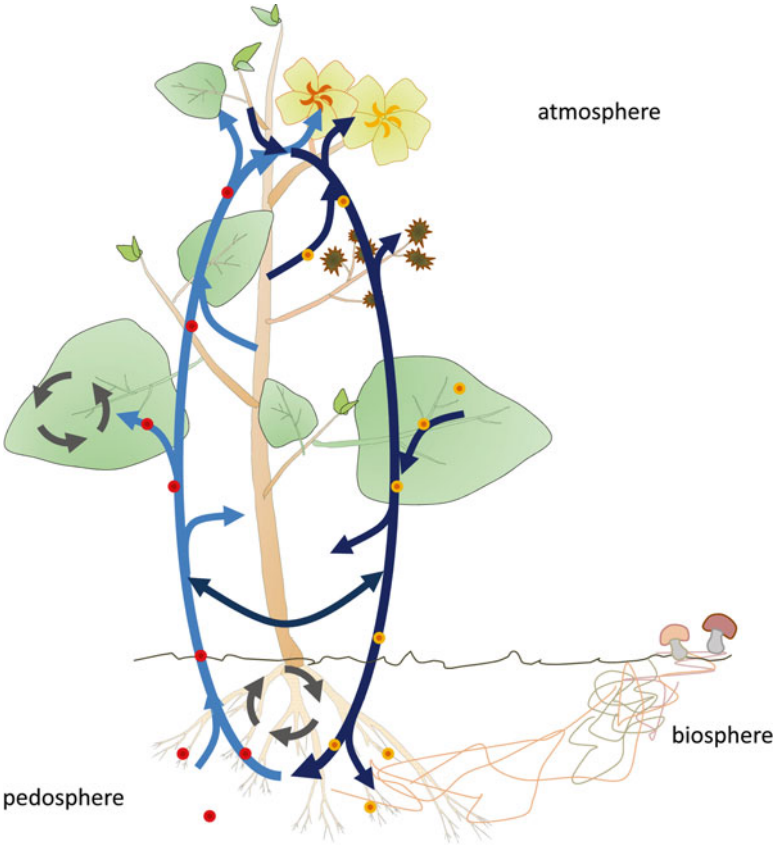


Fig. 1 Model of whole plant sulfur cycling. Sulfur cycling (*gray arrow circles*) within organs includes cellular and tissue sulfur cycling (Rennenberg and Herschbach 2014). The whole plant sulfur cycling and thus sulfur distribution is indicated by *light blue arrows* for xylem related flow and *dark blue arrows* for phloem related sulfur flow according to Herschbach et al. (2012). The whole plant sulfur flow combines cellular, tissue and organ sulfur cycling. The long-distance transport as the communication system needs transport of signals from roots to shoot (*red dots*) and from shoot to roots (*yellow dots*)

later in the year mature deciduous leaves are the source, while mature conifer leaves of the current year are sink organs (Oparka and Santa Cruz 2000; Herschbach et al. 2012). Stem tissues of bark and wood can be source organs in spring and sink organs during active growth and leaf senescence (Sauter and van Cleve 1994).

Lucas et al. (2013) have summarized the importance of long-distance transport paths as an inter-organ communication system. This communication system includes signaling from roots to shoot, for example by abscisic acid (ABA) in drought and salt signaling (Wilkinson and Davies 2010; Schachtman and Goodger 2008; Goodger and Schachtman 2010), and from shoot to roots, for example by signaling the nutrient demand (for examples see Chiou and Lin 2011; Kehr 2013; Gessler et al. 2004; Gojon et al. 2009; Liu et al. 2009). Moreover, signaling from and to other plant organs such as flowers, seeds, as well as bark and wood of perennial plants must be considered (Fig. 1). A further important function of the vascular system is the effective distribution of nutrients between plant organs (Fig. 1; Herschbach et al. 2012; Gessler et al. 2004). Regarding sulfur metabolism the following sulfur-containing compounds are detected and transported in the xylem and phloem: sulfate, cysteine, glutathione (GSH), methionine (Herschbach and Rennenberg 2001; Herschbach et al. 2012), γ-glutamylcysteine (γEC) (Schneider et al. 1994), *S*-methylmethionine (SMM) (Bourgis et al. 1999; Tan et al. 2010) and glucosinolates (Chen et al. 2001; Andersen et al. 2013). Thus, all these sulfur compounds are able to cycle within plants between roots and the shoot and can be distributed from places of surplus, i.e. sources, to places of demand, i.e. sinks (Fig. 1).

Root (In)dependency on Reduced S from the Shoot

In 1979 Rennenberg and co-workers showed that GSH functioned as a long-distance transport form of reduced sulfur from the shoot to roots (Rennenberg et al. 1979). In 1990 Brunold discussed the importance of shoot sulfate reduction as the main source of reduced sulfur for the entire plant (Brunold 1990). It is still a matter of debate whether roots are dependent or independent from the reduced sulfur allocated from the shoot. To address this question, two whole plant approaches with poplar were conducted and indicate partial independency of roots from shoot derived reduced sulfur. Feeding ^{35}S-GSH to mature poplar leaves indicated that root tips are not the preferential sink for reduced-S from the shoot (Herschbach et al. 2010). Instead, roots showing secondary growth accumulated the bulk of ^{35}S-GSH, while fine roots accumulated a comparable amount, but at a higher total root biomass. On the other hand, interruption of phloem transport by girdling, i.e. peeling off the bark from the stem of poplar, affected the GSH content in root tips and fine roots, but not in roots with secondary root growth (Herschbach et al. 2010). L-buthionine sulfoximine (BSO), a specific inhibitor of the first step in GSH synthesis, strongly reduced GSH levels in root tips, white roots without side roots, and fine roots irrespective as to whether phloem transport was interrupted by girdling (Xia and Herschbach, unpublished). Similar results were obtained in studies with other

plant species such as Arabidopsis (Koprivova et al. 2010) or tomato (Tyburski and Tretyn 2010). These observations strongly suggest that roots are able to synthesize sufficient amounts of GSH and are independent from shoot-derived GSH supply. The same conclusion was drawn from a split root experiment (Hubberten et al. 2012). In this experiment the root system of Arabidopsis plants was separated into two equal parts. One part was supplied with sufficient sulfur (+S) while the other root part was exposed to sulfur depletion (−S). If ^{35}S-sulfate was fed to the +S split root part both ^{35}S-sulfate and ^{35}S-GSH were detected in the −S split root part although only in scarce amounts. This finding indicates that sulfate was allocated from the +S split root part to the −S split root part. Whether GSH was also allocated in the same manner was tested in BSO treated seedlings. Although ^{35}S-GSH was visible in the corresponding shoot, ^{35}S-GSH was not detected in roots of the −S split root part. Hence, this experiment supports the idea that roots synthetize sufficient amounts of GSH for their own growth and development. This assumption is further supported by studies with Arabidopsis mutants manipulated in the first step of GSH synthesis (GSH1 or γ-EC synthetase) that either showed abolished root growth but not shoot development (*rml1*, Vernoux et al. 2000) or decreased root growth in length and lateral root development (Koprivova et al. 2010; Marquez-Garcia et al. 2014).

Sulfur Cycling: The Distribution System

Reduced sulfur supply from shoot to roots is carried out by phloem transport (Rennenberg et al. 1979). Together with sulfur allocation in the xylem a cycling pool of sulfur compounds provides oxidized and reduced sulfur to respective sinks (Herschbach et al. 2012). Such a cycling pool needs transport processes over the plasma membrane of cells and is also influenced by short-distance transport processes over compartment membranes within cells, which results in an intracellular cycling pool of sulfur (Rennenberg and Herschbach 2014). Individual cells are part of tissues consisting of similar or different cell types, which in turn make up specific organs. Sulfur compounds are able to cycle between cells, tissues and organs because these all form interconnected systems. Sulfur cycling at the tissue level has been shown with corn plants between mesophyll and bundle sheath cells for Cys and GSH (Burgener et al. 1998). Whereas sulfate reduction and assimilation up to Cys occurs in bundle sheath cells, the subsequent use of Cys in GSH synthesis is realized mainly in mesophyll cells. As another example of sulfur cycling between tissues, phloem to xylem exchange within the shoot and xylem to phloem exchange along the transport path of stems and in roots contributes to whole plant sulfur cycling (Herschbach et al. 2012). A brief overview of membrane crossing steps, which contribute to whole plant nutrient cycling is presented in Fig. 2 using sulfate as an example.

Sulfur cycling was intensively investigated with perennial plants regarding the annual growth cycle (Herschbach et al. 2012). Perennial plants need to store

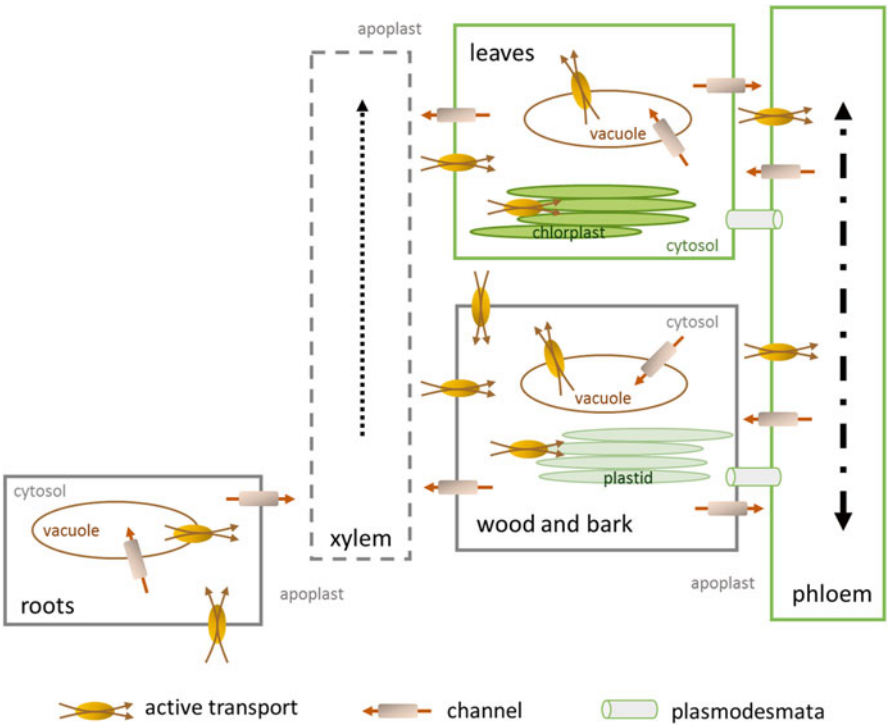


Fig. 2 Membrane crossing steps necessary for nutrient cycling within the whole plant drawn at the example of sulfate. Sulfatetransporters (SULTR) enable sulfate transport over membranes. However, as SULTR are proton co-transporters (Lass und Ulrich-Eberius 1984; Cram 1990) the transport is only possible along the proton motive force, i.e. from the apoplast into the cytosol and from vacuoles into the cytosol. Sulfate permeable channels may carry out sulfate release from cells into the apoplast and sulfate flow into the vacuole. Phloem loading and unloading may, however, also occur via plasmodesmata connecting sieve elements via companion cell with mesophyll cells (De Schepper et al. 2013)

nutrients during dormancy while they have to mobilize nutrients during spring to supply the newly sprouting shoot with carbohydrates, nitrogen and sulfur compounds. Sulfate contents together with sulfate transporter (SULTR) expression were investigated in leaves, bark and wood of field grown poplar. These investigations strongly indicate a control of sulfate cycling by SULTR expression (Dürr et al. 2010; Malcheska et al. 2013). During autumn, sulfate accumulates in bark and wood. Sulfate for storage can be taken out of the xylem or phloem sap. This needs on one hand xylem and phloem loading and on the other hand unloading into storage tissues. If all SULTRs are working as proton/co-transporters (Lass and Ulrich-Eberius 1984; Cram 1990) xylem loading from xylem parenchyma cells in roots may depend on sulfate permeable channels. SULTRs of xylem parenchyma cells in roots can transport sulfate back into cells for storage and may thus control xylem loading by sulfate retrieval (Fig. 2). Xylem unloading along the transport path can be carried out in the same way by sulfate uptake into xylem parenchyma cells.

During autumn only two SULTRs, Pta*SULTR2*;*1a* and Pta*SULTR3*;*2a*, are expressed in the wood of poplar and could therefore be responsible for sulfate storage in living cells of the wood (Malcheska et al. 2013). In the bark diminished expression of two phloem-localized SULTRs, Pta*SULTR1*;*1* and Pta*SULTR3*;*3a*, indicates reduction of phloem re-loading when the sulfate content of the bark increased during autumn (Dürr et al. 2010). Diminished expression of both group 4 SULTRs, which are assumed to be responsible for sulfate release out of vacuoles (Kataoka et al. 2004) furthermore suggests sulfate retention for storage in vacuoles (Dürr et al. 2010). In spring sulfate mobilization from wood and bark tissues is indicated by decreasing sulfate contents (Herschbach and Rennenberg 1996; Dürr et al. 2010; Malcheska et al. 2013) and by the observation of increased sulfate contents in xylem saps (Schupp et al. 1991; Rennenberg et al. 1994; Malcheska et al. 2013). At that time, SULTRs were not expressed in poplar wood and, thus, a complete prevention of sulfate uptake into xylem parenchyma cells can be assumed which would allow for sulfate accumulation in the xylem sap (Malcheska et al. 2013). Sulfate permeable channels must consequently provide sulfate release from storage cells of wood. Only few indications of sulfate permeable channels have been described so far (Piñeros et al. 2008; Barbier-Brygoo et al. 2011; Meyer et al. 2010). The sulfate released during spring from bark parenchyma cells may be channeled either into the phloem or via ray cells into the xylem and thereby to buds and developing leaves. Expression of Pta*SULTR3*;*3a* and Pta*SULTR4*;*2* increased in the bark during spring and furthermore expression of both SULTRs strongly correlates with sulfate contents (Dürr et al. 2010). Thus, phloem loading by Pta*SULTR3*;*3a* and increased sulfate release from vacuoles via Pta*SULTR4*;*2* seems to control sulfate mobilization, which was indicated by a decline in bark sulfate during spring. In conclusion, expression of SULTRs seems to regulate sulfate cycling at the whole plant level and, moreover, in perennial plants sulfate storage and mobilization during the annual growth cycle.

Two further examples that clearly illustrate the importance of specific transporters for sulfur cycling have been published in recent years. One described the consequences of enhanced *S*-methylmethionine (SMM) phloem loading capacity (Tan et al. 2010) and the other demonstrated the function of glucosinolate transporters 1 and 2 (GTR1 and GTR2) for organ-specific glucosinolate (GLS) accumulation (Andersen et al. 2013). The yeast MMP1 gene (SMM transporter) targeted to the phloem and seeds in pea plants (Tan et al. 2010) mediated enhanced phloem loading of SMM. Over-expression of this gene increased SMM in phloem exudates but SMM did not accumulate in roots; rather, SULTR expression as well as APR expression increased. The authors concluded that enhanced xylem loading could be responsible for unaffected SMM contents in roots of these pea mutants. Indeed, the SMM content in the xylem sap was 1.5-fold higher compared to wild type plants. In leaves, however, the down-regulation of APR and other genes of the sulfate reduction pathway corresponded to higher SMM contents that might function as a signal to reduce sulfate assimilation (Tan et al. 2010). As shoot biomass of transgenic pea plants and seed soluble and total N increased, this study shows that manipulation in long-distance transport can influence whole plant physiology. The second example

established the function of two glucosinolate transporters for organ-specific glucosinolate (GLS) distribution in Arabidopsis in a combination of grafting experiments by using knockout mutants and mutants deficient in glucosinolate synthesis (Andersen et al. 2013). The outcome of this study was that short chain aliphatic GLS are mainly synthesized and stored in rosette leaves while long chain GLS were synthesized in both roots and leaves but stored only in the roots. GTR1 and GTR2 loaded GLS not only into the phloem, but were also localized around the xylem at root branching points and may reload GLS into as yet unknown storage cells. In this way, a specific distribution pattern of glucosinolates, which are important defense compounds, is established and allows plants to react against specific pest infections either affecting the shoot or roots. Also this example demonstrates the importance of specific transporters for the whole plant physiology.

Signaling of Sulfur Demand: The Communication System

The long-distance transport paths, xylem and phloem, together function as a communication system to maintain nutrient homeostasis by adapting nutrient uptake to nutrient demand (Gojon et al. 2009; Liu et al. 2009). Signaling of sulfur demand has been studied for several decades. Initially, GSH was assumed to be the sole signal from shoot to roots that communicates the sulfur status and thus controls sulfate uptake by negative feedback control of the sulfate uptake system (Herschbach and Rennenberg 1991, 1994; Lappartient et al. 1999). Beside exogenous GSH application to roots, internal GSH contents were increased by gaseous H_2S or SO_2 application approaches. H_2S fumigation of tobacco, spinach and poplar led to higher GSH contents in roots while sulfate uptake decreased (Herschbach et al. 1995a, b, 2000). Plant species that contain secondary sulfur compounds such as glucosinolates or alliin sometimes seem to react differently (De Kok et al. 2000; Durenkamp et al. 2007; Koralewska et al. 2008, 2009). First confirmation of GSH as a systemic long-distance signal for sulfur demand was provided by a split root approach with *Brassica* (Lappartient and Touraine 1996). In this study, one part of the root system was supplied with sufficient sulfur (+S), while the other was exposed to sulfur deficiency (−S). Decreasing GSH concentrations in the +S root part combined with increasing sulfate uptake rates and declining GSH contents in phloem exudates upon whole plant sulfur deficiency were observed. Furthermore, if GSH was fed to one split root part sulfate uptake by the other root part was diminished. It was concluded that GSH functions as a systemic regulatory signal related to the sulfur demand (Lappartient and Touraine 1996). However, the authors could not exclude that sulfate transported from the +S split root part to the −S split root part led to enhanced GSH synthesis and thus GSH accumulation in the −S split root part (see discussion of GSH synthesis in roots). Furthermore, these experiments did not test if sulfate is involved in long-distance signaling from the +S split root part to the −S split root part. In addition, the transport of GSH was only concluded from GSH accumulation; transport of GSH as a whole molecule was not proved. More recently,

Hubberten and co-workers clearly showed that GSH is not the main sulfur form transported from the +S split root part to the −S split root part. Rather sulfate is the sulfur compound most likely transported from the +S split root to the −S split root part, where additional GSH seems to be synthesized (Hubberten et al. 2012).

A new direction in thinking for signaling sulfur demand has been developed in the work of Rouached et al. (2008). The authors analyzed over a broad range of environmental conditions the correlation between the expression of two SULTR genes, *SULTR1*;*1* and *SULTR1*;*2* responsible for sulfate uptake (Takahashi et al. 2000; Yoshimoto et al. 2002), and sulfate, GSH as well as *O*-acetylserine (OAS) concentrations in roots. Expression of *SULTR1*;*1* strongly correlated with sulfate in roots while *SULTR1*;*2* showed no correlation, either to sulfate, GSH or to OAS. As *SULTR1*;*1* expression also depends on the sulfate treatment in split root approaches, the authors concluded that *SULTR1*;*1* is locally controlled by sulfate availability while the unaffected *SULTR1*;*2* expression indicates systemic regulation to metabolic demand (Rouached et al. 2008). There were no indications of systemic signaling either from the +S split root part to the −S split root part or vice versa, i.e. no systemic induction of sulfate uptake or of any sulfur starvation marker was observed if the split root system was established shortly after seed germination with Arabidopsis (Hubberten et al. 2012). Rather, the sulfate content of shoots correlated to the size of the −S split root part and suggested that whole plant sulfur acquisition can be performed from local sulfate patches within the soil. However, −S sulfur responsive reactions may occur locally if the sulfate cycling pool falls below a certain threshold that causes local S deficiency by restricted sulfate withdraw from the cycling pool. Nevertheless, OAS accumulation observed in roots of −S Arabidopsis plants was omitted in −S split roots of split root plants. The authors concluded that OAS in −S split roots was consumed in GSH. Whether OAS constitutes a long-distance signal needs further analysis.

Further systemic signals of the sulfur demand could include plant hormones, as discussed for nitrogen (Collier et al. 2003) and phosphorus (Chiou and Lin 2011), as well as microRNAs (Liu et al. 2009). Cytokinins negatively affect *SULTR1*;*1* and *SULTR1*;*2* expression in Arabidopsis roots (Maruyama-Nakashita et al. 2004; Werner et al. 2010). The systemic significance of plant hormones during sulfur deficiency was analysed in poplar using a long-term sulfur depletion approach (Honsel et al. 2012). Although active cytokinins (Sakakibara 2006) increased under sulfur deficiency in young leaves and active cytokinins decreased in mature leaves, only inactive cytokinins increased in the roots (Honsel et al. 2012). However, beside a decline in sulfate and GSH none of the plant hormones revealed consistent changes in the long-distance transport fluids, xylem sap and phloem exudates (Honsel et al. 2012). Apparently, cytokinins and probably other plant hormones are not involved in systemically signaling the sulfur demand and rather may be local signals in poplar.

MicroRNA395 (miR395) is known to target genes of the primary sulfur metabolism (Kawashima et al. 2009; Honsel et al. 2012), but seems to be a local rather than a systemic signal although transport of miR395 from the shoot to roots has been reported (Buhtz et al. 2010). In Arabidopsis miR395 targets ATP sulfurylase 4

(*ATPS 4*) and *SULTR2;1* (Kawashima et al. 2009) while miR395 targets only *ATPS3/4* in poplar (Honsel et al. 2012). If wild type Arabidopsis scions were grafted onto *hen1-1* root stocks, miR395 sequence detection in the miR processing mutant root stock clearly indicates miR395 transport from shoot to roots (Buhtz et al. 2010). However, if target gene expression (*APS4* and *SULTR2;1*) was compared with miR395 expression in Arabidopsis at sulfur deficiency in roots, only *APS4* but not *SULTR2;1* seems the target of miR395 while transcripts of both target genes were down-regulated in leaves if miR395 expression was up-regulated (Liang et al. 2010). This result seems unexpected, but can be explained by local rather than systemic signaling of miR395. Considering that *SULTR2;1* in Arabidopsis is expressed in xylem parenchyma cells (Takahashi et al. 1997, 2000) whereas miR395 is expressed in companion cells (Kawashima et al. 2009), the spatial separation of the target gene *SULTR2;1* from miR395 expression supports local instead of systemic signaling by miR395. Further support of this assumption has been derived from split root experiments with Arabidopsis (Hubberten et al. 2012) and S starvation experiments with poplar (Honsel et al. 2012).

At the current state of knowledge, the sulfur demand at the whole plant level seems to be controlled and regulated in a complex manner including local as well as systemic signals. Apparently, the 'signal' that controls sulfur nutrition and, thus, adapts sulfate uptake to the sulfur demand does not exist. The combination of local plus systemic influences on sulfur acquisition needs to consider the cellular reaction cascade including transcription factors (Takahashi et al. 2011; Davidian and Kopriva 2010). In addition, the cycling pool of sulfur, consisting of different metabolites including precursors like OAS, operates at different levels from the cell to the whole plant and has to be taken into account. Therefore, interactive regulation of these different cycling pools can be expected.

Sulfur and Signaling of Environmental Stress

Water stress in the soil but also dry air leads to stomatal closure to prevent excessive water loss. ABA is widely accepted as a signal to induce stomatal closure (Hedrich 2012; Bauer et al. 2013; Goodger and Schachtmann 2010; Daszkowska-Golec and Szarejko 2013). Increasing ABA content in the apoplast around guard cells initiates the reaction cascade that opens anion channels releasing K^+ and malate to reduce guard cell turgor and results in stomatal closure (Hedrich 2012; Negi et al. 2014). However, plants sense soil drying first by the roots, while ABA is a signal for stomatal closure in leaves. Hence, ABA as well as pH increment in the xylem sap as a first signal of drought has been suggested, but is a matter of controversial discussion (Wilkinson and Davies 2002, 2010; Goodger and Schachtmann 2010). Recently, Ernst and co-workers (Ernst et al. 2010) investigated early signals in the xylem sap of *Zea mays* and observed that sulfate was the first compound found to be increased during moderate drought stress, when only a small reduction in stomatal conductance occurred. This is surprising because sulfate has not been established as a

compound relevant in stomatal closure so far. Furthermore, Meyer and co-workers (2010) characterized a channel, ATML12, in Arabidopsis that is sensitive and permeable for both malate and sulfate. This channel is strongly expressed in guard cells and *atatml12* mutants are impaired in dark and CO_2-mediated stomatal closure. These findings support the hypothesis that sulfate in the xylem sap could be a first stress related signal for stomatal closure. Sulfate is not a limiting nutrient for plants and thus enhanced sulfate uptake could result in increased sulfate content in the xylem sap during drought as suggested by Ernst et al. (2010). However, sulfate enrichment in the xylem sap can also be provided by sulfate release from xylem parenchyma cells as observed during spring in perennial plants after sulfate mobilization (Herschbach and Rennenberg 1996; Rennenberg et al. 1994; Malcheska et al. 2013). More recently, a dependency of sulfate transport into chloroplasts on adequate ABA synthesis during stress has been reported (Cao et al. 2014). Cys is needed in the last step of ABA synthesis for sulfurization of the molybdenum co-factor (MoCo) via MoCo sulfurase (ABA3) (Mendel and Hänsch 2002; Schwarz and Mendel 2006). This sulfurized MoCo co-factor is part of abscisic aldehyde oxidase (AAO3), catalysing the last step in ABA synthesis that converts abscisic aldehyde to abscisic acid. If sulfate transport into chloroplasts was prevented or reduced in *atsultr3*;*1* mutants, AAO activity and consequently ABA content decreased (Cao et al. 2014). As ABA induced *SULTR3*;*1* expression, a strong co-regulation for sufficient ABA synthesis under stress was suggested. Hence, sulfate may be both a long-distance signal during stress and a nutrient transported over short-distances to be used in ABA synthesis but also in the induction of ABA synthesis during stress. To resolve these functions of sulfate will be a great challenge for future studies.

Conclusions

Sulfur cycling and thus sulfur distribution via the long-distance transport system has been shown for sulfate especially during the annual growth cycle (Herschbach et al. 2012), for glucosinolates (Andersen et al. 2013) and SMM (Tan et al. 2010). As Cys, GSH and Met were also detected in xylem sap and phloem exudates it must be assumed that these sulfur compounds are also distributed via long-distance transport paths and thus, cycle in plants. Control of cycling by long-distance transport has been established for sulfate via SULTRs and for glucosinolates via GTR1 and GTR2 that control uptake from xylem or uptake into phloem sap. A number of transporters for sulfate have been established (see reviews by Takahashi et al. 2011), but specific Cys, Met and GSH transporters are scarcely analyzed. Although GSH transporters have been characterized (Bogs et al. 2003; Zhang et al. 2004; Cagnac et al. 2004; Osawa et al. 2006) they were not found to be specific for GSH. Studies on sulfate, glucosinolate and SMM indicate that long-distance transport and thus cycling of these compounds seems to be controlled by expression of specific transporters. Whether channels are also involved in the regulation of long-distance

transport, for example to release sulfur compounds into the xylem, has to be considered in future studies.

Long-distance transport is also important to signal the demand of nutrients between shoot and roots and vice versa. It seems obvious that signaling of sulfur demand can be local and/or systemic. Sulfur-containing metabolites, OAS as a precursor of Cys, plant hormones, miR395 as well as transcription factors may all be involved to adapt sulfur nutrition to the sulfur demand for growth and development (Davidian and Kopriva 2010; Takahashi et al. 2011; Hubberten et al. 2012). Sulfur-containing compounds such as sulfate, GSH and the precursor OAS are potential signals operating via their cycling pools in the xylem and phloem that also could constitute additional storage pools of sulfur (Herschbach et al. 2012). From studies with poplar, we suggest that the sulfate-to-GSH ratio may be more relevant to signal the sulfur status of the whole plant than one of these compounds alone (Herschbach et al. 2000). This needs to be analyzed in a broader range of species and under different environmental conditions considering a broader range of sulfur compounds. Further signaling compounds are phytohormones, microRNAs and transcription factors, which may be local or systemic signals. To data the 'systemic signal' that indicates the sulfur status and sulfur demand of plants has not been identified. Such a signal may not even exist and a complex signaling network may be more relevant to control sulfur nutrition of plants. Furthermore, understanding sulfur nutrient control and sulfur cycling at the whole plant level starts at the cellular level. It expands to sulfur cycling in tissues to organs up to the whole plant (Rennenberg and Herschbach 2014). Signaling at these different levels of sulfur cycling and its interaction are far from being clearly elucidated. These questions continue to be fascinating areas of research, even 35 years after Heinz Rennenberg first published on GSH transport from the shoot to the roots in the phloem of tobacco plants (Rennenberg et al.1979).

Acknowledgment The author thanks Heinz Rennenberg for critical reading of the manuscript and for supporting her research throughout the years. Financial support of the DFG is also gratefully acknowledged.

References

Andersen TG, Nour-Eldin HH, Fuller VL, Olsen CE, Burow M, Halkier BA (2013) Integration of biosynthesis and long-distance transport establish organ-specific glucosinolate profiles in vegetative Arabidopsis. Plant Cell 25:3133–3145

Barbier-Brygoo H, De Angeli A, Filleur S, Frachisse JM, Gambale F, Thomine S, Wege S (2011) Anion channels/transporters in plants: from molecular bases to regulatory networks. Annu Rev Plant Biol 62:25–51

Bauer H, Ache P, Lautner S, Fromm J, Hartung W, Al-Rasheid KAS, Sonnewald S, Sonnewald U, Kneitz S, Lachmann N, Mendel RR, Bittner F, Hetherington AM, Hedrich R (2013) The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. Curr Biol 23:53–57

Bogs J, Bouboulloux A, Cagnac O, Wachter A, Rausch T, Delrot S (2003) Functional characterization and expression analysis of a glutathione transporter, BjGT1, from *Brassica juncea*: evidence for regulation by heavy metal exposure. Plant Cell Environ 26:1703–1711

Bourgis F, Roje S, Nuccio ML, Fisher DB, Tarczynski MC, Li C, Herschbach C, Rennenberg H, Pimenta MJ, Shen T-L, Gage DA, Hanson AD (1999) S-Methylmethionine plays a major role in phloem sulfur transport and is synthesized by a novel type of methyltransferase. Plant Cell 11:1485–1497

Brunold C (1990) Reduction of sulfate to sulfide. In: Rennenberg H, Brunold C, De Kok LJ, Stulen I (eds) Sulfur nutrition and sulfur assimilation in higher plants. SPB Academic Publishing, The Hague, pp 13–31

Buhtz A, Pieritz J, Springer F, Kehr J (2010) Phloem small RNAs, nutrient stress responses, and systemic mobility. BMC Plant Biol 10:64

Burgener M, Suter M, Jones S, Brunold C (1998) Cyst(e)ine is the transport metabolite of assimilated sulfur from bundle-sheath to mesophyll cells in maize leaves. Plant Physiol 116:1315–1322

Cagnac O, Bourbouloux A, Chakrabarty D, Zhang M-Y, Delrot S (2004) AtOPT6 transports glutathione derivatives and is induced by primisulfuron. Plant Physiol 135:1378–1387

Cao M-J, Wang Z, Zhao Q, Mao J-L, Speiser A, Wirtz M, Hell R, Zhu J-K, Xiang C-B (2014) Sulfate availability affects ABA levels and germination response to ABA and salt stress in *Arabidopsis thaliana*. Plant J 77:604–615

Chen S, Petersen BL, Olsen CE, Schulz A, Halkier BA (2001) Long-distance phloem transport of glucosinolates in Arabidopsis. Plant Physiol 127:194–201

Chiou T-J, Lin S-I (2011) Signaling network in sensing phosphate availability in plants. Annu Rev Plant Biol 62:185–206

Collier MD, Fotelli MN, Nahm M, Kopriva S, Rennenberg H, Hanke DE, Geßler A (2003) Regulation of nitrogen uptake by *Fagus sylvatica* on a whole plant level – interactions between cytokinins and soluble N compounds. Plant Cell Environ 26:1549–1560

Cram WJ (1990) Uptake and transport of sulfate. In: Rennenberg H, Brunold C, De Kok LJ, Stulen I (eds) Sulfur nutrition and sulfur assimilation in higher plants. SPB Academic Publishing, The Hague, pp 3–11

Daszkowska-Golec A, Szarejko I (2013) Open or close the gate – stomata action under the control of phytohormones in drought stress conditions. Front Plant Sci 4:138

Davidian J-C, Kopriva S (2010) Regulation of sulfate uptake and assimilation – the same or not the same? Mol Plant 3:314–325

De Kok LJ, Westerman S, Stuiver CEE, Stulen I (2000) Atmospheric H2S as plant sulfur source: interaction with pedospheric sulfur nutrition – a case study with *Brassica oleracea* L. In: Brunold C, Rennenberg H, De Kok LJ, Stulen I, Davidian J-C (eds) Sulfur nutrition and sulfur assimilation in higher plants. Paul Haupt Publisher, Bern, pp 41–55

De Schepper V, Swaef TD, Bauweraerts I, Steppe K (2013) Phloem transport: a review of mechanisms and controls. J Exp Bot 64:4839–4850

Durenkamp M, De Kok LJ, Kopriva S (2007) Adenosine 5′-phosphosulphate reductase is regulated differently in *Allium cepa* L. and *Brassica oleracea* L. upon exposure to H_2S. J Exp Bot 58:1571–1579

Dürr J, Bücking H, Mult S, Wildhagen H, Palme K, Rennenberg H, Ditengou F, Herschbach C (2010) Seasonal and cell type specific expression of sulfate transporters in the phloem of *Populus* reveals tree specific characteristics for SO_4^{2-} storage and mobilization. Plant Mol Biol 72:499–518

Ernst L, Goodger JQD, Alvarez S, Marsh EL, Berla B, Lockhart E, Jung J, Li P, Bohnert HJ, Schachtman DP (2010) Sulfate as a xylem-born chemical signal precedes the expression of ABA synthetic genes in maize root. J Exp Bot 61:3395–3405

Gessler A, Kopriva S, Rennenberg H (2004) Regulation of nitrate uptake at the whole-tree level: interaction between nitrogen compounds, cytokinins and carbon metabolism. Tree Physiol 24:1313–1321

Gojon A, Nacry P, Davidian J-C (2009) Root uptake regulation: a central process for NPS homeostasis in plants. Curr Opin Plant Biol 12:328–338

Goodger JQD, Schachtmann DP (2010) Re-examining the role of ABA as the primary long-distance signal produced by water-stressed roots. Plant Signal Behav 5:1298–1301

Hedrich R (2012) Ion channels in plants. Physiol Rev 92:1777–1811

Herschbach C, Rennenberg H (1991) Influence of glutathione (GSH) on sulfate influx, xylem loading and exudation in excised tobacco roots. J Exp Bot 42:1021–1029

Herschbach C, Rennenberg H (1994) Influence of glutathione (GSH) on net uptake of sulfate and sulfate transport in tobacco plants. J Exp Bot 45:1069–1076

Herschbach C, Rennenberg H (1996) Storage and re-mobilization of sulfur in beech trees (*Fagus sylvatica*). Physiol Plant 97:125–132

Herschbach C, Rennenberg H (2001) Sulfur nutrition of deciduous trees. Naturwissenschaften 88:25–36

Herschbach C, De Kok LJ, Rennenberg H (1995a) Net uptake of sulfate and its transport to the shoot in spinach plants fumigated with H_2S or SO_2: does atmospheric sulfur affect the 'inter-organ' regulation of sulfur nutrition. Bot Acta 108:41–46

Herschbach C, De Kok LJ, Rennenberg H (1995b) Net uptake of sulfate and its transport to the shoot in tobacco plants fumigated with H_2S or SO_2. Plant Soil 175:75–84

Herschbach C, van der Zalm E, Schneider A, Jouanin L, De Kok LJ, Rennenberg H (2000) Regulation of sulfur nutrition in wild-type and transgenic poplar overexpressing γ-glutamylcysteine synthetase in the cytosol as affected by atmospheric H_2S. Plant Physiol 124:461–473

Herschbach C, Scheerer U, Rennenberg H (2010) Redox states of glutathione and ascorbate in root tips of poplar (*Populus tremula* x *P. alba*) depend on phloem transport from the shoot to the roots. J Exp Bot 61:1065–1074

Herschbach C, Gessler A, Rennenberg H (2012) Long-distance transport and plant internal cycling of N- and S-compounds. Prog Bot 73:161–188

Honsel A, Kojima M, Haas R, Frank W, Sakakibara H, Herschbach C, Rennenberg H (2012) Sulfur limitation and early sulfur deficient responses in poplar: significance of gene expression, metabolites and plant hormones. J Exp Bot 63:1873–1893

Hubberten H-M, Drozd A, Tran BV, Hesse H, Hoefgen R (2012) Local and systemic regulation of sulfur homeostasis in roots of *Arabidopsis thaliana*. Plant J 72:625–635

Kataoka T, Watanabe-Takahashi A, Hayashi N, Ohnishi M, Mimura T, Buchner P, Hawkesford MJ, Yamaya T, Takahashi H (2004) Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in Arabidopsis. Plant Cell Online 16:2693–2704

Kawashima CG, Yoshimoto N, Maruyama-Nakashita A, Tsuchiya YN, Saito K, Takahashi H, Dalmay T (2009) Sulfur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. Plant J 57:313–321

Kehr J (2013) Systemic regulation of mineral homeostasis by microRNAs. Front Plant Sci 4:145

Koprivova A, Mugford ST, Kopriva S (2010) Arabidopsis root growth dependence on glutathione is linked to auxin transport. Plant Cell Rep 29:1157–1167

Koralewska A, Stuiver CEE, Posthumus FS, Kopriva S, Hawkesford MJ, De Kok LJ (2008) Regulation of sulfate uptake, expression of the sulfate transporters *Sultr1*;*1* and *Sultr1*;*2*, and *APS reductase* in Chinese cabbage (*Brassica pekinensis*) as affected by atmospheric H_2S nutrition and sulfate deprivation. Funct Plant Biol 35:318–327

Koralewska A, Buchner P, Stuiver CEE, Posthumus FS, Kopriva S, Hawkesford MJ, De Kok LJ (2009) Expression and activity of sulfate transporters and APS reductase in curly kale in response to sulfate deprivation and re-supply. J Plant Physiol 166:168–179

Lappartient AG, Touraine B (1996) Demand-driven control of root ATP sulfurylase activity and SO_4^{2-} uptake in intact canola. The role of phloem-translocated glutathione. Plant Physiol 111:147–157

Lappartient AG, Vidmar JJ, Leustek T, Glass ADM, Touraine B (1999) Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. Plant J 18:89–95

Lass B, Ullrich-Eberius C (1984) Evidence for proton/sulfate cotransport and its kinetics in *Lemna gibba* G1. Planta 1:53–60

Liang G, Yang F, Yu D (2010) MicroRNA395 mediates regulation of sulfate accumulation and allocation in *Arabidopsis thaliana*. Plant J 62:1046–1057

Liu T-Y, Chang C-Y, Chiou T-J (2009) The long-distance signaling of mineral macronutrients. Curr Opin Plant Biol 12:312–319

Lucas WJ, Groover A, Lichtenberger R, Furuta K, Yadav SR, Helariutta Y, He XQ, Fukuda H, Kang J, Brady SM, Patrick JW, Sperry J, Yoshida A, López-Millán AF, Grusak MA, Kachroo P (2013) The plant vascular system: evolution, development and functions. J Integr Plant Biol 55:294–388

Malcheska F, Honsel A, Wildhagen H, Dürr J, Larisch C, Rennenberg H, Herschbach C (2013) Differential expression of specific sulfate transporters underlies seasonal and spatial patterns of sulfate allocation in trees. Plant Cell Environ 36:1285–1295

Marquez-Garcia B, Njo M, Beeckman T, Goormachtig S, Foyer CH (2014) A new role for glutathione in the regulation of root architecture linked to strigolactones. Plant Cell Environ 37:488–498

Marschner P (2012) Marschner's mineral nutrition of higher plants, 3rd edn. Elsevier, Amsterdam

Maruyama-Nakashita A, Yumiko N, Yamaya T, Takahashi H (2004) A novel regulatory pathway of sulfate uptake in Arabidopsis roots: implication of CRE1/WOL/AHK4-mediated cytokinin-dependent regulation. Plant J 38:779–789

Mendel RR, Hänsch R (2002) Molybdoenzymes and molybdenum cofactor in plants. J Exp Bot 53:1689–1698

Meyer S, Mumm P, Imes D, Endler A, Weder B, Al-Raseid KAS, Geider D, Marten I, Martinoia E, Hedrich R (2010) *At*ALMT12 represents an R-type anion channel required for stomatal movement in Arabidopsis guard cells. Plant J 63:1054–1062

Negi J, Hashimoto-Sugimoto M, Kusumi K, Iba K (2014) New approaches to the biology of stomatal guard cells. Plant Cell Physiol 55:241–250

Oparka KJ, Santa Cruz S (2000) The great escape: phloem transport and unloading of macromolecules. Annu Rev Plant Physiol Plant Mol Biol 51:323–347

Osawa H, Stacey G, Gassmann W (2006) ScOPT1 and AtOPT4 function as proton-coupled oligopeptide transporters with broad but distinct substrate specificities. Biochem J 393:267–275

Piñeros MA, Cançado GMA, Maron LG, Lyi SM, Menossi M, Kochian LV (2008) Not all ALMT1-type transporters mediate aluminum-activated organic acid responses: the case of ZmALMT1 – an anion-selective transporter. Plant J 53:352–367

Rennenberg H, Herschbach C (2014) A detailed view on sulphur metabolism at the cellular and whole plant level illustrates challenges in metabolite flux analyses. J Exp Bot 65:5711–5724

Rennenberg H, Schmitz K, Bergmann L (1979) Long-distance transport of sulfur in *Nicotiana tabacum*. Planta 147:57–62

Rennenberg H, Schupp R, Glavac V, Jochheim H (1994) Xylem sap composition of beech (*Fagus sylvatica* L.) trees: seasonal changes in the axial distribution of sulfur compounds. Tree Physiol 14:541–548

Rouached H, Wirtz M, Alary R, Hell R, Arpat AB, Davidian J-C, Fourcroy P, Berthomieu P (2008) Differential regulation of the expression of two high-affinity sulfate transporters, *SULTR1.1* and *SULTR1.2*, in Arabidopsis. Plant Physiol 147:897–911

Sakakibara H (2006) Cytokinins: activity, biosynthesis, and translocation. Annu Rev Plant Biol 57:431–449

Sauter JJ, van Cleve B (1994) Storage, mobilization and interrelations of starch, sugars, protein and fat in the ray storage tissue of poplar trees. Trees 8:297–304

Schachtman DP, Goodger JQD (2008) Chemical root to shoot signaling under drought. Trends Plant Sci 13:281–287

Schneider A, Kreuzwieser J, Schupp R, Sauter JJ, Rennenberg H (1994) Thiol and amino acid composition of the xylem sap of poplar trees (*Populus* x *canadensis* 'Robusta'). Can J Bot 72:347–351

Schupp R, Glavac V, Rennenberg H (1991) Thiol composition of xylem sap of beech trees. Phytochemistry 30:1415–1418

Schwarz G, Mendel RR (2006) Molybdenum cofactor biosynthesis and molybdenum enzymes. Annu Rev Plant Biol 57:623–647

Stroock AD, Pagay VV, Zwieniecki MA, Holbrook NM (2014) The physicochemical hydrodynamics of vascular plants. Annu Rev Fluid Mech 46:615–642

Takahashi H, Yamazaki M, Sasakura N, Watanabe A, Leustek T, Engler JA, Engler G, Van Montagu M, Saito K (1997) Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate-starved roots plays a central role in Arabidopsis thaliana. Proc Natl Acad Sci USA 94:11102–11107

Takahashi H, Watanabe-Takahashi A, Smith Frank W, Blake-Kalff M, Hawkesford MJ, Saito K (2000) The roles of three functional sulfate transporters involved in uptake and translocation of sulfate in *Arabidopsis thaliana*. Plant J 23:171–182

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu Rev Plant Biol 62:157–184

Tan Q, Zhang L, Grant J, Cooper P, Tegeder M (2010) Increased phloem transport of S-methylmethionine positively affects sulfur and nitrogen metabolism and seed development in pea plants. Plant Physiol 154:1886–1896

Tyburski J, Tretyn A (2010) Glutathione and glutathione disulfide affect adventitious root formation and growth in tomato seedling cuttings. Acta Physiol Plant 32:411–417

Vernoux T, Wilson RC, Seeley KA, Reichheld J-P, Muroy S, Brown S, Maughan SC, Cobbett CS, Van Montagu M, Inzé D, May MJ, Sung ZR (2000) The *ROOT MERISTEMLESS1/CADMIUM SENSITIVE2* gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. Plant Cell 12:97–109

Werner T, Nehnevajova E, Köllmer I, Novák O, Strnad M, Krämer U, Schmülling T (2010) Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in *Arabidopsis* and tobacco. Plant Cell 22:3905–3920

Wilkinson S, Davies WJ (2002) ABA-based chemical signaling: the co-ordination of responses to stress in plants. Plant Cell Environ 25:195–210

Wilkinson S, Davies WJ (2010) Drought, ozone, ABA and ethylene: new insights from cell to plant community. Plant Cell Environ 33:510–525

Yoshimoto N, Takahashi H, Smith FW, Yamaya T, Saito K (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots. Plant J 29:465–473

Zhang M-Y, Bourbouloux A, Cagnac O, Srikanth CV, Rentsch D, Bachhawat AK, Delrot S (2004) A novel family of transporters mediating the transport of glutathione derivatives in plants. Plant Physiol 134:482–491

GSH Partitioning Between the Nucleus and Cytosol in *Arabidopsis thaliana*

Ambra De Simone, Yingping Dong, Pedro Diaz Vivancos, and Christine H. Foyer

Abstract The thiol tripeptide, glutathione (GSH) is an essential redox metabolite in plant cells but little information is available concerning GSH partitioning between the cytosol and nucleus. In this article we discuss the evidence concerning the distribution of GSH between the nucleus and the cytosol. The glutathione redox potential was similar in the nucleus and cytosol of developing radicles of *Arabidopsis thaliana* seeds after germination. However, in the arrested embryonic root meristem of the *root meristemless 1* (*rml1)* mutant that have less than 5 % GSH of the wild type, GSH was predominantly localised in the nuclei. This was also the case in wild type roots treated with the auxin transport inhibitor, *N*-1-napthylphthalamic acid (NPA), which have decreased root glutathione levels. GSH was co-localised with nuclear DNA at G1 and G2 in *A. thaliana* cultures in which the cell cycle was synchronised. The functions of GSH are considered in terms of cell cycle regulation and the regulation of gene expression.

Introduction

Reduced glutathione (γ-glutamyl cysteinyl glycine; GSH) is an important metabolite in plants with essential roles in development and survival (Maughan and Foyer 2006; Noctor et al. 2011). GSH is synthesised from component amino acids in a two-step pathway. The first reaction is catalysed by γ-glutamyl cysteine synthetase (γ-ECS). This involves ATP-dependent amide bond formation between the cysteine and the carboxylate side chain of glutamic acid to form γ-glutamyl cysteine (γ-EC).

A. De Simone • C.H. Foyer (✉)
Centre for Plant Sciences, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK
e-mail: c.foyer@leeds.ac.uk

Y. Dong
Institute of South China Karst, Guizhou Normal University, 550001 Guiyang, China

P.D. Vivancos
CEBAS-CSIC, Department of Plant Breeding, Campus de Espinardo, P.O. Box 164, 30100 Murcia, Spain

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*, Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_4

The second step is catalysed by glutathione synthetase (GSH-S; Rennenberg 1982; Meister 1988; Noctor et al. 2002; Mullineaux and Rausch 2005; Wachter et al. 2005), which adds glycine on to γ-EC producing GSH. *Arabidopsis thaliana* knockouts for either of the synthetic enzymes results in lethal phenotypes. Knockout expression of *GSH1*, which encodes γ-ECS, causes lethality at the embryo stage (Cairns et al. 2006), whereas knockouts for *GSH2*, encoding GSH-S, produces a seedling-lethal phenotype (Pasternak et al. 2008). There are a number of *A. thaliana* genotypes with less severe mutations in the *GSH1* gene that result in lower levels of GSH deficiency relative to the wild type. The *rootmeristemless1 (rml1)* mutant, which contains less than 5 % of wild-type GSH is characterised by an arrest of growth, particularly in the roots (Vernoux et al. 2000). The *zinc tolerance induced by iron 1* (*zir1)* mutant, which contains only about 15 % of wild-type GSH is affected in Fe-mediated Zn-tolerance (Shanmugam et al. 2012), while the cadmium-sensitive 2 (*cad2*) mutant that has about 30 % of wild-type GSH shows enhanced sensitivity to cadmium (Howden et al. 1995; Cobbett et al. 1998).

Several lines of evidence demonstrate that GSH is required for the activation and maintenance cell division in the postembryonic *A. thaliana* root. Firstly, the *rootmeristemless1 (rml1)* mutant is unable to establish an active post-embryonic meristem in the root apex (Vernoux et al. 2000). Similarly, root growth is severely inhibited in the presence of the GSH-synthesis inhibitor buthionine sulfoximine (BSO) (Vernoux et al. 2000; Maughan et al. 2010). Thirdly, the *miao* mutant, which is defective in the chloroplast form of glutathione reductase (GR), the enzyme that catalyses the reduction of glutathione disulfide (GSSG) to GSH, displays strong root apical meristem (RAM) defects and root growth inhibition (Yu et al. 2013). A reducing environment within the chloroplasts was required for regulation of PLETHORA (PLT), a master regulator of root development (Yu et al. 2013).

Plants cells maintain a redox potential of approximately −310 mV in the cytosol (Schnaubelt et al. 2015). This value is considered to be critical for catalytic redox reactions involving electron transfer and thiol chemistry. Cellular redox homeostasis is maintained by GSH, glutaredoxins (Grx), thioredoxins (Trxs) and their light- or NADPH-dependent reductases. Together with ascorbate, these systems perform a buffering function against the oxidation generated by metabolism and during environmental stresses. The *rml1* mutants can produce leaves and flowers suggesting that progression through the cell cycle is not seriously affected by GSH depletion in the shoot apical meristem (Cheng et al. 1995). Crosses involving *rml1* and Arabidopsis mutants lacking two of the NADPH-thioredoxin reductase (NTR) genes (*ntra, ntrb)* produced a shoot meristemless phenotype (Reichheld et al. 2007), demonstrating an interplay between glutathione and reduced thioredoxin in the control of shoot meristem development. When the *ntra, ntrb* double mutants were crossed with the *cad2* mutants, the resultant triple mutants developed in the same manner as the wild type up to the rosette stage but were thereafter unable to sustain normal floral meristem development (Bashandy et al. 2010).

GSH has important functions in the nucleus (García-Giménez et al. 2013). GSH is localised in the nucleus during the proliferation of *Arabidopsis* cells in culture (Pellny et al. 2009; Diaz Vivancos et al. 2010a). The pattern of GSH co-localisation with nuclear DNA was similar to that previously described in mammalian cells

(Markovic et al. 2007; Pallardó et al. 2009). In both cases, high GSH levels in the nucleus were observed when the highest proportion of cells is in the G1 phase of the cell cycle (Markovic et al. 2007; Diaz Vivancos et al. 2010a).

Nuclear Glutathione in Intact Roots

Relatively little attention has been paid to the pool of GSH in the nucleus largely because small molecules like GSH can diffuse freely between the nucleus and cytosol through the nuclear pore complex (García-Giménez et al. 2013). However, GSH is always present in the nucleus in plant cells (Zechmann et al. 2008) and it accumulates in the nucleus during cell proliferation (Markovic et al. 2007; Pallardo et al. 2009; Diaz-Vivancos et al. 2010a, b). Several methods have been developed to monitor glutathione in living cells (Noctor et al. 2011, 2012). The glutathione redox potential of plant cells can be measured using redox-sensitive green fluorescent protein (roGFP; Meyer et al. 2007). The glutathione redox potentials of the cytosol (Fig. 1c) and nuclei (Fig. 1b) were similar and constant in growing regions of the developing radicles after germination (Fig. 1a). The relative contents of GSH in the

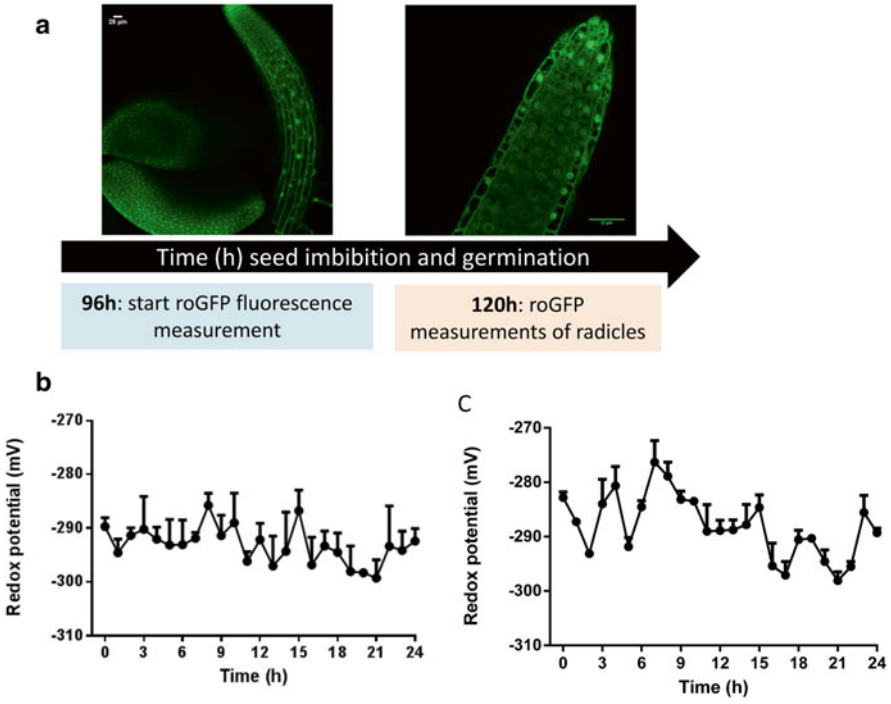


Fig. 1 Confocal microscopy images of of ro-GFP in the developing radicles of Arabidopsis seeds after germination (**a**) and the glutathione redox potentials of the nuclei (**b**) and cytosol (**c**) measured by ro-GFP. Measurements of roGFP fluorescence were performed over a 24 h period beginning 96 h after germination

nucleus and cytoplasm have also been measured in the developing lateral root meristem (Diaz-Vivancos et al. 2010a) by confocal microspcopy using a double staining procedure. Hoechst 33342 (Hoechst) was used to localise nuclear DNA (blue stain) and CellTracker green 5-chloromethylfluorescein diacetate (CMFDA; green stain) was used to detect GSH, as previously described (Markovic et al. 2007; Diaz-Vivancos et al. 2010a).

Lateral root initiation is promoted by auxin, which stimulates the G1-S transition in the xylem pericycle (Himanen et al. 2002). Lateral root meristem formation consists of two phases: pericycle activation and meristem establishment. Auxin induces the first asymmetrical, anticlinal division of the pericycle cells located adjacent to the xylem poles. The cyclin-dependent kinase inhibitor KRP2 is down-regulated following the addition of auxin (Himanen et al. 2002). GSH was localised in the nucleus during the first divisions of the pericycle cells leading to the formation of the lateral root primordium (Diaz-Vivancos et al. 2010a; Fig. 2a, b). After emergence, the green staining was distributed uniformly throughout the cells, particularly in the expansion zone (Fig. 2c). The distribution of GSH between the cytosol and nuclei in the cells of the lateral root (Fig. 2c) is similar to that of the primary

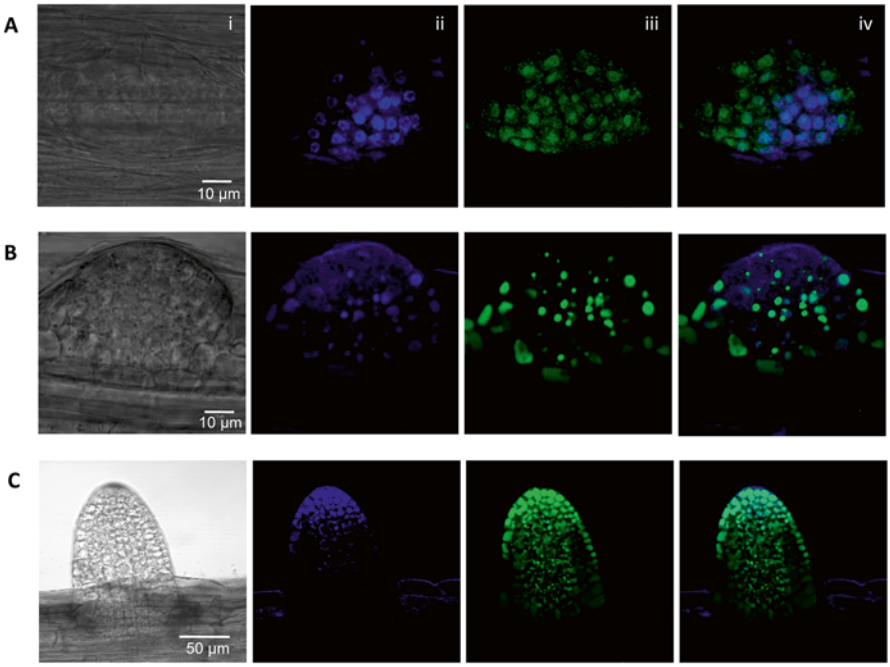


Fig. 2 Confocal microscopy images of nuclear and GSH staining during the formation of the lateral root primordium in *Arabidopsis* seedlings. The dividing pericycle cells are illustrated in **a**; the cells in newly emerged lateral root primordium are illustrated in **b**, while cells in the post-emergence primordium are illustrated in **c** Light microscopy images (*i*); staining with Hoechst 33342 (*blue*; *ii*) and CellTracker green 5-chloromethylfluorescein diacetate (*green*; *iii*); over-laid images of *blue* (nuclei) and *green* (GSH) staining (*iv*)

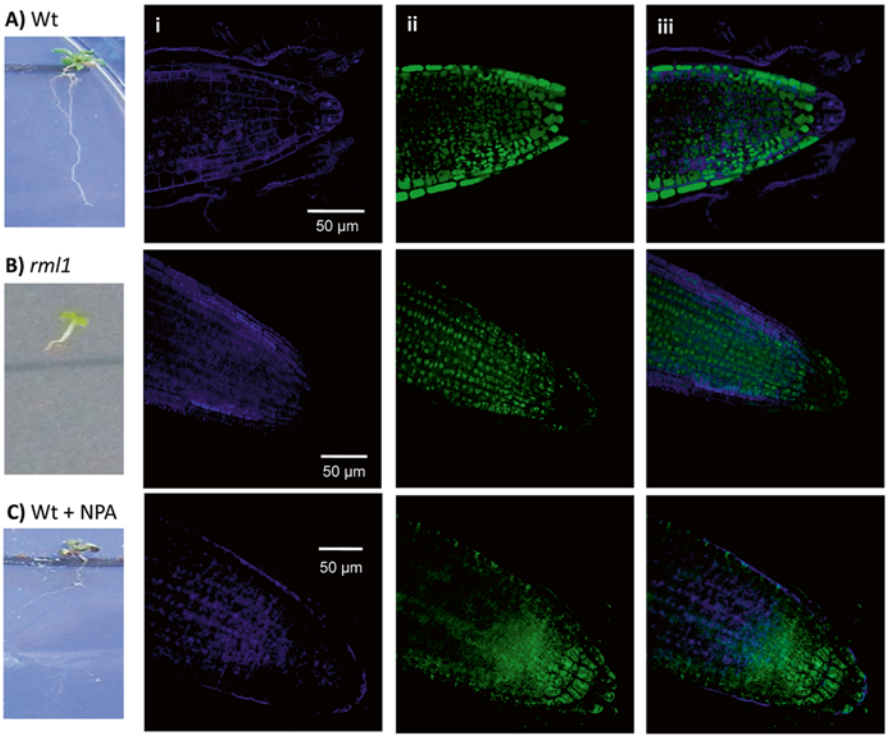


Fig. 3 A comparison of the intracellular distribution of GSH in the primary roots of the *Arabidopsis* wild type (Wt; **a**), the *rml1* mutant (**b**); and in the wild type treated with the auxin inhibitor *N-1-napthylphthalamic* acid (NPA; **c**). NPA treatment was performed in PANG2 medium containing 5 mM NPA for 4 days. Confocal microscopy images of the primary roots of the *Arabidopsis* seedlings stained with Hoechst 33342 (*blue*; *i*) and CellTracker green 5-chloromethylfluorescein diacetate (*green*; *ii*); over-laid images of blue (nuclei) and *green* (GSH) staining (*iii*)

root, as measured by the double staining procedures (Fig. 3a) and by ro-GFP (Fig. 1a). In contrast to the wild type (Fig. 3a), the roots of GSH-deficient *rml1* mutant (Fig. 3b) were only weakly stained by CMFDA (green stain), consistent with the depletion of the GSH pool in these mutants. However, the remaining GSH detected by the staining in the *rml1* roots was localised mainly in the nuclei of the cells, particularly in the cortex, endodermis, pericycle and vascular tissues of the primary roots (Fig. 3b).

Interplay Between Auxin and Intracellular Distribution of GSH

Literature evidence points to a link between glutathione and auxin metabolism and transport. For example, alterations in auxin abundance and the transport of auxin were suggested in the disrupted floral meristem of the *ntra, ntrb, cad2* triple mutants

(Bashandy et al. 2010) and also in the impaired pollen germination and pollen tube growth caused by glutathione depletion (Zechmann et al. 2011). Similarly, BSO-dependent inhibition of root growth was linked to decreased auxin transport (Koprivova et al. 2010). These findings suggest that redox mechanisms could be involved in the regulation of the expression of PIN proteins and auxin transport in shoot and root meristem development (Bashandy et al. 2010; Koprivova et al. 2010).

We have previously shown that the addition of the auxin transport inhibitor, *N-1-napthylphthalamic* acid (NPA), which blocks auxin transport from the root tip and causes auxin accumulation in the meristem (Himanen et al. 2002), had no effect on the ratio of GSH to glutathione disulphide (GSSG) in Arabidopsis roots (Schnaubelt et al. 2015). However, the total glutathione (GSH plus GSSG) pool was significantly decreased in the roots by NPA (Schnaubelt et al. 2015). These finding suggests a link between the level of glutathione and the presence of auxin in NPA treated roots. In agreement with this conclusion, the level of CMFDA staining was much lower in NPA-treated roots (Fig. 3c) than controls (Fig. 3a). Moreover, the green staining was largely localised in the nuclei of the NPA-treated roots. The intensity of the CMFDA staining was greatest in the tissues immediately behind the root tip (Fig. 3c). A transcriptome analysis of proliferating Arabidopsis cells (Diaz-Vivancos et al. 2010a) showed that the abundance of transcripts encoding auxin-related proteins such as AXR3 and AXR5, and PIN1, was higher when GSH was largely localised in the nucleus than when GSH was distributed evenly between the nucleus and the cytoplasm (Table 1). These findings suggest that the presence of GSH in the

Table 1 Transcripts encoding auxin-related proteins with fold change >2 in abundance when GSH is largely localised in the nucleus relative to when GSH is distributed evenly between the nucleus and cytoplasm (Diaz-Vivancos et al. 2010a)

Probe ID	Accession number	Gene name	Protein description
245076_at	At2g23170	GH3.3	Indole-3-acetic acid amido synthetase activity, involved in auxin homeostasis, response to auxin stimulus
245397_at	At4g14560	AXR5	Transcription factor activity, involved in response to auxin stimulus
248528_at	At5g50760	MFB16.16	Auxin-responsive family protein
253253_at	At4g34750	F11/11.5	Calmodulin binding, involved in response to auxin stimulus
258402_at	At3g15450	MJK13.11	Hormone metabolism, auxin responsive protein
259845_at	At1g73590	PIN1	Encodes an auxin efflux carrier involved in shoot and root development
263664_at	At1g04250	AXR3	Transcription regulator acting as repressor of auxin-inducible gene expression
267380_at	At2g26170	MAX1	Encodes a protein with similarity to thromboxane-A synthase, member of the CYP711A cytochrome P450 family, specific repressor of vegetative axillary buds generated by the axillary meristem

nucleus may promote the expression of genes involved in auxin transport and signalling. Similarly, the abundance of a large number of transcripts encoding auxin-associated proteins were changed in *rml1* roots (Schnaubelt et al. 2015). In particular, transcripts encoding PIN5 and AXR3 were decreased in *rml1* relative to the wild-type (Schnaubelt et al. 2015).

Nuclear GSH in Arabidopsis Cells Synchronously Dividing in Culture

We have previously reported the relative abundance of GSH in the nucleus and cytosol of Arabidopsis cells growing in culture without synchronisation using the CMFDA and Hoechst double staining technique (Pellny et al. 2009; Diaz-Vivancos et al. 2010a). The partitioning of GSH between the nucleus and the cytosol was also measured in cultures, in which synchronisation of the cell cycle was achieved by first growing the cells in sucrose-free media supplemented with aphidicolin (Fig. 4). In these studies, the early stationary phase cell suspensions (Pellny et al. 2009) were washed via vacuum-assisted filtration (Nalgene disposable filter unit, 0.2 μm pore size) with Murashige and Skoog (MS) media containing 0.5 mg l^{-1} 1-naphthalene acetic acid (NAA) and 0.05 mg l^{-1} kinetin but lacking sucrose (MS-sucrose medium), as described by. Aliquots (50 ml) of washed cell suspensions were transferred to

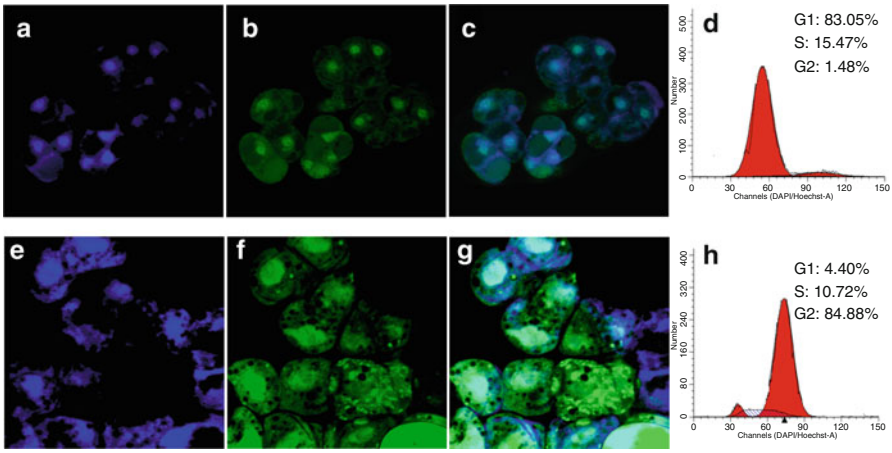
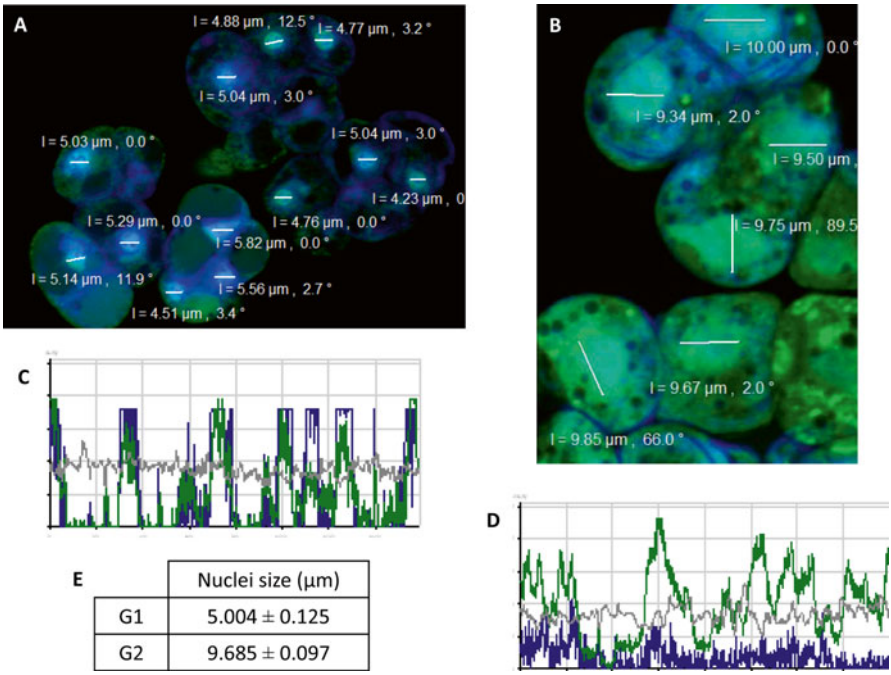


Fig. 4 Localisation of GSH in the nucleus of Arabidopsis cells at the G1 and G2 phases of the cell cycle. Staining with Hoechst 33342 was used to localise nuclei (*blue*; **a**, **c**, **e**, **g**) while staining with CellTracker green 5-chloromethylfluorescein diacetate (*green*; **b**, **c**, **f**, **g**) was used to detect GSH. Staining procedures were performed on living cells at the G1 (**d**) and G2 (**h**) phases of the cell cycle. Images **c** and **g** show the over-laid *blue* and *green* images

250 ml flasks, filtered and washed with MS-sucrose medium. The cells were then suspended in a final volume of 200 ml of MS-sucrose medium. Cells were incubated in light conditions at 22 °C at 120 rpm. Aphidicolin (0.4 μg ml^{-1}) was added to the media after 12 h and the cells were incubated for a further 12 h. Cells were then washed with MS-sucrose medium as above and then suspended in growth media containing MS salts, 0.5 mg l^{-1} NAA, 0.05 mg l^{-1} kinetin and 3 % w/v sucrose. The blue staining in the nuclei with Hoechst shown in Fig. 4a, e and the green CMFDA staining (Fig. 4b, f) were co-incident (Fig. 4c, g) when the majority of Arabidopsis cells were at the G1 phase (Fig. 4d). Similarly, the majority of the cellular GSH pool was co-localised with nuclear DNA at the G2 phase (Fig. 4h) of the cell cycle. Cell cycle phases were determined by flow cytometry as previously described in Diaz-Vivancos et al. (2010a). In addition, the peak area of the blue stain and that of the green stains were co-incident across the cells at G1 (Fig. 5a, c). However, at G2 (Fig. 5b), where the diameter of the nuclei was approximately twice the value measured at G1 (Fig. 5e), the percentage of the total cellular GSH pool that was associated with the nuclei was slightly decreased (by about 30 %).



E

	Nuclei size (μm)
G1	5.004 ± 0.125
G2	9.685 ± 0.097

Fig. 5 Co-localisation of GSH and nuclear DNA in Arabidopsis cells at the G1 (**a**) and G2 (**b**) phases of the cell cycle. Double staining with Hoechst 33342 to detect nuclear DNA (*blue*; **a**, **b**) and CellTracker green 5-chloromethylfluorescein diacetate (*green*; **a**, **b**) was used to detect GSH and to determine the relative distribution of GSH (*green lines*) between the nuclei (*blue lines*) and cytoplasm across the cells at G1 (**c**) and G2 (**d**). In **c** and **d**, the *grey line* indicates the background signal across the cells. The diameter of the nuclei was measured at both phases as illustrated on (**a**) and (**b**), and the average diameter for each stage is given in (**e**)

Conclusions and Perspectives

Of the many functions of GSH in plants, the maintenance of cellular redox homeostasis and mediation of redox signalling that controls growth, development and defence, are perhaps the most important (Noctor et al. 2011; Han et al. 2013a, b). GSH also has functions in the redox regulation of the cell cycle in animals and plants (Potters et al. 2002; Burhans and Heintz 2009). The finding that GSH is recruited into the nucleus at G1 (Pellny et al. 2009; Diaz-Vivancos et al. 2010a, b), which is shown in Fig. 5, suggests that GSH fulfils important functions in the nucleus during the cell cycle. In animals, the oxidation-dependent activation of mitogenic pathways controls the activity of cyclin-dependent kinases (CDKs) and phosphorylation of the retinoblastoma protein (pRB), thereby regulating S-phase entry (Menon et al. 2003; Menon and Goswami 2007; Burhans and Heintz 2009). Very low GSH levels, as found in the *rml1* mutants, decreased the abundance of transcripts encoding many core cell cycle components in the roots but not in the shoots (Schnaubelt et al. 2015). The transcripts that were decreased in abundance in *rml1* roots encode cyclins (CYCs) and CDKs that are necessary for the G2 to M transition including *Knolle*, *AtAUR1*, *AtAUR2*, *CKS2*, *PROLIFERA*, *ATBS1*, *BUBR1*, *OSD1* (*UVI4-Like*), *PCNA1*, *MITOTIC ARREST-DEFICIENT* (*MAD*)*2* (Schnaubelt et al. 2015).

A number of studies have linked low GSH levels to impaired auxin transport and signalling (Bashandy et al. 2010; Kopriva et al. 2010). Further evidence for a link between GSH levels and auxin was shown by Schnaubelt et al. (2015). The abundance of transcripts encoding transcription factors such as *MYB15* and *MYB75*, and transcripts involved in growth regulation such as *IAA17/AXR3*, *IAA20*, *SPT*, *HEC1* and *RSM1* were decreased in the *rml1* mutants relative to the wild type (Schnaubelt et al. 2015). However, with the exception of *PIN5,* the abundance of transcripts encoding PIN transporters was similar in the *rml1* mutants relative to the wild type (Schnaubelt et al. 2015). Figure 3b shows much of the available GSH is localised in the nuclei in the roots of *rml1* mutants in which the cell cycle has arrested before G2 to M transition (Schnaubelt et al. 2015), This finding is consistent with observations on 3T3 fibroblasts treated with BSO, which show that the GSH pool in the nucleus was more resistant to depletion compared to the cytosol (Markovic et al. 2009).

The data presented here show that GSH is present in the nucleus and that glutathione redox potential is similar in the cytosol and nuclei in the developing embryonic root of Arabidopsis seeds after germination. However, when the cellular GSH is low, as is found in the *rml1* mutants or in the wild type following NPA treatment, the nucleus is better able to retain GSH than the cytosol. GSH is also abundant in the nucleus early in cell proliferation. While the concept that cellular glutathione homeostasis is crucial to cell functions and defence is well accepted, relatively little attention has been paid to nuclear GSH. The roles of GSH in the nucleus remain to be identified particularly during the cell cycle. In the light of the evidence presented here, the functions of nuclear GSH are likely to receive increasing attention and interest in future years.

Acknowledgements This work was funded by EU FP7: KBBE-2012-6-311840 (ECOSEED: ADS). PDV acknowledges the CSIC and the Spanish Ministry of Economy and Competitiveness for his 'Ramon & Cajal' research contract, co-financed by FEDER funds. YPD thanks the China Scholarship Council (China) for a scholarship.

References

Bashandy T, Guilleminot J, Vernoux T, Caparros-Ruiz D, Ljung K, Meyer Y, Reichheld JP (2010) Interplay between the NADP-linked thioredoxin and glutathione systems in Arabidopsis auxin signaling. Plant Cell 22:376–391

Burhans WC, Heintz NH (2009) The cell cycle is a redox cycle: linking phase-specific targets to cell fate. Free Radic Biol Med 47:1282–1293

Cairns NG, Pasternak M, Wachter A, Cobbett CS, Meyer AJ (2006) Maturation of Arabidopsis seeds is dependent on glutathione biosynthesis within the embryo. Plant Physiol 141:446–455

Cheng JC, Seeley KA, Sung ZR (1995) RML1 and RML2, Arabidopsis genes required for cell-proliferation at the root-tip. Plant Physiol 107:365–376

Cobbett CS, May MJ, Howden R, Rolls B (1998) The glutathione-deficient, cadmium-sensitive mutant, cad2-1, of *Arabidopsis thaliana* is deficient in gamma-glutamylcysteine synthetase. Plant J 16:73–78

Diaz-Vivancos P, Dong Y-P, Ziegler K, Markovic J, Pallardó FV, Pellny TK, Verrier P, Foyer CH (2010a) Recruitment of glutathione into the nucleus during cell proliferation adjusts whole cell redox homeostasis in *Arabidopsis thaliana* and lowers the oxidative defence shield. Plant J 64:825–838

Diaz-Vivancos P, Wolff T, Markovic J, Pallardó FV, Foyer CH (2010b) A nuclear glutathione cycle within the cell cycle. Biochem J 431:169–178

García-Giménez JL, Markovic J, Dasí F, Queval G, Schnaubelt D, Foyer CH, Pallardó FV (2013) Nuclear glutathione. Biochim Biophys Acta 1830:3304–3316

Han Y, Chaouch S, Mhamdi A, Queval G, Zechmann B, Noctor G (2013a) Functional analysis of *Arabidopsis* mutants points to novel roles for glutathione in coupling H_2O_2 to activation of salicylic acid accumulation and signaling. Antioxid Redox Signal 18:2106–2121

Han Y, Mhamdi A, Chaouch S, Noctor G (2013b) Regulation of basal and oxidative stress-triggered jasmonic acid-related gene expression by glutathione. Plant Cell Environ 36:1135–1146

Himanen K, Boucheron E, Vanneste S, de Almeida EJ, Inzé D, Beeckman T (2002) Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell 14:2339–2351

Howden R, Andersen CR, Goldsbrough PB, Cobbett CS (1995) A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. Plant Physiol 107:1067–1073

Koprivova A, Mugford ST, Kopriva S (2010) Arabidopsis root growth dependence on glutathione is linked to auxin transport. Plant Cell Rep 29:1157–1167

Markovic J, Borrás C, Ortega A, Sastre J, Viña J, Pallardó FV (2007) Glutathione is recruited into the nucleus in early phases of cell proliferation. J Biol Chem 282:20416–20424

Markovic J, Mora NJ, Broseta AM, Gimeno A, de-la-Concepción N, Viña J, Pallardó FV (2009) Nuclear GSH depletion impairs cell proliferation in 3T3 fibroblasts. PLoS One 29, e6413

Maughan S, Foyer CH (2006) Engineering and genetic approaches to modulating the glutathione network in plants. Physiol Plant 126:382–397

Maughan SC, Pasternak M, Cairns N, Kiddle G, Brach T, Jarvisb R, Haasc F, Nieuwlanda J, Limb B, Müllerc C, Salcedo-Sorae E, Krusec C, Orseld M, Hellc R, Millerd AJ, Braye P, Foyer CH, Murray JAH, Meyerc AJ, Cobbettb CS (2010) Plant homologs of the *Plasmodium falciparum* chloroquine-resistance transporter, *Pf*CRT, are required for glutathione homeostasis and stress responses. Proc Natl Acad Sci U S A 107:2331–2336

Meister A (1988) Glutathione metabolism and its selective modification. J Biol Chem 263:17205–17208

Menon SG, Goswami PC (2007) A redox cycle within the cell cycle: ring in the old with the new. Oncogene 26:1101–1109

Menon SG, Sarsour EH, Spitz DR, Higashikubo R, Sturm M, Zhang H, Goswami PC (2003) Redox regulation of the G1 to S phase transition in the mouse embryo fibroblast cell cycle. Cancer Res 63:2109–2117

Meyer AJ, Brach T, Marty L, Kreye S, Rouhier N, Jacquot JP, Hell R (2007) Redox-sensitive GFP in Arabidopsis thaliana is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer. Plant J 52:973–986

Mullineaux PM, Rausch T (2005) Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. Photosynth Res 86:459–474

Noctor G, Gomez L, Vanacker H, Foyer CH (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signaling. J Exp Bot 53:1283–1304

Noctor G, Queval G, Mhamdi A, Chaouch S, Foyer CH (2011) Glutathione. In: Millar H (ed) The Arabidopsis book 9. American Society of Plant Biologists, Rockville, pp 1–32

Noctor G, Mhamdi A, Queval G, Chaouch S, Han Y, Neukermans J, Foyer CH (2012) Glutathione functions in plants: an integrated overview. Plant Cell Environ 35:454–484

Pallardó FV, Markovic J, Garcia JL, Viña J (2009) Role of nuclear glutathione as a key regulator of cell proliferation. Mol Asp Med 30:77–85

Pasternak M, Lim B, Wirtz M, Hell R, Cobbett CS, Meyer AJ (2008) Restricting glutathione biosynthesis to the cytosol is sufficient for normal plant development. Plant J 53:999–1012

Pellny TK, Locato V, Vivancos PD, Markovic J, De Gara L, Pallardo FV, Foyer CH (2009) Pyridine nucleotide cycling and control of intracellular redox state in relation to poly (ADP-ribose) polymerase activity and nuclear localisation of glutathione during exponential growth of Arabidopsis cells in culture. Mol Plant 2:442–456

Potters G, De Gara L, Asard H, Horemans H (2002) Ascorbate and glutathione: guardians of the cell cycle, partners in crime? Plant Physiol Biochem 40:537–548

Reichheld JP, Khafif M, Riondet C, Droux M, Bonnard G, Meyer Y (2007) Inactivation of thioredoxin reductases reveals a complex interplay between thioredoxin and glutathione pathways in Arabidopsis development. Plant Cell 19:1851–1865

Rennenberg H (1982) Glutathione metabolism and possible biological roles in higher-plants. Phytochemistry 21:2771–2781

Schnaubelt D, Queval G, Dong Y, Diaz-Vivancos P, Makgopa ME, Howell G, De Simone A, Bai J, Hannah MA, Foyer CH (2015) Low glutathione regulates gene expression and the redox potentials of the nucleus and cytosol in *Arabidopsis thaliana*. Plant Cell Environ. 38:266–279. doi:10.1111/pce.12252

Shanmugam V, Tsednee M, Yeh KC (2012) Zinc tolerance induced by iron 1 reveals the importance of glutathione in the cross-homeostasis between zinc and iron in *Arabidopsis thaliana*. Plant J 69:1006–10017

Vernoux T, Wilson RC, Seeley KA, Reichheld JP, Muroy S, Brown S, Maughan SC, Cobbett CS, Van Montagu M, Inze D, May MJ, Sung ZR (2000) The root meristemless1/cadmium sensitive2 gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. Plant Cell 12:97–110

Wachter A, Wolf S, Steininger H, Bogs J, Rausch T (2005) Differential targeting of GSH1 and GSH2 is achieved by multiple transcription initiation: implications for the compartmentation of glutathione biosynthesis in the Brassicaceae. Plant J 41:15–30

Yu X, Pasternak T, Eiblmeier M, Ditengou F, Kochersperger P, Sun J, Wang H, Rennenberg H, Teale W, Paponov I, Zhou W, Li C, Li X, Palmea K (2013) Plastid-localized glutathione reductase 2–regulated glutathione redox status is essential for Arabidopsis root apical meristem maintenance. Plant Cell 25:4451–4468

Zechmann B, Mauch F, Sticher L, Müller M (2008) Subcellular immunocytochemical analysis detects the highest concentrations of glutathione in mitochondria and not in plastids. J Exp Bot 59:4017–4027

Zechmann B, Koffler BE, Russell SD (2011) Glutathione synthesis is essential for pollen germination in vitro. BMC Plant Biol 11:54

Sulfur Metabolism in Hemiascomycetes Yeast

Jean-Marie Beckerich, Sophie Landaud, Djamila Onésime, and Agnès Hébert

Abstract Sulfur metabolism is a central function of the cell. It has been extensively studied in the model yeast *Saccharomyces cerevisiae*. A comparative genomic study carried out across the hemiascomycetes clade has shown that *S. cerevisiae* displayed specificities not shared by the other yeast species. For instance, an *O*-acetylserine pathway was shown to be present in many yeast species. The complex regulatory pathway seems also to be conserved, with the exception of MET28, whose presence seems to be restricted to *S. cerevisiae* and related species. In order to explore this pathway in two distant yeast species, *Kluyveromyces lactis* and *Yarrowia lipolytica*, transcriptomic and metabolomic studies have been carried out in different conditions of sulfur supply. These high-throughput techniques allowed confirmation of the data of the comparative genomics but also the investigation of new components and new functions linked to sulfur metabolism, for instance, the role of the O-acetylserine pathway in cysteine biogenesis and the role of the aminotransferases in the degradation of methionine were confirmed. The screening of the pools of metabolic intermediates affected by the sulfur supply allowed the identification of new components of the pathway in *Y. lipolytica* such as taurine and hypotaurine, which seemed to play a role of sulfur storage. These methods also allowed the identification of the set of transporters involved in sulfur metabolism. Eventually, the comparison of these results with the data accumulated in the model *S. cerevisiae* highlighted the large-scale conservation of this pathway but also the large diversity in the regulated steps inside the pathway.

J.-M. Beckerich (✉) • D. Onésime • A. Hébert
UMR MICALIS, INRA, AgroParisTech, CBAI, BP 01, 78850 Thiverval Grignon, France
e-mail: jean-marie.beckerich@grignon.inra.fr

S. Landaud
UMR GMPA, INRA, AgroParisTech, CBAI, BP01, 78850 Thiverval Grignon, France

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_5

Introduction

The clade of the hemiascomycetes (Saccharomycotina) is a large subphylum of the Ascomycota fungi. It consists of more than 1,000 species. They are mainly unicellular microorganisms although some of them are dimorphic. They display a large range of sizes from several μ^3 to more than 200 μ^3 (for instance, *Geotrichum candidum*). More specifically, they have been shown to use a large panel of sulfur compounds for growth, with the reference species, *Saccharomyces cerevisiae*, being one of the less efficient (Linder 2012). In our study, we will consider four species scattered along the evolutionary tree (Fig. 1): *S. cerevisiae* which is probably the

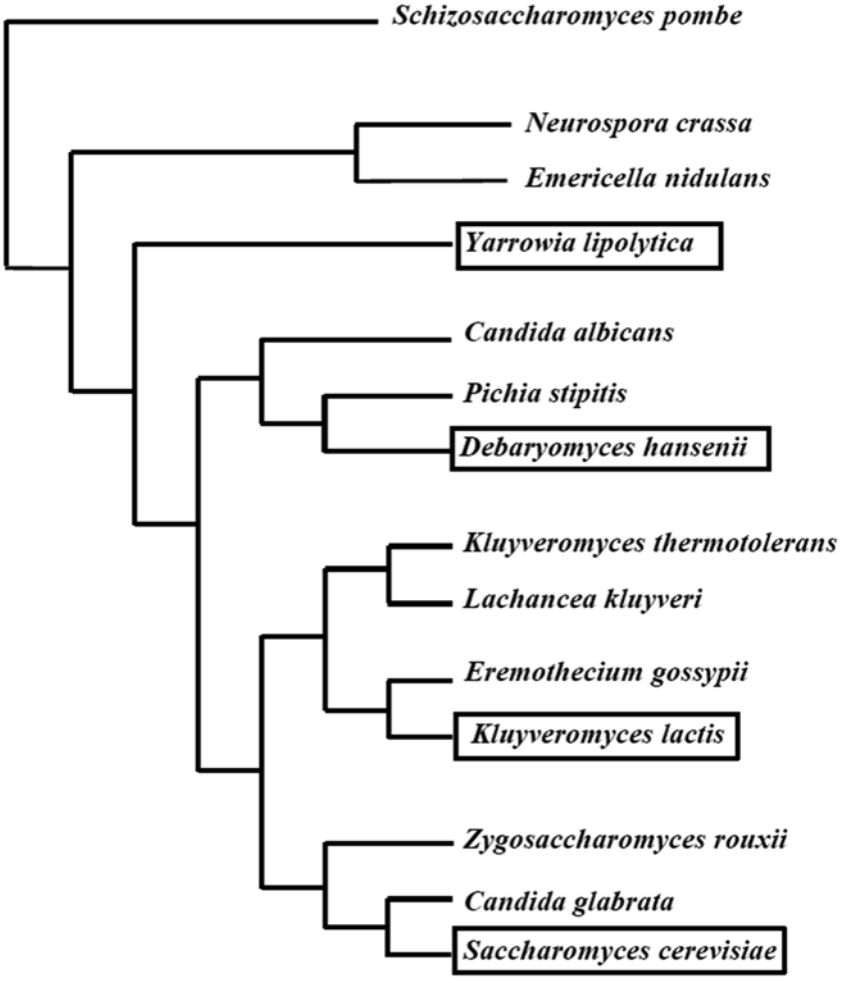


Fig. 1 Evolutionary tree of the hemiascomycetous yeasts adapted from Dujon (2006). The species of interest are *boxed*. The relative position of ascomycetous fungi and of *Schizosaccharomyces pombe* is shown

eukaryotic organism most extensively known with more than 80 % of its protein encoding genes having a functional characterization. *K. lactis* is a unicellular yeast relatively closely related to the *Saccharomyces* sensu *lato* clade but before the whole genome duplication event undergone by the members of this clade. *Debaryomyces hansenii* belongs to the GTC clade, which uses a modified genetic code with the CUG codon used as a serine codon. *Yarrowia lipolytica* is a dimorphic yeast at the root of the evolutionary tree. All these yeast species belong to the cheese ecosystem (for instance, Cholet et al. 2007). They have therefore an adaptation to this environment, osmotolerance to brine, and an ability to use the components of the curd (proteins, lipids and lactate). Lactose was eliminated by the growth of lactic acid bacteria during preparation of the curd. They contribute to the flavor of the cheeses, especially by the production of volatile sulfur compounds. Their genomes have been sequenced and annotated (Dujon et al. 2004). For *K. lactis* and *Y. lipolytica*, a range of genetic tools have been developed. In our laboratory, we have set up orfeomic DNA microarrays for these three species. Advances in metabolomics technologies (for review, Godat et al. 2010) have enabled the identification of metabolites and their quantification.

Using these tools, we have studied by transcriptomics and metabolomics the sulfur metabolism in these yeasts in comparison with the reference species, *S. cerevisiae*. These studies demonstrated conservation and divergences in this central part of the cell metabolism.

Sulfurmetabolism is very central to the functioning of the living cell (Fig. 2). It is involved in many different aspects of the cell metabolism. It plays an obvious role in the synthesis of two amino acids, cysteine and methionine and is also involved in the synthesis of thiol-containing compounds, which are important in the cell redox homeostasis and in the synthesis of iron-sulfur proteins. For instance, in the work of Petti et al. (2012), the pathways controlled by sulfur availability included cysteine and methionine biosynthetic processes, allantoin catabolic pathway, iron ion homeostasis, response to copper ion, electron transport chain (through probably the need for Fe/S proteins), ion transport, transmembrane transport, glutamate biosynthesis, redox processes, deoxyribonucleoside diphosphate process, autophagy, and chromatin organization (see also McIsaac et al. 2012). The work of Fauchon et al. (2002) has highlighted the importance of the sulfur assimilation for the cell by showing that during sulfur starvation, a new set of proteins was induced which had a lower content in sulfur amino acids than the bulk of proteins translated in normal conditions.

Genomic Studies

Firstly a genomic approach was carried out. The homologues of the genes involved in the biosynthesis of the sulfur amino acids were searched in the genomes. The genes identified in the different yeast species were named using the *S. cerevisiae* nomenclature (Hébert et al. 2011a). This pathway appeared to be highly conserved and it was easy to record homologous genes in all the scanned genomes. However

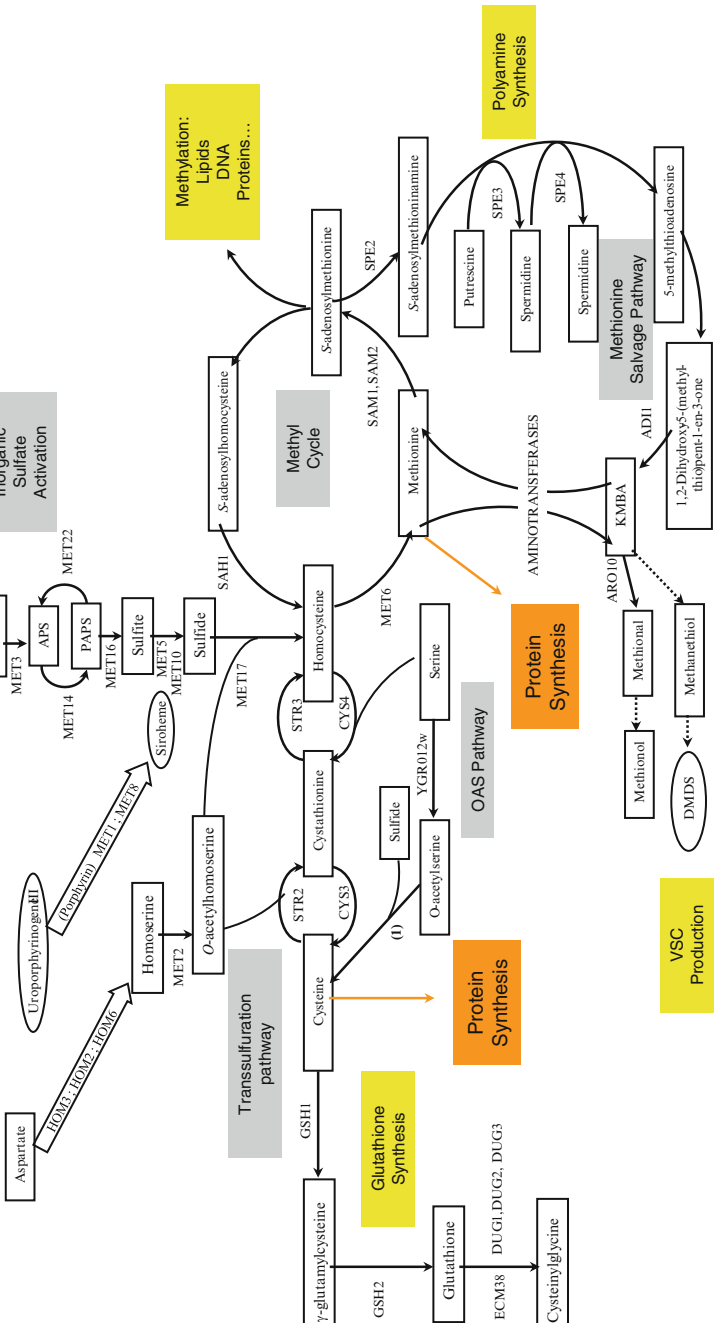


Fig. 2 General schematic of the sulfur metabolism in the hemiascomycetous yeasts (Adapted from A. Hébert, PhD thesis). The gene nomenclature in *S. cerevisiae* has been used

this work highlighted the existence of an *O*-acetyltransferase in the non-Saccharomyces species, opening up the possibility of an *O*-acetylserine (OAS) pathway for cysteine biosynthesis, as already described in *Schizosaccharomyces pombe* (Baudoin-Cornu and Labarre 2006). Evidence for a metabolism relying on the OAS pathway now has been shown (Sohn et al. 2014). For the catabolism of sulfur amino acids, the question was more complex. The first step is a transamination step catalyzed by an aminoacid aminotransferase but there is no specific methionine aminotransferase and according to the different species, this step can be catalyzed by a branched chain amino acid aminotransferase or by an aromatic acid aminotransferase. These enzymes could have a cytoplasmic or a mitochondrial location. Some of these aminotransferases have no attributed specificity such as the family of YER152c, which appeared to be present in all the studied yeast species.

As we have shown previously, sulfur metabolism is involved in many major processes of the living cell. A very versatile regulatory network should modulate the function of this complex pathway. This network has been subject to a number of studies (for review, Ouni et al. 2010). The central component of this network is the Met4 transcriptional factor. Met4 by itself is unable to bind to target DNA as it needs the help of several cofactors, viz. Met28, Met31, Met32 and Cbf1 (Lee et al. 2010). The recognition site of Cbf1 is CACGTG and there are therefore hundreds of such sites in the genome. Combination with Met4 gives to Cbf1 a more narrow range of specificity (Siggers et al. 2011). Met31 and Met32 are very homologous and have redundant specificities but Met32 seems to have a more specific role in cell cycle control. Therefore, Met4 acts as a complex with one of its cofactors and Met28. In conditions where there was no need for induction of this pathway, Met4 was inactivated by ubiquitinylation by the complex SCF^{Met30}. This complex is constituted of a Ring-finger protein Rbx1, a scaffold cullin Cdc53 and a linker protein Skp1 that associates to the F-box protein Met30. This complex mediated ubiquitinylation of Met4. Met4 is not degraded by the proteasome but it appeared to be stored ubiquitinylated as a complex with SCF^{Met30} (Flick et al. 2004; Tyrrel et al. 2010). It is protected from degradation by the interaction of an internal domain, UIM (Ubiquitin Interacting Motif) present in the Met4 sequence (Tyrrel et al. 2010) hiding the ubiquitin chain. In case of need for regulon induction, Met4 was released from the complex SCF^{Met30} and was deubiquinylated to become active (Yen et al. 2012). This was shown in the absence of Met4 *de novo* synthesis (Barbey et al. 2005). This platform is also used to modulate the level of the cofactors Met31 and Met32 because their ubiquitinylation and proteolysis by the proteasome required that these cofactors were associated by Met4 to the SCF^{Met30} complex.

In order to evaluate the conservation of this complex regulatory mechanism, a search to look for homologues of the different partners has been carried out from *S. cerevisiae* to *Emericella nidulans*. The best conserved sequences were those of the components of the SCF^{Met30} complex: Met30, Skp1, Rbx1, Cdc53 and Cdc34. On the contrary, it was difficult to identify the homologues of Met28 beyond the related parents of *S. cerevisiae*. For the transcription factors, Cbf1 appeared relatively well conserved. Met31/Met32 was present as a single copy by genome for the species other than *S. cerevisiae* and *Candidaglabrata*. These two homologous genes arose probably after the genome duplication in the *Saccharomyces* clade.

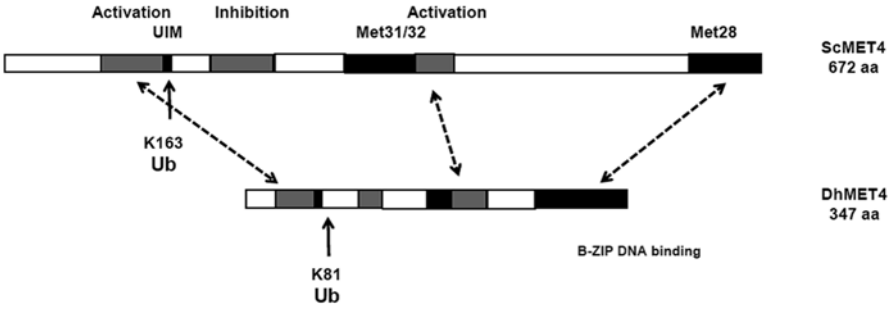


Fig. 3 Alignment of the domains between *S. cerevisiae* Met4 and *D. hansenii* Met4. The activation and inhibition domains are in *grey boxes*. UIM, Met31/32, Met28 interaction domains are in *black boxes* and also the B-ZIP domain. The lysine ubiquinylation sites are shown (According to Chandrasekaran and Skowyra (2008))

For Met4, two subfamilies of homologues were found differing by their size, a large Met4 family including homologues from *S. cerevisiae* to *K. lactis* with a size ranging from 500 to 700 aa and a short Met4 family from *D. hansenii* to *Y. lipolytica* with a size ranging from 319 to 371 aa. These latter were related to Cys3 from *Neurosporacrassa* and MetR from *E. nidulans*. We have taken as a reference the analysis of the subdomains of *S. cerevisiae* Met4 proposed by Chandrasekaran and Skowyra (2008) (see also Kaiser et al. 2006). When compared to *S. cerevisiae* Met4, the *D. hansenii* homologue retained most of the main domains, the activation domain (D82 to F100 in *S. cerevisiae*) can be found from D23 to L41 (Fig. 3). The UIM can be found by homology and by hydrophobic cluster analysis upstream of the lysine, which was ubiquitinylated. The Met31/Met32 binding domain of *S. cerevisiae* from L376 to H397 can be found from L182 to Q203 in *Debaryomyces*. It appeared therefore that the size is expanded from *Debaryomyces* to *Saccharomyces*. But what happened at the C-terminal end? In the short Met4 family, a canonical B-ZIP motif can be identified but in the large Met4 family, this motif appeared to be disrupted by a large insertion from D504 to Q599 in *S. cerevisiae*. The short Met4s can therefore interact directly with DNA without the need for Met28. The hypothesis that Met28 arose lately during evolution can therefore be put forward. As the other partners of the regulatory network have been identified by homology, the members of the short Met4 should retain the other properties of the large Met4 homologues and the complex regulatory mechanism depicted in *S. cerevisiae* can be kept for the other yeast species.

Physiological Studies

An important question is to identify the small molecule, which is the effector of the MET4 regulatory network. A large number of studies have been devoted to the description of the components of the network and to the identification of the related

sequence motifs in the promoters of the regulon but it was more difficult to identify the metabolite pool controlling the regulatory system. For many workers, it was obvious that *S*-adenosylmethionine (SAM) should be the effector of the pathway due to its crucial role in methyl cycle (for instance, Petti et al. 2012). In fact, only two reports have carefully studied this aspect, both leading to a different conclusion.

We have now evidence that the effector should be cysteine. Hansen and Johannsen (2000) have constructed a set of mutations along this pathway in *S. cerevisiae*. The regulation was tested by measuring the transcription of the MET17 gene controlling the incorporation of sulfur into homocysteine. The mutants *cys3*$^{-}$ and *cys4*$^{-}$, which impaired the transformation of methionine into cysteine, were unable to repress the MET17 gene indicating that cysteine is the effector of the regulon. More recently, Sadhu et al. (2014) screened for mutants able to express a GFP-MET3 fusion in the presence of repressing quantities of methionine in a *met17*$^{-}$ context. Among these mutants, cho2^{-} mutants have been identified which were defective in the first step of choline synthesis. Two subsequent methyl transfers led to synthesis of phosphatidylcholine (Hickman et al. 2011). This phospholipid synthesis accounts for about half of the phospholipids and therefore SAM should accumulate in these mutants blocking its recycling to homocysteine. Therefore neither methionine nor SAM, which were both present in large quantities, could repress GFP-MET3, but compounds in the other side of the transsulfuration pathway, cysteine and possibly glutathione. This central role of the cysteine pool in the regulation of the sulfur amino acid compounds appeared more significant in the species with an OAS pathway because the cysteine pool is at the crossroads between the transsulfuration pathway, the OAS pathway and the glutathione synthesis pathway. These results did not indicate which was the genetic target of this regulation by cysteine. One possibility would be that cysteine could control the stability of the complex SCF^{MET30}-Met4 as was shown in vitro by Chandrasekaran et al. (2006).

Up to now, all the biochemical and expression studies have been carried out on the reference species, *S. cerevisiae*. The genomic exploration of the other yeast species has, however, shown that there exists a large biodiversity among the hemiascomycetous species. We have therefore carried out transcriptomic and metabolomic studies in three yeast species scattered along the evolutionary tree, *K. lactis*, *D. hansenii* and *Y. lipolytica*. Our work has been focused on the sulfur metabolism. Moreover, as we are interested in the study of the cheese ecosystem, this liquid medium retains some properties of the curd, lactate and lactose as carbon sources, all the amino acids except methionine and cysteine, etc. The liquid Chemically Defined Medium (CDM) is relatively rich in carbon source (lactose, lactate), in amino acids and in vitamins (Mansour et al. 2008). This would limit the risk of starvation due to a component unrelated to sulfur metabolism. This synthetic liquid medium was used because the growth of cells was homogenous and the extraction procedure was simpler, especially for metabolomics. The cells were maintained for a least ten generations in the exponential phase of growth to secure a steady state of the pools of mRNAs and metabolites. The experiments have been carried out by comparing cells cultivated with high and low sulfur supplies. In this paper, we will

limit our discussion to the experiment with methionine, which has given the most important conclusions. In this experiment, a 10 mM high methionine culture has been compared to a 10 μM low methionine culture. The transcriptomic studies were carried out in the transcriptome platform in Jouy-en-Josas using Agilent microarrays designed with the help of Valentin Loux from Jouy-en-Josas. The statistical analysis was carried out by Julie Aubert from AgroParisTech-Paris. The metabolome studies were carried out in the laboratory of Christophe Junot in CEA-Saclay.

The objectives were the identification of the regulated steps of this metabolism and the identification of the expandable pools of metabolites. By looking for genes regulated by the sulfur supply, it was possible to identify new genes involved in these pathways and to discover new pathways regulated by sulfur availability. Transcriptomic studies are, for instance, the best means to identify the membrane transporters involved in sulfur metabolism. Our genomic studies had already raised some specific questions: Is the *O*-acetylserine pathway truly functional? What is the aminotransferase in charge of the methionine catabolism?

For *K. lactis* (Hébert et al. 2011b), in transcriptomics, all the pathways of sulfur assimilation, either inorganic sulfur or sulfonates appeared to be repressed by methionine. In the transsulfuration pathway, only CYS3 and STR3 were transcriptionally repressed. More surprisingly, DUG2 and DUG3, which catalyze cytoplasmic glutathione degradation, also appeared to be repressed. It was perhaps to counteract the consequence of the repression of GSH1, which catalyzes the first step of glutathione synthesis. The gene KLLA0A04906g encoding one of the two cytoplasmic aromatic amino acid aminotransferase homologues to ARO8 was induced confirming the work from Kagkli et al. (2006). The metabolomic studies have shown that the expandable pools were methionine (x6), cystathionine (x22) and cysteine (X4). The pools of the glutathione pathway, gamma-glutamylcysteine, glutathione and cysteinylglycine were increased also by methionine supply but by a lower factor. Due to the metabolite extraction method, the *S*-adenosylmethionine pool cannot be measured. An expandable pool of 5-methylthioadenosine (x8), which is an intermediate in the methionine salvage pathway, was observed. On the contrary, it was impossible to find a measurable pool of homocysteine although a homocysteine control appeared to be detectable by our analytical method.

The pool of another foreseen intermediate, 4-methylthio-oxobutyric acid (KMBA) was absent. It is the product of transamination of methionine, the first step of its catabolic pathway. The downstream products of this pathway, methional and methionol were also not detected and ARO10, the decarboxylase thought to be involved in this pathway, was not induced by methionine. These observations and the induction of the transporter PDR12 prompted the hypothesis that KMBA, a fusel acid which is toxic for the cell, was excreted by Pdr12 outside in the medium according to the conclusions of Hazelwood et al. (2006). KMBA in the external medium will be transformed chemically into methane-thiol, which is a potent cheese aroma and the precursor of various other volatile sulfur compounds (Bonnarme et al. 2004).

The same kinds of experiments have been carried out with *Y. lipolytica* (Hébert et al. 2013). For the transcriptomic experiments, comparing high to low methionine conditions, the pathways for sulfur assimilation were strongly repressed. One of the differences with *K. lactis* was that in *Y. lipolytica*, there were six JLP1 homologues for the sulfonates catabolism and all of them were repressed by methionine instead of only a single gene as in *K. lactis*. Such diversity in sulfonate dioxygenases is found in ascomycetous fungi such as *E. nidulans* or *N. crassa*. The transsulfuration pathway appeared not to be transcriptionally regulated but the cysteine synthase genes YALI0E08536g and YALI0F14047g were repressed. This could be indicative that in *Yarrowia*, the OAS pathway was playing a major role than the transsulfuration pathway for cysteine synthesis. Concerning methionine catabolism, BAT1 was the only amino acid transaminase induced by methionine confirming our former work (Bondar et al. 2005). As in *K. lactis*, the ARO10 homologue was not affected by methionine supply. Transcriptomic studies allowed us to link membrane transporters to a physiological function. The sulfate transporter SUL1/SUL2 (which is recorded in *S. cerevisiae* as a high affinity methionine permease) was repressed by methionine as expected but among the five homologues of MUP1 only two were strongly repressed by methionine and one was not affected. The physiological role of these different transporters is therefore questionable. A scavenging yeast such as *Yarrowia* displays numerous transporters. For instance, there are seven homologues of the general amino acid permease GAP1 and 16 homologues of the oligopeptide transporter OPT2. For the GAP1 homologues, one was induced and only one was repressed by methionine. The others should have evolved in other physiological functions as was shown by Kraidlova et al. (2011) in *Candidaalbicans*. For the OPT2 homologues, only two appeared sensitive to methionine repression. In contrast, homologues of permeases referenced in *S. cerevisiae* as allantoate permeases (six homologues for SEO1 and two for YIL166c) were repressed by methionine indicating that they belonged to the methionine regulon. Concerning the metabolomic approach, the expandable pools were those of methionine, cystathione and 5-methylthioadenosine. As in *K. lactis*, it was impossible to detect homocysteine and KMBA, which is the product of the induced aminotransferase BAT1. For KMBA, the hypothesis can be put forward that, being toxic for the cell, it was excreted but in *Y. lipolytica*, the PDR12 homologue, YALI0F17996g, was not induced by methionine. One of the six homologues of JEN1, recorded in *S. cerevisiae* to be a monocarboxylate/proton symporter, was largely induced by high methionine. This homologue, YALI0C15488g, displayed an induction factor of 27, the highest observed in the transcriptome experiment. Our hypothesis was that this gene was in charge to detoxify KMBA by excretion. Concerning the glutathione pool and its by-products pools, they appeared to be highly regulated in all the tested conditions.

Metabolomics allowed the detection of two new sulfur compounds, taurine and hypotaurine. These two compounds accumulated in high methionine growth conditions by 4.4 and 20 times, respectively. Taurine is a sulfonate produced from cysteine by three steps, two of these being enzymatically catalyzed. In *Y. lipolytica*, YALI0F11627g encoded a cysteine dioxygenase and YALI0C16753g encoded a

cysteine sulfinic acid decarboxylase (which is different from a canonical glutamate decarboxylase using the criteria defined by Agnello et al. 2013). These two genes were not induced by methionine even if their end product was largely accumulated. Taurine has already been found in higher eukaryotes (for review, Stipanuk and Ueki 2011). It is thought to play the role of osmoprotectant, antioxidant and membrane stabilizer and may also serve for sulfur storage. In *Y. lipolytica* seven taurine dioxygenases can be detected. All of them were repressed in high methionine. A genomic study among ascomycetes has shown that a complete set of genes for the synthesis and degradation of taurine can be found in the ascomycetous fungi and in a part of the hemiascomycetous yeasts from *D. hansenii* to *Y. lipolytica*. The biosynthesis gene set was absent from *K. lactis* to the *Saccharomyces* clade.

In order to genetically verify these results, a disruption of the YALI0F11627g gene encoding cysteine dioxygenase was carried out using the SEP method (Maftahi et al. 1996). The combined pools of taurine and hypotaurine were measured by HPLC on MCD supplemented or not with methionine (Fig. 4). The results were surprising and in the wildtype, these pools increased by a factor of 5 in high methionine. In the mutant strain, there was a wildtype pool of taurine but it decreased in the presence of methionine. Our current interpretation is that YALI0F11627g is involved in taurine biosynthesis but there is another unknown gene involved and this latter gene seemed to be controlled by the methionine supply. Another thiol dioxygenase, cysteamine dioxygenase, has been described in mammals (Dominy et al. 2007) but it was impossible to find a homologue of this gene in *Yarrowia*.

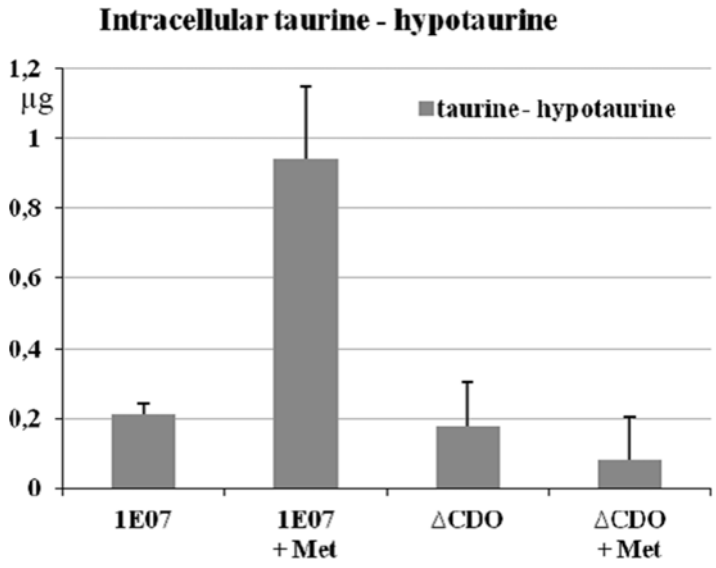


Fig. 4 Dosage of taurine: *Y. lipolytica* wild type and ΔCDO strains were grown overnight in MCD medium supplemented or not with 10 mM methionine. The content of taurine (in fact, taurine and hypotaurine were not separated) were measured by HPLC (B. Pollet, unpublished results)

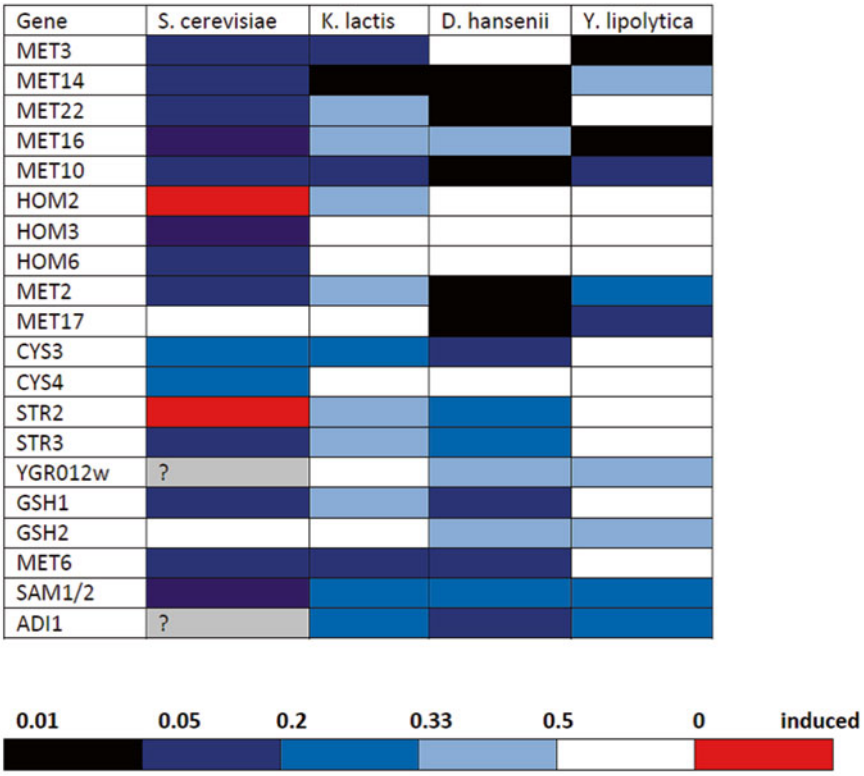


Fig. 5 Comparison of transcriptomic data from *S. cerevisiae*, *K. lactis*, *D. hansenii* (D. Onesime, unpublished data) and *Y. lipolytica*. Data for *S. cerevisiae* were extracted from literature (especially from Knijnenburg TA et al.). Data for *K. lactis* and *Y. lipolytica* have been published (Hébert et al. 2013)

Figure 5 shows a comparison between the expression levels of different genes of the sulfur aminoacid pathway. The data for *D. hansenii*, *K. lactis* and *Y. lipolytica* are more easily comparable as they were all obtained using the growth medium CDM. For *S. cerevisiae*, data were obtained from the compendium of Knijnenburg et al. (2009), which were acquired in different conditions, for instance, in chemostats. It appeared that in general the genes involved in inorganic sulfur assimilation such as MET14, MET16, MET22, MET10 were tightly repressed by methionine. In *D. hansenii*, surprisingly, MET3 was not repressed. The branch involved in homocysteine synthesis appeared very variously regulated in *S. cerevisiae*, HOM2 was reported to be induced by methionine but the other genes HOM3 and HOM6 were repressed; in *K. lactis* HOM2 only was repressed and this branch did not seem regulated in *D. hansenii* and *Y. lipolytica*. Downstream of this homoserine synthesis, MET2 appeared repressed in the four species and in *D. hansenii* and *Y. lipolytica*, MET17 was also tightly repressed. This could be explained because homoserine

was also the precursor of threonine and isoleucine. Concerning the transsulfuration pathway, even more surprising was that STR2 from *S. cerevisiae* leading from cysteine to cystathionine was induced in high methionine; the other three genes were repressed. For *K. lactis* and *D. hansenii*, STR2 and STR3 were repressed in high methionine; this seemed adequate if there was no need for homocysteine and methionine pools. CYS3 was repressed which could explain the increase of the cystathionine pool in the presence of external methionine. For *Yarrowia*, no strong regulation was detected and as discussed elsewhere, this could indicate that in this yeast, the transulfuration pathway was not the major pathway to incorporate sulfur but rather the OAS pathway. This could be also be explained by post-translational regulation of the enzymes of this pathway. It was the same for MET6 encoding the methionine synthase which was not transcriptionally regulated in *Yarrowia* but strongly repressed by methionine in the three other yeasts.

For the study of metabolite pools, we have no data for *Debaryomyces*. For *Saccharomyces*, the data from Lafaye et al. (2005) cannot be directly compared to the data in *K. lactis* and *Y. lipolytica*. The most obvious difference was that Lafaye et al. were able to detect quantifiable homocysteine pools and this was impossible in our conditions in *K. lactis* and *Y. lipolytica*. It was not due to the analytical method as the control homocysteine could be detected. However, in Lafaye et al., homocysteine could not be detected in standard conditions but only in methionine supplementation. It seemed that the control of the pools appeared more relaxed in *K. lactis* than *Y. lipolytica*. It was true for cysteine, glutathione cysteinylglycine and γ-glutamylcysteine and for thioadenosine.

By combining genomic, transcriptomic and metabolomic studies of the sulfur metabolism across the hemiascomycetous yeast clade, we were able to highlight conservation of fundamental mechanisms and biodiversity along this clade. This metabolism has been extensively studied in *S. cerevisiae*. By genomic studies, we were able to show that the same regulatory network can be found in the other yeast species and that we can apply the mechanism described in *S. cerevisiae* to the other yeasts. For the sulfur assimilation pathway as well as the sulfate transporters SUL, these genes are tightly regulated by the availability of sulfur amino acids. However, if we consider the genes regulated in each species, differences of the repression level or even evidence for repression are obvious. We have shown the existence of an OAS pathway among the non-Saccharomyces yeasts. The transcriptomic studies in *D. hansenii* and *Y. lipolytica* confirmed that the genes encoding cysteine synthases appeared to be repressed in high methionine as expected for a biosynthetic pathway contributing significantly to the cysteine pool. If we compare the transsulfuration pathway genes, their transcription appeared to be regulated in *K. lactis* and *D. hansenii* but not in *Y. lipolytica*. Concerning the metabolite pools, methionine, cystathionine and 5-methylthioadenosine displayed expandable pools. Cysteine appeared to be more tightly regulated which was coherent with the hypothesis that it is the effector of the regulation of the whole pathway. Glutathione appeared to be tightly regulated in *Y. lipolytica* as compared to *K. lactis*. Finally, these studies have demonstrated evidence for a complete taurine pathway in a large part of the hemiascomycetous clade.

Acknowledgments The major part of the experimental data presented in this text have been acquired during the EcoMet ANR program (ANR-06-PNRA-014). We want to thank our colleagues, P. Bonnarme and M.P. Forquin of the GMPA in Grignon, S. Casaregola of the CIRM-levures in Grignon, J. Aubert of the MIA UMR in Paris, V. Loux of the MIG lab in Jouy-en-Josas, and A. Roux and C. Junot of CEA-Saclay.

References

Agnello G, Chang LL, Lamb CM, Georgiou G, Stone EM (2013) Discovery of a substrate selectivity motif in amino acid decarboxylases unveils a taurine biosynthetic pathway in prokaryotes. ACS Chem Biol 8:2264–2271

Barbey R, Baudouin-Cornu P, Lee TA, Rouillon A, Zarzov P, Tyers M, Thomas D (2005) Inducible dissociation of SCF(Met30) ubiquitin ligase mediates a rapid transcriptional response to cadmium. EMBO J 24:521–532

Baudouin-Cornu P, Labarre J (2006) Regulation of cadmium stress response through SCF-like ubiquitin ligases: comparison between *Saccharomyces cerevisiae*, *Schizosacchromyces pombe* and mammalian cells. Biochimie 88:1673–1685

Bondar DC, Beckerich JM, Bonnarme P (2005) Involvement of a branched-chain aminotransferase in production of volatile sulfur compounds in *Yarrowia lipolytica*. Appl Environ Microbiol 71:4585–4591

Bonnarme P, Amarita F, Chambellon E, Semn E, Spinnler HE, Yvon M (2004) Methylthioacetaldehyde, a possible intermediate metabolite for the production of volatile sulphur compounds from L-methionine by *Lactococcus lactis*. FEMS Microbiol Lett 236:85–90

Chandrasekaran S, Skowyra D (2008) The emerging regulatory potential of SCF^{Mert30}-mediated polyubiquitination and proteolysis of the Met4 transcriptional activator. Cell Div 3:11

Chandrasekaran S, Deffenbaugh AE, Ford DA, Bailly E, Mathias N, Skowyra D (2006) Destabilization of binding to cofactors and SCFMet30 is the rate-limiting regulatory step in degradation of polyubiquitinated Met4. Mol Cell 24:689–699

Cholet O, Hénaut A, Casaregola S, Bonnarme P (2007) Gene expression and biochemical analysis of cheese-ripening yeast: focus on catabolism of L-methionine, lactate and lactose. Appl Environ Microbiol 73:2561–2570

Dominy JE Jr, Simmons CR, Hirschberger LL, Hwang J, Coloso RM, Stipanuk MH (2007) Discovery and characterization of a second mammalian thiol dioxygenase, cysteamine dioxygenase. J Biol Chem 282:25189–25198

Dujon B (2006) Yeasts illustrate the molecular mechanisms of the eukaryotic genome evolution. Trends Genet 22:375–387

Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, Lafontaine I, De Montigny J, Mark C, Neuvéglise C, Talla E, Goffard N, Frangeul L, Aigle M, Anthouard V, Babour A, Barbe V, Barnay S, Blanchin S, Beckerich JM, Beyne E, Bleykasten C, Boisramé A, Boyer J, Cattolica L, Confanioleri F, De Daruvar A, Despons L, Fabre E, Fairhead C, Ferry-Dumazet H, Groppi A, Hantraye F, Hennequin C, Jauniaux N, Joyet P, Kachouri R, Kerrest A, Koszul R, Lemaire M, Lesur I, Ma L, Muller H, Nicaud JM, Nikolski M, Oztas S, Ozier-Kalogeropoulos O, Pellenz S, Potier S, Richard GF, Straub ML, Suleau A, Swennen D, Tekaia F, Wésolowski-Louvel M, Westhof E, Wirth B, Zeniou-Meyer M, Zivanovic I, Bolotin-Fukuhara M, Thierry A, Bouchier C, Caudron B, Scarpelli C, Gaillardin C, Weissenbach J, Wincker P, Souciet JL (2004) Genome evolution in yeasts. Nature 430:35–44

Fauchon M, Lagniel G, Aude JC, Lombardia L, Soularue P, Petat C, Marguerie G, Sentenac A, Werner M, Labarre J (2002) Sulfur sparing in the yeast proteome in response to sulfur demand. Mol Cell 9:713–723

Flick K, Ouni I, Wohlschlegel JA, Capati C, McDonald WH, Yates JR, Kaiser P (2004) Proteolysis-independent regulation of the transcription factor Met4 by a single Lys 48-linked ubiquitin chain. Nat Cell Biol 6:634–641

Godat E, Madalinski G, Muller L, Heilier JF, Labarre J, Junot C (2010) Mass spectrometry-based methods for the determination of sulfur and related metabolite concentrations in cell extracts. Methods Enzymol 473:41–76

Hansen J, Johannesen PF (2000) Cysteine is essential for transcription regulation of the sulfur assimilation genes in *Saccharomyces cerevisiae*. Mol Gen Genet 263:535–542

Hazelwood LA, Tai SL, Boer VM, de Winde JH, Pronk JT, Daran JM (2006) A new physiological role for Pdr12p in *Saccharomyces cerevisiae*: export of aromatic and branched organic acids produced by amino acid catabolism. FEMS Yeast Res 6:937–945

Hébert A, Casaregola S, Beckerich JM (2011a) Biodiversity in sulfur metabolism in hemiascomycetous yeasts. FEMS Yeast Res 11:366–378

Hébert A, Forquin-Gomez MP, Roux A, Aubert J, Junot C, Loux V, Heilier JF, Bonnarme P, Beckerich JM, Landaud S (2011b) Exploration of the sulfur metabolism in the yeast *Kluyveromyces lactis*. Appl Microbiol Biotechnol 91:1409–1423

Hébert A, Forquin-Gomez MP, Roux A, Aubert J, Junot C, Heilier JF, Landaud S, Bonnarme P, Beckerich JM (2013) New insight into sulfur metabolism as revealed by studies of *Yarrowia lipolytica*. Appl Environ Microbiol 79:1200–1211

Hickman MJ, Petti AA, Ho-Shing O, Silverman SJ, McIsaac RS, Lee TA, Botstein D (2011) Coordinated regulation of sulfur and phospholipid metabolism reflects the importance of methylation in the growth of yeast. Mol Biol Cell 22:4192–4204

Kagkli DM, Bonnarme P, Neuvéglise C (2006) L-methionine degradation pathway in *Kluyveromyces lactis*: identification and functional analysis of the genes encoding L-methionine aminotransferase. Appl Environ Microbiol 72:3330–3335

Kaiser P, Su N-Y, Yen JL, Ouni I, Flick K (2006) The yeast ubiquitin ligase SCF^{Met30}: connecting environmental and intracellular conditions to cell division. Cell Div 1:16

Knijnenburg TA, Daran JM, van den Broek MA, Daran-Lapujade PA, de Winde JH, Pronk JT, Reinders MJ, Wessels LF (2009) Combinatorial effects of environmental parameters on transcriptional regulation in *Saccharomyces cerevisiae*: a quantitative analysis of a compendium of chemostat-based transcriptome data. BMC Genomics 10:53

Kraidlova L, Van Zeebroeck G, Van Dijck P, Sychrová H (2011) The *Candida albicans* GAP gene family encodes permeases involved in general and specific amino acid uptake and sensing. Eukaryot Cell 10:1219–1229

Lafaye A, Junot C, Pereira Y, Lagniel G, Tabet JC, Ezan E, Labarre J (2005) Combined proteome and metabolite-profiling analyses reveal surprising insights into yeast sulfur metabolism. J Biol Chem 280:24723–24730

Lee TA, Jorgensen P, Bognar AL, Peyraud C, Thomas D, Tyers M (2010) Dissection of combinatorial control by the Met4 transcriptional complex. Mol Biol Cell 21:456–469

Linder T (2012) genomics of alternative sulfur utilization in ascomycetous yeasts. Microbiology 158:2585–2597

Maftahi M, Gaillardin C, Nicaud JM (1996) Sticky-end polymerase chain reaction method for systematic gene disruption in *Saccharomyces cerevisiae*. Yeast 12:859–868

Mansour S, Beckerich JM, Bonnarme P (2008) Lactate and aminoacid catabolism in the cheese ripening yeast *Yarrowia lipolytica*. Appl Environ Microbiol 74:6505–6512

McIsaac RS, Petti AA, Bussemaker HJ, Botstein D (2012) Perturbation-based analysis and modeling of combinatorial regulation in the yeast sulfur assimilation pathway. Cell 154:2993–3007

Ouni I, Flick K, Kaiser P (2010) A transcriptional activator is part of an SCF ubiquitin ligase to control degradation of its cofactors. Mol Cell 40:954–964

Petti AA, McIsaac RS, Ho-Shing O, Bussemaker HJ, Botstein D (2012) Combinatorial control of diverse metabolic and physiological functions by transcriptional regulators of the yeast sulfur assimilation pathway. Mol Biol Cell 23:3008–3024

Sadhu MJ, Moresco JJ, Zimmer AD, Yates JR 3rd, Rine J (2014) Multiple inputs control sulfur-containing amino-acid synthesis in *Saccharomyces cerevisiae*. Mol Biol Cell 25:1653–1665

Siggers T, Duyzend MH, Reddy J, Khan S, Bulyk ML (2011) Non-DNA-binding cofactors enhance DNA-binding specificity of a transcriptional regulatory complex. Mol Syst Biol 7:555

Sohn MJ, Yoo SJ, Oh D-B, Kwon O, Lee SY, Sibirny AA, Kangf HA (2014) Novel cysteine-centered sulfur metabolic pathway in the thermotolerant methylotrophic yeast *Hansenula polymorpha*. PLoS One 9:e100725

Stipanuk MH, Ueki I (2011) Dealing with methionine/homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur. J Inherit Metab Dis 34:17–32

Tyrrell A, Flick K, Kleiger G, Zhang H, Deshaies RJ, Kaiser P (2010) Physiologically relevant and portable tandem ubiquitin-binding domain stabilizes polyubiquitylated proteins. Proc Natl Acad Sci U S A 107:19796–19801

Yen JL, Flick K, Papagiannis CV, Mathur R, Mathur R, Tyrrell A, Ouni I, Kaake RM, Huang L, Kaiser P (2012) Signal-induced disassembly of the SCF ubiquitin ligase complex by Cdc48/p97. Mol Cell 48:288–297

Small World: A Plant Perspective on Human Sulfate Activation

Jonathan W. Mueller, Nathanael O'Neill, and Naeem Shafqat

Abstract The metabolism of sulfur has been widely studied with major progress in plant model systems. In plants and humans alike, activation occurs in canonical steps starting from the highly inert oxy-anion sulfate by the action of the enzymes ATP sulfurylase and APS kinase, resulting in the production of the atypical nucleotides adenosine-5′-phosphosulfate (APS) and PAPS (3′-phospho-APS). This review compares novel insights into structure, mechanism and regulation of plant ATP sulfurylases and APS kinases with findings from human sulfation pathways to highlight the benefit of "looking over the fence" and engaging in truly interdisciplinary research.

Introduction

Research into sulfur metabolism has a long history and in recent years has seen a steady growth within the plant community, but not to the same degree in biomedical research. There is, however, a strong and growing interest in human sulfate activation as defects in the genes responsible for this process have demonstrated essential roles of sulfation pathways in bone and cartilage formation (Faiyaz ul Haque et al. 1998) as well as steroid homeostasis (Noordam et al. 2009).

In plants and humans alike, activation occurs in canonical steps starting from the highly inert oxy-anion sulfate by the action of the enzymes ATP sulfurylase and APS kinase, resulting in the production of the atypical nucleotides adenosine-5′-phosphosulfate (APS) and PAPS (3′-phospho-APS) (Mueller and Shafqat 2013). The evolutionary history of the genes of sulfate activation is complex with multiple origins in the lineages and several gene fusions with other enzymes of the pathway (Patron et al. 2008). While plant ATP sulfurylase and APS kinase represent separate polypeptides, the human bifunctional enzymes consist of a C-terminal ATP sulfurylase and an

J.W. Mueller (✉) • N. O'Neill • N. Shafqat
Centre for Endocrinology, Diabetes, and Metabolism (CEDAM), School of Clinical and Experimental Medicine, University of Birmingham, Institute of Biomedical Research, Birmingham B15 2TT, UK
e-mail: j.w.mueller@bham.ac.uk

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_6

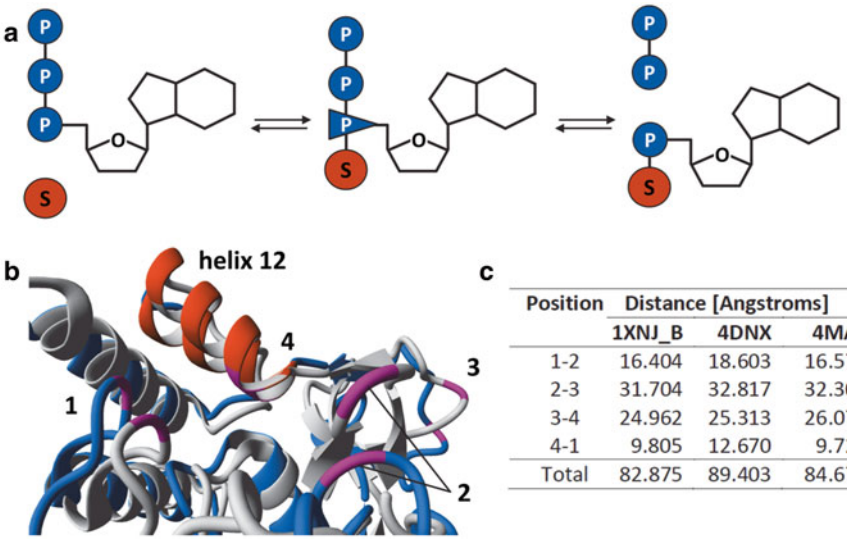
N-terminal APS kinase domain; they are referred to as PAPS synthases (Mueller and Shafqat 2013; van den Boom et al. 2012). There are more sulfate activating complexes in bacteria and archaea (Mougous et al. 2006; Sun and Leyh 2006). However this mini-review focuses on human-plant comparisons only with the enzymatic components of these sulfate activation systems.

What Can We Learn from Plant ATP Sulfurylase for the Mechanism and Function of Human PAPS Synthases?

ATP sulfurylase catalyzes the first step of sulfate activation; the transfer of an AMP moiety of ATP on sulfate. Formation of the mixed sulfo-phospho anhydride bond is highly endergonic (Mueller and Shafqat 2013), so that thermodynamics favors the reverse reaction, the formation of ATP and sulfate from APS and PP_i. It is worth noting that it is this very reaction that forms the chemical basis for pyrophosphate detection in various next-generation DNA sequencing techniques (Wu et al. 2011). The different ATP sulfurylase structures presently known have shown the versatility of the ATP sulfurylase fold that has been observed as mono-functional dimers, bi-functional dimers like in the human PAPS synthases, mono-functional heterodimers as well as hexameric mono-functional enzymes (Grum et al. 2010; Herrmann et al. 2014).

ATP is bound by ATP sulfurylase in a unique, strained conformation allowing for the in-line attack of sulfate on the alpha-phosphorus. This substitution mechanism leads directly to the formation of APS, with PP_i as the leaving group and without covalent adenylyl-enzyme intermediates (schematically depicted in Fig. 1a). It was initially proposed based on an enzyme-APS-PPi complex of yeast ATP sulfurylase (Ullrich et al. 2001) and recently confirmed for ATP sulfurylase from soybean (Herrmann et al. 2014) and the respective enzyme from the sulfur-oxidizing purple sulfur bacterium *Allochromatium vinosum* (Parey et al. 2013). The substitution mechanism relies on reactive groups in close contact with both substrates to provide protein-ligand interactions that stabilize a pentavalent transition state and enhance reaction rates (Herrmann et al. 2014). These residues, including two histidine residues in close proximity of the gamma-phosphate (H252 and H255 in 4MAF), two arginine residues and an asparagine residue close to the alpha-phosphate (R248, N249 and R349 in 4MAF) as well as a glutamine residue close to the sulfate binding site (Q246 in 4MAF) are invariant across the ATP sulfurylases from many organisms. Interestingly, none of these sulfurylase structures contained magnesium bound close to the nucleotide (Mueller and Shafqat 2013), in stark contrast to other nucleotidyl transfer enzymes or an ADP complex of yeast ATP sulfurylase (Ullrich et al. 2001).

The recent crystal structure 4DNX of the ATP sulfurylase homologue from *Allochromatium vinosum* (Parey et al. 2013) suggested the existence of an open



Position	Distance [Angstroms]		
	1XNJ_B	4DNX	4MAF
1-2	16.404	18.603	16.573
2-3	31.704	32.817	32.305
3-4	24.962	25.313	26.074
4-1	9.805	12.670	9.727
Total	82.875	89.403	84.679

Fig. 1 **a** Schematic representation of the reaction mechanism of sulfotransferases. *Encircled* P and S represent phosphate and sulfate moieties, respectively. The "*P*" in a triangle represents the trigonal-bipyramidal transition state. See main text for more details. **b** Open and closed conformations of ATP sulfurylases. Following alignment of different ATP sulfurylase structures, four positions were defined around the mouth of the catalytic cavity (depicted in *magenta* and numbered 1–4). These included one position within the alpha-helix 12 (depicted in *orange*). The distance between the alpha-carbon atoms of adjacent residues was measured within the YASARA structural viewer. Structurally equivalent positions 1–4 are ASP461, GLU531, GLU578, GLY588 in 1XNJB (shown in *gray*) and GLY236, GLY302, ASP350, GLY 361 in 4DNX (shown in *blue*). **c** Atomic distances between structurally equivalent positions in different ATP sulfurylase structures. 1XNJ, human PAPSS1; Harjes et al. 2005), 4DNX, ATP sulfurylase from *Allochromatium vinosum* (Parey et al. 2013), 4MAF, ATP sulfurylase from soybean (Herrmann et al. 2014)

state at the substrate binding site that retained some binding affinity to APS. To our knowledge, this is the first real "apo" structure of a sulfurylase with water only in the catalytic cavity; all previous structures without nucleotide ligand had some charge-compensating ions bound instead. We have aligned this structure to the ATP sulfurylase domain of human PAPSS1 as well as to the sulfurylase structure from soybean (Herrmann et al. 2014). Within this alignment, we defined four positions including one within the opening alpha-helix 12 (Fig. 1b) and summed up their distances. Clearly, 4DNX shows the widest opening with 4DNX 89.4 Å compared to 82.9 or 84.7 Å in 1XNJ and 4MAF, respectively. However, as the previously reported apo form of PAPSS1 still had a chloride ion bound in the sulfurylase active site, it may have already mimicked the substrate-bound closed state. At the moment, it is impossible to state whether the open conformation seen in 4DNX really is connected to induced fit-binding or due to variations in charged residues around this binding site.

Novel Insights for Human PAPS Synthases from the Regulation of Plant APS Kinases

As the equilibrium of the ATP sulfurylase reaction lies far on the side of sulfate and ATP, more energy-dissipating reactions have to take place to draw overall PAPS synthesis to completion. This is, on the one hand, the cleavage of the released pyrophosphate by ubiquitous pyrophosphatases. On the other hand, APS is phosphorylated at the ribose 3′-OH group yielding active sulfate in the form of PAPS. For the purpose of this review, we compare redox regulation, cytoplasmic regulation as well as nucleotide binding of human and plant APS kinase-containing proteins.

For bi-functional human PAPS synthases, it is well established that the APS kinase reaction is the rate-limiting step in PAPS production (Lansdon et al. 2004). Hence, the recent report of a redox-regulated APS kinase from Arabidopsis (Ravilious et al. 2012) is of interest also for a functional understanding of the human PAPS synthase protein. This structure showed a C86–C119 disulfide-linked dimer of Arabidopsis APS kinase 1 with the N-terminus of one subunit flopped over to the other domain (Fig. 2a) (Ravilious et al. 2012). Such conformation of a flopped N-terminus was already observed in structures of the isolated APS kinase domain of human PAPSS1 (2OFX and 2OFW) (Sekulic et al. 2007). Arabidopsis contains a total of four APS kinase genes, but redox regulation was shown here for the APS kinase isoform1 only (Ravilious et al. 2012). Nonetheless, this AtAPSK1 was able to maintain wild-type levels of growth and development in an Arabidopsis triple AtAPSK knockout (Mugford et al. 2010) and the redox cysteines were noted to be conserved in all the three chloroplast isoforms, but not in the cytoplasmic AtAPSK3 (see Table 1 for an overview of the various APS kinases as well as ATP sulfurylases).

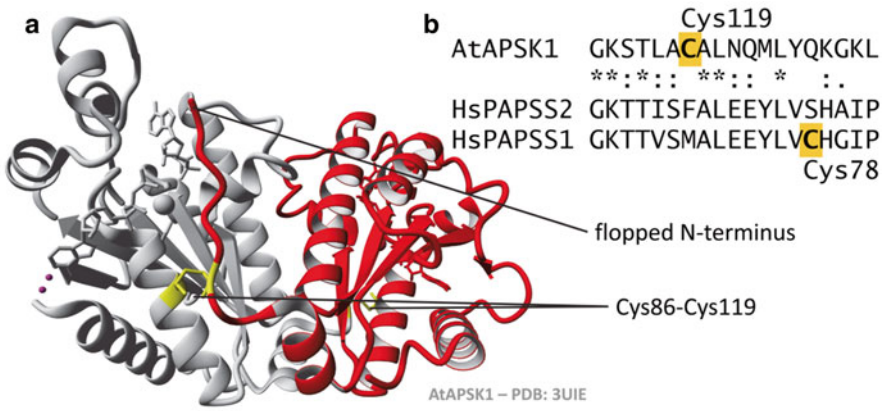


Fig. 2 APS kinase 1 from *Arabidopsis thaliana* is redox-regulated. **a** Structural representation of the cystine-linked AtAPK1 dimer (Ravilious et al. 2012). Protein subunits are colored in *grey* and *red* with bound APS and AMPPNP. The cystine bridges are shown in *yellow*. **b** Alignment of AtAPK1 with human PAPS synthases 1 and 2. Cys78, confined to PAPSS1-type PAPS synthases, is located within the same alpha-helix that contains Cys119 in AtAPK1

Table 1 Sulfate activating complexes in plants and animals

Organism	Name	UniProt ID	Amino acids (aa)	aa identity[a]	Localisation signal
APS kinase					
Arabidopsis thaliana	APK1	Q43295	276	90/170 (53 %)	Chloroplast
	APK2	O49196	293	97/180 (54 %)	Chloroplast
	APK3	Q9SRW7	208	91/178 (51 %)	Cytosol
	APK4	Q84JF0	310	87/179 (49 %)	Chloroplast
Oryza sativa (*Japonica*)	APK	Q6ZL22	345	100/204 (49 %)	–
	APK	Q2R0R8	304	100/202 (50 %)	Chloroplast
	APK	A3AF71	228	93/182 (51 %)	–
Saccharomyces cerevisiae	Met14	W7RB78	202	99/175 (57 %)	–
Mus musculus	PAPSS1	Q60967	624	167/201 (83 %)	Cytosol/nucleus[b]
	PAPSS2	O88428	621	195/201 (97 %)	Cytosol/nucleus[b]
Homo sapiens	PAPSS1	O43252	624	167/201 (83 %)	Cytosol/nucleus[b]
	PAPSS2	O95340	614	201/201 (100 %)	Cytosol/nucleus[b]
ATP sulfurylase (ATS)					
Arabidopsis thaliana	ATP sulf 1	Q9LIK9	463	246/390 (63 %)	Chloroplast
	ATP sulf 2	Q43870	476	239/390 (61 %)	Cytosol/chloroplast
	ATP sulf 3	O23324	465	241/390 (62 %)	Chloroplast
	ATP sulf 4	Q9S7D8	469	238/393 (61 %)	Mitochondrion/ chloroplast
Oryza sativa (*Japonica*)	H0215A08.5	Q01N37	474	234/381 (61 %)	–
Saccharomyces cerevisiae	Met3	W7RIF2	511	106/376 (28 %)	Cytosol

(continued)

Table 1 (continued)

Organism	Name	UniProt ID	Amino acids (aa)	aa identity[a]	Localisation signal
Mus musculus	PAPSS1	Q60967	624	299/388 (77 %)	Cytosol/nucleus[b]
	PAPSS2	O88428	621	360/393 (92 %)	Cytosol/nucleus[b]
Homo sapiens	PAPSS1	O43252	624	300/388 (77 %)	Cytosol/nucleus[b]
	PAPSS2	O95340	614	388/388 (100 %)	Cytosol/nucleus[b]

[a]Percent amino acid identity is expressed for the APS kinase and ATP sulfurylase domain separately. Human PAPSS2 was chosen as a point for comparison because it may be evolutionary older than its PAPSS1 counterpart (van den Boom et al. 2012). Stretches of protein corresponding to Thr16 to Val216 and Ile224 to Leu611 of human PAPSS2A were compared for APS kinase and ATP sulfurylase proteins, respectively

[b]Cellular localisation of human PAPS synthases was recently been studied (Schroder et al. 2012)

The respective cysteines involved in disulfide formation are conserved in APS kinases from different plant species, but not in APS kinases outside the plant world. Can one exclude redox regulation for human enzymes then? Detailed sequence comparisons between many PAPSS1 and PAPSS2 sequences using machine learning techniques revealed a group of 23 residues that are crucial for assigning sequences to these two PAPS synthase sub-families (van den Boom et al. 2012). Strikingly, four cysteine residues were among them and two of these cysteines (Cys78 and Cys207) are found within the APS kinase domain, Cys78 even within the same alpha-helix like Cys119 from AtAPSK1 (Fig. 2b). It remains to be seen whether the human PAPS synthase proteins are also subject to redox regulation.

APS kinases and APS reductases seem to be redox-controlled in a reciprocal manner and this may have implications in controlling sulfur partitioning between the primary and secondary metabolism in plants (Kopriva et al. 2012). Sulfur flux analysis in the human system would be a highly interesting new tool in studying human sulfation pathways; this is however hampered by the large chemical variety of sulfated compounds in human cell lines. Such development might become crucial to finally understand the different phenotypes of the small, but ever growing number of patients with PAPSS2 mutations who show remarkably different phenotypes.

Different localization of enzyme isoforms is a recurring theme when trying to understand different functionality of the respective genes. For human PAPS synthases, we showed recently that these enzymes are also regulated on the level of cellular localization; PAPS synthases shuttle between cytoplasm and nucleus (Schroder et al. 2012). Within the APS kinase domain of PAPSS1 and –S2, we identified both, an N-terminal nuclear localization signal of the KKxK type as well as an atypical nuclear export signal at the dimer interface (Schroder et al. 2012). While

the KKxK motif is absent from all AtAPKs, the atypical nuclear export signal is highly conserved in sequence in all four AtAPKs. Here it would surely be of interest to determine the exact localization of cytosolic AtAPK3 within the plant cell.

Recently we have described human PAPS synthases as surprisingly fragile; PAPSS2 is partially unfolded at physiological temperatures and the intermediate of PAPS biosynthesis, APS, was identified as a specific stabilizer of PAPS synthases (van den Boom et al. 2012). Quantitative binding data for APS nucleotide binding to AtAPSK1 was reported recently: the apo-enzyme weakly bound APS ($Kd = 66.7 \pm 10.5$ μM); the affinity increased significantly when the enzyme was preloaded with ADP (3.3 ± 0.70 μM), and even further in the presence of magnesium (0.60 ± 0.20 μM) (Ravilious and Jez 2012). Assuming similar binding affinities for human PAPS synthases, we hypothesized the specific molecular effect of APS to be due to the formation of an enzyme-ADP-APS complex within the APS kinase domain at low micro-molar concentrations of APS (Mueller and Shafqat 2013). Indeed, we could show that all our preparations of recombinant human PAPS synthases contained one ADP molecule per protein dimer (Mueller and Shafqat 2013) as has been reported previously for human PAPSS1 (Harjes et al. 2005). On the other hand, assessing stability and stabilization by its ligands similar to PAPSS2 may also be studied for plant APS kinases.

A Broader Look at Sulfation Pathways

Sulfur pathways are more complex in plants than in humans (Takahashi et al. 2011). APS may represent a metabolic branch point in plants (Chan et al. 2013) where the competing enzymatic activities of APS reductases and APS kinases determine to which extent sulfur flows into the primary, meaning reductive, metabolism or the secondary metabolism that includes transfer of the sulfate group to hydroxyl or amino groups of plant hormones or other secondary metabolites such as the large group of glucosinolates. Humans lack the ability to reduce sulfate and hence PAPS usage is limited to sulfation processes only, catalyzed by a multitude of sulfotransferase enzymes.

Ensembl lists a total of 62 and 63 sulfotransferase genes for the human and mouse genomes, respectively (Flicek et al. 2014) (Ensembl release 76); where some 46 represent obvious orthologues. While the sulfotransferase repertoire is seemingly less diverse in plants with 18 genes in Arabidopsis (Chan et al. 2013; Klein and Papenbrock 2004), studying these enzymes may turn out to be a valuable resource for future evolutionary studies. Moreover, the traditional classification into soluble (cytosolic) and membrane-spanning (Golgi-residing) enzymes may need revision as some of the plant sulfotransferases seem to be localized to other cellular compartments (Klein and Papenbrock 2004).

Once sulfation has occurred, the nucleotide 3′-phospho-adenosine-5′-phosphate is produced as a by-product. Dedicated phosphatases exist in all kingdoms of life to degrade this nucleotide to AMP and ortho-phosphate (Cummings et al. 2014;

Hudson and York 2012). The many names for the respective phosphatase from Arabidopsis (FIERY1, ROTUNDA 1, SAL1, SUPO1, ALX8 and others) reflect the highly pleiotropic phenotypes of SAL1 mutants, affecting drought tolerance, leaf shape and root growth, as well as the enormous interest in this gene (Estavillo et al. 2011; Hirsch et al. 2011; Lee et al. 2012; Robles et al. 2010). When SAL1 gets inactivated during drought or high light stress, PAP accumulates in plants and elicits a stress response, most likely by inhibiting XRN exoribonucleases (Estavillo et al. 2011). In mice, the knockout of the homologous phosphatase BPNT1 causes a translation defect due to incomplete processing of ribosomal RNAs, most likely again via XRN inhibition (Hudson et al. 2013). As of now, no human individuals are described who harbor BPNT1 mutations.

Finally, various proteins degrade sulfate esters again, known as sulfatases, and this protein family has received recent attention in the biomedical research community (Purohit and Foster 2012). It remains to be seen what a large sulfurylase domain-containing protein [UniProt: F4JD48] as well as the APS-hydrolyzing Fhit proteins (Guranowski et al. 2010) in Arabidopsis still hold for us.

Conclusions

Taken together, our studies of human sulfation pathways have highlighted PAPS synthases to be regulated at the levels of cellular localization and ligand-regulated protein stability. Studying the homologous proteins from plants has given insights into the catalytic mechanism and possible novel ways of redox regulation. With the many components of plant and human sulfation pathways, this research field will surely stay interesting and hold great promises and surprises in the next couple of years and "looking over the fence" may be inspiring and insightful.

Acknowledgement W. Arlt is cordially acknowledged for her generous support and critical review of the manuscript. JWM also wants to acknowledge David Lodge from the University of Birmingham for various insights. JWM is a recipient of the Marie Curie IEF award 625451 SUPA-HD: Sulfation pathways in Health and Disease. Finally, we thank the Biochemical Society, UK, for a travel award (to JWM) to attend the 9th International Workshop on Sulfur Metabolism in Plants, Freiburg, Germany.

References

Chan KX, Wirtz M, Phua SY, Estavillo GM, Pogson BJ (2013) Balancing metabolites in drought: the sulfur assimilation conundrum. Trends Plant Sci 18:18–29

Cummings JA, Vetting M, Ghodge SV, Xu C, Hillerich B, Seidel RD, Almo SC, Raushel FM (2014) Prospecting for unannotated enzymes: discovery of a 3′,5′-nucleotide bisphosphate phosphatase within the amidohydrolase superfamily. Biochemistry 53:591–600

Estavillo GM, Crisp PA, Pornsiriwong W, Wirtz M, Collinge D, Carrie C, Giraud E, Whelan J, David P, Javot H, Brearley C, Hell R, Marin E, Pogson BJ (2011) Evidence for a SAL1-PAP

chloroplast retrograde pathway that functions in drought and high light signaling in Arabidopsis. Plant Cell 23:3992–4012

Faiyaz ul Haque M, King LM, Krakow D, Cantor RM, Rusiniak ME, Swank RT, Superti-Furga A, Haque S, Abbas H, Ahmad W, Ahmad M, Cohn DH (1998) Mutations in orthologous genes in human spondyloepimetaphyseal dysplasia and the brachymorphic mouse. Nat Genet 20:157–162

Flicek P, Amode MR, Barrell D, Beal K, Billis K, Brent S, Carvalho-Silva D, Clapham P, Coates G, Fitzgerald S, Gil L, Giron CG, Gordon L, Hourlier T, Hunt S, Johnson N, Juettemann T, Kahari AK, Keenan S, Kulesha E, Martin FJ, Maurel T, McLaren WM, Murphy DN, Nag R, Overduin B, Pignatelli M, Pritchard B, Pritchard E, Riat HS, Ruffier M, Sheppard D, Taylor K, Thormann A, Trevanion SJ, Vullo A, Wilder SP, Wilson M, Zadissa A, Aken BL, Birney E, Cunningham F, Harrow J, Herrero J, Hubbard TJ, Kinsella R, Muffato M, Parker A, Spudich G, Yates A, Zerbino DR, Searle SM (2014) Ensembl 2014. Nucleic Acids Res 42:D749–D755

Grum D, van den Boom J, Neumann D, Matena A, Link NM, Mueller JW (2010) A heterodimer of human 3′-phospho-adenosine-5′-phosphosulphate (PAPS) synthases is a new sulphate activating complex. Biochem Biophys Res Commun 395:420–425

Guranowski A, Wojdyla AM, Zimny J, Wypijewska A, Kowalska J, Jemielity J, Davis RE, Bieganowski P (2010) Dual activity of certain HIT-proteins: A. thaliana Hint4 and C. elegans DcpS act on adenosine 5′-phosphosulfate as hydrolases (forming AMP) and as phosphorylases (forming ADP). FEBS Lett 584:93–98

Harjes S, Bayer P, Scheidig AJ (2005) The crystal structure of human PAPS synthetase 1 reveals asymmetry in substrate binding. J Mol Biol 347:623–635

Herrmann J, Ravilious GE, McKinney SE, Westfall CS, Lee SG, Baraniecka P, Giovannetti M, Kopriva S, Krishnan HB, Jez JM (2014) Structure and mechanism of soybean ATP sulfurylase and the committed step in plant sulfur assimilation. J Biol Chem 289:10919–10929

Hirsch J, Misson J, Crisp PA, David P, Bayle V, Estavillo GM, Javot H, Chiarenza S, Mallory AC, Maizel A, Declerck M, Pogson BJ, Vaucheret H, Crespi M, Desnos T, Thibaud MC, Nussaume L, Marin E (2011) A novel fry1 allele reveals the existence of a mutant phenotype unrelated to 5′- >3′ exoribonuclease (XRN) activities in *Arabidopsis thaliana* roots. PLoS One 6:e16724

Hudson BH, York JD (2012) Roles for nucleotide phosphatases in sulfate assimilation and skeletal disease. Adv Biol Regul 52:229–238

Hudson BH, Frederick JP, Drake LY, Megosh LC, Irving RP, York JD (2013) Role for cytoplasmic nucleotide hydrolysis in hepatic function and protein synthesis. Proc Natl Acad Sci U S A 110:5040–5045

Klein M, Papenbrock J (2004) The multi-protein family of Arabidopsis sulphotransferases and their relatives in other plant species. J Exp Bot 55:1809–1820

Kopriva S, Mugford SG, Baraniecka P, Lee BR, Matthewman CA, Koprivova A (2012) Control of sulfur partitioning between primary and secondary metabolism in Arabidopsis. Front Plant Sci 3:163

Lansdon EB, Fisher AJ, Segel IH (2004) Human 3′-phosphoadenosine 5′-phosphosulfate synthetase (isoform 1, brain): kinetic properties of the adenosine triphosphate sulfurylase and adenosine 5′-phosphosulfate kinase domains. Biochemistry 43:4356–4365

Lee BR, Huseby S, Koprivova A, Chetelat A, Wirtz M, Mugford ST, Navid E, Brearley C, Saha S, Mithen R, Hell R, Farmer EE, Kopriva S (2012) Effects of fou8/fry1 mutation on sulfur metabolism: is decreased internal sulfate the trigger of sulfate starvation response? PLoS One 7:e39425

Mougous JD, Lee DH, Hubbard SC, Schelle MW, Vocadlo DJ, Berger JM, Bertozzi CR (2006) Molecular basis for G protein control of the prokaryotic ATP sulfurylase. Mol Cell 21:109–122

Mueller JW, Shafqat N (2013) Adenosine-5′-phosphosulfate–a multifaceted modulator of bifunctional 3′-phospho-adenosine-5′-phosphosulfate synthases and related enzymes. FEBS J 280:3050–3057

Mugford SG, Matthewman CA, Hill L, Kopriva S (2010) Adenosine-5′-phosphosulfate kinase is essential for Arabidopsis viability. FEBS Lett 584:119–123

Noordam C, Dhir V, McNelis JC, Schlereth F, Hanley NA, Krone N, Smeitink JA, Smeets R, Sweep FC, Claahsen-van der Grinten HL, Arlt W (2009) Inactivating PAPSS2 mutations in a patient with premature pubarche. N Engl J Med 360:2310–2318

Parey K, Demmer U, Warkentin E, Wynen A, Ermler U, Dahl C (2013) Structural, biochemical and genetic characterization of dissimilatory ATP sulfurylase from *Allochromatium vinosum*. PLoS One 8:e74707

Patron NJ, Durnford DG, Kopriva S (2008) Sulfate assimilation in eukaryotes:fusions, relocations and lateral transfers. BMC Evol Biol 8:39

Purohit A, Foster PA (2012) Steroid sulfatase inhibitors for estrogen- and androgen-dependent cancers. J Endocrinol 212:99–110

Ravilious GE, Jez JM (2012) Nucleotide binding site communication in Arabidopsis thaliana adenosine 5′-phosphosulfate kinase. J Biol Chem 287:30385–30394

Ravilious GE, Nguyen A, Francois JA, Jez JM (2012) Structural basis and evolution of redox regulation in plant adenosine-5′-phosphosulfate kinase. Proc Natl Acad Sci U S A 109:309–314

Robles P, Fleury D, Candela H, Cnops G, Alonso-Peral MM, Anami S, Falcone A, Caldana C, Willmitzer L, Ponce MR, Van Lijsebettens M, Micol JL (2010) The RON1/FRY1/SAL1 gene is required for leaf morphogenesis and venation patterning in Arabidopsis. Plant Physiol 152:1357–1372

Schroder E, Gebel L, Eremeev AA, Morgner J, Grum D, Knauer SK, Bayer P, Mueller JW (2012) Human PAPS synthase isoforms are dynamically regulated enzymes with access to nucleus and cytoplasm. PLoS One 7:e29559

Sekulic N, Dietrich K, Paarmann I, Ort S, Konrad M, Lavie A (2007) Elucidation of the active conformation of the APS-kinase domain of human PAPS synthetase 1. J Mol Biol 367:488–500

Sun M, Leyh TS (2006) Channeling in sulfate activating complexes. Biochemistry 45:11304–11311

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu Rev Plant Biol 62:157–184

Ullrich TC, Blaesse M, Huber R (2001) Crystal structure of ATP sulfurylase from *Saccharomyces cerevisiae*, a key enzyme in sulfate activation. EMBO J 20:316–329

van den Boom J, Heider D, Martin SR, Pastore A, Mueller JW (2012) 3′-Phosphoadenosine 5′-phosphosulfate (PAPS) synthases, naturally fragile enzymes specifically stabilized by nucleotide binding. J Biol Chem 287:17645–17655

Wu H, Wu W, Chen Z, Wang W, Zhou G, Kajiyama T, Kambara H (2011) Highly sensitive pyrosequencing based on the capture of free adenosine 5′ phosphosulfate with adenosine triphosphate sulfurylase. Anal Chem 83:3600–3605

Auxin Response Factors and Aux/IAA Proteins Potentially Control –S Responsive Expression of *SULTR1;1*

Akiko Maruyama-Nakashita

Abstract Sulfur is an essential nutrient for plants and its deficiency (–S) severely affects plant growth. To acquire limited sulfur under –S, plants have evolved signal transduction pathways resulting in enhanced sulfate uptake and assimilation. The transcript level of the high affinity sulfate transporter *SULTR1;1* is dramatically induced by –S. The –S-induced expression of *SULTR1;1* is dependent on SLIM1 transcription factor which controls a broad range of –S responsive gene expression. Previously we identified the sulfur-responsive element of *SULTR1;1* (SURE11) which includes a 6 bp sequence, GAGACA, identical to the binding sequence of auxin response factors (ARFs). ARFs are a family of transcription factors that promote or repress the expression of auxin responsive genes. The function of ARFs is inhibited by the hetero-dimerization with Aux/IAA proteins that cannot bind to the sequence. Though *SULTR1;1* expression was not modulated by exogenously applied auxin, the identity between *SURE*-core sequence and the ARF binding sequence suggests that one of the ARF works to induce *SULTR1;1* expression under –S. In this paper, we attempt to predict which ARFs and Aux/IAA proteins potentially control –S-induced expression of *SULTR1;1* by using microarray data on *slim1* and the parental plants. Five ARF and seven Aux/IAA proteins were up- or down-regulated by –S in parental plants. Among them, none of the ARF modified its –S response in *slim1*, but five Aux/IAA proteins lost their –S response in *slim1*, including IAA13 and IAA28 whose positive function in sulfur assimilation had been reported. These results indicated that this method could be useful in predicting candidates for regulatory genes working in –S responsive gene expression.

Sulfur is an essential nutrient for plants and its deficiency severely affects the plant growth, crop yield and quality. Sulfate, the major form of sulfur that plants can utilize for the synthesis of cysteine and methionine, is taken up from plant roots by the activity of sulfate transporters. In Arabidopsis, two high-affinity sulfate transporters,

A. Maruyama-Nakashita (✉)
Laboratory of Plant Nutrition, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan
e-mail: amaru@agr.kyushu-u.ac.jp

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*, Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_7

SULTR1;1 and SULTR1;2, expressed in the epidermis and cortex of roots, play essential roles in the uptake of sulfate (Takahashi et al. 2000; Vidmar et al. 2000; Shibagaki et al. 2002; Yoshimoto et al. 2002, 2007; Maruyama-Nakashita et al. 2003). Gene expression of both *SULTR1;1* and *SULTR1;2* is dramatically induced by the depletion of sulfate (−S) in a promoter-dependent manner, thereby the sulfate uptake activity is induced by –S (Takahashi et al. 2000; Shibagaki et al. 2002; Yoshimoto et al. 2002; Maruyama-Nakashita et al. 2004a, b). However, the signal transduction pathways from perception of the sulfur status to the regulation of these transporters are not fully understood at the molecular level.

To uncover the regulatory mechanisms of sulfur response, the *cis*-acting element involved in the –S response was investigated. For *SULTR1;1*, deletion and gain-of-function analysis using the luciferase reporter gene in transgenic *Arabidopsis* revealed that the 16 bp sulfur responsive element (SURE11) from −2777 to −2761 of the promoter was sufficient and necessary for the –S-responsive expression, which was reversed when supplied with sulfate, cysteine and glutathione (GSH) (Maruyama-Nakashita et al. 2005). Base substitution analysis indicated the significance of a 7 bp sequence (GGAGACA) as a core element. The core sequences exist in the promoter regions of several –S-inducible genes, suggesting a common mechanism for –S regulation.

The core sequence of SURE11 includes the ARF binding sequence (GAGACA), which has previously been reported as an auxin response element (Ulmasov et al. 1997, 1999; Hagen and Guilfoyle 2002). However, SURE11 was not responsive to naphthalene acetic acid (NAA), indicating its specific function in the sulfur response (Maruyama-Nakashita et al. 2005). In *Arabidopsis*, 23 genes are reported as ARF family transcription factors, but their function in the regulation of auxin responsive gene expression has not been investigated for all the family members (Okushima et al. 2005). There is a possibility that an ARF-like transcription factor binds to the *SURE* core sequence and induces –S-dependent expression of *SULTR1;1*. Generally, ARF regulates the transcription of auxin responsive genes in a dimer form and the heterodimerization between Aux/IAA and ARF inhibits the access of ARF to the binding sequence by inhibiting the dimerization between ARFs (Guilfoyle and Hagen 2007). Though the ARF binding sequence in the SURE11 is not repeated, a similar regulatory circuit to control *SULTR1;1* expression by ARF and Aux/IAA proteins could exist.

Another regulatory protein of –S-inducible expression of *SULTR1;1* is SLIM1 transcription factor (Maruyama-Nakashita et al. 2006). SLIM1 controls both the activation of several sulfur assimilatory genes including sulfate transporters and degradation of glucosinolates, and the repression of glucosinolates synthetic genes under –S conditions (Maruyama-Nakashita et al. 2006). As SLIM1 controls both –S up-regulated and down-regulated gene expression, there should be other regulators connecting SLIM1 and each metabolic process. In this report, to isolate the transcription factors that directly control *SULTR1;1* expression, the ARF and Aux/IAA proteins whose expression is modulated by sulfate availability and SLIM1 were investigated using the microarray data obtained from *slim1* mutants (Maruyama-Nakashita et al. 2006).

In the Arabidopsis genome, 23 genes of ARF and 29 genes of Aux/IAA exist (Table 1). Among them, probes for 16 and 22 genes of ARF and Aux/IAA, respectively, exist on Affymetrix ATH1 GeneChip microarray (Table 1). The previous

Table 1 Selection of ARF and Aux/IAA proteins responsive to sulfur deficiency in a SLIM1-dependent manner

	ARF	AuX/IAA
Arabidopsis Genome	**23**	**29**
Genechip	**16**	**22**
Signals can be trusted	**13**	**18**
Sulfur responsive in parental plants ($P<0.1$)	**5**	**7**
Sulfur response is different between parental and *slim1* plants ($P<0.1$)	**0**	**5**

ARF and Aux/IAA proteins were selected whose transcript levels were influenced by sulfur deficiency (−S) and SLIM1 existence. The previous GeneChip data were used for the selection (Maruyama-Nakashita et al. 2006). $P_{SULTR1;2}$-*GFP* (parental), *slim1-1*, *slim1-2* plants were grown vertically on an agar medium containing 1500 μM or 15 μM of sulfate (+S, −S) for 10 days, and duplicated root RNA samples were used for the Affymetrix ATH-1 GeneChip analysis. "Signals can be trusted" means signals were present or marginal at least once over the 12 experiments. Among the genes whose signals can be trusted, −S-responsive genes were selected by comparing the gene expression between parental plants grown under +S and –S conditions. The genes whose responses to –S were modified in *slim1* mutants were selected by comparing the –S/+S ratio of transcript levels between parental and *slim1* plants. Student's *t*-test was performed and the genes showing probability values less than 0.1 were selected

Table 2 ARF and IAA proteins responsive to sulfur deficiency

ARF			Aux/IAA		
Affymetrix ID	AGI code	Gene name	Affymetrix ID	AGI Code	Gene name
247468_at	AT5G62000	ARF2	257766_at	AT3G23030	IAA2
256311_at	AT1G30330	ARF6	**257769_at**	**AT3G23050**	**IAA7**
254194_at	AT4G23980	ARF9	**255788_at**	**AT2G33310**	**IAA13**
251289_at	AT3G61830	ARF18	**246376_at**	**AT1G51950**	**IAA18**
256010_at	AT1G19220	ARF19	**246861_at**	**AT5G25890**	**IAA28**
			253423_at	**AT4G32280**	**IAA29**
			247906_at	AT5G57420	IAA33

Genes selected as "sulfur responsive in parental plants" in Table 1 were listed. The genes selected as "sulfur response is different between parental and *slim1* plants" in Table 1 were shown in *bold characters*

GeneChip experiment was performed using duplicated root RNA samples of $P_{SULTR1;2}$-*GFP* (parental line), *slim1-1*, *slim1-2* plants grown on an agar medium containing 1500 μM (+S) or 15 μM (−S) of sulfate for 10 days. Among the 12 experiments, signals for 13 and 18 genes of ARF and Aux/IAA, respectively, were detected as statistically trusted data whose signals were present or marginal at least once over the 12 experiments (Table 1). From these genes, −S responsive genes were selected by comparing the signals of parental plants grown under +S and –S conditions, which included five *ARF* genes, *ARF2*, *ARF6*, *ARF9*, *ARF18* and *ARF19* and seven Aux/IAA genes, *IAA2*, *IAA7*, *IAA13*, *IAA18*, *IAA28*, *IAA29* and *IAA33* (Tables 1 and 2; Fig. 1). Among the genes obtained, *ARF2*, *IAA13* and *IAA28* were reported as –S up-regulated genes (Nikiforova et al. 2003, 2005). Though the up-regulation of *ARF2* and *IAA13* by –S were consistent with the previous reports, the

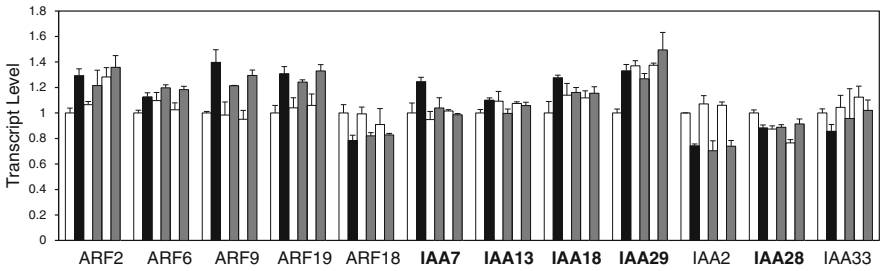


Fig. 1 Relative transcript levels of ARF and Aux/IAA proteins responsive to sulfur deficiency. Average values of relative transcript levels of the genes selected as "sulfur responsive in parental plants" in Table 1 were presented. Each bar graph represents relative transcript levels in parental plants grown under + S (*white*) or –S (*black*), those in *slim1-1* grown under + S (*white*) or –S (*gray*), those in *slim1-2* grown under + S (*white*) or –S (*gray*), from left to right. The relative transcript levels calculated are those of parental plants grown under + S as 1.0. *Error bars* are standard errors. The genes selected as "sulfur response is different between parental and *slim1* plants" in Table 1 are shown in *bold characters*

down-regulation of *IAA28* was not (Fig. 1, Nikiforova et al. 2003, 2005). This may be due to differences in culture conditions: here plants were grown vertically on an agar medium containing 1500 or 15 μM sulfate for 10 days, whereas Nikiforova et al. (2003, 2005) grew plants horizontally on an agar medium containing 750 and 65 μM sulfate. Another microarray analysis with –S treated roots demonstrated the down-regulation of *IAA28* by –S which also treated the plants on agar media (Winter et al. 2007; Iyer-Pascuzzi et al. 2011).

The genes whose response to –S were modified in *slim1* mutants were selected by comparing the –S/+S ratio of transcript levels in parental and *slim1* plants. From this, five genes of Aux/IAA proteins, IAA7, IAA13, IAA18, IAA28 and IAA29, were obtained as the –S responsive SLIM1-dependent genes (Tables 1 and 2, Fig. 1). Their expression in root epidermis suggested that they can directly control *SULTR1;1* expression by accessing the proteins binding to SURE11 (Arabidopsis eFP Browser, Winter et al. 2007; Birnbaum et al. 2003; Nawy et al. 2005). Among the IAA proteins selected, IAA13 and IAA28 demonstrated a positive function in sulfur assimilation (Falkenberg et al. 2008), indicating that this method could be useful in predicting candidates for regulatory genes working in –S responsive gene expression. As Aux/IAA proteins do not have DNA binding domains, they should affect the activity or the interaction capacity of the ARF-like transcription factors binded to SURE11.

There are some reports suggesting that an increase of auxin in sulfur-starved plants may mediate the signals for the regulation of –S-responsive genes (Kutz et al. 2002; Nikiforova et al. 2003; Kasajima et al. 2007). The transcript level of nitrilase 3 was increased two-fold by –S in parental plants but the same increase was not observed in *slim1* mutants (Maruyama-Nakashita et al. 2006). The transcript levels of five IAA proteins, *IAA7*, *IAA13*, *IAA18*, *IAA28* and *IAA29*, were modulated by auxin in a very similar way with –S treatment, such that *IAA7*, *IAA13*, *IAA18* and

IAA29 were up-regulated but *IAA28* was down-regulated by auxin (Arabidopsis eFP Browser, Winter et al. 2007; Goda et al. 2008). Though the transcript level of *SULTR1;1* was not influenced by auxin (Maruyama-Nakashita et al. 2005; Goda et al. 2008), auxin and its signal transduction can be an important component in plant adaptation to –S.

References

Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN (2003) A gene expression map of the *Arabidopsis* root. Science 302:1956

Falkenberg B, Witt I, Zanor MI, Steinhauser D, Mueller-Roeber B, Hesse H, Hoefgen R (2008) Transcription factors relevant to auxin signalling coordinate broad-spectrum metabolic shifts including sulphur metabolism. J Exp Bot 59:2831–2846

Goda H, Sasaki E, Akiyama K, Maruyama-Nakashita A, Nakabayashi K, Li W, Ogawa M, Yamauchi Y, Preston J, Aoki K, Kiba T, Takatsuto S, Fujioka S, Asami T, Nakano T, Kato H, Mizuno T, Sakakibara H, Yamaguchi S, Nambara E, Kamiya Y, Takahashi H, Hirai MY, Sakurai T, Shinozaki K, Saito K, Yoshida S, Shimada Y (2008) The AtGenExpress hormone and chemical treatment data set: experimental design, data evaluation, model data analysis and data access. Plant J 55:526–542

Guilfoyle TJ, Hagen G (2007) Auxin response factors. Curr Opin Plant Biol 10:453–460

Hagen G, Guilfoyle TJ (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol 49:373–385

Iyer-Pascuzzi AS, Jackson T, Cui H, Petricka JJ, Busch W, Tsukagoshi H, Benfey PN (2011) Cell identity regulators link development and stress responses in the *Arabidopsis* root. Dev Cell 21:770–782

Kasajima I, Ohkama-Ohtsu N, Ide Y, Hayashia H, Yoneyama T, Suzukia Y, Naito S, Fujiwara T (2007) The BIG gene is involved in regulation of sulfur deficiency-responsive genes in *Arabidopsis thaliana*. Physiol Plant 129:351–363

Kutz A, Muller A, Hennig P, Kaiser WM, Piotrowski M, Weiler EW (2002) A role for nitrilase 3 in the regulation of root morphology in sulphur-starving *Arabidopsis thaliana*. Plant J 30:95–106

Maruyama-Nakashita A, Inoue E, Watanabe-Takahashi A, Yamaya T, Takahashi H (2003) Transcriptome profiling of sulfur responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways. Plant Physiol 132:597–605

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Yamaya T, Takahashi H (2004a) Induction of SULTR1;1 sulfate transporter in *Arabidopsis* roots involves protein phosphorylation/dephosphorylation circuit for transcriptional regulation. Plant Cell Physiol 45:340–345

Maruyama-Nakashita A, Nakamura Y, Yamaya T, Takahashi H (2004b) A novel regulatory pathway of sulfate uptake in *Arabidopsis* roots: implication of CRE1/WOL/AHK4-mediated cytokinin-dependent regulation. Plant J 38:779–789

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel *cis*-acting element conferring sulfur deficiency response in *Arabidopsis* roots. Plant J 42:305–314

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006) Central transcriptional regulator of plant sulfur response and metabolism. Plant Cell 18:3235–3251

Nawy T, Lee JY, Colinas J, Wang JY, Thongrod SC, Malamy JE, Birnbaum K, Benfey PN (2005) Transcriptional profile of the Arabidopsis root quiescent center. Plant Cell 17:1908–1925

Nikiforova V, Freitag J, Kempa S, Adamik M, Hesse H, Höfgen R (2003) Transcriptome analysis of sulfur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. Plant J 33:633–650

Nikiforova V, Kopka J, Tolstikov V, Fiehn O, Hopkins L, Hawkesford MJ, Hesse H, Höfgen R (2005) Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidopsis plants. Plant Physiol 138:304–318

Okushima Y, Overvoorde PJ, Arima K, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Lui A, Nguyen D, Onodera C, Quach H, Smith A, Yu G, Theologis A (2005) Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in Arabidopsis thaliana: unique and overlapping functions of ARF7 and ARF19. Plant Cell 17:444–463

Shibagaki N, Rose A, McDermott JP, Fujiwara T, Hayashi H, Yoneyama T, Davies JP (2002) Selenate-resistant mutants of *Arabidopsis thaliana* identify Sultr1;2, a sulfate transporter required for efficient transport of sulfate into roots. Plant J 29:475–486

Takahashi H, Watanabe-Takahashi A, Smith FW, Blake-Kalff M, Hawkesford MJ, Saito K (2000) The roles of three functional sulfate transporters involved in uptake and translocation of sulfate in *Arabidopsis thaliana*. Plant J 23:171–182

Ulmasov T, Hagen G, Guilfoyle TJ (1997) ARF1, a transcription factor that binds auxin response elements. Science 276:1865–1868

Ulmasov T, Hagen G, Guilfoyle TJ (1999) Activation and repression of transcription by auxin response factors. Proc Natl Acad Sci U S A 96:5844–5849

Vidmar JJ, Tagmount A, Cathala N, Touraine B, Davidian J-CE (2000) Cloning and characterization of a root specific high-affinity sulfate transporter from *Arabidopsis thaliana*. FEBS Lett 475:65–69

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart N (2007) An "electronic fluorescent pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS One 2:e718

Yoshimoto N, Takahashi H, Smith FW, Yamaya T, Saito K (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots. Plant J 29:465–473

Yoshimoto N, Inoue E, Watanabe-Takahashi A, Saito K, Takahashi H (2007) Posttranscriptional regulation of high-affinity sulfate transporters in Arabidopsis by sulfur nutrition. Plant Physiol 145:378–388

SULTR1;2 in S Nutrient-Status Control in Arabidopsis

Thomas Leustek and Zhi-Liang Zheng

Abstract An essential life process for plants is the assimilation of sulfur, which is available to plant roots as sulfate that is taken up into cells where it is reduced and assimilated. Plants can sense the availability of sulfur, but the sensor has not been identified. Two *Arabidopsis thaliana* mutants were isolated (termed *sel1-15* and *sel1-16*), that show increased expression of a sulfur-deficiency-activated gene beta glucosidase 28 (*BGLU28*) when grown on medium with high sulfate content (Zhang et al. 2014). The mutants are missense alleles of *SULTR1;2* encoding a high affinity sulfate transporter. Although the mutants are defective in sulfate transport they show higher expression of *BGLU28* and other sulfur-deficiency-activated genes even when they are treated with a very high dose of sulfate such that the intracellular concentration of sulfate and its metabolite glutathione (GSH) are identical to wild type. The reduced sensitivity to inhibition of gene expression is also observed in *sel1* mutants fed with the sulfur assimilation products cysteine and GSH. The results suggest that SULTR1;2 may have a second role in addition to its known function as a high affinity sulfate transporter. It may also have a regulatory role in response to sulfur nutrient status.

Plants assimilate sulfur (S) in a pathway that reduces sulfate (SO_4^{2-}) to sulfide (S^{2-}) and then incorporates S^{2-} into cysteine (Leustek et al. 2000; Takahashi et al. 2011). Cysteine is the first organic form that is produced when S^{2-} is covalently bonded to *O*-acetylserine (OAS). Cysteine is used for the synthesis of methionine, proteins and many S-containing compounds including vitamins, co-factors and secondary compounds. Some secondary compounds include glucosinolates and glutathione (GSH). Both play critical roles in response to biotic and abiotic stresses (Leustek

T. Leustek (✉)
Department of Plant Biology and Pathology, Rutgers University,
New Brunswick, NJ 08901, USA
e-mail: leustek@aesop.rutgers.edu

Z.-L. Zheng
Department of Biological Sciences, City University of New York-Lehman College,
Bronx, NY 10468, USA
e-mail: zhiliang.zheng@lehman.cuny.edu

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_8

et al. 2000; Rausch and Wachter 2005). SO_4^{2-} is taken up from the rhizosphere by roots and is moved throughout the plant. However, not all the SO_4^{2-} that is taken up is reduced, and most serves as a major intracellular solute.

Uptake of SO_4^{2-} is mediated by membrane-localized transporters, encoded in various plant species by gene families (Takahashi et al. 2011; Maruyama-Nakashita et al. 2004b; Buchner et al. 2004; Rouached et al. 2009). Arabidopsis has 12 confirmed *SULTR* genes organized into four groups (Buchner et al. 2004; Takahashi et al. 2006, 2011). *SULTR* transporters have been implicated in specific functions such as SO_4^{2-} transport from roots to shoots (Takahashi et al. 2000; Kataoka et al. 2004a; Cao et al. 2013), vacuolar export of SO_4^{2-} (Kataoka et al. 2004b), phloem transport and internal SO_4^{2-} redistribution (Yoshimoto et al. 2003), and uptake from the rhizosphere (Takahashi et al. 2000; Shibagaki et al. 2002; Yoshimoto et al. 2002; Maruyama-Nakashita et al. 2004b; Rouached et al. 2008; Barberon et al. 2008). *SULTR1;1* and *SULTR1;2* are high affinity sulfate transporters involved in SO_4^{2-} uptake from the rhizosphere. They show unequal functional redundancy, with *SULTR1;2* playing a predominant role (Yoshimoto et al. 2002; Rouached et al. 2008; Barberon et al. 2008).

The structure of SULTR proteins and their membrane topology is only partially understood. The Arabidopsis transporters are predicted to contain up to 12 transmembrane (TM) helices (Fig. 1), although topology prediction varies markedly depending on the algorithm used (Shibagaki et al. 2002).

All SULTR proteins contain a conserved C-terminal STAS domain (sulfate transporter and anti-sigma factor antagonist) also found in a wide range of membrane proteins from both prokaryotes and eukaryotes. In one such protein the STAS domain was found to be cytoplasmically located (Zheng et al. 2001; Lohi et al.

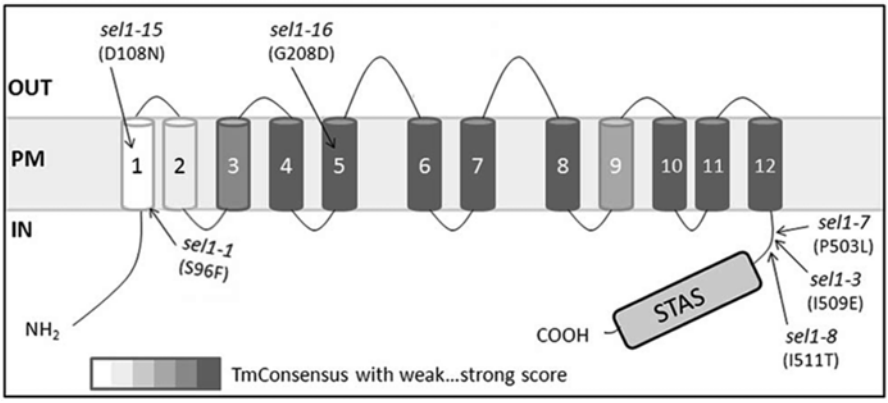


Fig. 1 Predicted SULTR1;2 topology showing 12 transmembrane helices (TM) derived from 12 different topology algorithms. The model is depicted embedded within the plasma membrane (PM), which is the membrane in which SULTR1;2 is localized. The darkness of TM shading is indicative of higher prediction scores. TM1 and TM2 have the lowest prediction scores. The *arrows* indicate the positions of known *sel1* missense mutations. This figure was modified from Zhang et al. (2014)

2003). Three studies reveal the critical role that the STAS domain, and the region connecting it to the remainder of the protein, plays in SULTR1;2 protein stability and transport function (Shibagaki and Grossman 2004, 2006; Rouached et al. 2005). The STAS domain is also the ligand for protein-protein interaction with cytosolically localized OAS (thiol)lyase (OASTL), the enzyme responsible for cysteine synthesis (Shibagaki and Grossman 2010).

The function of SULTR1;2 was initially identified based on a forward genetic screen for selenate-resistant Arabidopsis (Shibagaki et al. 2002; El Kassis et al. 2007). For this reason the mutants were designated *sel*. Resistance is caused by the reduced ability of the mutants to take up selenate (SeO_4^{2-}), a toxic SO_4^{2-} analog. Genomic inactivation of *SULTR1;2* caused by T-DNA insertions are also SeO_4^{2-}-resistant (Shibagaki et al. 2002; Maruyama-Nakashita et al. 2003; El Kassis et al. 2007), further supporting the idea that this phenotype is caused by elimination of high affinity SO_4^{2-} transport.

Plants have the ability to sense and adapt to the level of S in their environment. When S is limiting they are able to regulate S acquisition (increased expression of high affinity sulfate transporters), assimilation (increase in SO_4^{2-} reduction and assimilation enzymes), scavenging (increase in enzymes for catabolism of S compounds), unessential usage (decrease in synthesis of secondary metabolites), interconnected metabolisms (altered carbon and nitrogen metabolism), and morphology (modification of root architecture) (Hirai et al. 2003; Leustek et al. 2000; Maruyama-Nakashita et al. 2003; Scheible et al. 2004; Wang et al. 2003). Recent studies have revealed that ~1,500 Arabidopsis genes respond to S starvation (Hirai et al. 2003, 2004, 2005; Maruyama-Nakashita et al. 2003; Nikiforova et al. 2003; Hoefgen and Nikiforova 2008). One of the regulated genes that shows one of the largest increases in expression after S starvation is *BGLU28* encoding a member of beta-glucosidase family, which is likely to be involved in glucosinolate degradation (Maruyama-Nakashita et al. 2003, 2006; Nikiforova et al. 2003; Amtmann and Armengaud 2009). The promoter of this gene (Dan et al. 2007) was used to identify mutants that misregulate the S-deficiency response (Zhang et al. 2014).

In addition to the transcriptomic response to S deficiency, the rate with which cysteine is produced is regulated through the control of the cysteine synthase complex consisting of SAT and OASTL. OASTL is the enzyme catalyzing cysteine synthesis (Leustek et al. 2000; Hirai et al. 2004; Hawkesford and De Kok 2006; Yi et al. 2010; Wirtz et al. 2012). Less well understood, but of potential significance, is the recently described interaction between the sulfate transporter SULTR1;2 and OASTL (Shibagaki and Grossman 2010).

The mechanism for perception and signaling of S nutrient status in plants remains an open question. Several different S-assimilation metabolites have been suggested to act as signals including O-acetyl-L-serine (OAS), acting as a positive signal for transcription, cysteine and GSH functioning as negative regulators (Lappartient et al. 1999; Buchner et al. 2004; Rouached et al. 2008), and SO_4^{2-} concentration acting as a negative signal (Reuveny and Filner 1977). The question of which molecule is the signal is complicated by the fact that all possible candidates are also S-assimilation substrates, products, or intermediates that can be interconverted and

are subject to changes in metabolic flux. Therefore, the cellular concentration of potential signaling compounds cannot be separated from S metabolism, which is itself controlled by gene expression. To date, all efforts to identity the signal molecule have not overcome this fundamental dilemma.

Because of the difficulties associated with the physiological approach to identifying the S signaling compound, recent studies have focused on the genetic identification of components in the sensory and transduction pathway that controls the S response in plants. First, using a biochemical approach it was found that protein phosphorylation/dephosphorylation is likely to be involved in signaling based on the finding that phosphatase inhibitors mitigate the S-starvation response (Maruyama-Nakashita et al. 2004a). Second, a *cis* element has been identified called SURE that is necessary for S-deficiency control of many S-response genes including *BLGU28* (Maruyama-Nakashita et al. 2005). Third, the cysteine synthase (CS) enzyme complex controls cysteine homeostasis (Hirai et al. 2004; Yi et al. 2010; Takahashi et al. 2011; Wirtz et al. 2012). One of the CS complex enzymes, OASTL forms a physical interaction with sulfate transporter SULTR1;2 suggesting a possible mechanism for S-response regulation (Shibagaki and Grossman 2010).

Forward genetic approaches have also been used to identify genes needed for the S response by isolation of mutants that mis-regulate a reporter gene linked to an S-starvation-activated promoter (Ohkama-Ohtsu et al. 2004; Maruyama-Nakashita et al. 2006; Dan et al. 2007). This strategy has identified *OSH1*, a thiol reductase (Ohkama-Ohtsu et al. 2004); *BIG*, a calossin-like protein involved in polar auxin transport (Kasajima et al. 2007); *SLIM1*, a transcriptional regulator (Maruyama-Nakashita et al. 2006); and *SULTR1;2* (Zhang et al. 2014).

The *slim1* mutant is unable to induce expression of many S-response genes and microRNA-395, a known post-transcriptional regulator of S-assimilation enzyme production (Kawashima et al. 2009; Maruyama-Nakashita et al. 2006). However, there are some genes that show normal induction in the *slim1* mutants, indicating the existence of a *SLIM1*-independent signaling pathway. Currently there is no further information on how *OSH1*, *BIG*, *SLIM1*, and *SULTR1;2* co-ordinate S signaling, nor how these putative regulators interact (Kopriva and Rennenberg 2004; Maruyama-Nakashita et al. 2004b; Rouached et al. 2009; Gojon et al. 2009).

Using a genetic screen for mutants that misregulate a SURE-containing S-response gene *BGLU28*, we have identified two novel *SEL1* alleles (*sel1-15* and *sel-16*) with missense mutations in TM1 and TM5, respectively (Fig. 1). These mutants are also defective in high affinity SO_4^{2-} transport (Zhang et al. 2014). In contrast to the interpretation of several prior studies that attributed the elevated expression of many S-response genes in *SULTR1;2* mutants to the transport defect (Maruyama-Nakashita et al. 2003; El Kassis et al. 2007; Lee et al. 2012), our physiological studies suggest that the *sel1-15*/*16* mutants likely impact both transport and S sensing. This is because under a high SO_4^{2-} dose (10 mM) the mutants and wild type (WT) had similar contents of internal SO_4^{2-} (Fig. 2a) and GSH (Fig. 2a). The likely explanation for this effect is that at such a high external SO_4^{2-} concentration low affinity sulfate transporters bypass SULTR1;2 and so overcome the *sel1* transport defect. Despite the comparable internal S pools between mutant and WT,

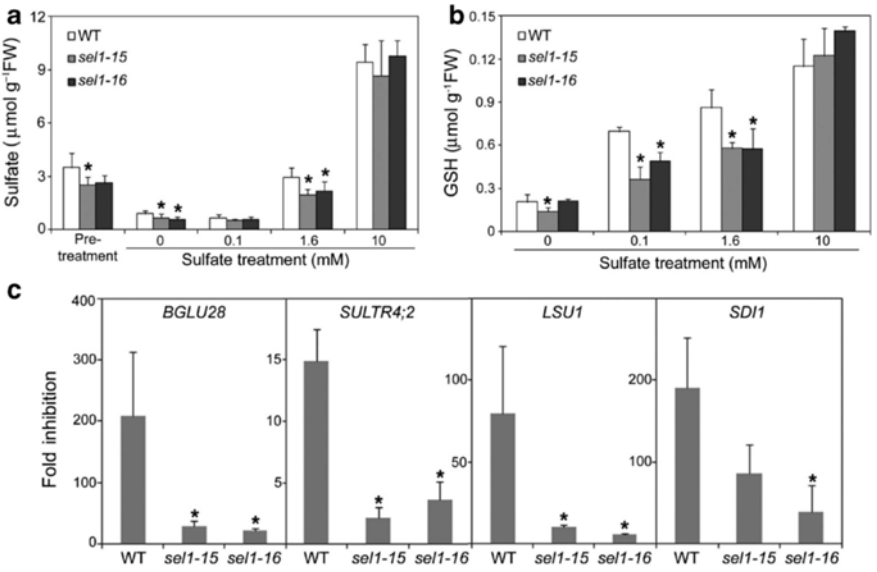


Fig. 2 SULTR1;2 acts as a putative sulfur transceptor. **a** When seedlings are treated with different sulfate doses for 48 h the *sel1-15* and *sel1-16* mutants show the same internal sulfate content as WT at the 10 mM condition. The data shown was derived from whole seedlings. **b** When seedlings are treated with different sulfate doses for 48 h the *sel1-15* and *sel1-16* mutants show the same GSH content as WT at the 10 mM condition. The data shown was derived from seedling roots. **c** When the seedlings were treated with 10 mM sulfate for 48 h at the reduction in gene expression sensitivity to the sulfate treatment is evident in the roots of *sel1-15* and *sel1-16* alleles. The *asterisk* (*) indicates values that are significantly different from wild type (WT). In addition to *BGLU28*(AT2G44460), the expression of three other S-responsive genes were examined by QPCR including *SULTR4*;*2* (AT3G12520), *LSU1* (AT3G49580), and *SDI1* (AT5G48850). This figure was adapted from (Zhang et al. 2014)

when plants are treated with 10 mM SO_4^{2-}*sel1-15/16* plants still show reduced repressability of four key S-response genes, *BGLU28*, *SULTR4*;*2*, *LSU1* and *SDI1* (Fig. 2c) (Zhang et al. 2014). This data suggests that SULTR1;2 plays a role in sensing S level or in regulating S response. Further evidence for this hypothesis comes from the effect of treating the mutants with 1 mM cysteine or 1 mM GSH, both treatments resulting in the same internal GSH content in the mutants as in WT (the results for cysteine are shown in Fig. 3a). Both cysteine and GSH are transported into cells via a SULTR1;2-independent mechanism (Zhang et al. 2014). Yet, *sel1-15/16* show reduced sensitivity to both compounds with respect to repression of expression of four S-response genes compared to WT (Fig. 3b, c). Taken together these genetic and physiological studies suggest that SULTR1;2 has a signaling or sensing function independent of its transport function.

Dual function in transport and sensing roles is the property of a class of proteins called transceptors (Thevelein and Voordeckers 2009). In yeast and mammals, several dual-function metabolite transporters/sensors have been identified including

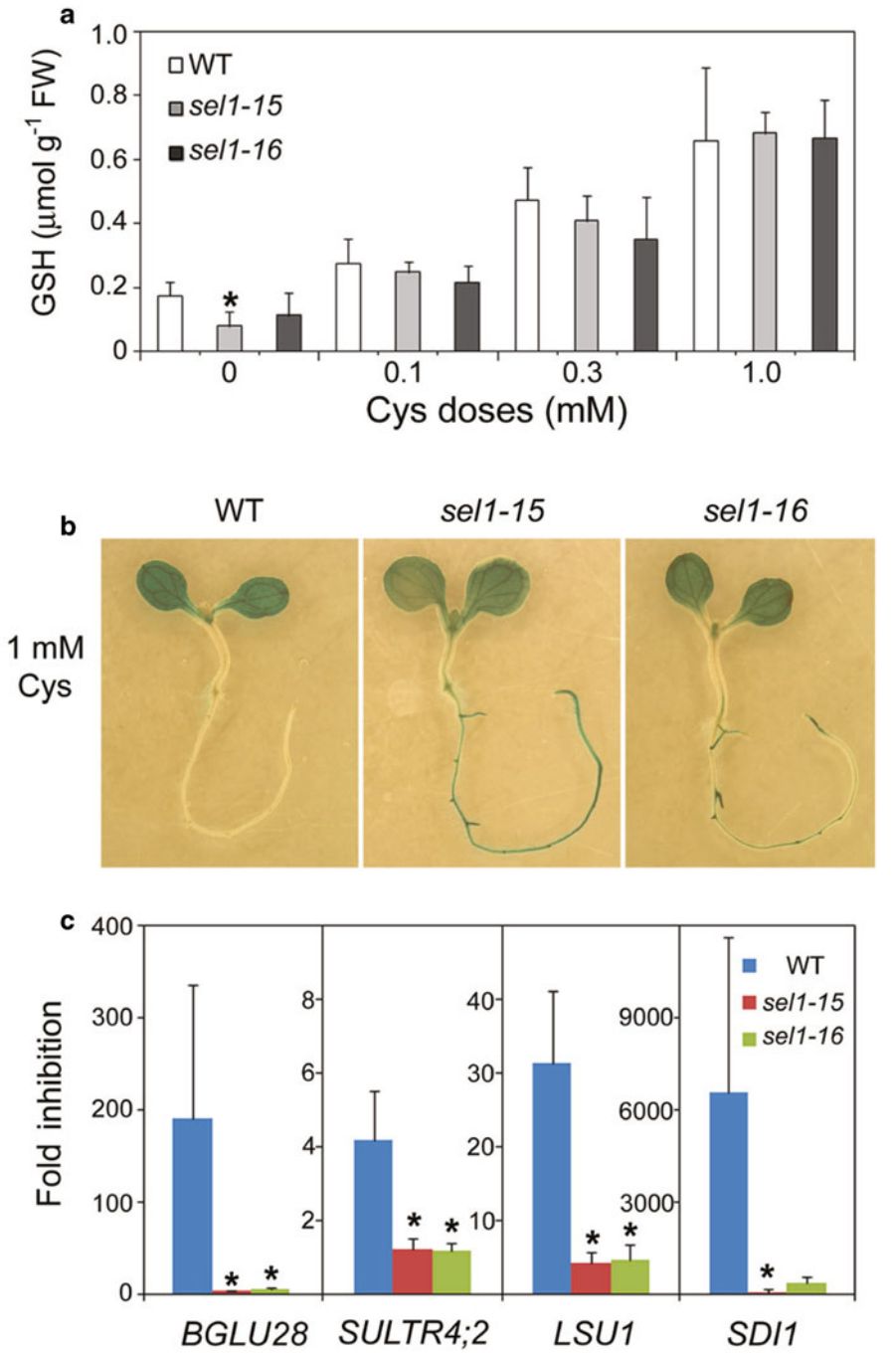


Fig. 3 SULTR1;2 acts as a putative S-Transceptor. **a** Seedlings treated with different L-Cysteine doses for 48 h at the 1 mM dose the *sel1-15* and *sel1-16* mutants show the same GSH content as WT. The data shown was derived from seedling roots. **b** *BGLU28* promoter: GUS activity in plants

the amino acid transporter/receptor Gap1 (Van Zeebroeck et al. 2009), glucose transporter/sensor GLUT2 (Leturque et al. 2009) and phosphate transceptor Pho84 (Popova et al. 2010; Schothorst et al. 2013; Conrad et al. 2014). Of particular note, very recently yeast SO_4^{2-} transporters Sul1/2 were also been suggested to be transceptors (Schothorst et al. 2013; Conrad et al. 2014). Although there are not many examples of transceptors in plants, excellent work has been reported on the Arabidopsis nitrate transporters NRT1.1 and NRT2.1, which have such a regulatory role, with NRT1.1 being the first demonstrated nitrate receptor/sensor (Munos et al. 2004; Little et al. 2005; Remans et al. 2006; Ho et al. 2009). Recent genetic and structure-functional studies revealed the importance of NRT1.1 phosphorylation in controlling structural flexibility and transport (Ho et al. 2009; Tsay 2014; Sun et al. 2014; Parker and Newstead 2014).

Results from our prior genetic and physiological studies (Zhang et al. 2014) have led to the hypothesis that the high-affinity SO_4^{2-} transporter SULTR1;2 may have dual functions both in transport and sensing/signaling of S status. It should be noted that SULTR1;2 cannot function in isolation as an S sensor/signaling component based on the observations that the *sel1-15*/16 missense mutations and a T-DNA knockout mutant *sel1-18* show reduced sensitivity to S, but do not entirely abolish the S-limitation response (Zhang et al. 2014). Nevertheless, this work has started to provide intriguing insights into S sensing in plants. In contrast to the recently defined nitrate sensing mechanism for NRT1.1, how SULTR1;2 mediates SO_4^{2-} transport and sensing/signaling in particular at the PM (where sensing, transport and signaling occur) remains to be investigated. The results reported here raise several questions. Specifically, are the transport and sensing/signalling function of SULTR1;2 coupled or are they separate functions (uncoupled), meaning that sensing/signaling is not dependent on the transport function of SULTR1;2? Does SULTR1;2 form a sensor/signalling complex with other proteins? As to the former, our results suggest that transport and sensing may be uncoupled based on evidence that the *sel1-15*/*16* alleles show reduced sensitivity to very high sulfate (10 mM) and to two forms of assimilated S that are not transported by SULTR1;2, GSH and cysteine. For the latter, it is possible that SULTR1;2 may have a redundant sensing/signalling function with SULTR1;1 or even other SO_4^{2-} transporters and also interact with OASTL (Shibagaki and Grossman 2010) in S sensing/signaling. Many of these questions should be readily assessed through further biochemical and cell biological studies. Such studies promise to advance our understanding of SULTR1;2-mediated transport/sensing/signaling mechanisms.

Fig. 3 (continued) treated with 1 mM L-cysteine for 48 h. **c** When the seedlings were treated with 1 mM L-cysteine for 48 h the reduction in gene expression sensitivity to the L-cysteine treatment is evident in the roots of *sel1-15* and *sel1-16* alleles. The *asterisk* (*) indicates values that are significantly different from wild type (WT). The expression of four different S-response genes was measured by QPCR as described in Fig. 2 legend. This figure was adapted from (Zhang et al. 2014)

References

Amtmann A, Armengaud P (2009) Effects of N, P, K and S on metabolism: new knowledge gained from multi-level analysis. Curr Opin Plant Biol 12:275–283

Barberon M, Berthomieu P, Clairotte M, Shibagaki N, Davidian JC, Gosti F (2008) Unequal functional redundancy between the two *Arabidopsis thaliana* high-affinity sulphate transporters SULTR1;1 and SULTR1;2. New Phytol 180:608–619

Buchner P, Takahashi H, Hawkesford MJ (2004) Plant sulphate transporters: co-ordination of uptake, intracellular and long-distance transport. J Exp Bot 55:1765–1773

Cao MJ, Wang Z, Wirtz M, Hell R, Oliver DJ, Xiang CB (2013) SULTR3;1 is a chloroplast-localized sulfate transporter in *Arabidopsis thaliana*. Plant J 73:607–616

Conrad M, Schothorst J, Kankipati HN, Van Zeebroeck G, Rubio-Texeira M, Thevelein JM (2014) Nutrient sensing and signaling in the yeast Saccharomyces cerevisiae. FEMS Microbiol Rev 38:254–299

Dan H, Yang G, Zheng ZL (2007) A negative regulatory role for auxin in sulphate deficiency response in *Arabidopsis thaliana*. Plant Mol Biol 63:221–235

El Kassis E, Cathala N, Rouached H, Fourcroy P, Berthomieu P, Terry N, Davidian JC (2007) Characterization of a selenate-resistant Arabidopsis mutant. Root growth as a potential target for selenate toxicity. Plant Physiol 143:1231–1241

Gojon A, Nacry P, Davidian JC (2009) Root uptake regulation: a central process for NPS homeostasis in plants. Curr Opin Plant Biol 12:328–338

Hawkesford MJ, De Kok LJ (2006) Managing sulphur metabolism in plants. Plant Cell Environ 29:382–395

Hirai MY, Fujiwara T, Awazuhara M, Kimura T, Noji M, Saito K (2003) Global expression profiling of sulfur-starved Arabidopsis by DNA macroarray reveals the role of *O*-acetyl-l-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J 33:651–663

Hirai MY, Yano M, Goodenowe DB, Kanaya S, Kimura T, Awazuhara M, Arita M, Fujiwara T, Saito K (2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A 101:10205–10210

Hirai MY, Klein M, Fujikawa Y, Yano M, Goodenowe DB, Yamazaki Y, Kanaya S, Nakamura Y, Kitayama M, Suzuki H, Sakurai N, Shibata D, Tokuhisa J, Reichelt M, Gershenzon J, Papenbrock J, Saito K (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in arabidopsis by integration of metabolomics and transcriptomics. J Biol Chem 280:25590–25595

Ho CH, Lin SH, Hu HC, Tsay YF (2009) CHL1 functions as a nitrate sensor in plants. Cell 138:1184–1194

Hoefgen R, Nikiforova VJ (2008) Metabolomics integrated with transcriptomics: assessing systems response to sulfur-deficiency stress. Physiol Plant 132:190–198

Kasajima I, Ohkama-Ohtsu N, Ide Y, Hayashi H, Yoneyama T, Suzuki Y, Naito S, Fujiwara T (2007) The *BIG* gene is involved in regulation of sulfur deficiency-response genes in *Arabidopsis thaliana*. Physiol Plant 129:351–363

Kataoka T, Hayashi N, Yamaya T, Takahashi H (2004a) Root-to-shoot transport of sulfate in Arabidopsis. Evidence for the role of SULTR3;5 as a component of low-affinity sulfate transport system in the root vasculature. Plant Physiol 136:4198–4204

Kataoka T, Watanabe-Takahashi A, Hayashi N, Ohnishi M, Mimura T, Buchner P, Hawkesford MJ, Yamaya T, Takahashi H (2004b) Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in Arabidopsis. Plant Cell 16:2693–2704

Kawashima CG, Yoshimoto N, Maruyama-Nakashita A, Tsuchiya YN, Saito K, Takahashi H, Dalmay T (2009) Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. Plant J 57:313–321

Kopriva S, Rennenberg H (2004) Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism. J Exp Bot 55:1831–1842

Lappartient AG, Vidmar JJ, Leustek T, Glass AD, Touraine B (1999) Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. Plant J 18:89–95

Lee BR, Huseby S, Koprivova A, Chetelat A, Wirtz M, Mugford ST, Navid E, Brearley C, Saha S, Mithen R, Hell R, Farmer EE, Kopriva S (2012) Effects of fou8/fry1 mutation on sulfur metabolism: is decreased internal sulfate the trigger of sulfate starvation response? PLoS One 7:e39425

Leturque A, Brot-Laroche E, Le Gall M (2009) GLUT2 mutations, translocation, and receptor function in diet sugar managing. Am J Physiol Endocrinol Metab 296:E985–E992

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol 51:141–165

Little DY, Rao H, Oliva S, Daniel-Vedele F, Krapp A, Malamy JE (2005) The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. Proc Natl Acad Sci U S A 102:13693–13698

Lohi H, Lamprecht G, Markovich D, Heil A, Kujala M, Seidler U, Kere J (2003) Isoforms of SLC26A6 mediate anion transport and have functional PDZ interaction domains. Am J Physiol Cell Physiol 284:C769–C779

Maruyama-Nakashita A, Inoue E, Watanabe-Takahashi A, Yamaya T, Takahashi H (2003) Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways. Plant Physiol 132:597–605

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Yamaya T, Takahashi H (2004a) Induction of SULTR1;1 sulfate transporter in Arabidopsis roots involves protein phosphorylation/dephosphorylation circuit for transcriptional regulation. Plant Cell Physiol 45:340–345

Maruyama-Nakashita A, Nakamura Y, Yamaya T, Takahashi H (2004b) Regulation of high-affinity sulphate transporters in plants: towards systematic analysis of sulphur signalling and regulation. J Exp Bot 55:1843–1849

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel cis-acting element conferring sulfur deficiency response in Arabidopsis roots. Plant J 42:305–314

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006) Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. Plant Cell 18:3235–3251

Munos S, Cazettes C, Fizames C, Gaymard F, Tillard P, Lepetit M, Lejay L, Gojon A (2004) Transcript profiling in the chl1-5 mutant of Arabidopsis reveals a role of the nitrate transporter NRT1.1 in the regulation of another nitrate transporter, NRT2.1. Plant Cell 16:2433–2447

Nikiforova V, Freitag J, Kempa S, Adamik M, Hesse H, Hoefgen R (2003) Transcriptome analysis of sulfur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. Plant J 33:633–650

Ohkama-Ohtsu N, Kasajima I, Fujiwara T, Naito S (2004) Isolation and characterization of an Arabidopsis mutant that overaccumulates *O*-acetyl-L-Ser. Plant Physiol 136:3209–3222

Parker JL, Newstead S (2014) Molecular basis of nitrate uptake by the plant nitrate transporter NRT1.1. Nature 507:68–72

Popova Y, Thayumanavan P, Lonati E, Agrochao M, Thevelein JM (2010) Transport and signaling through the phosphate-binding site of the yeast Pho84 phosphate transceptor. Proc Natl Acad Sci U S A 107:2890–2895

Rausch T, Wachter A (2005) Sulfur metabolism: a versatile platform for launching defence operations. Trends Plant Sci 10:503–509

Remans T, Nacry P, Pervent M, Filleur S, Diatloff E, Mounier E, Tillard P, Forde BG, Gojon A (2006) The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. Proc Natl Acad Sci U S A 103:19206–19211

Reuveny Z, Filner P (1977) Regulation of adenosine triphosphate sulfurylase in cultured tobacco cells. Effects of sulfur and nitrogen sources on the formation and decay of the enzyme. J Biol Chem 252:1858–1864

Rouached H, Berthomieu P, El Kassis E, Cathala N, Catherinot V, Labesse G, Davidian JC, Fourcroy P (2005) Structural and functional analysis of the C-terminal STAS (sulfate transporter and anti-sigma antagonist) domain of the *Arabidopsis thaliana* sulfate transporter SULTR1.2. J Biol Chem 280:15976–15983

Rouached H, Wirtz M, Alary R, Hell R, Arpat AB, Davidian JC, Fourcroy P, Berthomieu P (2008) Differential regulation of the expression of two high-affinity sulfate transporters, SULTR1.1 and SULTR1.2, in Arabidopsis. Plant Physiol 147:897–911

Rouached H, Secco D, Arpat AB (2009) Getting the most sulfate from soil: regulation of sulfate uptake transporters in Arabidopsis. J Plant Physiol 166:893–902

Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelasch D, Thimm O, Udvardi MK, Stitt M (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiol 136:2483–2499

Schothorst J, Kankipati HN, Conrad M, Samyn DR, Van Zeebroeck G, Popova Y, Rubio-Texeira M, Persson BL, Thevelein JM (2013) Yeast nutrient transceptors provide novel insight in the functionality of membrane transporters. Curr Genet 59:197–206

Shibagaki N, Grossman AR (2004) Probing the function of STAS domains of the Arabidopsis sulfate transporters. J Biol Chem 279:30791–30799

Shibagaki N, Grossman AR (2006) The role of the STAS domain in the function and biogenesis of a sulfate transporter as probed by random mutagenesis. J Biol Chem 28:22964–22973

Shibagaki N, Grossman AR (2010) Binding of cysteine synthase to the STAS domain of sulfate transporter and its regulatory consequences. J Biol Chem 285:25094–25102

Shibagaki N, Rose A, McDermott JP, Fujiwara T, Hayashi H, Yoneyama T, Davies JP (2002) Selenate-resistant mutants of *Arabidopsis thaliana* identify Sultr1;2, a sulfate transporter required for efficient transport of sulfate into roots. Plant J 29:475–486

Sun J, Bankston JR, Payandeh J, Hinds TR, Zagotta WN, Zheng N (2014) Crystal structure of the plant dual-affinity nitrate transporter NRT1.1. Nature 507:73–77

Takahashi H, Watanabe-Takahashi A, Smith FW, Blake-Kalff M, Hawkesford MJ, Saito K (2000) The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana*. Plant J 23:171–182

Takahashi H, Yoshimoto N, Saito K (2006) Anionic nutrient transport in plants: the molecular basis of the sulfate transporter gene family. Genet Eng 27:67–80

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu Rev Plant Biol 62:157–184

Thevelein JM, Voordeckers K (2009) Functioning and evolutionary significance of nutrient transceptors. Mol Biol Evol 26:2407–2414

Tsay YF (2014) Plant science: how to switch affinity. Nature 507:44–45

Van Zeebroeck G, Bonini BM, Versele M, Thevelein JM (2009) Transport and signaling via the amino acid binding site of the yeast Gap1 amino acid transceptor. Nat Chem Biol 5:45–52

Wang R, Okamoto M, Xing X, Crawford NM (2003) Microarray analysis of the nitrate response in Arabidopsis roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol 132:556–567

Wirtz M, Beard KF, Lee CP, Boltz A, Schwarzlander M, Fuchs C, Meyer AJ, Heeg C, Sweetlove LJ, Ratcliffe RG, Hell R (2012) Mitochondrial cysteine synthase complex regulates *O*-acetylserine biosynthesis in plants. J Biol Chem 287:27941–27947

Yi H, Galant A, Ravilious GE, Preuss ML, Jez JM (2010) Sensing sulfur conditions: simple to complex protein regulatory mechanisms in plant thiol metabolism. Mol Plant 3:269–279

Yoshimoto N, Takahashi H, Smith FW, Yamaya T, Saito K (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots. Plant J 29:465–473

Yoshimoto N, Inoue E, Saito K, Yamaya T, Takahashi H (2003) Phloem-localizing sulfate transporter, Sultr1;3, mediates re-distribution of sulfur from source to sink organs in Arabidopsis. Plant Physiol 131:1511–1517

Zhang B, Pasini R, Dan H, Joshi N, Zhao Y, Leustek T, Zheng ZL (2014) Aberrant gene expression in the Arabidopsis SULTR1;2 mutants suggests a possible regulatory role for this sulfate transporter in response to sulfur nutrient status. Plant J 77:185–197

Zheng J, Long KB, Shen W, Madison LD, Dallos P (2001) Prestin topology: localization of protein epitopes in relation to the plasma membrane. Neuroreport 12:1929–1935

Comparison of Nitrite Reductase (*Ac*NiR1) with Sulfite Reductase (*Ac*SiR1) in *Allium cepa* (L.)

Srishti Joshi, Susanna C.S. Leung, John A. McCallum, and Michael T. McManus

Abstract Sulfite reductase (SiR) and nitrite reductase (NiR) belong to a family of oxidoreductases with conserved siroheme and iron-sulfur cluster domains. While functional redundancy has been characterized extensively between the two enzymes in prokaryotes, not much work has been done in characterizing the functional redundancy between assimilatory SiR and NiR in plants. Because of the cytotoxic nature of sulfite and nitrite, the substrates for SiR and NiR respectively, proper functioning of these two proteins is essential for plant survival. Here, we describe the preliminary characterization of *Ac*NiR1 in a sulfur-accumulating species onion (*Allium cepa* L.) and provide evidence for its functional redundancy with *Ac*SiR1.

The sulfur and nitrogen assimilatory pathways are two major metabolic pathways in plants which assimilate and incorporate these macronutrients. Proper functioning of these two multi-step pathways has been shown to be essential for plant survival. However, some of the intermediates of the N and S assimilation pathways such as sulfite and nitrite are strong nucleophiles and can cause extensive damage to the plant upon accumulation (Gruhlke and Slusarenko 2012). Adaptive measures to counter the accumulation of sulfite include its oxidation to the less toxic state, sulfate, by peroxisome-localised sulfite oxidase (Brychkova et al. 2007). Sulfite is also diverted to other pathways such as formation of sulfolipids by UDP-sulfoquinovose synthase (Sanda et al. 2001). In contrast, not many nitrate consumption pathways exist in plants other than the recently elucidated nitrate reductase (NR)-dependent

Michael T. McManus: Author was deceased at the time of publication

S. Joshi • S.C.S. Leung • M.T. McManus (✉)
Institute of Fundamental Sciences, Massey University,
Private Bag 11 222, Palmerston North, New Zealand
e-mail: m.t.mcmanus@massey.ac.nz

J.A. McCallum
New Zealand Institute for Plant and Food Research Ltd, Canterbury Agriculture & Science Centre, Christchurch Mail Centre, Private Bag 4704, Christchurch, New Zealand

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_9

Table 1 Specific activity was determined for sulfite and nitrite for recombinant AcSiR1 and AcNiR1 separately. Change in absorbance was measured for AcSiR1 assay at 546 nm and for AcNiR1 at 548 nm

	Enzyme activity (nmol min^{-1} mg^{-1} protein)	
Enzyme	Sulfite as substrate	Nitrite as substrate
Recombinant AcSiR1	235	19
Recombinant AcNiR1	78	2,696

conversion of nitrite to nitric oxide (Zhao et al. 2009). Thus the substrate redundancy between SiR and NiR could also be exploited as an alternative pathway for consumption of both sulfite and nitrite by plants (Crane and Getzoff 1996). In onion, a preferentially sulfur-accumulating species, this redundancy could have a further important role during N or S stress where either enzyme could be recruited to provide an increased reduction of sulfite or nitrite (Table 1).

At a structural level, both enzymes share the same prosthetic groups with a siroheme and 4Fe-4S cluster and both utilize reduced ferredoxin as an electron donor for the six electron reduction of sulfite or nitrite. Alignment of SiR and NiR sequences from different monocot and dicot species including onion, not only shows the four highly conserved cysteine residues which form the point of the 4Fe-4S ligation (Crane and Getzoff 1996), but also the conserved α-helix and β-strands flanking the conserved cysteine residues in the predicted secondary structure (Fig. 1).

A sequence alignment of *Ac*SiR1 and *Ac*NiR1 using the PROMALS server shows 27.15 % as identity between the two sequences with four conserved cysteine residues as well as secondary structure conservation (Fig. 2). Phylogenetic comparison of *Ac*SiR1 and *Ac*NiR1 with other monocot and dicot sequences (Fig. 3), shows both enzymes group distinctly. This distance from other monocot sequences might be due to a lack of annotated SiR sequences from species other than the grasses in the NCBI database.

To check for redundancy between the two enzymes in vitro, *Ac*SiR1 and *Ac*NiR1, constructs with a glutathione-S-transferase (GST) tag, were ligated into the pGEX-6P-3 vector and grown in *E. coli* BL21 strain and recombinant protein purified using glutathione-sepharose 4B. The recombinant proteins were then assayed for activity against sulfite and nitrite. The assay for sulfite reductase measured the total cysteine accumulated in the assay (Brychkova et al. 2012) while the nitrite reductase activity measured the decrease in nitrite using the Griess reagent (Ferrari and Warner 1971). Both recombinant *Ac*SiR1 and *Ac*NiR1 were found to be active and each enzyme reduced both substrates. However, a higher rate of conversion was observed for the preferred physiological substrate in each case.

In summary, similarities in silico between SiR and NiR from different plant species have been investigated with specific structural and functional comparisons between *Ac*SiR1 and *Ac*NiR1. Recombinant *Ac*SiR1 and *Ac*NiR1 have been shown to be active and able to reduce both nitrite and sulfite. This suggests that in addition

Fig. 1 CLUSTAL alignment of SiR and NiR sequences from various monocot and dicot showing conserved residues (highlighted in *red box*) and conserved secondary structure in last line consensus_ss. In the secondary structure, the *blue* colour and letter *e* stands for α-helix and the *red* colour and letter *h* for β sheet. The sequences are grouped into two based on sequence similarity with the group representative sequence ID in *magenta*

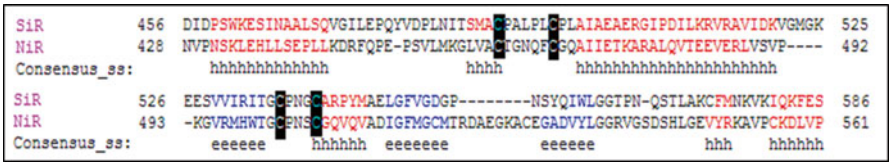
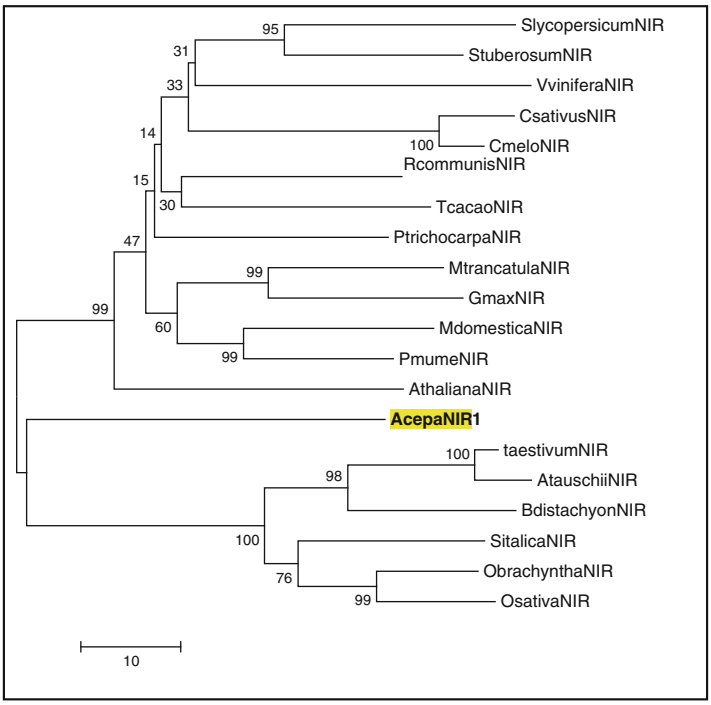


Fig. 2 CLUSTAL alignment of AcNiR1 and AcSiR1 sequences showing conserved cysteine residues highlighted in *black*. In the secondary structure, the *blue* colour and letter *e* denotes the α-helix, while the *red* colour and letter *h* denotes the β-sheet

Fig. 3 Evolutionary relationships of SiR and NiR protein sequences as compared in Fig. 1, and analysed using MEGA6 software. The evolutionary history was inferred using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the number of differences method and are expressed as the number of amino acid differences per sequence



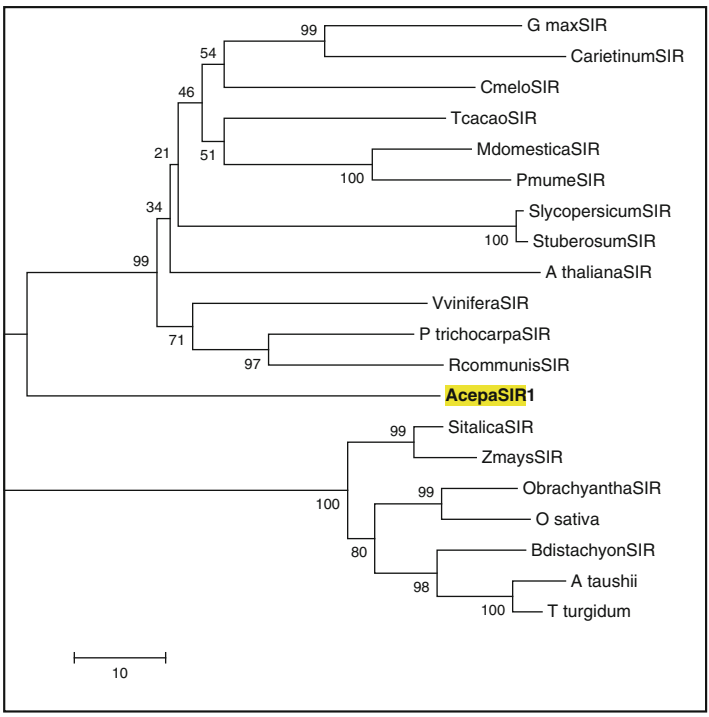


Fig. 3 (continued)

to the different diversions for consumption of sulfite and nitrite described above, reduction of sulfite through *Ac*NiR1 and nitrite through *Ac*SiR1 could also be a possible mechanism to either efficiently detoxify the plant by rapidly consuming sulfite or nitrite, or as a means for recruitment of either enzyme into the N or S assimilation pathways under nutrient stress.

References

Brychkova G, Xia Z, Yang G, Yesbergenova Z, Zhang Z, Davydov O, Fluhr R, Sagi M (2007) Sulfite oxidase protects plants against sulfur dioxide toxicity. Plant J 50:696–709

Brychkova G, Yarmolinsky D, Ventura Y, Sagi M (2012) A novel in-gel assay and an improved kinetic assay for determining in vitro sulfite reductase activity in plants. Plant Cell Physiol 53:1507–1516

Crane BR, Getzoff ED (1996) The relationship between structure and function for the sulfite reductases. Curr Opin Struct Biol 6:744–756

Ferrari TE, Warner JE (1971) Intact tissue assay for nitrite reductase in barley aleurone layers. Plant Physiol 47:790–794

Gruhlke MCH, Slusarenko AJ (2012) The biology of reactive sulfur species (RSS). Plant Physiol Biochem 59:98–107

Sanda S, Leustek T, Theisen MJ, Garavito RM, Benning C (2001) Recombinant Arabidopsis SQD1 converts udp-glucose and sulfite to the sulfolipid head group precursor UDP-sulfoquinovose in vitro. J Biol Chem 276:3941–3946

Zhao MG, Chen L, Zhang LL, Zhang WH (2009) Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing tolerance in Arabidopsis. Plant Physiol 151:755–767

Metabolic Analysis of Sulfur Metabolism During Leaf Senescence

Mutsumi Watanabe and Rainer Hoefgen

Abstract Plants have a constitutive demand for sulfur to synthesize sulfur-containing amino acids, numerous essential metabolites and secondary metabolites for growth and development. Leaf senescence in plants is a highly coordinated physiological process and is critical for nutrient redistribution from senescing leaves to newly formed organs including developing seeds which act as sinks. In order to study the metabolism and recycling of sulfur-containing compounds during leaf senescence, we analyzed the changes of sulfur-containing metabolites using the model plant *Arabidopsis thaliana*.

Cysteine (Cys) synthesis in plants and the respective derived downstream metabolites provide for most non-ruminant animals the only and indispensable source of reduced sulfur as a food or feed constituent. Methionine (Met) is an essential amino acid and Cys is a semi-essential amino acid as animals can convert Met to Cys. However, in many crops, especially cereals, seed protein is deficient in the essential amino acids lysine, tryptophan and especially Met (Galili and Hoefgen 2002; Lee et al. 2001). Therefore, increasing Cys and Met levels in seed protein is a pertinent goal for plant breeding and agricultural biotechnology. Several efforts have been made to enrich the essential amino acids by manipulating sink strength and source provision (Hesse et al. 2001, 2004; Hesse and Hoefgen 2003; Nguyen et al. 2012). In order to improve this manipulation strategy, a better understanding of source-sink relationships of sulfur metabolism including uptake, assimilation, remobilization and accumulation is required. Sulfate as a sulfur source is taken up via the roots, assimilated mainly in photosynthetic leaf tissues and then remobilized during senescence from source leaves to several sink tissues such as developing leaves, roots for growth and seeds for nutrient storage. It was reported that 60 % of total sulfur was remobilized during the senescence process in Arabidopsis leaves (Himelblau and Amasino 2001). However, how sulfur/sulfate is metabolized during the senescence process has not been well studied. To address this issue we conducted a

M. Watanabe • R. Hoefgen (✉)
Max Planck Institute of Molecular Plant Physiology,
Am Muehlenberg 1, 14476 Potsdam-Golm, Germany
e-mail: hoefgen@mpimp-golm.mpg.de

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_10

comprehensive profiling of metabolites including pigments, lipids, sugars, amino acids, organic acids, nutrient ions and secondary metabolites and determined approximately 260 metabolites in Arabidopsis leaves during developmental senescence (Watanabe et al. 2013). Here, we focus on the metabolic changes of sulfur-related metabolites during leaf senescence. In Arabidopsis, sulfur metabolism is very complex and contains a large variety of sulfur metabolites (Nikiforova et al. 2004) such as glutathione (GSH) for antioxidant, Met-derived *S*-adenosylmethionine (SAM) for C1 metabolism and methylations, sulfolipids (SGDGs; sulfoquinovosyldiacylglycerols) for lipid metabolism and glucosinolates (GLSs) for plant defense against pest and diseases (Fig. 1). Protein contains a predominant proportion of the organic sulfur as Cys and Met residues.

For the metabolite profiles of leaf senescence, rosette leaves (leaf nos. 1–12) were harvested at four different stages: 0 % senescent 20 % senescent, 50 % senescent and 100 % senescent, respectively (Fig. 2a). The numbers of green and yellowish rosette leaves were counted to ascertain the percentage of leaves which

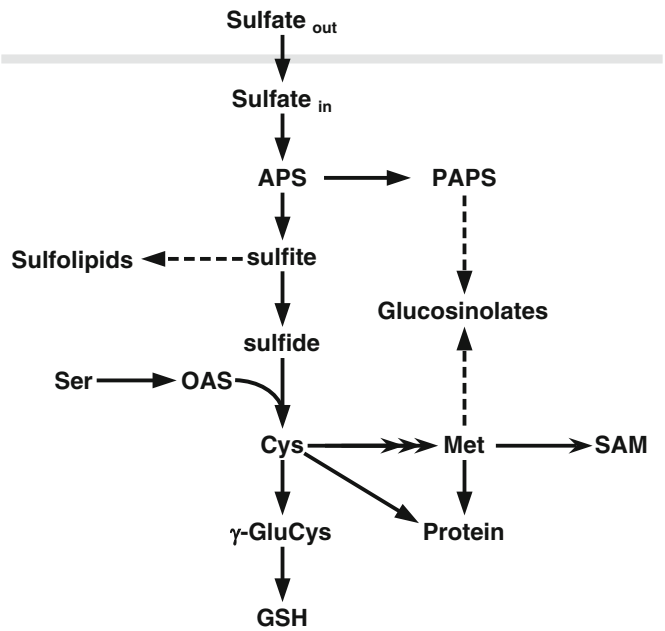


Fig. 1 Sulfur metabolism in *Arabidopsis thaliana*. Sulfate is taken up and activated to adenosine 5′-phosphosulfate (*APS*). APS is further converted to sulfite and 3′-phosphoadenosine 5′-phosphosulfate (*PAPS*). PAPS serves as a substrate for sulfotransferases producing numerous sulfated metabolites including glucosinolates. Sulfite is then reduced to sulfide. In a side reaction, sulfite is also utilized to synthesize sulfolipids. Cysteine (*Cys*) is produced using sulfide and *O*-acetylserine (*OAS*) as activated serine (*Ser*). Cysteine is used for numerous downstream products such as gamma-glutamylcysteine (*γ-GluCys*), glutathione (*GSH*), methionine (*Met*) and *S*-adenosylmethionine (*SAM*). Protein contains a predominant proportion of the organic sulfur as Cys and Met residues

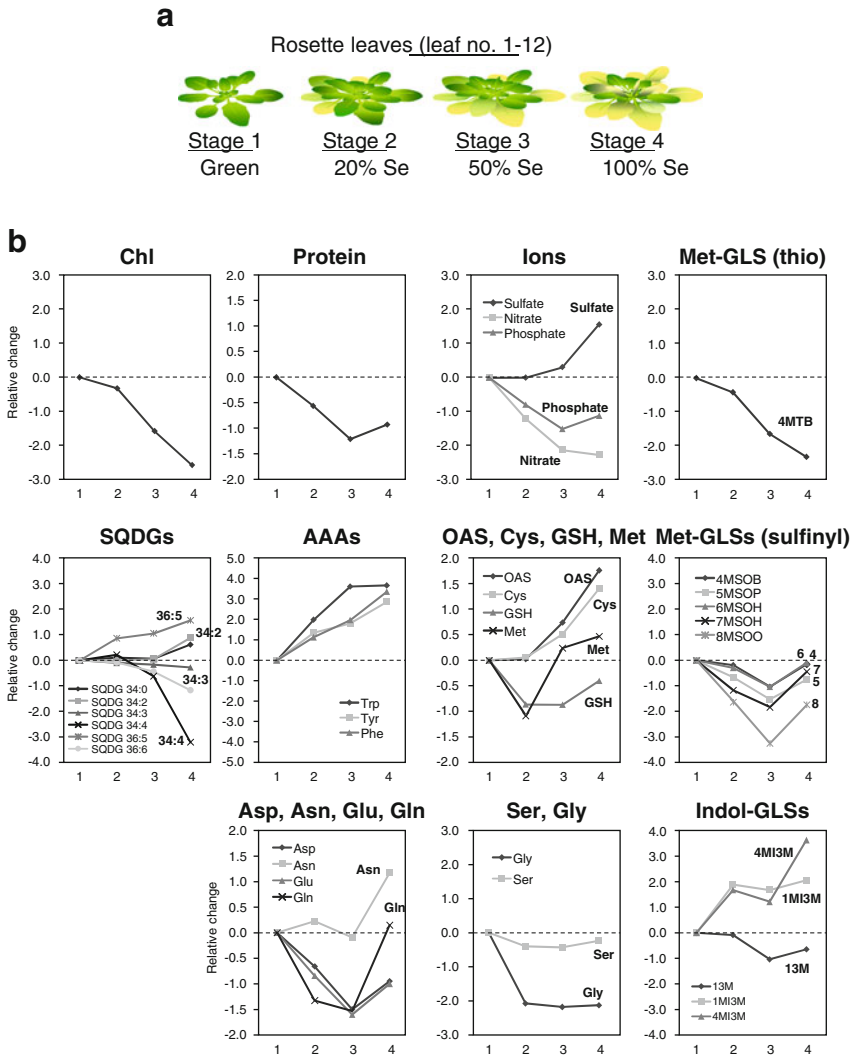


Fig. 2 Metabolite changes in leaves during developmental senescence of *Arabidopsis thaliana*. **a** The leaf senescence in Arabidopsis is schematically depicted. Leaf yellowing due to degradation of chlorophyll in the leaves is a visible phenotype during senescence process (This figure is modified from Watanabe et al. 2013 (www.plantphysiol.org; Copyright American Society of Plant Biologists)). **b** Metabolite changes during leaf senescence are depicted. Metabolite contents in rosette leaves during developmental senescence are presented as fold-change from stage 1 in log 2 scale. No change to stage 1 is indicated by the *dashed line*, decrease by negative and increase by positive values. *Chl* chlorophyll, *SQDG* sulfoquinovosyldiacylglycerol, *AAAs* aromatic amino acids, *GLS* glucosinolate

were senescent. Chlorophyll content as an indicator metabolite of senescence was decreasing during progressing leaf senescence (Fig. 2b). During leaf senescence the loss of chlorophyll is generally correlated with the reduction of the protein content due to the degradation of the highly abundant chloroplast protein Rubisco (ribulose-1,5-bisphosphat-carboxylase-oxygenase; Peoples and Dalling 1978), which was also observed in this study (Fig. 2b). Protein degradation thus provides an important source for the accumulation of amino acids. However, besides by protein degradation the changes of amino acid contents are further affected by various metabolic processes such as transport from source to sink tissue, interconversion to their transportable forms (e.g. conversions of Glu and Asp to Gln and Asn, respectively) and consumption of especially aromatic amino acids (AAAs) for the synthesis of secondary metabolites including flavonoids during leaf senescence (Fig. 2b). The loss of chlorophyll content was also associated with a decline in most chloroplast-localized lipid species such as the mono- and di-galactosyldiacylglycerols (MGDGs and DGDGs) and phosphatidylglycerols (PGs) (Watanabe et al. 2013). Sulfur-containing lipids, SQDGs, which are a minor fraction of chloroplast lipids, displayed a mixed pattern of changes (Fig. 2b). SQDGs are not limiting under optimal growth conditions in Arabidopsis, but SQDGs play a key role in maintaining the proper balance of anionic charge in the thylakoid membrane with substitution of PGs, especially under conditions of phosphate starvation (Yu and Benning 2003). As the most abundant sulfolipid, SQDG34:3 did not display any change while the amounts of other SQDGs showed a mixed pattern of changes (Fig. 2b). It can thus be deduced that a SQDGs-PGs substitution does not occur during leaf senescence despite the low phosphate content in senescencing leaves which is probably caused by efficient mobilization of phosphate from the senescing leaf tissues to sinks (Fig. 2b). Sulfate was increased in leaves during senescence, while other nutrient ions such as nitrate and phosphate were decreased (Fig. 2b), which is consistent with the assumed general mobility of nutrient ions. Nitrogen and phosphorus were reported to be highly mobile nutrients while sulfur is relatively immobile in plants, which is known from the fact that sulfate starvation symptoms typically start in young rather than old leaves, while nitrate and phosphate starvation symptoms are prevalent in old leaves (Bennett 1993; Marschner 1995). It can thus be further concluded that the sink demand for sulfate under optimal sulfate availability conditions can be met by either de novo uptake of sulfate or supply of reduced sulfur compounds from source tissues. Unlike in the case of sulfate starvation where sulfur containing metabolites were all decreased with a concomitant accumulation of *O*-acetylserine (OAS) (Nikiforova et al. 2004, 2005; Hoefgen and Nikiforova 2008), the sulfur metabolites did not show such a co-behavior during leaf senescence (Fig. 2b). Cys and OAS were significantly increased, but GSH was rather decreased, Met showed a bi-phasic pattern and serine as a substrate of OAS was not changed, suggesting that the accumulation level of each sulfur metabolite is regulated individually during leaf senescence.

The regulation of metabolite accumulation is expected to be complex since the steady-state levels of metabolites depend not only on the relative rate of their anabolism and catabolism but also their rate of transport. Furthermore, it needs to be

considered that the metabolism and the accumulation of sulfur metabolites are subcellularly localized in plant cells (Takahashi et al. 2011). Different behaviors of sulfur metabolites were also observed during the diurnal rhythm of plants (Espinoza et al. 2010) and upon light-dark transition (Caldana et al. 2011), where accumulation of OAS was observed without any changes in other sulfur metabolites such as sulfate, sulfite, sulfide, Cys, and GSH (Hubberten et al. 2012). For a better understanding of sulfur metabolism, not only the study of the interactions between various sulfur metabolic pathways but also between subcellular compartments is required.

GSLs, which are sulfur-containing secondary metabolites, are involved in plant defense against various insects and pathogens (Halkier 1999; Rask et al. 2000) and are also a major sink for reduced sulfur in Arabidopsis (Wittstock and Halkier 2002; Halkier and Gershenzon 2006). Sulfinyl-Met-derived-GLSs (sulfinyl Met-GLSs; 4MSOB, 5MSOP, 6MSOH, 7MSOH and 8MSOO for 4-methylsulfinylbutyl-, 5-methylsulfinylpentyl-, 6-methylsulfinylhexyl-, 7-methylsulfinylheptyl-, and 8-methylsulfinyloctyl-, respectively), which are known to be abundant in leaves (Brown et al. 2003), showed a bi-phasic pattern with maximum reduction at the third stage (Fig. 2b), while thio-Met-GLS, (4MTB for 4-methylthiobutyl-GLS), which are known to be abundant in seeds (Brown et al. 2003), was continuously decreased throughout leaf senescence (Fig. 2b). In contrast to the decreasing trend of Met-GLSs, indole-derived-glucosinolates (indole-GLSs; 1- and 4-methoxyindol-3-ylmethyl glucosinolates; 1MI3M and 4MI3M) were significantly increased (Fig. 2b). These different accumulation patterns indicated that the synthesis or metabolic fluxes of Met-GLSs and indole-GLSs were differently regulated, which is consistent with reports that different MYB transcription factors are involved in the regulation of either branch of GLS synthesis (Hirai et al. 2007; Sonderby et al. 2010; Frerigmann and Gigolashvili 2014). Furthermore, the different accumulation patterns of both types of GLSs might in conclusion suggest different physiological roles of the respective GLS classes, at least during senescence.

Metabolite profiles in this study revealed that the various sulfur-containing metabolites accumulated differently during leaf senescence. However, whether these distinct changes in metabolite contents in senescing leaves were caused by the changes in anabolism/catabolism or transport still remains unresolved. In order to address this issue, further detailed analyses, such as flux analysis or transport assays with isotope labeling, are required. Finally, analyzing the absolute amounts of sulfur-containing metabolites, proteins and other cell components is a key requirement in order to understand the differential distribution of the various cellular components and transport metabolites during developmental plant senescence.

Acknowledgements We thank the Max Planck Society (MPG) for funding. We gratefully acknowledge the contributions of Takayuki Tohge, Salma Balazadeh, Alexander Erban, Patrick Giavalisco, Joachim Kopka, Bernd Mueller-Roeber and Alisdair R. Fernie in obtaining the metabolite profiles and discussing the results.

References

Bennett WF (1993) Nutrient deficiencies and toxicities in crop plants. American Phytopathological Society (APS) Press, St. Paul

Brown PD, Tokuhisa JG, Reichelt M, Gershenzon J (2003) Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. Phytochemistry 62:471–481

Caldana C, Degenkolbe T, Cuadros-Inostroza A, Klie S, Sulpice R, Leisse A, Steinhauser D, Fernie AR, Willmitzer L, Hannah MA (2011) High-density kinetic analysis of the metabolomic and transcriptomic response of Arabidopsis to eight environmental conditions. Plant J 67:869–884

Espinoza C, Degenkolbe T, Caldana C, Zuther E, Leisse A, Willmitzer L, Hincha DK, Hannah MA (2010) Interaction with diurnal and circadian regulation results in dynamic metabolic and transcriptional changes during cold acclimation in Arabidopsis. PloS One 5: e14101

Frerigmann H, Gigolashvili T (2014) MYB34, MYB51, and MYB122 distinctly regulate indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. Mol Plant 7:814–828

Galili G, Hoefgen R (2002) Metabolic engineering of amino acids and storage proteins in plants. Metab Eng 4:3–11

Halkier BA (1999) Glucosinolates. In: Ikan R (ed). Naturally occurring glycosides. Wiley: Chichester, UK, p 193–223

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. Annu Rev Plant Biol 57:303–333

Hesse H, Hoefgen R (2003) Molecular aspects of methionine biosynthesis. Trends Plant Sci 8:259–262

Hesse H, Kreft O, Maimann S, Zeh M, Willmitzer L, Hofgen R (2001) Approaches towards understanding methionine biosynthesis in higher plants. Amino Acids 20:281–289

Hesse H, Kreft O, Maimann S, Zeh M, Hoefgen R (2004) Current understanding of the regulation of methionine biosynthesis in plants. J Exp Bot 55:1799–1808

Himelblau E, Amasino RM (2001) Nutrients mobilized from leaves of *Arabidopsis thaliana* during leaf senescence. J Plant Physiol 158:1317–1323

Hirai MY, Sugiyama K, Sawada Y, Tohge T, Obayashi T, Suzuki A, Araki R, Sakurai N, Suzuki H, Aoki K, Goda H, Nishizawa OI, Shibata D, Saito K (2007) Omics-based identification of Arabidopsis Myb transcription factors regulating aliphatic glucosinolate biosynthesis. Proc Natl Acad Sci U S A 104:6478–6483

Hoefgen R, Nikiforova VJ (2008) Metabolomics integrated with transcriptomics: assessing systems response to sulfur-deficiency stress. Physiol Plant 132:190–198

Hubberten HM, Klie S, Caldana C, Degenkolbe T, Willmitzer L, Hoefgen R (2012) Additional role of O-acetylserine as a sulfur status-independent regulator during plant growth. Plant J 70:666–677

Lee SI, Kim HU, Lee YH, Suh SC, Lim YP, Lee HY, Kim HI (2001) Constitutive and seed-specific expression of a maize lysine-feedback-insensitive dihydrodipicolinate synthase gene leads to increased free lysine levels in rice seeds. Mol Breed 8:75–84

Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic, London, p 1–889

Nguyen HC, Hoefgen R, Hesse H (2012) Improving the nutritive value of rice seeds: elevation of cysteine and methionine contents in rice plants by ectopic expression of a bacterial serine acetyltransferase. J Exp Bot 63:5991–6001

Nikiforova VJ, Gakiere B, Kempa S, Adamik M, Willmitzer L, Hesse H, Hoefgen R (2004) Towards dissecting nutrient metabolism in plants: a systems biology case study on sulphur metabolism. J Exp Bot 55:1861–1870

Nikiforova VJ, Kopka J, Tolstikov V, Fiehn O, Hopkins L, Hawkesford MJ, Hesse H, Hoefgen R (2005) Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidopsis plants. Plant Physiol 138:304–318

Peoples MB, Dalling MJ (1978) Degradation of ribulose 1,5-bisphosphate carboxylase by proteolytic enzymes from crude extracts of wheat leaves. Planta 138:153–160

Rask L, Andreasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J (2000) Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol 42:93–113

Sonderby IE, Geu-Flores F, Halkier BA (2010) Biosynthesis of glucosinolates – gene discovery and beyond. Trends Plant Sci 15:283–290

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu Rev Plant Biol 62:157–184, In: Merchant SS, Briggs WR, Ort D (eds)

Watanabe M, Balazadeh S, Tohge T, Erban A, Giavalisco P, Kopka J, Mueller-Roeber B, Fernie AR, Hoefgen R (2013) Comprehensive dissection of spatiotemporal metabolic shifts in primary, secondary, and lipid metabolism during developmental senescence in Arabidopsis. Plant Physiol 162:1290–1310

Wittstock U, Halkier BA (2002) Glucosinolate research in the Arabidopsis era. Trends Plant Sci 7:263–270

Yu B, Benning C (2003) Anionic lipids are required for chloroplast structure and function in Arabidopsis. Plant J 36:762–770

Apoplastic Iron Concentration in Maize Roots Grown Under Sulfate Deprivation

Filippa Maniou, Styliani N. Chorianopoulou, and Dimitris L. Bouranis

Abstract Apoplastic iron (Ai) represents all iron (Fe) fractions located within the space delimited by the plasma membranes and the plant surface; Fe precipitations attached to the root surface (Di) are not included. Total extractable Fe (TEi) includes Ai and Di. Seven-day-old maize plants were grown hydroponically for 19 days under sulfate deprivation against a control. TEi and Ai concentrations were determined and their kinetics studied in each root type of maize plants grown under sulfate deprivation in samples taken at various hours (0.5, 1, 2, 3, 6) and days (7, 8, 9, 10, 17, 26) during each treatment. In order to remove any iron precipitations from the root surface, a treatment with dithionite-citrate-bicarbonate (DCB) was performed. After 10 days under sulfate deprivation, the total root iron (ROOTi) was lower than the control by 28 %, allocated as internal iron (INTi) by 96 %, Ai by 2 % and Di by 2 %. The Ai within the root system was allocated by 58 % in the embryonic roots (unchanged), 16 % in the mesocotyl roots (increased) and 26 % in the crown roots (decreased). After 19 days of sulfate deprivation, the ROOTi was lower than the control by 97 %, allocated as INTi by 57 %, Ai by 20.6 % and Di by 22.4 %. The Ai within the root system was allocated by 42 % in the embryonic roots (decreased), 34 % in the mesocotyl roots (increased) and 24 % in the crown roots (decreased). Crown roots suffered the largest decrease compared to the embryonic system, whilst the mesocotyl roots were richer in Ai.

Maize plants grown in a nutrient solution with ferric EDTA as the source of iron utilize it relatively poorly. Maize as a graminaceous plant utilizes strategy II for Fe uptake; the phytosiderophore deoxymugineic acid (DMA) is excreted from roots and the Fe-DMA is transported into the plant via the YELLOW STRIPE1 (YS1) protein. DMA is derived from nicotianamine (NA). Graminaceous plants possess the unique characteristic of being able to convert NA to phytosiderophores. Methionine is the precursor of NA, which means that NA pools depend on the S status of the root. From the above it appears that the Fe-chelator DMA as Fe-acceptor

F. Maniou • S.N. Chorianopoulou • D.L. Bouranis (✉)
Plant Physiology Laboratory, Crop Science Department, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece
e-mail: bouranis@aua.gr

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_11

is not strong enough to capture iron from the Fe-donor Fe(III)-EDTA. On the other hand, the formation of a ferric iron pool in the free spaces of maize roots has been reported (Bienfait et al. 1985). The intimate relationship between Fe and S in maize under iron deprivation has been reported (Astolfi et al. 2004). The formation of this pool depends on the source of iron in the nutrient solution. Sulfate deprivation alters root cation exchange capacity, as pectin esterification and feruloylation of root cell walls are affected by the deficiency in sulfate (Bouranis et al. 2009), whilst the expression of ferritin genes *ZmFe1* and *ZmFe2* in the roots of S-deprived young maize plants has been reported (Chorianopoulou et al. 2012). In this work the kinetics of iron in the free space (Ai) of maize roots experiencing sulfate deprivation are presented, along with the corresponding external iron depositions (Di).

Maize was grown in a full nutrient solution (C) from day 7 onwards: the solution was adjusted to a pH of 5.5 and well aerated, the source of iron was ferric EDTA and the available iron per plant was 70 μmol. Sulfate deprivation (−S) also began on day 7. The root system of maize includes one primary (PR) and several seminal roots (SR), comprising the embryonic root system, and the crown roots, i.e. the successive nodal roots (CR1, CR2, CR3), which along with the lateral roots comprise the post-embryonic system. The cultivar used in this study also produces mesocotyl roots (MR). However, in general maize does not produce mesocotyl roots. The location of Di on the surface area of the maize roots was visualized by means of ferrocyanide, which reacts with ferric iron to produce ferric ferrocyanide, a blue pigment known as Prussian Blue (Perl's Prussian Blue staining; PPB; Meguro et al. 2007).

Staining with PPB revealed that (1) there were iron depositions on the root surface, (2) the allocation of the depositions along the root axis was not uniform, (3) the allocation differed in each root type, and (4) sulfate deprivation affected the allocation of iron depositions (Table 1). PPB was mainly located in the root sector that carries lateral and emerging lateral roots. An arbitrary scale was adopted to depict the staining gradient, which shows that under sulfate deprivation more depositions were concentrated in the root sector carrying the lateral roots. Di is a fraction of iron deposited onto the root surface and composed of hydrous ferric oxyhydroxides containing ferric phosphates. This fraction is a nanoporous material that presents high surface area to volume ratio and high density of point defects and is therefore an excellent adsorber. According to Bienfait et al. (1985), during nitrate uptake a pH gradient forms in the solution surrounding the plant roots which excrete HO^- ions, the pH being more alkaline in this free space. Accordingly, it will be in the solution surrounding the roots that the equilibrium between hydroxyl, chelator, and ferric ions will be most strongly shifted toward formation of a ferric hydroxide complex. In aerated solutions, with NO_3^- as a source of nitrogen, crystal growth is then a plausible mechanism for apoplastic free space iron pool formation. To visualize the pH on the surface area of the roots bromocresol purple was used; this appears yellow at pH below 6 and becomes purple at pH 6.5 (Weisenseel et al. 1979). At the employed concentration the pH indicator had no harmful effects on the roots. Staining the various root types with bromocresol purple (Table 2) and comparing with Table 1, in most cases purple staining coincided with dark PPB coloration.

Table 1 The allocation of Di along root axis, as revealed by the allocation of PPB staining. A dash indicates no staining with Prussian Blue; 1–3: an arbitrary staining scale from light to deep colour was adopted to simulate colour intensity

C

days		PR				SR				CR1				CR2				MR			
		B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A
d7	0	-	-	-	-	-	-		-												
	0.5h	-	-	-	-	-	-		-												
	1h	-	-	-	-	-	-		1												
	2h	-	1	-	1	-	-		1												
	3h	-	1	1	1	-	1		1												
	6h	-	1	2	-	-	2		-												
d8		-	2	2	-	-	2		-												
d9		-	1	1	-	-	1		-		-										
d10		-	2	1	-	-	2	1	-		-										
d17		-	2	-	-	-	2	-	-		-							1	1		-
d26		2	2	-	-	2	2	-	-	2	1	-	-	2	1	-	-	3	2	-	-

-S

days		PR				SR				CR1				CR2				MR			
		B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A
d7	0	-	-	-	-	-	-	-	-												
	0.5h	-	-	-	-	-	-	-	-												
	1h	-	1	1	-	-	-		-												
	2h	-	1	2	1	-	1		1												
	3h	-	1	2	-	-	1		1												
	6h	-	1	1	-	-	1		-												
d8		-	2	3	-	-	2		-												
d9		-	2	1	-	-	2		-		-										
d10		-	3	1	-	-	3	1	-		-										
d17		-	3	-	-	-	2	-	-		-							1	1		-
d26		-	3	1	-	2	3	1	-	-	2	1	-	-	2	1	-	2	2		-

Table 2 Estimation of pH along root axis, as revealed by the allocation of bromocresol purple staining. Yellow: pH <6, purple pH >6.5

C

days		PR				SR				CR1				CR2				MR			
		B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A
d7	0	1	1		1	1	2		1												
	0.5h	1	1		1	1	1		1												
	1h	1	1	1	1	1	1		1												
	2h	1	1	1	1	1	1		1												
	3h	1	1	1	1	1	1		1												
	6h	1	1	1	1	1	1		1												
d8		1	1	1	1	1	1		1		1										
d9		1	2	1	1	1	2	1	1		1										
d10		1	2	2	1	1	2	1	1		1										
d17		2	2	1	1	2	2	1	1		1								1		
d26		2	2	1	1	2	2	1	1	1	2	1	1	1	1	1	1	1	1		1

-S

days		PR				SR				CR1				CR2				MR			
		B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A	B	LR	ELR	A
d7	0	1	1		1	1	2		1												
	0.5h	1	1	1	1	1	1		1												
	1h	1	1	1	1	1	1		1												
	2h	1	2	2	1	1	1		1												
	3h	1	1	1	1	1	1		1												
	6h	2	2	1	1	1	1		1												
d8		1	1	1	1	1	1		1		1										
d9		1	2	1	1	1	2	1	1		1										
d10		1	1	1	1	1	1	1	1		1										
d17		2	2	1	1	1	2	1	1	1	1		1						1		
d26		2	2	1	1	2	2	1	1	1	1	1	1	1	1		1	1	1		

Table 3 Kinetics of iron accumulation at the whole plant level, along with its allocation in root, shoot and seed, in plants grown under full nutrition *vs* sulfate deprivation. Δx/x: percentage change of −S value relative to the corresponding control value

		ROOTi	SHOOTi	SEEDi	PLANTi	ROOTi	SHOOTi
	Day	μmol organ^{-1}				%	
C	7	0.07	0.03	0.05	0.15	47	20
	8	0.90	0.03	0.05	0.98	92	3
	9	1.38	0.04	0.04	1.47	94	3
	10	1.50	0.06	0.07	1.62	92	3
	17	2.50	0.20		2.70	93	7
	26	8.70	0.50		9.20	95	5
−S	8	1.14	0.04	0.04	1.22	94	3
	9	0.93	0.04	0.04	1.01	92	4
	10	0.92	0.07	0.04	1.02	90	6
	17	1.80	0.20		2.00	90	10
	26	0.30	0.30		0.60	50	50
Δx/x	8	26	39	−22	24		
	9	−33	−7	−7	−31		
	10	−39	18	−45	−37		
	17	−28	0		−26		
	26	−97	−40		−93		

The kinetics of iron allocation within the various organs (Table 3) revealed that (1) seeds provided iron up to day 10, (2) sulfate deprivation forced seeds to provide more iron, thereby depleting it, (3) during the first day of sulfate deprivation the accumulation of iron increased in both root and shoot, (4) during normal growth 90 % of iron was allocated to the root, and (5) sulfate deprivation reduced root iron dramatically during the third week of deprivation. Applying the Bienfait method (Bienfait et al. 1985) to each root type the total extractable iron (TEi) was determined; dithionite reduces the ferric iron and the ferrous iron reacts with bipyridyl producing a purple colour. Release of K^+ during this reductive mobilization of iron from the roots was used as an indicator of tissue damage and so the extraction method was adapted to 7 min. The difference between total iron of the root (ROOTi) and TEi represents the internal iron (INTi) (Table 4). Under full nutrition, TEi increased along with INTi, which increased in an exponential fashion. Sulfate deprivation decreased TEi dramatically in favor of INTi. After day 17 INTi also reduced dramatically. The Di fraction was removed with dithionite in bicarbonate solution, i.e. the DCB treatment; dithionite reduces ferric iron and the ferrous iron is complexed and removed with citrate (Taylor and Crowder 1983). The Bienfait method was then applied to determine the remaining TEi, i.e. the Ai fraction. In this way ROOTi was distinguished into three fractions: Di, Ai and INTi. Under full nutrition, TEi was composed mainly of Di, which fluctuated whilst Ai increased progressively (Table 5). Under sulfate deprivation, Di fraction was reduced drastically, with Ai increasing on the first day but decreasing significantly after day 10, compared with

Table 4 Kinetics of the contribution of TEi and INTi to the total iron of root system under full nutrition *vs* sulfate deprivation

		ROOTi	TEi	INTi	TEi	INTi
	Day	µmol root^{-1}			% ROOTi	
C	7	0.07	0.02	0.05	29	71
	8	0.90	0.75	0.15	84	16
	9	1.38	1.28	0.10	93	7
	10	1.50	1.10	0.40	73	27
	17	2.50	0.44	2.07	17	83
	26	8.70	3.13	5.57	36	64
	7	0.07	0.02	0.05	29	71
−S	8	1.14	0.18	0.96	16	84
	9	0.93	0.15	0.78	16	84
	10	0.92	0.08	0.84	9	91
	17	1.80	0.07	1.73	4	96
	26	0.30	0.13	0.17	43	57
Δx/x	8	27	−76	553		
	9	−33	−88	684		
	10	−39	−93	110		
	17	−28	−83	−16		
	26	−97	−96	−97		

Table 5 Kinetics of the contribution of Di and Ai fractions to the TEi fraction of root system under full nutrition *vs* sulfate deprivation

		TEi	Di	Ai	Di	Ai
	Day	µmol root^{-1}			%TEi	
C	7	0.02	0.010	0.010	50	50
	8	0.75	0.750	0.003	100	0
	9	1.28	1.255	0.026	98	2
	10	1.10	1.076	0.024	98	2
	17	0.44	0.386	0.049	89	11
	26	3.13	2.982	0.146	95	5
−S	8	0.18	0.175	0.005	97	3
	9	0.15	0.127	0.026	83	17
	10	0.08	0.057	0.023	71	29
	17	0.07	0.036	0.038	49	51
	26	0.13	0.067	0.062	52	48
Δx/x	8	−76	−77	67		
	9	−88	−90	0		
	10	−93	−95	−4		
	17	−83	−91	−22		
	26	−96	−98	−58		

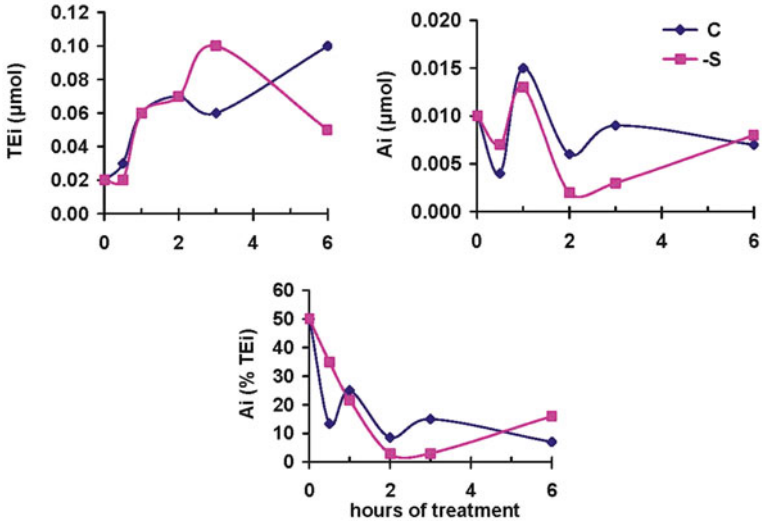


Fig. 1 Kinetics of TEi and Ai fractions during the first 6 h of each treatment

the control. Within the first 6 h in full nutrition at day 7, TEi of the root system was accumulated at a mean rate of 25 nmol h^{-1} for the first 2 h of the treatment and 7.5 nmol h^{-1} for the next 4 h. Under sulfate deprivation, TEi accumulated with a mean rate of 27 nmol h^{-1} for the first 3 h of sulfate deprivation and then decreased with a mean rate of 17 nmol h^{-1} (Fig. 1). The rate of accumulation of Ai fluctuated around 8 nmol h^{-1}, the fluctuation being intense for the first 2 h of the treatment. With sulfate deprivation, Ai accumulation decreased between the second and fifth hours compared with the control. During the first 6 h of each treatment, iron accumulated in the surface of the various root types and Ai was diminished fluctuating (under full nutrition) or steadily (under sulfate deprivation) for the first 2 h. The allocation of TEi and Ai within each root type is given in Table 6.

Summarizing, under full nutrition: (1) the presence of ferric iron was located with PPB staining onto the surface of the root sectors LR and ELR in all root types, (2) the staining intensity increased with time, (3) the root apex remained unstained in all root types, (4) pH increased on the surface of LR and ELR sectors of primary root and secondary roots and onto the LR sector of CR1 (pH >6.5), (5) the iron depositions were greater compared with the apoplastic iron in every root type, and (6) at day 17, the internal iron was more than the sum of apoplastic iron plus the external depositions of iron in the primary root, the seminal roots and the mesocotyl roots and less in the crown roots. At day 26 the internal iron was more in the seminal and the mesocotyl roots and less in all other types.

Under sulfate deprivation: (1) TEi was affected negatively in all root types, (2) Di was affected negatively in all root types, (3) at day17 the INTi fraction was more than the TEi one in the primary root, the seminal and the mesocotyl roots, whilst it was less in the crown roots. At day 26 INTi was more only in the primary and less in the other root types.

Table 6 The percentage allocation of TEi and Ai fractions within each root type under full nutrition *vs* sulfate deprivation

			PR	SR	CR1	CR2	MR	CR3	PR	SR	CR1	CR2	MR	CR3
	Day	Hour	%TEi						%Ai					
C		0	42	58					50	50				
		0.5	30	70					25	75				
	7	1	32	68					33	67				
		2	39	61					33	67				
		3	48	52					33	67				
		6	40	60					29	71				
	8		40	57	3				33	67	0			
	9		48	50	3				19	60	21			
	10		42	54	4				37	57	6			
	17		35	37	15	4	8		16	42	20	14	9	
	26		44	29	16	8	2	1	13	36	23	16	10	3
−S		0.5	25	75					0	100				
		1	38	62					31	69				
	7	2	45	55					0	100				
		3	40	60					33	67				
		6	45	55					0	100				
	8		45	49	6				20	79	1			
	9		39	52	9				23	62	15			
	10		41	47	12				21	64	15			
	17		28	40	13	12	7		16	42	13	13	16	
	26		28	40	17	6	8		18	24	34	16	8	

References

Astolfi S, Zuchi S, Cesco S, Varanini Z, Pinton R (2004) Influence of iron nutrition on sulphur uptake and metabolism in maize (*Zea mays* L.) roots. Soil Sci Plant Nutr 50:1079–1083

Bienfait HF, van den Breil W, Mesland-Mul NT (1985) Free space iron pools in roots. Plant Physiol 78:596–600

Bouranis DL, Mataranga M, Malaganis Y, Gomez LD, Flemetakis E, Chorianopoulou SN, Hawkesford MJ (2009) Effects of sulphate-deprivation on β-galactosidase, β-glucosidase, pectin-methylesterase, and pectin-acetylesterase gene expression in maize root types. In: Sirko A, De Kok LJ, Haneklaus S, Hawkesford MJ, Rennenberg H, Saito K, Schnug E, Stulen I (eds) Sulfur metabolism in plants – regulatory aspects significance of sulfur in food chain, agriculture and environment. Backhuys Publishers, Leiden, pp 93–96, Margraf Publishers, Weikersheim

Chorianopoulou SN, Dimou M, Kazila C, Maniou P, Katinakis P, Bouranis DL (2012) Expession of ferritins *ZmFer1* and *ZmFer2* genes in shoots and roots of S-deprived young maize plants. In: Sulfur metabolism in plants: mechanisms and application to food security, and responses to climate change, De Kok LJ, Tausz M, Hawkesford MJ, Hoefgen R, McManus MT, Norton RM, Rennenberg H, Saito K, Schnug E, Tabe L (eds). Springer Science+Business Media Dordrecht, pp 163–168

Meguro R, Asano Y, Odagiri S, Li C, Iwatsuki H, Shoumura K (2007) Nonheme-iron histochemistry for light and electron microscopy: a historical, theoretical and technical review. Arch Histol Cytol 70:1–19

Taylor GJ, Crowder AA (1983) Use of the DCB technique for extraction of hydrous iron oxides from roots of wetland plants. Am J Bot 70:1254–1257

Weisenseel MH, Dorn A, Jaffle LF (1979) Natural H^+ currents traverse growing roots and root hairs of barley (*Hordeum vulgare*). Plant Physiol 64:512–518

Suitability of the Ratio Between Reduced and Oxidized Glutathione as an Indicator of Plant Stress

Elke Bloem, Silvia Haneklaus, and Ewald Schnug

Abstract It is generally assumed that plant stress is related to the release of reactive oxygen species (ROS) that need to be detoxified. Glutathione exists in reduced (GSH) and oxidized (GSSG) forms, and is involved in the detoxification of ROS during which GSH is oxidized to build GSSG. The glutathione status of the cell is thought to be a sensitive stress marker, and a change in the GSSG content or the GSH/GSSG ratio can indicate stress. However glutathione is also a very rapidly changing pool as an important interface between different metabolic pathways. Therefore whether the ratio between reduced and oxidized glutathione is a suitable indicator for stress under various experimental stress conditions is questionable. The early recognition of stress can be important in agriculture to be able to identify and counteract a special stress early enough to prevent yield losses. In this paper results are compiled from experiments where stress was triggered in greenhouse trials. Severe sulfur deficiency, drought, and elicitation of stress by methyljasmonate (MeJA) application and fungal infection as well as a disturbed mineral nutrition caused by high EDTA soil application were investigated as stress factors. The impact of plant part, age, and plant species was addressed. The results reveal that only under severe stress conditions does the GSH/GSSG ratio significantly decrease. Even under non-stress conditions, the ratio can be quite different depending on other factors such as plant part, age or species making the GSH/GSSG ratio alone unsuitable to be an early stress indicator.

Many different factors affect plant growth and several stress factors were evaluated with respect to its effect on the release of ROS as part of the plant's defense response. It is an obvious question under which conditions plants suffer from stress as fast acclimation of metabolic fluxes and long-term (1–7 days) adaptation (eg. leaf size and thickness) are processes by which plants adapt to changing environmental

E. Bloem (✉) • S. Haneklaus
Federal Research Centre for Cultivated Plants, Institute for Crop and Soil Science, Julius Kühn-Institut (JKI), Bundesallee 50, 38116 Braunschweig, Germany
e-mail: elke.bloem@jki.bund.de

E. Schnug
Institute for Crop and Soil Science, Julius Kühn-Institut (JKI), Braunschweig, Germany

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_12

conditions (Lichtenthaler 1996) which are by definition not stresses. Lichtenthaler (1996) gave the following definition: "*Stress in the context of a plant is any unfavourable condition or substance that affects or blocks plant metabolism, growth, or development*". A reduction in biomass production is an indication that plants suffer from a stress treatment.

Numerous physiological stress parameters such as chilling, drought, desiccation and salt cause an increased production of activated oxygen species (Zhang and Kirkham 1996; Sgherri and Navari-Izzo 1995; Gossett et al. 1994). Preservation of the redox status and detoxification of ROS is vital for the cell as ROS can induce lipid peroxidation and oxidative damage of proteins and DNA (Gill and Tuteja 2010). ROS formation is controlled by an antioxidant system consisting of the low molecular mass antioxidants ascorbic acid, glutathione and tocopherol (Blokhina et al. 2003). One important function of reduced glutathione (GSH) is keeping the redox homeostasis of the cell (Foyer and Noctor 2011). Under stress conditions when ROS were produced, GSSG was reported to accumulate at higher levels in plant cells (Gómez et al. 2004; Vanacker et al. 2000). This oxidation process is catalyzed by different enzymes such as dehydroascorbate reductase, glutathione *S*-transferase or glutathione peroxidase (Rahantaniaina et al. 2013) and a complex cascade of reactions occur under stress. In literature the GSH/GSSG ratio is mentioned to be a possible indicator for plant stress prediction (Tausz et al. 2004; Rahantaniaina et al. 2013). The determination and interpretation of enzyme activities as stress indicators is much more complex because of the involvement of different enzymes in the detoxification of ROS and the high variability of enzyme activities.

When plants are cultivated under optimum growth conditions the GSH/GSSG ratio is high (Rahantaniaina et al. 2013) and usually more than 97 % is present in the reduced form (Vanacker et al. 2000). This ratio can change considerably in relation to environmental conditions, plant age and under stress (see Table 2).

Rahantaniaina et al. (2013) pointed out that a stress-induced accumulation of GSSG is not necessarily connected to a reduction in GSH but that the GSH pool remains rather constant. This indicates a stress-induced GSH neo-synthesis. Therefore it is necessary to determine changes in the content of GSH as well as GSSG and not only the total glutathione content when glutathione is determined as an indicator for stress. Moreover, a stress-related change in glutathione compartmentation was observed by Queval et al. (2011), who found GSSG accumulation particularly in the vacuole. The GSH/GSSG ratio of the whole tissue delivers no indication about compartment-specific ratios. In the present study the question was addressed whether the GSH/GSSG ratio can be used as an indicator for stress under various stress conditions and in different plants.

Three trials were included in this evaluation to highlight the impact of different stress parameters, plant species, and plant parts on the GSH and GSSG content. Sulfur (S) deficiency was investigated because it was shown in the past that the total glutathione content decreases with S deficiency (Bloem et al. 2004) while GSSG has been supposed to increase with stress (Tausz et al. 2004). Further on the impact of drought stress, MeJA application and fungal infection on the glutathione system were investigated. The general impact of stress on the glutathione content has been

shown before (Bloem et al. 2004; Loggini et al. 1999; Tausz et al. 2004) but data are missing if this response is sensitive enough to be used as a stress indicator. EDTA is not a classical stress factor but acts as a complexing agent changing the availability of microelements such as Fe and Pb considerably (Vassil et al. 1998). EDTA application is relevant in agriculture as it enters the environment in significant amounts via sewage sludge application and irrigation of wastewater. The phytotoxic effect of high soil EDTA concentrations is mainly caused by the uptake of protonated EDTA causing significant water losses from the plant tissue ending up with the formation of necrotic lesions (Vassil et al. 1998).

Trial 1: *Sinapis alba* and *Brassica juncea* were grown in a soil sand mixture. At an age of 25 days after sowing (DAS) drought stress was induced by applying no further water until the evapo-transpiration of the plants was less than 80 % of that of the control plants, indicating distinct stomata closure. Further on the plants received 65–70 % of the amount of irrigation water that was applied to the control plants which were watered according to their daily uptake rate. MeJA was applied as spray application (4 mL of a 2 mM solution containing 0.2 % Triton X) 3 days before harvest. Plants were harvested at 63 DAS at full flowering. Trial 2: Three different plant species (*Zea mays, Brassica napus, Helianthus annuus*) were grown in sand culture fertilized sufficiently with all essential plant nutrients. Five different levels of EDTA (ethylenediaminetetraacetic acid), corresponding to 0, 50, 150, 550, 1050 kg ha^{-1} EDTA, were applied to the pots. Different plant species showed differences in their sensitivity towards EDTA and results are presented here for the level, which expressed the first significant effect on vegetative growth. Maize was harvested when the third leaf collar was visible, oilseed rape at early stem elongation and sunflower when their internodes were visibly elongated. Vegetative plant parts and roots were harvested and analyzed. Trial 3: Two different tomato varieties (*Solanum lycopersicum,* variety *Ranger* and *Harzfeuer*) were grown in sand culture and S deficiency (0 mg S per pot) as well as fungal infection (root inoculation with *Verticillium dahliae*) was analyzed in comparison to a control (50 mg S per pot). The variety *Ranger* is supposed to be resistant to *V. dahlia* while *Harzfeuer* is recorded as non-resistant. After root inoculation a first harvest was conducted at the start of flowering and the glutathione content was determined. At fruit development the final harvest was conducted because S deficiency interfered with fruit development. At this time biomass development was recorded. All plants without S fertilization showed severe symptoms of S deficiency. At harvest the plants were divided into leaf, stem, fruit and root material. Fungal infection caused only weak visual symptoms, however, no influence on yield was observed. In all trials biomass development was determined at harvest and plant parts were collected and ground in liquid nitrogen. Glutathione extraction was performed on the fresh material according to Rellan-Alvarez et al. (2006) and the content of oxidized and reduced glutathione determined by LC-MS-MS. The GSH/GSSG ratio was calculated.

The impact of stress treatments on biomass production (Table 1) and on the GSH/GSSG ratio (Table 2) is shown. Reduction in biomass development in comparison to control plants is a clear indication that plants were affected by the treatment. A significant biomass reduction was observed with nearly all stress treatments with exception of fungal infection of tomato plants with *V. dahliae* and MeJA

Table 1 Impact of stress treatment on biomass production of greenhouse-grown plants

Variant	Stress factor	Species	Growth stage	Plant part	Biomass (g FW/pot)		Biomass reduction
					control	stress	(%)
1	Drought	*S. alba*	Flowering	Vegetative	136.5 a	81.5 b	40.3
2		*B. juncea*	Flowering	Vegetative	137.3 a	76.4 b	44.4
3	MeJA	*S. alba*	Flowering	Vegetative	136.5 a	99.1 b	27.4
4		*B. juncea*	Flowering	Vegetative	137.3 a	127.5 a	7.1
5	EDTA (1050 kg/ha)	*Z. mays*	Third leaf	Vegetative	162.5 a	110.5 b	32.0
			Collar visible	Roots	62.3 a	26.9 b	56.8
6	EDTA (550 kg/ha)	*B. napus*	Early stem	Vegetative	113.4 a	64.9 b	42.8
			Elongation	Roots	43.7 a	11.0 b	74.8
7	EDTA (150 kg/ha)	*H. annuus*	Visible	Leaves	21.9 a	8.6 b	60.7
			Elongated	Stems	21.1 a	11.9 b	43.6
			Internodes	Roots	9.3 a	4.3 b	53.8
8	Sulfur deficiency	*S. lycopersicum* *Harzfeuer*	Fruit development	Vegetative	237.2 a	65.6 b	72.3
				Roots	46.6 a	8.9 b	80.9
9		*Ranger*		Vegetative	169.5 a	61.9 b	63.5
				Roots	57.7 a	11.9 b	79.4
10	Fungal infection	*Harzfeuer*	Fruit development	Vegetative	237.2 a	218.3 a	8.0
				Roots	46.6 a	36.6 a	21.5
11		*Ranger*		Vegetative	169.5 a	160.9 a	5.1
				Roots	57.7 a	61.3 a	−6.2

application to *B. juncea* where only a slightly decrease in biomass production was found (Table 1). Root development even increased slightly (by 6.2 %) in one tomato variety (*Ranger*) after fungal infection. Symptoms of fungal infection were observed in the present trial only very early in plant development but no differences were found in relation to variety or later in plant development. Both varieties were able to cope and to suppress the infection under the chosen experimental conditions.

A very high reduction in biomass was determined with S deficiency as well as with EDTA soil application, which caused a disturbance in the mineral nutrition. Microelements such as Fe and Pb build EDTA complexes and consequently their availability and plant uptake is significantly increased (Vassil et al. 1998). In the present trial it was most likely the uptake of protonated EDTA that caused a negative impact on plant performance as no critical heavy metal concentration was found in the soil or was determined in the plant material.

At least in the case of drought, EDTA application and S deficiency where yield reductions of more than 40 % were determined, the applied stress should be high enough to have an impact on the anti-oxidative system.

The relative GSSG content is supposed to increase with stress as during ROS detoxification GSH is oxidized to build GSSG. Therefore the GSH/GSSG ratio

Table 2 Change in the total glutathione content and in the GSH to GSSG ratio in relation to stress treatment, plant part and species/variety

Variant[a]	Plant part	Total glutathione [nmol g^{-1} fw]		GSH content [nmol g^{-1} fw]		GSSG content [nmol g^{-1} fw]		GSH/GSSG ratio	
		control	stress	control	stress	control	stress	control	stress
1	Veg.	**330a**	**228b**	**320a**	**214b**	10.2a	14.4a	**31.1a**	**16.8b**
2	Veg.	189a	197a	179a	176a	10.2a	21.1a	18.5a	10.2a
3	Veg.	330a	330a	320a	315a	**10.2b**	**15.3a**	31.1a	23.3a
4	Veg.	**189a**	**60.9b**	**179a**	**50.1b**	10.2a	10.8a	**18.5a**	**4.5b**
5	Veg.	56.4a	75.7a	53.0a	62.2a	**3.4b**	**10.6a**	15.5a	7.6a
	Root	**17.0b**	**60.2a**	**14.6b**	**55.1a**	**2.4b**	**5.1a**	**6.0b**	**11.0a**
6	Veg.	**90.4b**	**155.7a**	62.8a	65.9a	**27.6b**	**89.8a**	**2.3a**	**0.83b**
	Root	**71.5b**	**122.2a**	**56.9b**	**95.7a**	**14.7b**	**26.6a**	3.8a	3.6a
7	Leaf	85.8a	108.5a	48.1a	39.4a	37.7a	69.1a	1.23a	0.68a
	Stem	30.8a	44.7a	24.6a	35.6a	**6.2b**	**9.1a**	3.8a	4.0a
	Root	36.8a	35.5a	26.0a	22.2a	10.8a	13.3a	2.5a	1.6a
8[b]	Leaf	33.5a	39.3a	**0.81b**	**4.7a**	32.7a	34.6a	**0.026b**	**0.135a**
	Root	16.6a	21.3a	0.30a	0.35a	16.3a	20.9a	0.018a	0.017a
9[b]	Leaf	41.5a	36.3a	**1.2b**	**3.3a**	40.3a	33.0a	**0.031b**	**0.102a**
	Root	23.3a	27.2a	**0.12b**	**0.15a**	23.1a	27.1a	0.005a	0.006a
10[b]	Leaf	33.5a	20.1b	0.81a	0.97a	**32.7a**	**19.2b**	**0.026b**	**0.051a**
	Root	16.6a	8.3b	0.30a	0.22a	**16.3a**	**8.1b**	**0.018b**	**0.027a**
11[b]	Leaf	41.5a	33.1a	1.2a	0.99a	40.3a	32.1a	0.031a	0.031a
	Root	**23.2a**	**16.1b**	0.12a	0.15a	**23.1a**	**16.0b**	**0.005b**	**0.009a**

Different lower case letters indicate significant differences in the total glutathione content or GSH/GSSG ratio in relation to stress treatment at the $LSD_{5\%}$ level

[a]Details of the variants are explained in Table 1

[b]Glutathione was determined at the beginning of flowering in variant 8–11

should decrease under stress. Drought caused such a decrease in the GSH/GSSG ratio in *S. alba* and *B. juncea* in the compiled trials (Table 2) but this decrease was only statistically significant in *S. alba*. The total glutathione content which was much higher in *S. alba* compared to *B. juncea* dropped significantly because of a loss in reduced GSH in *S. alba* while no shift occurred in *B. juncea*.

Also with MeJA application the investigated mustard varieties showed distinctive differences: in *S. alba* no differences were found with MeJA application in the total glutathione and GSH content nor in the GSH/GSSG ratio, only the GSSG content increased with stress. In contrast *B. juncea* showed a significant decrease in the ratio, which was accompanied by a decrease in total glutathione caused by a decrease in GSH (Table 2). MeJA is a natural plant hormone that is supposed to be produced under biotic and abiotic stress conditions to induce the production of defense compounds such as phytoalexins. MeJA application under experimental conditions seemed to simulate a stress situation for the plant quite well but the investigated crops showed differing response: *S. alba* reacted with a yield reduction while *B. juncea* showed no yield reduction but a metabolic response with a

remarkably increased proportion of GSSG causing a decreased GSH/GSSG ratio (Tables 1 and 2). This increased proportion of GSSG was caused by a reduction in GSH with stress while the GSSG content remained constant.

EDTA soil application generally caused an increase in the total glutathione content in all tested plant species, which was partly caused by a significant increase in GSSG with stress. However, the GSH/GSSG ratio did decrease in vegetative plant parts but this decrease was not always significant because of the high variability in glutathione data. The investigated crops showed differences in their sensitivity towards EDTA: sunflower was the most sensitive crop and reacted already to a dose of 150 kg ha^{-1} with a yield reduction of more than 50 %. The data underline that sunflowers did not respond to EDTA stress by their glutathione metabolism (Table 2). Maize, that was the least sensitive investigated crop, was able to increase the total glutathione contents in roots considerably by 3.5-fold. The data give room to speculate that it is probably the capability of a crop to respond flexibly to stress under participation of the glutathione metabolism, which determines the sensitivity of a crop.

The total glutathione content did not change significantly with S deficiency (Table 2). While most S containing metabolites decrease with S deficiency glutathione can react in diverse ways depending on factors such as plant species, growth stage and conditions as well as plant health status (Bloem et al. 2007). In contrast, S deficiency led to an increase in the GSH/GSSG ratio in the leaves of tomato plants while in the roots no changes at all were determined. The reason for this is maybe the fact that the tomato plants already had a very high proportion of oxidized GSSG of more than 90 % at early flowering. In addition, inoculation of tomato plants with *V. dahliae* did not cause a distinct change in the total glutathione content. Only in the roots of *Ranger* did a significant decrease in total glutathione caused by a decrease in GSSG occur with infection.

The GSH/GSSG ratio increased in tomato plants that were inoculated with *V. dahliae*. In both tomato varieties such an increase was observed. The variety *Ranger* is reported to have a higher resistance against *V. dahliae*, but no differences in the GSH/GSSG ratio were observed between the two varieties. Plant age as well as plant species seem to be important for changes in the ratio. The tomato plants revealed a very high proportion of oxidized glutathione at the start of flowering while in comparison *S. alba* and *B. juncea* had a much higher proportion of reduced GSH at flowering. Plants that reveal such a high proportion of GSSG seem to be less sensitive to react to stress by their glutathione system. The results show that the ratio between reduced and oxidized glutathione it not always changing in the same direction with stress. Generally the distribution of reduced and oxidized glutathione differed in the various trials and crops. Only with S deficiency and fungal infection did the GSH/GSSG ratio significantly increase in leaves. However, in that trial it is probable that the very high proportion of oxidized glutathione already in the control plants is the reason that the plants cannot react to stress by further increasing the GSSG content. All other stress treatments increased the proportion of oxidized GSSG.

Water deficit and MeJA application decreased the GSH/GSSG ratio (increased the proportion of GSSG) in *S. alba* and *B. juncea* but this decrease was only

significant in *B. juncea* with MeJA application and in *S. alba* with drought. Both varieties show a different reaction with respect to total glutathione. Drought significantly reduced the total glutathione content in *S. alba* while MeJA did the same in *B. juncea*. In both cases a reduction in GSH was the reason for the reduction in total glutathione while GSSG remained constant. Therefore different varieties show a specific response to different stress treatments and it is not possible to predict the impact on the glutathione system.

A quite diverse response was also found for different plant species in response to soil EDTA application: While in sunflowers the biomass dropped at a concentration of 150 kg ha^{-1} EDTA, *B. napus* reacted to a concentration of 550 kg ha^{-1} and maize to 1050 kg ha^{-1}. Sunflowers were already suffering from the application of EDTA when maize was still growing without any visible signs of stress. In maize only a quite high EDTA level affected plant growth. A significant decrease in the GSH/GSSG ratio in relation to EDTA was found in leaves but this decrease was only significant in oilseed rape. In roots no comparable decrease was found; in contrast, the GSH/GSSG ratio increased in roots of maize (Table 2). The results show that generally stress is better indicated in vegetative plant parts which perform photosynthesis. Only in case of fungal infection with *V. dahliae* where the roots were wounded during root inoculation was a significant shift in the glutathione content found in the roots.

The ratio between reduced and oxidized glutathione proved to be influenced by several factors such as plant species, plant variety, plant part, growth stage, S nutrition and extent of stress or concentration of the stressor. Therefore changes in the ratio of reduced to oxidized glutathione can indicate stress but need to be evaluated carefully and should be accompanied by additional parameters to verify effects. The ratio alone is not a suitable indicator to evaluate plant stress. Moreover an increased proportion of GSSG was only observed in connection with extreme stress. A suitable stress indicator should indicate the onset of stress to enable the farmer to react early enough to treat and correct the situation. Therefore the relation between reduced and oxidized glutathione is only of limited value for early stress prediction. However, the redox state of the glutathione system is a useful tool in scientific experimentation to determine changes in plant metabolism under environmental stress conditions, especially if the data were collected together with other biochemical data.

References

Bloem E, Riemenschneider A, Volker J, Papenbrock J, Schmidt A, Salac I, Haneklaus S, Schnug E (2004) Sulphur supply and infection with *Pyrenopeziza brassicae* influence L-cysteine desulphydrase activity in *Brassica napus* L. J Exp Bot 55:2305–2312

Bloem E, Haneklaus S, Salac I, Wickenhäuser P, Schnug E (2007) Facts and fiction about sulfur metabolism in relation to plant-pathogen interactions. Plant Biol 9:596–607

Blokhina O, Virolainen E, Fagerstedt V (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot 91:179–194

Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol 155:2–18

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930

Gómez LD, Vanacker H, Buchner P, Noctor G, Foyer CH (2004) Intercellular distribution of glutathione synthesis in maize leaves and its response to short-term chilling. Plant Physiol 134:1662–1671

Gossett DR, Millhollon EP, Lucas MC (1994) Antioxidant response to NaCl stress in salt tolerant and salt-sensitive cultivars of cotton. Crop Sci 34:706–714

Lichtenthaler HK (1996) Vegetation stress: an introduction to the stress concept in plants. J Plant Physiol 148:4–14

Loggini B, Scartazza A, Brugnoli E, Navari-Izzo F (1999) Antioxidative defense system, pigment composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. Plant Physiol 119:1091–1099

Queval G, Jaillard D, Zechmann B, Noctor G (2011) Increased intracellular H_2O_2 availability preferentially drives glutathione accumulation in vacuoles and chloroplasts. Plant Cell Environ 34:21–32

Rahantaniaina MS, Tuzet A, Mhamdi A, Noctor G (2013) Missing links in understanding redox signalling via thiol/disulfide modulation: how is glutathione oxidized in plants? Frontiers Plant Sci 4:477

Rellán-Álvarez R, Hernández LE, Abadia J, Álvarez-Fernández A (2006) Direct and simultaneous determination of reduced and oxidized glutathione and homoglutathione by liquid chromatography-electrospray/mass spectrometry in plant tissue extracts. Anal Biochem 356:254–264

Sgherri CLM, Navari-Izzo F (1995) Sunflower seedlings subjected to increasing water deficit stress: oxidative stress and defence mechanisms. Physiol Plant 93:25–30

Tausz M, Sircelj H, Grill D (2004) The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid? J Exp Bot 55:1955–1962

Vanacker H, Carver TLW, Foyer CH (2000) Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hyper-sensitive response in the barley-powdery mildew interaction. Plant Physiol 123:1289–1300

Vassil AD, Kapulnik Y, Raskin I, Salt DE (1998) The role of EDTA in lead transport and accumulation by Indian mustard. Plant Physiol 117:447–453

Zhang J, Kirkham MB (1996) Antioxidant responses to drought in sunflower and sorghum seedlings. New Phytol 132:361–373

Part II
Genes and Proteins

OAS Cluster Genes: A Tightly Co-regulated Network

Fayezeh Aarabi, Hans-Michael Hubberten, Elmien Heyneke, Mutsumi Watanabe, and Rainer Hoefgen

Abstract The carbon input for cysteine synthesis is provided by *O*-acetyl-L-serine (OAS). Upon sulfur limitation, OAS levels increase, leading to the hypothesis that OAS regulates the expression of genes responsive to sulfur deficiency. Recently this hypothesis received support from a global co-expression analysis under conditions of variable OAS levels and constant sulfur status. This study identified six OAS cluster genes (*SDI-1*, *SDI-2*, *LSU1*, *APR3*, *ChaC* and *SHM7*), but the function of these genes and the signaling cascades regulating their expression remain uncharacterized. In our study, we provide additional proofs for the OAS-responsiveness of these genes by performing in silico analysis of their promoter regions together with the analysis of promoter::GUS lines.

Introduction

Several studies proposed the assumption that *O*-acetyl-L-serine (OAS) could be an indicator for sulfur limitation as during sulfur starvation the level of OAS increased because sulfide, the co-substrate for cysteine biosynthesis, was limited. It has also been shown that OAS affects the activity of APS-reductase (APR) and sulfate transporters directly in *Lemna minor* L. (Neuenschwander et al. 1991) and that the expression of several genes of the sulfur-reducing pathway were influenced (Smith et al. 1997; Koprivova et al. 2000; Kopriva et al. 2002; Hesse and Hoefgen 2003; Maruyama-Nakashita et al. 2003). Hirai et al. (2004) observed that OAS had similar effects to short-term -S treatment and considered OAS as a regulator of global transcript/metabolite profiles under short-term sulfur starvation. Moreover, a correlation has been observed between genes being expressed upon sulfur starvation and genes that were activated after feeding with OAS in *Arabidopsis thaliana* roots and

F. Aarabi • H.-M. Hubberten • E. Heyneke
Max Planck Institute of Molecular Plant Physiology, Science Park Potsdam-Golm, 14424 Potsdam, Germany

M. Watanabe • R. Hoefgen (✉)
Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, 14476 Potsdam-Golm, Germany
e-mail: hoefgen@mpimp-golm.mpg.de

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*, Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_13

leaves (Hirai et al. 2003). Further, a metabolite-gene network analysis revealed a correlation between the OAS level and the expression level of some sulfur starvation marker genes such as *SULTR1*;*1*, *SULTR2*;*1*, *SULTR4*;*2*, *APR3*, *ATPS3*, *SERAT3*;*1*, *SERAT3*;*2*,12-oxophytodienoate reductase-1 (*OPR1*), nitrilase 3 and two putative myrosinases (At1g32860, At2g44460) (Hirai et al. 2005). These genes were clustered together with OAS by batch-learning self-organizing mapping (BL-SOM), suggesting a co-regulation of their expression with OAS (Hirai et al. 2005).

Identification of OAS Responsive Genes

A general problem when analyzing the OAS response under sulfur starvation is that other metabolites with potential signaling function such as thiols like cysteine and glutathione are simultaneously altered (Lappartient et al. 1999; Vauclare et al. 2002; Marayuma-Nakashita et al. 2004). Hubberten et al. (2012) evaluated experimental datasets with the aim of separating the specific OAS response from pleiotropic effects by investigating conditions where exclusively the OAS content was altered. They identified OAS responsive genes by analyzing the Arabidopsis transcriptome in four different experiments where the OAS content displayed alterations. Identification of OAS as a putative signal was based on two high-throughput 'omics' time-course experiments of combined transcript and metabolite profiling. Two sets of data were obtained. First, the transition of plants from light to darkness at two different temperatures, 4 °C and 20 °C, where the level of OAS increased after 10 min in darkness (Caldana et al. 2011). Second, during a day-night cycle OAS levels were found to peak in the middle of the night and to be low during daytime (Espinoza et al. 2010). Further computational approaches applying two different correlation analyses were used to identify and validate OAS responsive genes (Hubberten et al. 2012) (Fig. 1). First, OAS to gene correlations were computed to find genes exhibiting expression patterns comparable to changes in OAS levels. Second, these inferred genes were used as guide-genes in a co-expression analysis to identify gene to gene correlations. Finally, a targeted experimental approach employing an inducible expression of SERAT leading to an accumulation of OAS was used in order to test the putative signaling function of OAS (Fig. 1). Eventually six genes were identified as being activated under all conditions. These genes are annotated as sulfur-deficiency-induced 1 (*SDI1*), sulfur-deficiency-induced 2 (*SDI2*), adenosine-5′-phosphosulfate reductase 3 (*APR3*), low-sulfur-induced 1 (*LSU1*), serine hydroxymethyltransferase 7 (*SHM7*) and ChaC-like protein. This set of genes was termed "OAS module" and selected for further elucidation (Table 1).

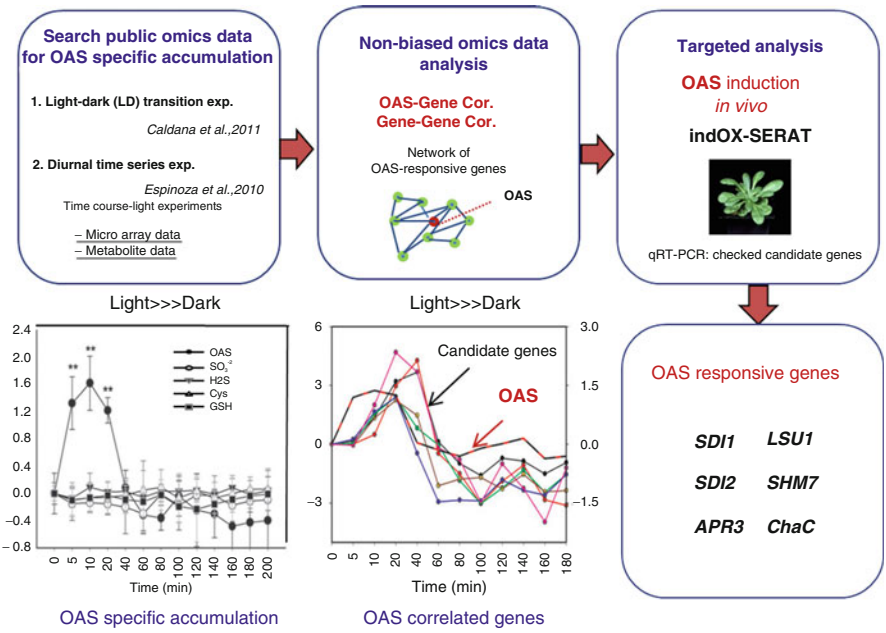


Fig. 1 Identification of OAS responsive genes (Hubberten et al. 2012; Caldana et al. 2011; Espinoza et al. 2010)

Table 1 List of the selected OAS responsive genes

Gene name	AGI ID	Annotation/function
APR3	At4g21990	Known: APS reductase, sulfate assimilation[a]
SHM7	At1g36370	Glycine hydroxymethyltransferase activity
LSU1	At3g49580	*Low sulfur-induced1*
ChaC	At5g26220	Known: glutathione degradation[b]
SDI1	At5g48850	*Sulfurdeficiency-induced 1*-unknown function
SDI2	At1g04770	*Sulfurdeficiency-induced 2*-unknown function

[a]Vauclare et al. 2002
[b]Paulose et al. 2013

In Silico Promoter Analysis of OAS Module Genes

Thus far the only identified transcription factor specifically regulating transcription of genes involved in sulfate uptake and assimilation is SLIM1 (sulfur limitation 1, a member of the EIN3-LIKE (EIL) family), which was found during a screening for mutations influencing the activity of the *SULTR1*;*2* promoter in *Ax thaliana*

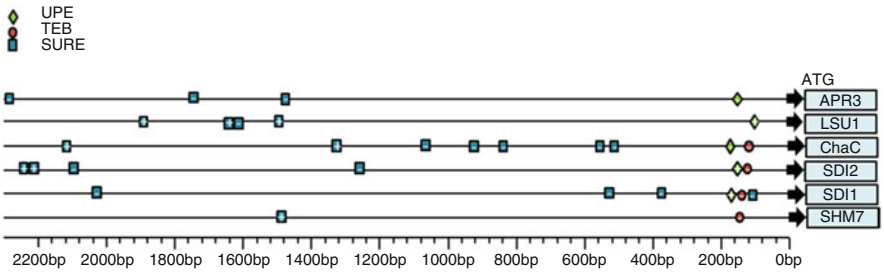


Fig. 2 Position of UPE-like motifs and SURE elements in the promoter regions of the OAS responsive genes (Hubberten et al. 2012)

(Maruyama-Nakashita et al. 2006; Lewandowska et al. 2010). SLIM1 is suggested as a central transcriptional regulator, which controls both the activation of sulfate acquisition and degradation of glucosinolates under – S conditions (Maruyama-Nakashita et al. 2006) and is responsible for increasing the expression of sulfate transporters and other genes induced by sulfate deficiency. However, it seems that SLIM1 cannot be the only factor involved in the regulation of the pathway by sulfate starvation because, for example, the induction of APR mRNA is not compromised in slim1 mutants and therefore its transcript levels are not regulated by SLIM1 (Takahashi et al. 2011). Following the analysis of the promoter region of UP9C (a tobacco gene which is strongly induced by sulfur limitation), a 20-nt sequence UPE-box (UP9-binding element) was identified which is also present in the promoters of several Arabidopsis genes, including some OAS cluster genes such as *APR3*, *LSU1*, *LSU2* and *ChaC* (Wawrzyńska et al. 2010; Hubberten et al. 2012). Further, Hubberten et al. (2012) identified that at least parts of the UPE box (TEB boxes) could also be detected in the promoter regions of SDI1, SDI2 and SHM7 (Fig. 2). The UPE-box, consisting of two parallel TEB sequences (tobacco ethylene-insensitive 3-like binding), proved to be necessary to bind the transcription factor belonging to the EIL family such as SLIM1 (Lewandowska et al. 2010). Furthermore, another well-characterized cis-acting element, SURE (sulfur response element), has been identified in the promoter region of several sulfur starvation responsive genes (Maruyama-Nakashita et al. 2005). A core motif of this cis-element, GAGAC, was shown to be involved in the regulation of gene expression by sulfate deficiency. In silico analysis of the promoter regions of OAS responsive genes revealed the presence of core SURE elements in all these genes. The core SURE element is mostly scattered in the 5′ parts of the promoters furthest from the start codon (Fig. 2). Thus, the functionality of these elements still needs to be investigated.

Histochemical GUS Assay Promoter Lines

In order to check the response of the OAS module genes to sulfur starvation and OAS, promoter::GUS lines of these genes were constructed with a Gateway cassette within GUS expression vector pKGWFS7. 5′-upstream regions of the genes starting

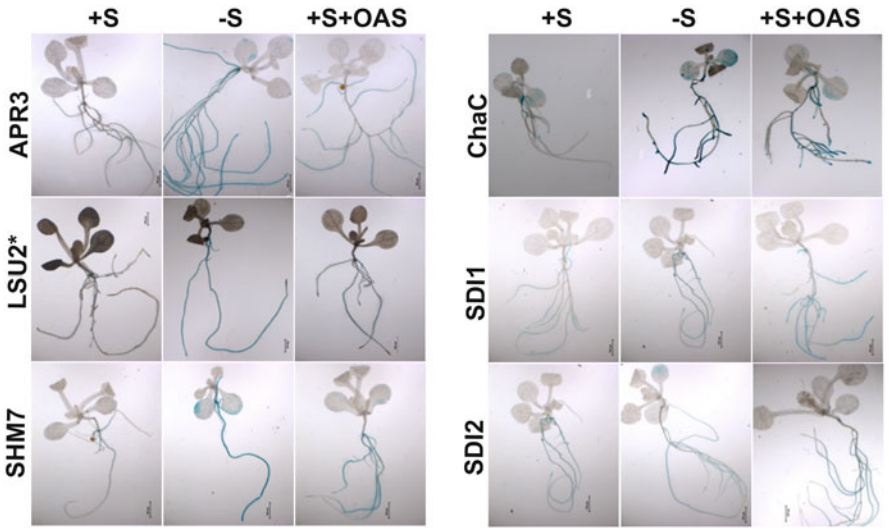


Fig. 3 GUS staining pattern of the promoters. Ten day old seedlings were treated for 48 h under either −S or +S supplemented with 1 mM OAS for 12 h. * A similar GUS expression pattern could be observed for *LSU1* as *LSU2* but is not included in the figure

from the position −2,000 and terminating at the translation initiation site of the genes were selected. Transgenic plants were selected and used for histochemical GUS assay. Ten day old seedlings were transferred to 24 well plates containing either +S (300 μM sulfate) or −S (0 μM sulfate) liquid media and were cultured with gentle shaking (150 rpm) for 2 days. Half of the +S plants were then treated with 1 mM OAS for 12 h. Following the histochemical GUS assay of −S/+S/OAS treated promoter-GUS lines, GUS activity in most seedlings grown at −S and upon OAS treatment increased strongly in the roots, while activity in the cotyledons, compared to the full nutrition condition, was much less (Fig. 3). This pattern is mainly observed in *APR3*, *ChaC*, *SHM7*, *LSU1* and *LSU2* promoter::GUS lines. The intensity of GUS expression was higher in promoter lines of *APR3*, *SHM7* and *ChaC* compared to *LSU* and *SDI* isoforms.

Functional Characterization of OAS Responsive Genes

Apart from *APR3*, which is involved in primary sulfur assimilation, the functional roles of the other genes remain mostly unknown and need further elucidation. The *LSU* family consists of four isoforms in Arabidopsis and at least two of them, *LSU1* (At3g49580) and *LSU2* (At5g24660), are strongly expressed under S deprivation (Wawrzyńska et al. 2010). Moniuszko et al. (2013) observed a direct interaction between one of the tobacco LSU-like isoforms, *UP9C*, with *ACO2A*, a tobacco isoform of 1-aminocyclopropane-1-carboxylic acid oxidase, which is involved in

the regulation of ethylene signaling leading to an elevated level of ethylene in response to S deficiency. Furthermore, a correlation of sulfur starvation to ethylene was predicted by Nikifrova et al. (2003). However, more experiments are still under investigation in order to fully characterize the function of this family in Arabidopsis and in response to sulfur starvation. Among the *SHM* family members, the function of *SHM1* is well characterized in photorespiration for one-carbon metabolism and is reported to be functional for the conversion of glycine into serine (Voll et al. 2006; Engel et al. 2011). Moreover, Moreno et al. (2005) considered a critical role for SHM1 in controlling the cell damage promoted by abiotic stresses (Moreno et al. 2005; Engel et al. 2011). However, little is known about the function of other isoforms of the family. The OAS responsiveness of *SHM7* and its high expression under –S renders it a good candidate for functional analysis. Further, the mutants of SHM7 have been observed to accumulate more sulfur in the shoot (www.ionomicshub.com; Hubberten et al. 2012). Recently there is evidence for the *ChaC*-like protein (GGCT2;1) to have a detoxification role on heavy metals by recycling Glu by its γ-glutamylcotransferase (GGCT) activity leading to the degradation of GSH (Paulose et al. 2013). Little is known about the function of *SDI1* and *SDI2* genes which are members of the MS5-like proteins family. However, *SDI1* and *SDI2* are indicative for low sulfur availability to the plant as their expression is strongly upregulated under sulfur starvation (Howarth et al. 2009).

Conclusions

OAS regulated genes were undoubtedly identified and validated through a stringent global co-expression analysis and also found to be consistently co-regulated under various different conditions in publicly available transcript data from Arabidopsis, rice, populus and medicago (Mutwil et al. 2008; Hubberten et al. 2012). Referring to the GUS staining results obtained from promoter GUS lines of OAS-responsive genes, a general induction of GUS expression could be clearly observed under sulfate depletion conditions as well as in the sulfate-rich conditions supplemented with OAS. This observation also supports the fact that OAS can induce the sulfate starvation response (Hirai et al. 2003; Hubberten et al. 2012). The task will be to further characterize the expression properties of these genes and to assign their molecular role in the response network to sulfur starvation, respectively, OAS induction.

Acknowledgements We thank the Max Planck Society (MPG) for financial support. We are grateful to Eugenia Maximova for assistance with the microscopic analyses.

References

Caldana C, Degenkolbe T, Cuadros-Inostroza A, Klie S, Sulpice R, Leisse A, Steinhauser D, Fernie AR, Willmitzer L, Hannah MA (2011) High-density kinetic analysis of the metabolomic and transcriptomic response of Arabidopsis to eight environmental conditions. Plant J 67:869–884

Engel N, Ewald R, Gupta KJ, Zrenner R, Hagemann M, Bauwe H (2011) The presequence of Arabidopsis serine hydroxymethyltransferase SHM2 selectively prevents import into mesophyll mitochondria. Plant Physiol 157:1711–1720

Espinoza C, Degenkolbe T, Caldana C, Zuther E, Leisse A, Willmitzer L, Hincha DK, Hannah MA (2010) Interaction with diurnal and circadian regulation results in dynamic metabolic and transcriptional changes during cold acclimation in Arabidopsis. PLoS One 5:e14101

Hesse H, Hoefgen R (2003) Molecular aspects of methionine biosynthesis. Trends Plant Sci 8:259–262

Hirai MY, Fujiwara T, Awazuhara M, Kimura T, Noji M, Saito K (2003) Global expression profiling of sulfur-starved Arabidopsis by DNA macroarray reveals the role of O-acetyl-l-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J 33:651–663

Hirai MY, Yano M, Goodenowe DB, Kanaya S, Kimura T, Awazuhara M, Arita M, Fujiwara T, Saito K (2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A 101:10205–10210

Hirai MY, Klein M, Fujikawa Y, Yano M, Goodenowe DB, Yamazaki Y, Kanaya S, Nakamura Y, Kitayama M, Suzuki H, Sakurai N, Shibata D, Tokuhisa J, Reichelt M, Gershenzon J, Papenbrock J, Saito K (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in Arabidopsis by integration of metabolomics and transcriptomics. J Biol Chem 280:25590–25595

Howarth JR, Parmar S, Barraclough PB, Hawkesford MJ (2009) A sulphur deficiency-induced gene, sdi1, involved in the utilization of stored sulphate pools under sulphur-limiting conditions has potential as a diagnostic indicator of sulphur nutritional status. Plant Biotechnol J 7:200–209

Hubberten HM, Klie S, Caldana C, Degenkolbe T, Willmitzer L, Hoefgen R (2012) An additional role of O-acetylserine as a sulphur status independent regulator during plant growth. Plant J 70:666–677

Kopriva S, Suter M, von Ballmoos P, Hesse H, Krahenbuhl U, Rennenberg H, Brunold C (2002) Interaction of sulfate assimilation with carbon and nitrogen metabolism in *Lemna minor*. Plant Physiol 130:1406–1413

Koprivova A, Suter M, den Camp RO, Brunold C, Kopriva S (2000) Regulation of sulfate assimilation by nitrogen in Arabidopsis. Plant Physiol 122:737–746

Lappartient AG, Vidmar JJ, Leustek T, Glass AD, Touraine B (1999) Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. Plant J 18:89–95

Lewandowska M, Wawrzyńska A, Moniuszko G, Lukomska J, Zientara K, Piecho M, Hodurek P, Zhukov I, Liszewska F, Nikiforova V, Sirko A (2010) A contribution to identification of novel regulators of plant response to sulfur deficiency: characteristics of a tobacco gene UP9C, its protein product and the effects of UP9C silencing. Mol Plant 3:347–360

Maruyama-Nakashita A, Inoue E, Watanabe-Takahashi A, Yarnaya T, Takahashi H (2003) Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways. Plant Physiol 132:597–605

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel cis-acting element conferring sulfur deficiency response in Arabidopsis roots. Plant J 42:305–314

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006) Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. Plant Cell 18:3235–3251

Moniuszko G, Skoneczny M, Zientara-Rytter K, Wawrzyńska A, Głów D, Cristescu S, Harren F, Sirko A (2013) Tobacco LSU-like protein couples sulphur-deficiency response with ethylene signalling pathway. J Exp Bot 64:5173–5182

Moreno JI, Martín R, Castresana C (2005) Arabidopsis SHMT1, a serine hydroxymethyltransferase that functions in the photorespiratory pathway influences resistance to biotic and abiotic stress. Plant J 41:451–463

Mutwil M, Obro J, Willats WG, Persson S (2008) GeneCAT–novel webtools that combine BLAST and co-expression analyses. Nucleic Acids Res 36:W320–W326

Neuenschwander U, Suter M, Brunold C (1991) Regulation of sulfate assimilation by light and *O*-acetyl-l-serine in *Lemna minor* L. Plant Physiol 97:253–258

Nikiforova VJ, Freitag J, Kempa S, Adamik M, Hesse H, Hoefgen R (2003) Transcriptome analysis of sulphur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. Plant J 33:633–650

Paulose B, Chhikara S, Coomey J, Jung H, Vatamaniuk O, Dhankher O (2013) A γ-glutamyl cyclotransferase protects Arabidopsis plants from heavy metaltoxicity by recycling glutamate to maintain glutathione homeostasis. Plant Cell 25:4580–4595

Smith FW, Hawkesford MJ, Ealing PM, Clarkson DT, Vanden Berg PJ, Belcher AR, Warrilow AG (1997) Regulation of expression of a cDNA from barley roots encoding a high affinity sulphate transporter. Plant J 12:875–884

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu Rev Plant Biol 62:157–184

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos P, Krahenbuhl U, den Camp RO, Brunold C (2002) Flux control of sulfate assimilation in *Arabidopsis thaliana*: adenosine 5′-phosphosulfate reductase is more susceptible than ATP sulfurylase to negative control by thiols. Plant J 31:729–740

Voll LM, Jamai A, Renné P, Voll H, McClung CR, Weber APM (2006) The photorespiratory Arabidopsis shm1 mutant is deficient in SHM1. Plant Physiol 140:59–66

Wawrzyńska A, Lewandowska M, Sirko A (2010) *Nicotiana tabacum* EIL2 directly regulates expression of at least one tobacco gene induced by sulphur starvation. J Exp Bot 61:889–900

More Than a Substrate: The *O*-Acetylserine Responsive Transcriptome

Hans-Michael Hubberten, Mutsumi Watanabe, Monika Bielecka, Elmien Heyneke, Fayezeh Aarabi, and Rainer Hoefgen

Abstract Sulfate is an important macro nutrient for plant growth. Sulfate starvation negatively influences crop yield and plant performance. Systems analysis at the transcriptome and metabolome level of *Arabidopsis thaliana* exposed to sulfate deprivation recently provided further evidence that *O*-acetyl-L-serine (OAS) acts as a signal within plant sulfate deprivation response, but also under conditions when the sulfate status is not disturbed, such as reactive oxygen species (ROS) accumulation, light to dark shifts or during the diurnal cycle. Here we compare the transcriptomes of roots and whole seedlings exposed to sulfate starvation with that of plants where OAS accumulation was induced by overexpression of a serine acetyltranferase gene (*SERAT2;1*). The results provide evidence for a sulfate-independent signalling role of OAS and for the modular response of plants to sulfate starvation.

Introduction

Plants respond dynamically to limited sulfate at the cellular level and in coordinating responses between various organs, in particular shoot demand for sulfate and uptake by the root system (Watanabe et al. 2012; Hubberten et al. 2009; Whitcomb et al. 2014). Reduced sulfate availability at the root-soil interface first leads to metabolic changes in the primary sulfate assimilation pathway. For the root system, evidence recently accumulated that the primary response, which is the induction of the root sulfate transporters (Table 1), is a local and root autonomous response (Hubberten et al. 2009, 2012b). Free sulfate levels in tissues progressively decrease

H.-M. Hubberten • E. Heyneke • F. Aarabi
Max Planck Institute of Molecular Plant Physiology, Science Park Potsdam-Golm, 14424 Potsdam, Germany

M. Bielecka
Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Wroclaw Medical University, Borowska 211a, 50-556 Wroclaw, Poland

M. Watanabe • R. Hoefgen (✉)
Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, 14476 Potsdam-Golm, Germany
e-mail: hoefgen@mpimp-golm.mpg.de

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*, Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_14

Table 1 Genes expressed due to SERAT overexpression (A), seedling starvation (B) and root starvation (C) for sulfate and the respective joint responses according to Fig. 1. For the conditions B and C only genes related to sulfate metabolism (grey) are listed. Marked in black are the core genes of the OAS cluster (Hubberten et al. 2012a)

Condition	AGI code	Affy No.	Gene name	Expression		
				A	B	C
A	At1g15010	260744_at	expressed protein (related to drought tolerance)	4.7	0.3	0.5
	At4g30290	253608_at	xyloglucan endotransglucosylase/hydrolase 19 (XTH19)	3.6	1.0	1.4
	At1g10585	263210_at	basic helix-loop-helix (bHLH) DNA-binding protein, target of JAM1; JAM2; JAM3	3.0	1.0	0.5
	At3g07600	259251_at	Heavy metal transport/detoxification superfamily protein	2.7	1.6	1.5
	At4g39320	252944_at	microtubule-associated protein-related	2.7	0.7	1.0
	At1g73805	260068_at	Calmodulin binding protein-like (SARD1)	2.3	0.4	0.7
	At1g21110	261450_s_at	*O*-methyltransferase family protein (IGMT3)	2.2	1.6	1.5
	At5g65140	247228_at	trehalose-6-phosphate phosphatase J (TPPJ)	2.4	0.7	1.0
	At5g44050	249071_at	MATE efflux family protein	2.7	1.4	1.5
	At2g18690	266017_at	expressed protein	1.9	0.7	1.1
	At1g64405	259735_at	expressed protein	2.1	1.8	1.4
	At5g23130	249872_at	Peptidoglycan-binding LysM domain-containing protein	2.1	1.0	1.1
	At1g17460	261086_at	TRF-like 3 (TRFL3)	2.4	1.1	1.6
	At1g32860	261187_at	Glycosyl hydrolase superfamily protein	2.2	0.8	1.4
	At5g62730	247447_at	Major facilitator superfamily protein	2.6	1.2	1.5
	At5g09440	245885_at	EXORDIUM like 4 (EXL4)	2.3	1.2	1.5
	At1g49620	257483_at	kip-related protein 7 (KRP7)	2.2	0.8	0.9
	At2g45560	267505_at	cytochrome P450 (CYP76C1)	2.2	0.7	1.0
	At2g37980	266100_at	*O*-fucosyltransferase family protein	2.1	1.0	1.4
	At3g60440	251418_at	Phosphoglycerate mutase family protein	2.0	0.8	1.3
	At5g04250	245699_at	Cysteine proteinases superfamily protein	2.0	1.2	1.2
	At1g18610	255769_at	Galactose oxidase/kelch repeat superfamily protein	2.0	0.8	1.3
	At5g46910	248814_at	Transcription factor jumonji (jmj) family protein	2.0	1.2	1.2
	At2g37760	267181_at	NAD(P)-linked oxidoreductase superfamily protein (AKR4C8)	2.0	1.4	1.3
	At1g74150	259904_at	Galactose oxidase/kelch repeat superfamily protein	2.0	1.2	1.4
	At3g55500	251791_at	expansin A16 (EXPA16)	2.0	1.0	0.9
	At1g63530	261545_at	hypothetical protein	2.1	1.0	2.0
	At4g30520	253620_at	Leucine-rich repeat protein kinase family protein (SARK)	1.8	1.4	1.2
	At2g34660	267319_at	multidrug resistance-associated protein 2 (MPR2)	1.8	1.8	1.5
	At3g01840	259004_at	Protein kinase superfamily protein	1.8	0.7	0.6

(continued)

Table 1 (continued)

A/B/C	At5g26220	246884_at	ChaC-like family protein	6.0	184.3	86.2
	At5g48850	248676_at	SDI1/MS5-1	4.5	161.8	88.8
	At2g44460	267389_at	BGLU28	3.9	551.9	250.3
	At4g21990	254343_at	APS reductase 3 (APR3)	2.9	10.8	4.5
	At1g04770	261177_at	SDI2	2.8	9.5	4.6
	At1g36370	260126_at	SHM7	3.0	19.8	5.7
	At3g49580	252269_at	LSU1	2.4	105.8	92.9
A/B	At1g55920	260602_at	serine acetyltransferase 2;1 (SERAT2;1)	13.4	2.1	1.3
	At4g04610	255284_at	APS reductase 1 (APR1)	2.9	4.9	1.6
	At1g64780	262883_at	ammonium transporter 1;2 (ATAMT1;2)	2.0	3.0	1.1
	At3g58060	251620_at	Cation efflux family protein	2.0	3.9	0.4
A/C	At2g02010	265221_s_at	glutamate decarboxylase 4 (GAD4)	2.3	1.5	2.7
	At5g07500	250583_at	PEI1/AtTZF6	2.8	1.5	5.4
	At2g03020 At4g16540	266772_s_at	Heat shock protein HSP20/alpha crystallin family	2.1	0.7	2.5
	At4g22620	254323_at	RAG1 / SAUR-like	2.1	1.7	3.9
	At2g45570	267559_at	cytochrome P450 (CYP76C2)	1.8	0.5	2.2
B/C	At4g08620	255105_at	SULTR1;1	nd	29.3	91.3
	At1g78000	262133_at	SULTR1;2	nd	16.6	4.5
	At3g12520	256244_at	SULTR4;2	nd	10.9	6.2
	At1g62180	264745_at	APS reductase 2 (APR2)	nd	4.5	2.6
	At1g64660	261957_at	MGL (methionine-gamma-lyase)	nd	2.9	2.7
	At5g10180	250475_at	SULTR2;1	nd	2.6	6.9
	At2g17640	264594_at	SERAT3;1	nd	2.5	2.6
B	At5g44120	249082_at	At12S4 (aleurone layer, seed storage protein, cruciferin)	nd	16.7	0.3
	At1g03880	265095_at	At12S3 (aleurone layer, seed storage protein, cruciferin)	nd	15.0	0.5
	At1g77990	262134_at	SULTR2;2	nd	12.0	1.6
	At5g13550	245855_at	SULTR4;1	nd	3.5	1.9
	At4g14680	245254_at	ATPS3	nd	2.9	2.0
	At5g28020	246701_at	BSAS4;1	nd	2.1	1.6
	At4g27150	253894_at	At2S2 (2S albumin seed storage protein)	nd	2.0	1.9
C	At3g15990	258287_at	SULTR3;4	nd	0.9	2.88
	At1g03890	265094_at	At12S2 (aleurone layer, seed storage protein, cruciferin)	nd	1	2.5

and hence all downstream intermediates including sulfite, sulfide, cysteine, methionine, *S*-adenosylmethionine (SAM) and glutathione (GSH), as well as sulfolipids, display reduced concentrations (Nikiforova et al. 2004, 2005; Krueger et al. 2009, 2010). Each of these metabolites, especially the cytosolic sulfate level, could potentially be considered as a signal for the sulfate starvation response (Hopkins et al. 2005; Lee et al. 2012) and induce the expression of sulfate starvation response genes (Table 1). In contrast to these decreases, the precursor of cysteine synthesis,

O-acetyl-L-serine (OAS), accumulates in an inverse pattern to cellular sulfate contents and as such also provides a potential signal within the sulfate starvation response (Saito 2000; Nikiforova et al. 2004, 2005). This assertion was recently substantiated (Hubberten et al. 2012a). It was further shown that OAS transiently accumulates under various conditions and triggers the expression of a consistent set of genes, even without contemporaneous changes in sulfate, sulfide or sulfur-containing metabolites as e.g. cysteine and GSH. We termed the set of genes coordinately regulated by OAS in disparate conditions, the OAS cluster. Moreover, a coexpression analysis using ATTED-II (Obayashi et al. 2007) revealed additional genes stably coexpressed with the core OAS cluster genes, among them further sulfate metabolism-related genes (*APR1*, *APR2*, *SULTR4*;*1*, *SULTR4*;*2*) (Hubberten et al. 2012a; Watanabe et al. 2012). In conclusion, the evidence strongly supports that assertion that OAS functions as a signal in plant sulfate starvation responses. However, the OAS cluster genes are also induced under conditions when OAS accumulates independently of the plant sulfate status such as during the diurnal cycle during the night (Espinoza et al. 2010), by treatment with drugs such as menadione leading to the production of reactive oxygen species (ROS) (Lehmann et al. 2009), or when plants are shifted from light to dark (Caldana et al. 2011) (for more details see chapter Aarabi et al. 2015, this volume; Hubberten et al. 2012a). Thus, OAS also has a signaling function outside of the sulfate starvation response.

The *O*-Acetyl-L-Serine (OAS) Responsive Transcriptome in Relation to the Sulfate Starvation Transcriptome

It has to be noted that certain parts of the sulfate starvation response such as the increase in sulfate uptake capacity occur before the accumulation of OAS, thus indicating further induction and signaling pathways to be present (Hopkins et al. 2005). The transcription factor SLIM1 (At1g73730) regulates a set of sulfate starvation responsive genes and is necessary for upregulation of the sulfate uptake system (Maruyama-Nakashita et al. 2006). Despite the fact that the promoters of all OAS cluster genes contain putative SLIM1 binding sites, such as UPE or TEB boxes (Lewandowska et al. 2010; Hubberten et al. 2012), the response of the respective genes in SLIM1 mutants is not uniform and rather suggests an inhibitory effect (*ChaC*, *SDI1*, *LSU1*, *GAD3*, *PEI1*, auxin response protein At4g22620) or no effect (*APR3*, *SDI2*) of SLIM1 on the expression of the OAS cluster genes under sulfate starvation conditions while only *SHM7* and *BGLU28* show a clear SLIM1 dependency (data not shown). This indicates signaling systems independent of SLIM1. Thus, we have to assume multiple, probably overlapping regulatory systems acting in a dynamic fashion on distinct parts of metabolism, which resembles a modular setup. Such a modular response was also observed in response to mineral nutrient depletion which, for example, identified nutrient depletion induced senescence (NuDIS) as an intrinsic part of the response at the molecular level for various individual mineral nutrient deprivations (Watanabe et al. 2010, 2012; Whitcomb et al. 2014).

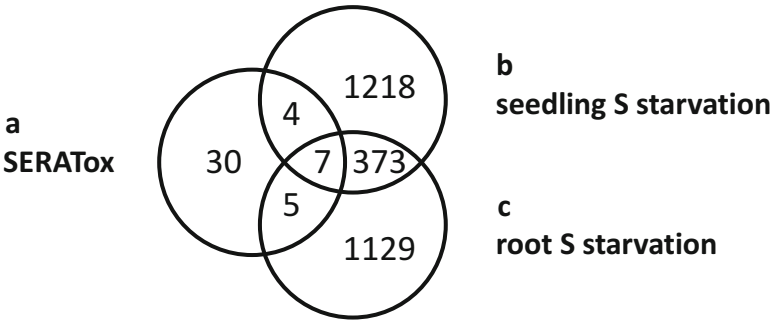


Fig. 1 Venn diagram displaying the overlap of the transcriptomes between SERAT overexpression lines (**a**), seedlings exposed to sulfur starvation (**b**), and roots exposed to sulfate deprivation (**c**). For all cases, genes with an expression of greater than twofold compared to controls were selected for the comparison. The numbers of genes were 46 (**a**), 1,602 (**b**) and 1,514 (**c**). The intersections show the number of genes shared by the respective conditions, respectively the genes entirely specific for either condition. Inducible SERAT overexpression was achieved by fusing the plastidic SERAT2;1 gene to a dexamethasone inducible promotor system and induction with dexamethasone sprayed on the plants grown in soil for 6 h (Hubberten et al. 2012a). Seedlings were grown in submerged shaking cultures where after 1 week precultivation under full nutrient conditions the culture medium of one batch was replaced by zero sulfate (Bielecka et al. 2015). Root tissues was harvested from seedlings grown on agar plates with 1,500 μM sulfate and then transferred to 15 μM sulfate (Maruyama-Nakashita et al. 2006)

We therefore compared the transcriptome of inducibly overexpressed *SERAT2*;*1* seedlings (Hubberten et al. 2012a) with two transcriptome data sets obtained from sulfate-starved plants (Fig. 1 and Table 1). More specifically, we compared the OAS response transcriptome, SERATox, (A) to a seedling sulfur starvation transcriptome (B) (Bielecka et al. 2015), and to a root sulfur starvation transcriptome (C) (Maruyama-Nakashita et al. 2006). A threshold of greater than two was chosen in comparison to the respective controls. The core OAS cluster genes were again identified in the intersection of all three conditions (see Table 1A, B, C). The overlap of SERATox-responsive genes with the seedling experiment additionally includes two sulfate pathway genes (*APR1*, *SERAT2*;*1*) and two transporters, an ammonium transporter (At1g64780) and a cation efflux transporter (At3g58060) (Table 1A, B). While in the root sulfate starvation transcriptome, five additional genes were identified to overlap with the OAS transcriptome (Table 1A, C). The zinc finger protein *PEI1cAtTZF6* (At5g07500) is significantly induced in both datasets. PEI1 is classified as an arginine-rich zinc finger protein. In addition to its role in Arabidopsis embryo development, it is speculated to be involved in the response to various stress conditions. PEI1 localizes under stress conditions or during seed germination to so-called processing bodies and to stress granules. These are ribonucleoprotein particles involved in RNA degradation which influence gene regulation through both targeted and bulk RNA degradation, both of which occur in stressed tissues (Bogamuwa and Jang 2014; Nikiforova et al. 2005). It is thus possible to speculate that PEI1 plays a role in targeted degradation of specific mRNAs during sulfate starvation and OAS accumulation. Induction of glutamate dehydrogenase GAD4

(At2g02010) might provide a link to the accumulation of gamma-aminobutyric acid (GABA) and alanine in sulfate-starved plants, an effect which is abundant under various stress conditions (Nikiforova et al. 2005; Miyashita and Good 2008; Minocha et al. 2014). As GABA might act as a regulator of a stress-induced calcium response or a direct regulator of gene expression through its interrelation with the putrescine/polyamine stress signaling response (Kinnersley and Turano 2000; Minocha et al. 2014), it is possible to speculate that OAS-induced gene expression might in this way be linked to the general stress response symptoms that we observe under sulfate starvation. RAG1/SAUR-like (At4g22620) is an integrator of auxin and gibberellin signals and influences side root branching. Thus, OAS controlled induction of RAG1 expression in sulfate-starved root tissues might affect the root growth responses to sulfate starvation, which were also linked previously to OAS accumulation (Hubberten et al. 2009, 2012b; Gruber et al. 2013).

The root (C) and seedling (B) transcriptomes (Fig. 1) display a joint induced expression of 380 genes in both, root and leaf tissues. However, in both cases about 75 % of the transcripts are specific for either tissue. In Table 1 only those genes that are related to sulfate metabolism are depicted. In both roots and seedlings, genes of the sulfate uptake system and assimilation pathways are induced, but also MGL, which is supposed to be involved in methionine degradation (Table 1B, C). In whole seedlings, which are in largely represented by leaf material, the expression of further genes of sulfate uptake, mobilization and assimilation are induced, unexpectedly, together with seed storage proteins (Table 1B) while only SULTR3;4 and another seed storage protein are expressed specifically in the root (Table 1C). This corroborates the previously identified root-specific function of SULTR3;4 in root to shoot transfer of sulfate (Rouached et al. 2011; Rashid et al. 2013).

As OAS accumulation in SERAT-induced plants is independent of changes in sulfate or sulfate-derived metabolite levels, it is proposed that OAS acts as a signal to induce at least those genes in the intersections with seedlings or roots under sulfate starvation and furthermore, that OAS accumulation during sulfate starvation is causative for the expression of these subsets of genes.

The *O*-Acetyl-L-Serine (OAS) Responsive Transcriptome Independent of the Sulfate Starvation Transcriptome

Despite the fact that OAS accumulation is an intrinsic feature of sulfate starvation in plants, there are a set of 30 genes (when applying a threshold of greater than two), which are responsive to OAS accumulation but not sulfate starvation (Table 1A). Most of these genes show no response to sulfur starvation besides At1g15010, a gene encoding for an unknown protein related to drought tolerance which appears to be downregulated as well as At1g73805, a gene encoding a calmodulin binding protein-like protein (SARD1) (Wan et al. 2012). Although it is a calmodulin-like protein, SARD1 does not bind calmodulin but has been shown to specifically bind

to the promoter of the isochorismate synthase 1 gene, which is involved in salicylic acid biosynthesis in plant defence against pathogens. The only gene being upregulated in root tissues is At1g63530, coding for a protein of unknown function. When defining the various gene sets responsive to certain conditions as modules, it has to be assumed that sulfate module-specific factors inhibit the expression of these genes under sulfate starvation conditions leading to the observed antagonistic behavior despite the presence of OAS. This suggests a complex, multicomponent regulatory stress response network determining the interaction between these modules.

Among the 30 OAS responsive genes are no further genes of primary sulfate metabolism. Of the OAS responsive genes, for example EXL4 (At5g09440) has been shown to play a function as an apoplastic protein in cell expansion in the apical meristem and the flower and is probably linked to brassinosteroid signaling (Schröder et al. 2009; Lisso et al. 2013). When EXL4 is knocked out, growth depression and a reduction in biomass were observed. Thus, EXL4 regulation might point to one of the sulfate starvation-independent functions of OAS. Whether induction of EXL4 links OAS signaling to brassinosteroid signaling needs to be explored. This function in promoting cell growth is further supported by the OAS-specific induced expression of XTH19 (At4g30290) and of expansin A16 (At3g55500) (Maris et al. 2011 JXB; Sakakibara et al. 2006). Trehalose phosphate phosphatase is discussed as a regulator in plants related to carbohydrate signaling and is interestingly also linked to the expression of EXO and EXO-like proteins such as EXL4 (Li et al. 2008; Vandesteene et al.2012; Lisso et al. 2013). The extracellular EXO protein is essential for cell expansion and promotes shoot and root growth. In particular, EXL4 is hypothesized to control plant carbon status and to be linked to trehalose phosphate regulation of the plant's sucrose status and to brassinosteroid regulation. The trehalose phosphate phosphatase J (*AtTPPJ*) (At5g65140) is known to respond to nitrate deficiency or to cadmium stress in roots, the latter resulting in an increased need for GSH. Further, it is naturally expressed during plant senescence and in siliques and developing seeds. While OAS accumulation leads to a slight upregulation of *AtTPPJ*, sulfate starvation has no effect on the expression of this gene and also SLIM1 seems not to control this gene. Thus, the increased demand for GSH and hence sulfate during cadmium stress seems not to be signaled via SLIM1 or other, OAS-independent sulfate starvation response signals. Whether OAS is involved in this signaling system has yet to be evaluated.

Among the OAS-induced genes, further genes are present which are linked to plant stress signaling and to hormone response. The *SARD1* gene (At1g73805) is involved in salicylic acid biosynthesis, a known stress hormone (Stamm and Kumar 2013), and the bHLH transcription factor At1g10585 is involved in jasmonic acid signaling and a target gene of the JAZ/JAM system (Sasaki-Sekimoto et al. 2013). The gene At1g15010, an as yet uncharacterized protein and the strongest responder to OAS, is thought to be involved in plant drought stress response and ABA synthesis as a target of the RING-H2 gene, *XERICO*, (At2g04240), a DELLA protein-induced gene whose overexpression results in ABA accumulation and confers drought and salt tolerance (Ko et al. 2006). Thus, accumulation of OAS without concomitant changes in the plant sulfate status appears to induce genes which are

linked to a variety of hormonal and stress-related responses as well as to the control of cell growth. It has previously been shown that sulfate starvation is linked to plant hormone signaling pathways such as jasmonic acid, auxins, and polyamines (putrescine, spermidine; Nikiforova et al. 2003; Falkenberg et al. 2008) and it is likely that OAS is involved in or modulates the sulfate starvation-related stress responses.

Why OAS accumulates without concurrent changes in other sulfur-related metabolites is still an open question. SERAT is assumed to interact with *O*-acetylserine(thiol)lyase (OASTL) to form OAS as part of the cysteine synthase complex (CSC; Hoefgen and Hesse 2008). OAS is then further converted to cysteine by free OASTL. We must, based on the above-mentioned data, assume that the activity of the CSC or of the individual proteins is modulated by further proteins. A cyclophylin (CYP20-3) has been identified to bind to SERAT2;1 (Dominguez-Solis et al. 2009) and a Recognition of Peronospora Parasitica 1 (RPP1)-like disease resistance gene has been identified to bind to a mutated OASTL affecting plant senescence (Tahir et al. 2013). We postulate further modulations of the SERAT, OASTL, or the CSC to allow OAS accumulation even under conditions of sufficient sulfide availability.

The plant system as a whole integrates these various inputs and seeks to maintain homeostasis and perform its natural developmental processes (Watanabe et al. 2010, 2013). When homeostasis cannot be maintained despite induction of rescue responses, e.g. because of continued sulfate starvation, the plant system induces salvage pathways or, finally, enters into senescence (NuDIS). We therefore suggest a modular response system to plant mineral nutrient depletion which consists of overlapping and specific elements in a dynamic response space (Whitcomb et al. 2014). Although a considerable amount of omics and targeted data on different treatments and mutants has accumulated, we are still far from a complete understanding of the wiring of sulfate metabolism and sulfate deprivation responses even in the model plant species, *Arabidopsisthaliana*. Certain response and regulatory modules are emerging, such as the OAS cluster, but the greater task will be to corroborate these and integrate them into a comprehensive network model with predictive power.

Acknowledgements We thank Sarah J Whitcomb for discussion and help with preparing the manuscript. We thank the Max Planck Society for financial contribution. Additional funding was received for HMH by the DFG grant HO1916/3-2, for FA by the Ministry of Science, Research and Technology of Iran and for EH by the EU – ITN grant 264296 (acronym: BIONUT).

References

Aarabi et al (2015) Chapter 13: OAS cluster genes: a tightly co-regulated network

Bielecka M, Watanabe M, Morcuende R, Scheible WR, Hawkesford MJ, Hesse H, Hoefgen R (2015) Transcriptome and metabolome analysis of plant sulfate starvation and resupply provides novel information on transcriptional regulation of metabolism associated with sulfur, nitrogen and phosphorus nutritional responses in Arabidopsis. Front Plant Sci 5:805

Bogamuwa SP, Jang JC (2014) Tandem CCCH zinc finger proteins in plant growth, development and stress response. PCP 55:1367–1375

Caldana C, Degenkolbe T, Cuadros-Inostroza A, Klie S, Sulpice R, Leisse A, Steinhauser D, Fernie AR, Willmitzer L, Hannah MA (2011) High-density kinetic analysis of the metabolomic and transcriptomic response of Arabidopsis to eight environmental conditions. Plant J 67:869–884

Dominguez-Solis JR, He Z, Lima A, Ting J, Buchanan BB, Luan S (2009) A cyclophilin links redox and light signals to cysteine biosynthesis and stress responses in chloroplasts. Proc Natl Acad Sci U S A 106:1292–1292

Espinoza C, Degenkolbe T, Caldana C, Zuther E, Leisse A, Willmitzer L, Hincha DK, Hannah MA (2010) Interaction with diurnal and circadian regulation results in dynamic metabolic and transcriptional changes during cold acclimation in Arabidopsis. PLoS One 5(11):e14101. doi:10.1371/journal.pone.0014101

Falkenberg B, Witt I, Zanor MI, Steinhauser D, Mueller-Roeber B, Hesse H, Hoefgen R (2008) Transcription factors relevant to auxin signaling coordinate broad-spectrum metabolic shifts including sulphur metabolism. J Exp Bot 59:2831–2846

Gruber BD, Giehl RF, Friedel S, von Wirén N (2013) Plasticity of the *Arabidopsis* root system under nutrient deficiencies. Plant Physiol 163:161–179

Hoefgen R, Hesse H (2008) Sulfur and cysteine metabolism. In: Jez J (ed) Sulfur: a missing link between soils, crops and nutrition, vol 50. American Society of Agronomy, Madison, pp 83–104

Hopkins L, Parmar S, Blaszczyk A, Hesse H, Hoefgen R, Hawkesford MJ (2005) *O*-acetylserine and the regulation of expression of genes encoding components for sulfate uptake and assimilation in potato. Plant Physiol 13:433–440

Hubberten HM, Hesse H, Hoefgen R (2009) Lateral root growth in sulfur enriched patches. In: Sirko A, De Kok LJ, Haneklaus S, Hawkesford MJ, Rennenberg H, Saito K, Schnug E, Stulen I (eds) Sulfur metabolism in plants: regulatory aspects, significance of sulfur in the food chain, agriculture and the environment. Backhuys Publishers, Leiden, pp 105–108, Margraf Publishers, Weikersheim

Hubberten HM, Klie S, Caldana C, Degenkolbe T, Willmitzer L, Hoefgen R (2012a) Additional role of O-acetylserine as a sulfur status-independent regulator during plant growth. Plant J 70:666–677

Hubberten HM, Drozd A, Tran BV, Hesse H, Hoefgen R (2012b) Local and systemic regulation of sulfur homeostasis in roots of *Arabidopsis thaliana*. Plant J 72:625–635

Kinnersley AM, Turano FJ (2000) Gamma aminobutyric acid (GABA) and plant responses to stress. Crit Rev Plant Sci 19:479–509

Ko JH, Yang SH, Han KH (2006) Upregulation of an Arabidopsis RING-H2 gene, XERICO, confers drought tolerance through increased abscisic acid biosynthesis. Plant J 47:343–355

Krueger S, Niehl A, Lopez-Martin MC, Steinhauser D, Donath A, Hildebrandt T, Romero LC, Hoefgen R, Gotor C, Hesse H (2009) Sub-cellular analysis of Arabidopsis serine acetyltransferase mutants: implications of compartmentation for cysteine biosynthesis. Plant Cell Environ 32:349–367

Krueger S, Donath A, Lopez-Martin MC, Hoefgen R, Gotor C, Hesse H (2010) Impact of sulfur starvation on cysteine biosynthesis in T-DNA mutants deficient for compartment-specific serine-acetyltransferase. Amino Acids 39:1029–1042

Lee BR, Huseby S, Koprivova A, Chételat A, Wirtz M, Mugford ST, Navid E, Brearley C, Saha S, Mithen R, Hell R, Farmer EE, Kopriva S (2012) Effects of fou8/fry1 mutation on sulfur metabolism: is decreased internal sulfate the trigger of sulfate starvation response? PLoS One 7:e39425

Lehmann M, Schwarzlaender M, Obata T, Sirikantaramas S, Burow M, Olsen CE, Tohge T, Fricker MD, Moller BL, Fernie AR, Sweetlove LJ, Laxa M (2009) The metabolic response of Arabidopsis roots to oxidative stress is distinct from that of heterotrophic cells in culture and highlights a complex relationship between the levels of transcripts, metabolites, and flux. Mol Plant 2:390–406

Lewandowska M, Wawrzynska A, Moniuszko G, Lukomska J, Zientara K, Piecho M, Hodurek P, Zhukov I, Liszewska F, Nikiforova V, Sirko A (2010) A contribution to identification of novel regulators of plant response to sulfur deficiency: characteristics of a tobacco gene UP9C, its protein product and the effects of UP9C silencing. Mol Plant 3:347–360

Li P, Ma S, Bohnert HJ (2008) Coexpression characteristics of trehalose-6-phosphate phosphatase subfamily genes reveal different functions in a network context. Physiol Plant 133:544–556

Lisso J, Schröder F, Müssig C (2013) EXO modifies sucrose and trehalose responses and connects the extracellular carbon status to growth. Front Plant Sci 4:219. doi:10.3389/fpls.2013.00219

Maris A, Kaewthai N, Eklöf JM, Miller JG, Brumer H, Fry SC, Verbelen JP, Vissenberg K (2011) Differences in enzymic properties of five recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of *Arabidopsis thaliana*. J Exp Bot 62:261–271

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006) Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. Plant Cell 18:3235–3251

Minocha R, Majumdar R, Minocha SC (2014) Polyamines and abiotic stress in plants: a complex relationship. Front Plant Sci 5:175. doi:10.3389/fpls.2014.00175

Miyashita Y, Good AG (2008) Contribution of the GABA shunt to hypoxia-induced alanine accumulation in roots of *Arabidopsis thaliana*. Plant Cell Physiol 49:92–102

Nikiforova VJ, Freitag J, Kempa S, Adamik M, Hesse H, Hoefgen R (2003) Transcriptome analysis of sulphur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. Plant J 33:633–650

Nikiforova VJ, Gakiere B, Kempa S, Adamik M, Willmitzer L, Hesse H, Hoefgen R (2004) Towards dissecting nutrient metabolism in plants: a systems biology case study on sulphur metabolism. J Exp Bot 55:1861–1870

Nikiforova VJ, Kopka J, Tolstikov V, Fieh O, Hopkins L, Hawkesford MJ, Hesse H, Hoefgen R (2005) Systems re-balancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of *Arabidopsis* plants. Plant Physiol 138:304–318

Obayashi T, Kinoshita K, Nakai K, Shibaoka M, Hayashi S, Saeki M, Shibata D, Saito K, Ohta H (2007) ATTED-II: a database of co-expressed genes and cis-elements for identifying co-regulated gene groups in Arabidopsis. Nucleic Acids Res 35:D863–D869

Rashid A, Badhan A, Deyholos M, Kav N (2013) Proteomic profiling of the aleurone layer of mature *Arabidopsis thaliana* seed. Plant Mol Biol Rep 31:464–469

Rouached H, Secco D, Arpat B, Poirier Y (2011) The transcription factor PHR1 plays a key role inthe regulation of sulfate shoot-to-root flux upon phosphate starvation in Arabidopsis. BMC Plant Biol 11:19. doi:10.1186/1471-2229-11-19

Saito K (2000) Regulation of sulfate transport and synthesis of sulfur-containing amino acids. Curr Opin Plant Biol 3:188–195

Sakakibara H, Takei K, Hirose N (2006) Interactions between nitrogen and cytokinin in the regulation of metabolism and development. TiPS 11:440–448

Sasaki-Sekimoto Y, Jikumaru Y, Obayashi T, Saito H, Masuda S, Kamiya Y, Ohta H, Shirasu K (2013) Basic helix-loop-helix transcription factors JASMONATE-ASSOCIATED MYC2-LIKE1 (JAM1), JAM2, and JAM3 are negative regulators of jasmonate responses in Arabidopsis. Plant Physiol 163:291–304

Schröder F, Lisso J, Lange P, Müssig C (2009) The extracellular EXO protein mediates cell expansion in Arabidopsis leaves. BMC Plant Biol. doi:10.1186/1471-2229-9-20

Stamm P, Kumar PP (2013) Auxin and gibberellin responsive Arabidopsis SMALL AUXIN UP RNA36 regulates hypocotyl elongation in the light. Plant Cell Rep 32:759–769

Tahir J, Watanabe M, Jing HC, Hunter DA, Tohge T, Nunes-Nesi A, Brotman Y, Fernie AR, Hoefgen R, Dijkwel PP (2013) Activation of R-mediated innate immunity and disease susceptibility is affected by mutations in a cytosolic *O*-acetylserine (thiol) lyase in Arabidopsis. Plant J 73:118–130

Vandesteene L, López-Galvis L, Vanneste K, Feil R, Maere S, Lammens W, Rolland F, Lunn JE, Avonce N, Beeckman T, Van Dijck P (2012) Expansive evolution of the trehalose-6-phosphate phosphatase gene family in Arabidopsis. Plant Physiol 160:884–896

Wan D, Li R, Zou B, Zhang X, Cong J, Wang R, Xia Y, Li G (2012) Calmodulin-binding protein CBP60g is a positive regulator of both disease resistance and drought tolerance in Arabidopsis. Plant Cell Rep 31:1269–1281

Watanabe M, Hubberten HM, Saito K, Hoefgen R (2010) General regulatory patterns of plant mineral nutrient depletion as revealed by *serat* quadruple mutants disturbed in cysteine synthesis. Mol Plant 3:438–466

Watanabe M, Hubberten HM, Hoefgen R (2012) Plant response to mineral ion availability: transcriptome responses to sulfate, selenium and iron. In: De Kok LJ, Tausz M, Hawkesford MJ, Hoefgen R, McManus MT, Norton RM, Rennenberg H, Saito K, Schnug E, Tabe L (eds) Sulfur metabolism in plants, Proceedings of the International Sulfur Workshop. Springer, Netherlands, pp 123–134

Watanabe M, Balazadeh S, Tohge T, Erban A, Giavalisco P, Kopka J, Mueller-Roeber B, Fernie AR, Hoefgen R (2013) Comprehensive dissection of spatio-temporal metabolic shifts in primary, secondary and lipid metabolism during developmental senescence in *Arabidopsis thaliana*. Plant Physiol 162:1290–1310

Whitcomb SJ, Heyneke E, Aarabi F, Watanabe M, Hoefgen R (2014) Mineral nutrient depletion affects plant development and crop yield. In: Hawkesford, MJ, Kopriva S, De Kok LJ (eds) Nutrient use efficiency in plants: concepts and approaches. Springer, Cham (ZG), Switzerland, pp 205–228

The CBL-SnRK3 Network: Connections to Sulfur Metabolism

Elmien Heyneke, Mutsumi Watanabe, Fayezeh Aarabi, and Rainer Hoefgen

Abstract The CBL-SnRK3 signalling system has received much attention relating to its role in nutrient signalling, largely in respect of N and K, while its role in S and P has not received any attention so far. Increasing evidence indicates that CBL-SnRK3 signalling could also play a role in sulfur starvation responses. In the following paper we introduce the CBL-SnRK3 network and highlight the transcriptional response of SnRKs during sulfur starvation.

Introduction

The CBL-SnRK3 signalling system recently arose as a plant-specific and Ca^{2+}-dependent interaction network mediating various stress responses including nutrient stress responses. It is well known that stresses elicit spatial and temporal changes in intracellular free Ca^{2+} concentrations in the cytosol which function as specific stress signals. This so called Ca^{2+} signature is perceived by calcium sensor proteins which transduce the signals and regulate stress response genes by switching them on or off in key regulatory pathways. The calcineurin b-like (CBL) family of proteins is well known for functioning as sensor relay proteins. Sensor relay proteins do not have kinase activity and must interact with their protein kinases to regulate their activity after binding Ca^{2+}. Unlike SnRK1 and SnRK2, the SnRK3 sub-family is calcium-dependent, and activation of these kinases can only occur after interaction with calcium-binding proteins. Currently the Arabidopsis genome contains 26 SnRK3 and 10 CBL proteins (Fig. 1). In the following chapter the possible involvement of the CBL-SnRK3 signalling system in sulfur starvation responses will be discussed.

E. Heyneke • F. Aarabi
Max Planck Institute of Molecular Plant Physiology, Science Park Potsdam-Golm, 14424 Potsdam, Germany

M. Watanabe • R. Hoefgen (✉)
Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, 14476 Potsdam-Golm, Germany
e-mail: hoefgen@mpimp-golm.mpg.de

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*, Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_15

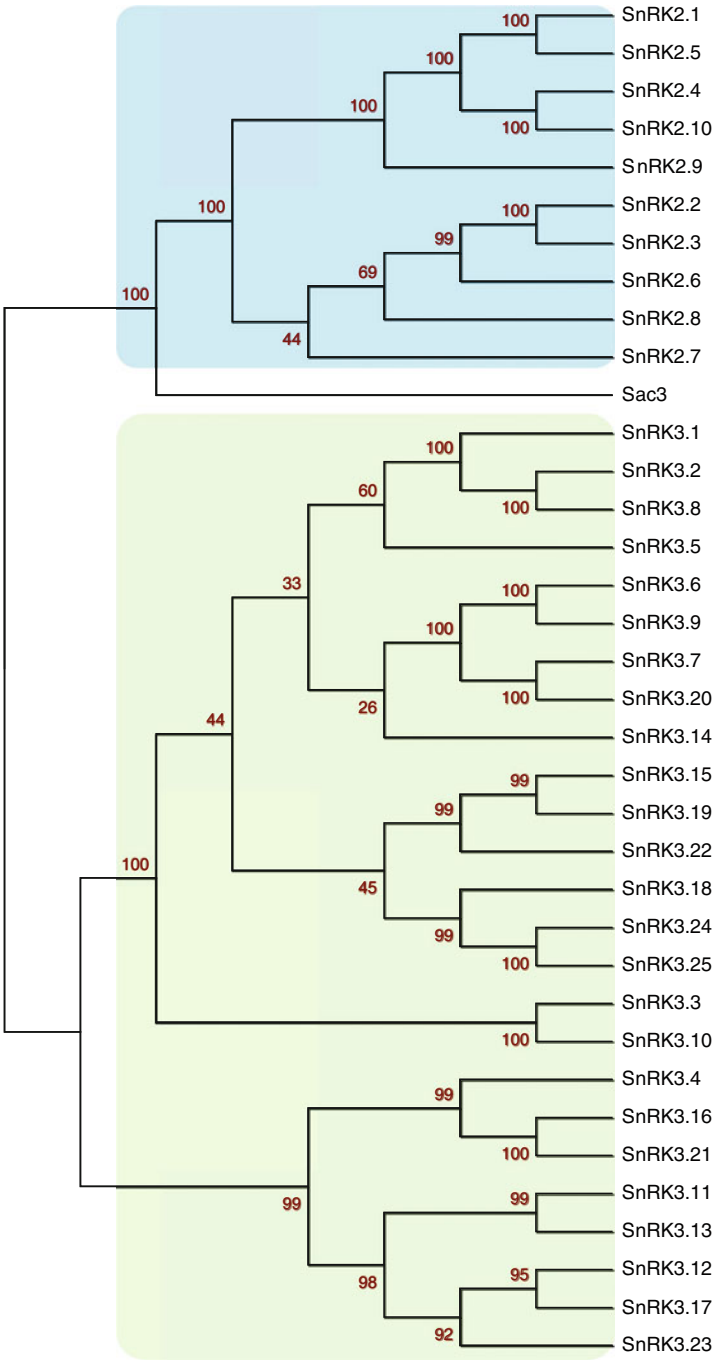


Fig. 1 Phylogenetic tree diagram depicting the relationships between members of the SNRK families of *A. thaliana* and Sac genes from *C. reinhardtii*. Protein sequences were obtained from

Structural Features of CBL Proteins

CBL proteins were first identified by their similarity to calcineurin B and neuronal calcium sensors in animals (Kudla et al. 1999). These rather small (23.5–32 kDa) proteins are specific to plants and up to now the CBL gene family of the Arabidopsis genome comprises ten members (Kolukisaoglu et al. 2004). As sensor relay proteins, CBLs comprise four calcium binding elongation factor hand domains, which are highly conserved amongst CBLs and responsible for binding calcium. Despite the CBLs being highly conserved between members in the family, some CBLs with different EF-hand compositions may bind Ca^{2+} in a dissimilar mode. For instance, the first and fourth EF hand motifs of the AtCBL2 protein both bind two Ca^{2+} ions with the second and third EF motifs remaining open indicating the inability of these EF hands to bind Ca^{2+} when the protein is not in complex with its interacting kinase (Nagae et al. 2003). Upon interaction with SnRK3.15, these EF hands in the AtCBL2 protein were able to bind Ca^{2+} at all four EF hand motifs, indicating complex formation and activation of the CBL-SnRK3 complex upon CBL phosphorylation (Akaboshi et al. 2008). Furthermore, it is assumed that this phosphorylation occurs on the C-terminus of the CBL protein which prevents binding of this C-terminus into the hydrophobic crevice of the CBL. CBL-SnRK3 protein interaction (Akaboshi et al. 2008). It is believed that all these attributes could result in prolonged activity of the complexes even at low Ca^{2+} concentrations or when the Ca^{2+} signature has faded. The sub-cellular localization of the CBL proteins are determined by their N-terminal domains. The CBLs can be categorized according to their sub-cellular localization and thereby can be divided into two groups: the CBLs that localize to the plasma membrane (CBL1, CBL4, CBL5, CBL8 and CBL9) and the CBLs that localize to the tonoplast (CBL2, CBL3, CBL6, CBL7 and CBL10) (D'Angelo et al. 2006; Batistic et al. 2008, 2010; Waadt et al. 2008).

Structural Features of SnRK3 Sub-family Proteins

The serine/threonine protein kinases are a relatively large group of kinases including 38 members that can be sub-divided into 3 sub-families of which the SnRK3 monophyletic group is the largest being represented by 26 members in the Arabidopsis genome (Kudla et al. 2010). These kinases are specific to plants but are

Fig. 1 (continued) the NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/) and PlantsP functional genomics of plant phosphorylation database (http://plantsp.genomics.purdue.edu). The alignment was performed using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) software. The phylogenetic tree was compiled from aligned sequences using the MEGA (6.0) program (Tamura et al. 2013) using the p-distance method and bootstrap method with the number of bootstrap replications set to 500 as test of phylogeny.

related to the sucrose non-fermenting (Snf) kinases from yeast and similar to the AMP-activated kinases (AMPK) from animals (Kudla et al. 2010). The SnRK3 sub-family of kinases is particularly unique since they are believed to be the exclusive targets of CBLs. The SnRK3 sub-family consists of an N-terminal kinase domain containing, 11 sub-domains which are conserved amongst SnRKs and a regulatory domain on the C-terminus that is made up of a junction domain and an interaction domain. The interaction domain contains a NAF/FISL domain to which CBL proteins can bind to relieve auto-inhibition by dissociating the kinase domain from the C-terminal regulatory domain, which acts as a pseudo-substrate and serves as an auto-inhibitory domain (Weinl and Kudla 2009). This interaction activates the kinase (Albrecht et al. 2001). This interaction also assists the kinase in recognizing its substrate with definite specificity. Adjacent to the NAF domain, a protein phosphatase interaction motif is responsible for the interaction with protein phosphatase 2Cs (PP2Cs) (Weinl and Kudla 2009). Sequence variations in the PPI motifs determine which SnRK3 interacts with the phosphatases ABA-insensitive 1 (ABI1) and ABA insensitive 2 (ABI2) (Sanchez-Barrena et al. 2013).

This multi-faceted mode of regulation demonstrates that a SnRK3 protein, depending on the identity of its interacting CBL protein, represents dual-functioning protein kinases that regulate targets in different compartments of the cell (Batistic et al. 2010). Because of the interaction, CBLs and SnRK3s display broad functional redundancy. One CBL or SnRK3 protein may participate in several different physiological processes. On the other hand, different CBLs and SnRK3s may function in the same pathway at the same time (Liu et al. 2013). Concluding which of the several CBLs is probably the primary upstream regulator of which of the several SnRK3s in response to sulfur deficiency has to be explored.

The Connection of CBL-SnRK3 to Sulfur Metabolism

Increasing evidence has shown that members of the different SnRK sub-families play central roles in deciphering stress signals. The participation of some members of the SnRK3 sub-family in nitrate and potassium signalling has been documented. Numerous labs contributed to unravelling the role of CBL-SnRK3 interaction in response to low potassium response (Cheong et al. 2007; Lee et al. 2007; Li et al. 2006; Luan et al. 2009; Xu et al. 2006) and recent studies identified SnRK3s as crucial components in nitrate sensing (Hu et al. 2009; Ho et al. 2009; Ho et al. 2010). How plants sense, and more importantly, distinguish and respond to the plethora of Ca^{2+} signals that are produced under stress remains an open question. Several studies also investigated the Ca^{2+} signature in response to potassium and nitrate starvation (Lee et al. 2007). However, up to now no studies have investigated the character of Ca^{2+} signature upon sulfur or phosphate starvation.

The response of *Chlamydomonas reinhardtii* to sulfur starvation has been studied quite extensively. The SNF1-related protein kinase 2.2 (SnRK2.2) gene, previously known as Sulfur Acclimation 3 (SAC3) gene, negatively regulates sulfur

deficiency responsive gene expression in *C. reinhardtii* (Davies et al. 1999). Kimura et al. (2006) considered that the regulatory function of *C. reinhardtii* SAC3 towards sulfur limitation was also conserved in vascular plants with the SnRK2 sub-family genes being the closest homologs in Arabidopsis (Fig. 1). *C. reinhardtii* has only one SAC3 gene whereas Arabidopsis contains ten homologs that could control sulfur starvation responses (Fig. 1). Kimura et al. (2006) showed that repression of the SnRK2.3 gene reduced sulfur transporter transcript accumulation and an enhanced level of *O*-acetyl-l-serine (OAS) accumulated upon sulfur starvation in these mutants when compared to wild type plants. The role of SnRK3 protein kinases in response to sulfur acclimation have not been analyzed yet.

Through analyzing micro array data (http://www.ebi.ac.uk/arrayexpress/GEO – GSE30098) from a meta-analysis performed as a time-course of the expression of whole Arabidopsis roots for 3, 12, 24, 48 and 72 h on media lacking sulfur (Iyer-Pascuzzi et al. 2011), the transcriptional response of the SnRK3 sub-family members to sulfur deprivation indicated that these kinases are regulated at the transcriptional level by S deprivation. Many of the SnRK3 sub-family genes were either up- or down-regulated in this micro array dataset (Fig. 2).

The PLACE (http://www.dna.affrc.go.jp/PLACE/) (Higo et al. 1999) search tool was used to scan the promoter regions of each SnRK3 sub-family member for the presence of putative *cis*-acting regulatory elements identical or similar to the motifs registered in PLACE. Promoter *cis*-element analysis revealed that the known regulatory motif, SURECORE (GAGAC), a sulfur responsive *cis* element, was present in the promoter regions of most of the SnRK3 sub-family members (Fig. 3). This *cis* element was first identified in the promoter region of sulfate transporter 1;1

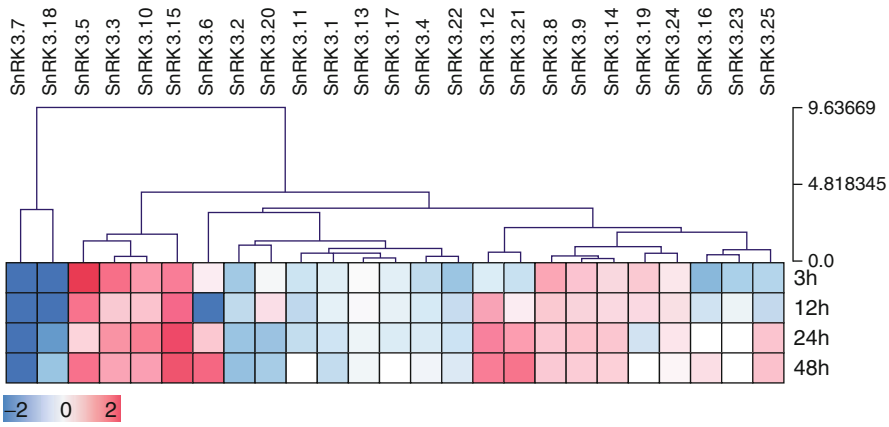


Fig. 2 Expression levels as Log2 fold-changes of SnRK3 sub-family genes upon sulfur starvation. A sulfur starvation time-course experiment published in the group of Phillip Benfey (Iyer-Pascuzzi et al. 2011) was used to evaluate the transcriptional behavior of the SnRK3 genes under sulfur starvation in root material of *Arabidopsis thaliana*. Array data were obtained from the EMBL array express webpage (http://www.ebi.ac.uk/arrayexpress/GEO – GSE30098); Log2 fold-changes relative to the 0 h sulfur starvation time-point are presented by the false colour code (*red* >2; *blue* <-2).

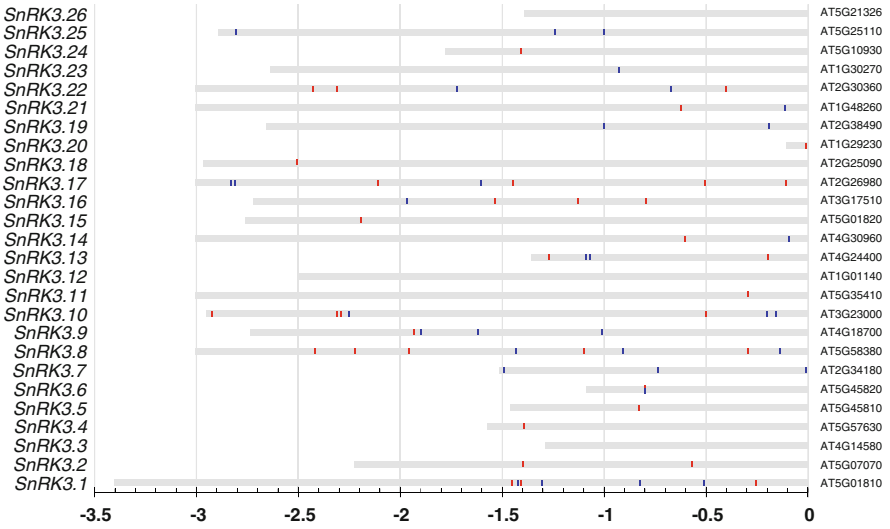


Fig. 3 The sequences of the promoter regions of SnRK3 sub-family members. Schematic diagram of the 3.5-kb promoter region of SnRK3 sub-family members of *Arabidopsisthaliana*, showing the SURECORE cis-acting element position (*red boxes* denote SURECORE elements on the reverse strand; *blue boxes* denote SURECORE (GAGAC) elements on the forward strand)

(Maruyama-Nakashita et al. 2004) and has since then been identified in the promoter of many sulfur starvation response genes and is essential for the induction in gene transcription when plants are sulfur-deprived.

The transcriptional response of a gene to a specific stress does not necessarily mean that the gene may have a significant effect in conferring stress tolerance. However, through examining these stress-induced transcriptional patterns and through the identification of putative *cis*-elements in their promoter sequences investigators can get a first clue to predicting the putative function of these genes.

Apart from playing a role in the nutrient stress responses already mentioned, SnRK3s also play important roles in other abiotic stress responses which include adaptive responses (mainly by maintaining ion homeostasis) to drought, cold, and salt stress, but also in response to ABA and sugar as well as pH changes (Coello et al. 2011; Luan et al. 2009). The SOS (salt-overly-sensitive) signalling pathway has so far been the best example in explaining how CBL-SnRK3 complexes act to regulate these stress responses by restoring ion homeostasis under salinity stress by regulating the intracellular Na^+ and K^+ homeostasis through control of the SOS1 Na^+/H^+ antiporter (Zhu 2000). Moreover, it is now also known that SnRK3s take part in ABA signalling (Lumba et al. 2014). ABA plays an important role in adaptive responses to abiotic stress. It is known that ABA also effects S homeostasis through increasing the levels of GSH. GSH is a significant sink for reduced sulfur and vital for maintaining the redox state by acting as a redox buffer in plants. ABA also affects other enzymes of the sulfate assimilation pathway such as ATPS, SERAT and the cytosolic OASTL (Barroso et al. 1999; Ruiz and Blumwald 2002;

Koprivova et al. 2008). Vice versa, sulfate supply also affects ABA synthesis through the availability of cysteine (Cao et al. 2014).

Signal transduction pathways in response to nutrient deprivation are only now beginning to be revealed. Transcriptional responses deduced from microarray analysis and in silico evidence are providing insights into the components involved in these networks. Spatio-temporal transcriptional analysis as performed in Phillip Benfey's lab (Iyer-Pascuzzi et al. 2011) is an invaluable tool for uncovering early and late signals that may partake in these signal transduction networks. Integrating expression profiling information and focussing on systematic and integrative analysis is still an incisive way to determine the plant's response to numerous stresses.

References

Akaboshi M, Hashimoto H, Ishida H, Saijo S, Koizumi N, Sato M, Shimizu T (2008) The crystal structure of plant-specific calcium-binding protein AtCBL2 in complex with the regulatory domain of AtCIPK14. J Mol Biol 377:246–257

Albrecht V, Ritz O, Linder S, Harter K, Kudla J (2001) The NAF domain defines a novel protein–protein interaction module conserved in Ca^{2+}-regulated kinases. EMBO J 20:1051–1063

Barroso C, Romero LC, Cejudo FJ, Vega JM, Gotor C (1999) Salt-specific regulation of the cytosolic O-acetylserine(thiol)lyase gene from *Arabidopsis thaliana* is dependent on abscisic acid. Plant Mol Biol 40:729–736

Batistic O, Sorek N, Schültke S, Yalovsky S, Kudla J (2008) Dual fatty acyl modification determines the localization and plasma membrane targeting of CBL/CIPK Ca^{2+} signaling complexes in Arabidopsis. Plant Cell 20:1346–1362

Batistic O, Waadt R, Steinhorst L, Held K, Kudla J (2010) CBL-mediated targeting of CIPKs facilitates the decoding of calcium signals emanating from distinct cellular stores. Plant J 61:211–222

Cao MJ, Wang Z, Zhao Q, Mao JL, Speiser A, Wirtz M, Hell R, Zhu JK, Xiang CB (2014) Sulphate availability affects ABA levels and germination response to ABA and salt stress in *Arabidopsis thaliana*. Plant J 77:604–615

Cheong YH, Pandey GK, Grant JJ, Batistic O, Li L, Kim BG (2007) Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis. Plant J 52:223–239

Coello P, Hey SJ, Halford NG (2011) The sucrose non-fermenting-1-related (SnRK) family of protein kinases: potential for manipulation to improve stress tolerance and increase yield. J Exp Bot 62:883–893Davies, J.P., Yildiz, F.H., and Grossman, A.R. (1999). Sac3, an Snf1-like Serine/Threonine Kinase That Positively and Negatively Regulates the Responses of Chlamydomonas to Sulfur Limitation. The Plant Cell Online 11, 1179-1190.

D'Angelo C, Weinl S, Batistic O (2006) Alternative complex formation of the Ca^{2+} -regulated protein kinase CIPK1 controls abscisic acid-dependent and independent stress responses in Arabidopsis. Plant J 48:857–872

Davies JP, Yildiz FH, Grossman AR (1999) Sac3, an Snf1-like serine/threonine kinase that positively and negatively regulates the responses of chlamydomonas to sulfur limitation. Plant Cell Online 11:1179–1190

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database. Nucleic Acids Res 27:297–300

Ho CH, Lin SH, Hu HC, Tsay YF (2009) CHL1 functions as a nitrate sensor in plants. Cell 138:1184–1194

Ho C-H, Tsay Y-F (2010) Nitrate, ammonium, and potassium sensing and signaling. Curr Opin in Plant Biol 13:604–610

Hu HC, Wang YY, Tsay YF (2009) AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. Plant J 57:264–278

Iyer-Pascuzzi AS, Jackson T, Cui H, Petricka JJ, Busch W, Tsukagoshi H, Benfey PN (2011) Cell identity regulators link development and stress responses in the *Arabidopsis* root. Dev Cell 21:770–782

Kimura N, Shibagaki N, Ohkama-Ohtsu N, Hayashi H, Yoneyama T, Davies JP, Fujiwara T (2006) Arabidopsis SNRK2.3 protein kinase is involved in the regulation of sulphur-responsive gene expression and *O*-acetyl-l-serine accumulation under limited sulphur supply. Soil Sci Plant Nutr 52:211–220

Kolukisaoglu Ü, Weinl S, Blazevic D, Batistic O, Kudla J (2004) Calcium sensors and their interacting protein kinases: genomics of the Arabidopsis and rice CBL-CIPK signaling networks. Plant Physiol 134:43–58

Koprivova A, North K, Kopriva S (2008) Complex signaling network in regulation of adenosine 5′ -phosphosulphate reductase by salt stress in Arabidopsis roots. Plant Physiol 146:1408–1420

Kudla J, Xu Q, Harter K, Gruissem W, Luan S (1999) Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. Proc Natl Acad Sci U S A 96:4718–4723

Kudla J, Batistič O, Hashimoto K (2010) Calcium signals: the lead currency of plant information processing. Plant Cell 22:541–563

Lee SC, Lan WZ, Kim BG, Li L, Cheong YH, Pandey GK, Lu G, Buchanan BB, Luan S (2007) A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc Natl Acad Sci U S A 104:15959–15964

Li L, Kim BG, Cheong YH, Pandey GK, Luan S (2006) A Ca^{2+} signaling pathway regulates a K^+ channel for low-K^+ response in *Arabidopsis*. Proc Natl Acad Sci U S A 103:12625–12630

Liu L-L, Ren H-M, Chen L-Q, Wang Y, Wu W-H (2013) A protein kinase, calcineurin B-like protein-interacting protein kinase9, interacts with calcium sensor calcineurin B-like protein3 and regulates potassium homeostasis under low-potassium stress in arabidopsis. Plant Physiol 161:266–277

Luan S, Lan W, Chul Lee S (2009) Potassium nutrition, sodium toxicity, and calcium signaling: connections through the CBL-CIPK network. Curr Opin Plant Biol 12:339–346

Lumba S, Toh S, Handfield LF, Swan M, Liu R, Youn JY, Cutler SR, Subramaniam R, Provart N, Moses A, Desveaux D, McCourt P (2014) A mesoscale abscisic acid hormone interactome reveals a dynamic signaling landscape in Arabidopsis. Dev Cell 29:360–372

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Yamaya T, Takahashi H (2004) Induction of SULTR1;1 sulphate transporter in Arabidopsis roots involves protein phosphorylation/dephosphorylation circuit for transcriptional regulation. Plant Cell Physiol 45:340–345

Nagae M, Nozawa A, Koizumi N, Sano H, Hashimoto H, Sato M (2003) The crystal structure of the novel calcium-binding protein AtCBL2 from *Arabidopsis thaliana*. J Biol Chem 278:42240–42246

Ruiz JM, Blumwald E (2002) Salinity-induced glutathione synthesis in *Brassica napus*. Planta 214:965–969

Sanchez-Barrena MJ, Martinez-Ripoll M, Albert A (2013) Structural biology of a major signaling network that regulates plant abiotic stress: the CBL–CIPK mediated pathway. Int J Mol Sci 14:5734–5749

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis Version 6.0. Mol Biol Evol 30:2725–2729

Waadt R, Schmidt LK, Lohse M, Hashimoto K, Bock R, Kudla J (2008) Multicolour bimolecular fluorescence complementation reveals simultaneous formation of alternative CBL/CIPK complexes in planta. Plant J 56:505–516

Weinl S, Kudla J (2009) The CBL-CIPK Ca^{2+}-decoding signaling network: function and perspectives. New Phytol 184:517–528

Xu J, Li HD, Chen LQ, Wang Y, Liu LL, He L (2006) A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporter AKT1 in Arabidopsis. Cell 125:1347–1360

Zhu JK (2000) Genetic analysis of plant salt tolerance using Arabidopsis. Plant Physiol 124:941–948

Hydrogen Sulfide and Reactive Friends: The Interplay with Reactive Oxygen Species and Nitric Oxide Signalling Pathways

John T. Hancock and Matthew Whiteman

Abstract Hydrogen sulfide (H_2S) is now considered to be a signalling molecule in a range of organisms. In animals and plants there are characterised mechanisms for generation and removal suggesting that it can be made and have an effect when required. In plants H_2S has been found to help mediate a host of physiological events, from seed germination to flower senescence. Furthermore, H_2S has been found to be involved in a range of stress responses. What is clear, however, is that such physiological events and responses also involve reactive oxygen species (ROS) and nitric oxide (NO). Therefore, considering the mechanisms by which H_2S acts, interactions with other reactive molecules must be taken into account. H_2S may affect the enzymes involved in ROS and NO accumulation, or may have a direct reaction with ROS or NO. Furthermore, ROS, NO and H_2S are all able to partake in the modification of thiol groups, suggesting that the final outcome will be dependent on the concentrations and locations of molecules such as ROS, NO and H_2S. It has been suggested that one of the ways in which H_2S may have its effects is in the modulation of ROS and NO metabolism, keeping it in check until required to relay a signal. What is clear is that future work needs to consider all these reactive compounds as a group, to unravel how they truly interact and bring about a co-ordinated response.

Introduction

Many compounds, which at first sight do not seem to be obvious candidates, over the last few years have been implicated in cell signalling events. These include chemicals which fall under the umbrella of being reactive oxygen species (ROS),

J.T. Hancock (✉)
Faculty of Health and Applied Sciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY, UK
e-mail: john.hancock@uwe.ac.uk

M. Whiteman
Exeter Medical School, University of Exeter, Exeter, UK

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_16

such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-) and singlet oxygen (O_2^1; Hancock 2009) as well as those known as reactive nitrogen species (RNS: Wilson et al. 2008) the most notable of which would be nitric oxide (NO), recognised as such many years ago (Palmer et al. 1987). Hydrogen sulphide (H_2S) should also now be included amongst such compounds and is often referred to as a gasotransmitter (Wang 2002, 2003). As with the others it would not be an obvious contender to be part of a signalling pathway, but if it is to be included how does it fit into the signal transduction pathways being unravelled?

The first obstacle for considering hydrogen sulfide as a signalling component is the fact that it is inherently poisonous. It is known to be an inhibitor of cytochrome oxide of mitochondria (Dorman et al. 2002) and therefore is toxic to many organisms including humans. However, H_2S is often present in the environment as discussed by Lisjak et al. (2013). It is generated by many industries and human activities, such as waste treatment plants, with warnings being given in some areas because of its toxic nature. It was even used as a chemical weapon (Szinicz 2005), highlighting its dangerous potential. Naturally it is released by volcanoes, from underwater vents (Martin et al. 2008) and is generated by bacteria, which has been known for a long time (Clarke 1953) but a look at ROS and NO should be reassuring here. ROS were first highlighted as being part of the suite of compounds generated during the respiratory burst (Wientjes and Segal 1995) where they are thought to be instrumental in the killing of invading pathogens. Enzymes such as NADPH oxidase are known to be part of a dedicated defense mechanisms of white blood cells where the poisonous nature of ROS are harnessed. NO too has been implicated in this activity (Fang 2004). As can be seen in Fig. 1, the chemistry of ROS and NO are quite complicated (as discussed by Kolluru et al. 2013), allowing for the generation of extremely reactive compounds such as the hydroxyl radical and peroxynitrite. Even so, ROS and NO are embraced as signalling molecules, so why not H_2S? It is a matter of the amount that is present and exactly where it is present, as discussed further below. To emphasis the first point, low levels of H_2S have been shown to be used by mitochondria as a reducing source, feeding electrons to ubiquinone and therefore on to Complex III and Complex IV (Bouillaud et al. 2013). Both complexes pump protons across the inner mitochondrial membrane creating a protomotive force for the generation of ATP. Therefore, low levels of H_2S can be instrumental in the generation of ATP (at least in animals), as can be seen in Fig. 2. To date there seems to be no parallel studies in plants. However, if levels of H_2S rise too far Complex IV becomes inhibited and the activity of the mitochondria ceases (Dorman et al. 2002). Therefore, it is the balance of the levels of H_2S which is critical here, as would be the case if hydrogen sulfide were acting as a signalling molecule.

H_2S is of course a compound which organisms have had to tolerate during millions of years of evolution, and is used still by organisms living where H_2S is generated, such as around thermal vents (Martin et al. 2008). In the same way, both ROS and NO have also been tolerated. ROS was an inevitable consequence of the release of oxygen into the atmosphere, and this continues to this day. It has been estimated that a small but significant proportion of the oxygen that we breathe is used in side reactions where ROS are the result (Turrens 2004), and, of course, similar ROS generation can be seen

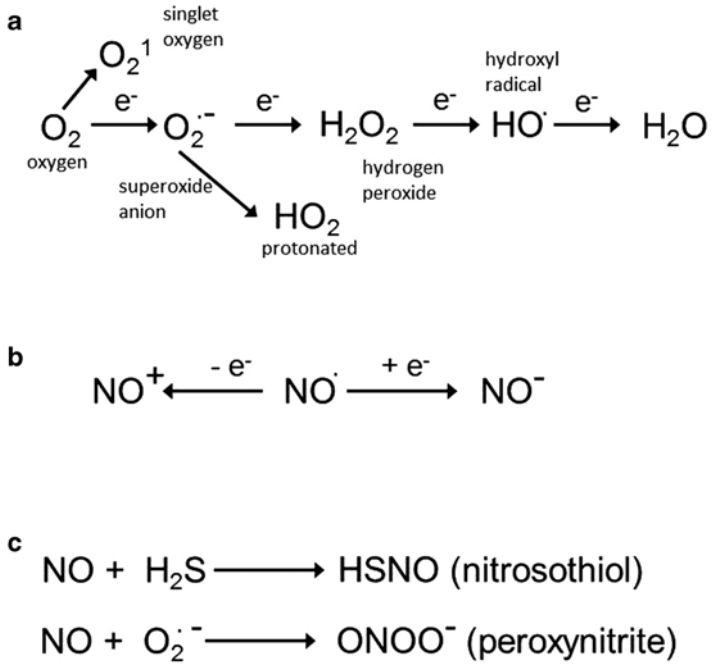


Fig. 1 The chemistry of reactive oxygen species and nitric oxide, with some interactions highlighted. (**a**) The progressive reduction of molecular oxygen; (**b**) the reactive forms of nitric oxide; (**c**) interaction of nitric oxide with hydrogen sulfide and superoxide, leading to the formation of new compounds

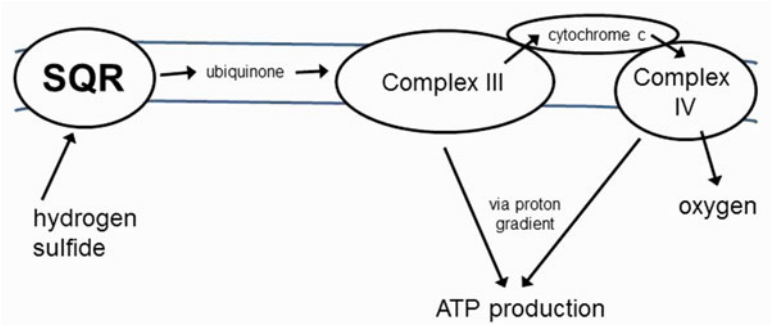


Fig. 2 Hydrogen sulfide can feed reducing equivalents to the electron transport chain of mitochondria. Hydrogen sulfide can donate electrons to sulfide quinone reductase (*SQR*) and so to the mitochondrial electron transport complexes, leading to ATP production

at other electron transport chains where reduced compounds are in contact with oxygen. Despite this ROS have been harnessed as signalling molecules, probably because organisms had to create mechanisms to tolerate toxic compounds such as ROS, NO and H_2S leading to their adoption as signalling molecules.

Why H_2S Should Be Considered as a Signalling Molecule?

There is now a considerable body of literature which considers H_2S to be a signalling molecule (Mustafa et al. 2009; Hancock et al. 2011; Li et al. 2011; Wang et al. 2012; Lisjak et al. 2011; Garcia-Mata and Lamattina 2013; Kimura 2013; Li 2013). The ability of H_2S to be considered as a signalling component will be discussed here, but to be a signalling compound and to be part of a signalling pathway a compound should fulfil certain criteria (Hancock 2010) including:

- the capacity to be made where and when required – there would be little point in allowing a compound to be made anywhere and at any time if it is supposed to be relaying a specific message,
- the ability to allow the message to be moved, either between cells or between locations within the same cell – if a required message was not allowed to move then the need for a signalling component disappears,
- the cessation of a signal when not required – signal transduction pathways tend to relay a specific message for a short period of time, allowing cells to respond only when needing to,
- the signalling component needs to relay a specific and unique message otherwise there is the capacity for misinterpretation and the wrong response, possibly leading to cellular dysfunction,
- a signal has to elicit a response else it would have little purpose.

So can H_2S fulfil these criteria, and if so, if it is to be considered a signalling molecule, how does it fit into the rest of a cell's repertoire of signals? There are dedicated enzymes which can make H_2S, such as cystathione ϒ-lyase (CSE), cystathione β-synthase (CBS) and 3-mercaptopyruvate sulfurtansferase (3-MST) in animals (Prabhakar 2012) and desulhydrases in plants (Alvarez et al. 2010). Therefore in theory H_2S can be made when and where needed, assuming the enzymes are in the right place and active. However, it should be remembered that sulfide is also an intermediate in sulphate metabolism in plants (Calderwood and Kopriva 2014), as can be seen in Fig. 3. Therefore it is likely that there is always some sulfide present in plant cells, so any signalling would need to take this into account, as discussed

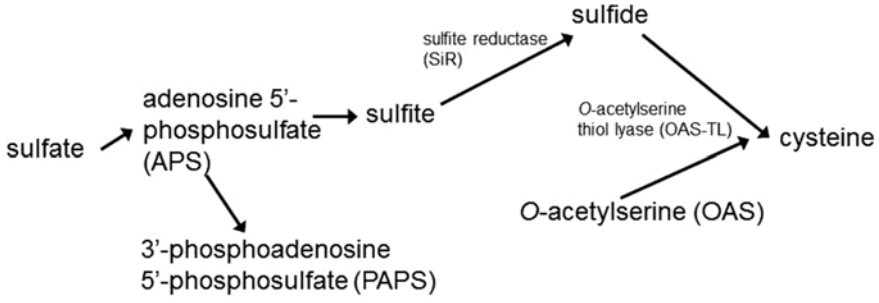


Fig. 3 Sulfide is an intermediate in sulphate metabolism in plants, leading to the formation of cysteine

below. Furthermore, there are enzymes which can remove H_2S in plants, such as *O*-acetylserine (thiol) lyase (Youssefian et al. 1993; Tai and Cook 2000), and therefore if H_2S is acting as a signal it can be removed when no longer needed and the relevant signalling can cease. Of course, in animals the H_2S may be being removed by the mitochondria (Bouillaud et al. 2013) which may also be the case for plant cells so keeping H_2S levels low.

In the gaseous state movement of H_2S should not be a problem as it is able to dissolve and move freely around the aqueous phase of an organism. It is not charged so it would be assumed that if not disassociated it would be free also to move through the hydrophobic phase of membranes and therefore could freely move in and out of cells, or in and out of organelles within cells. Of course, such discussion assumes that the H_2S would not react with the biological material it is passing through. History has rehearsed such arguments when it was assumed that superoxide, being charged, could not cross membranes, until it was realised that in the protonated form it probably could (Gus'kova et al. 1984). On the other hand, it was assumed that NO, not being charged, could freely move across membranes, but a quick look at Fig. 1 shows that there are charged forms of NO, and so this may be a false assumption. Therefore some caution is probably required when discussing H_2S because of its chemistry and reactivity.

However, putting all the evidence together it could be that H_2S is a good candidate as a signalling molecule, or at least as good as ROS or NO. H_2S has dedicated enzymes to make and remove it. It is toxic, but as long as the levels can be maintained within limits, like NO and ROS, signalling may be a role in which H_2S partakes. But is there a response, and does it impact on cell physiology?

The answer to that is a resounding yes. Table 1 shows that H_2S appears to have a role in plants from germination (Dooley et al. 2013, for example), to death (for example, on flower senescence: Zhang et al. 2011). In normal physiology H_2S has a role in the germination of seeds, controlling root development (for example, Lin et al. 2012), modulating stomatal apertures (for example, Lisjak et al. 2010) and interestingly has also been shown to prolong the storage for fruits once removed from the plant (Hu et al. 2012). What is very apparent from the list is the number of stress responses that H_2S seems to be involved in, for example, oxidative stress (Zhang et al. 2008), osmotic stress (Zhang et al. 2009b), and stress caused by a range of heavy metals (for example, Li et al. 2012c) as well as heat (Li et al. 2012b) and cold (Stuiver et al. 1992).

However, the responses are not always clear cut. Groups have reported for example that H_2S causes stomatal opening (Lisjak et al. 2010, 2013) and stomatal closing (Garcia-Mata and Lamattina 2010, 2013) which suggests that either one aspect is wrong or there is something odd going on here. However, the list of stresses in which H_2S is implicated, does point to a possible role in not just plants but also in animals. In many cases the role of H_2S seems to be in the alleviation of a stress or a disease state and it has been implicated in lowering of symptoms of atherosclerosis (Mani et al. 2013; Xu et al. 2014), a reduction in vascular inflammation (Liu et al. 2013) and in the onset of diabetes (Whiteman et al. 2010). Others have also reported that H_2S can contribute to a lessening of diseases in animals (Wang 2013; Ahmed 2013; Nagpure and Bian 2013; Al-Magableh et al. 2013), while it has been reported

Table 1 Some physiological events in which hydrogen sulfide has been implicated

Physiological event	Reference(s)
Normal physiology	
Seed germination	Zhang et al. (2008, 2010c), Li et al. (2012a), Dooley et al. (2013)
Root development	Zhang et al. (2009a), Lin et al. (2012)
Stomatal apertures	Garcia-Mata and Lamattina (2010, 2013), Lisjak et al. (2011, 2013), Liu et al. (2011), Jin et al. (2013)
Flower senescence	Zhang et al. (2011)
Post-harvest	Hu et al. (2012)
Stress responses	
Osmotic stress	Zhang et al. (2009b)
Salt stress	Wang et al. (2012), Lisjak et al. (2013)
Oxidative stress	Zhang et al. (2008), Shan et al. (2011)
Cadmium stress	Li et al. (2012c)
Aluminium stress	Zhang et al. (2010b), Chen et al. (2012)
Chromium stress	Zhang et al. (2010a)
Copper stress	Zhang et al. (2008)
Lead stress	Ali et al. (2014)
Pathogen challenge	Bloem et al. (2004, 2011, 2012)
Heat stress	Li et al. (2012b, 2013a, b)
Freezing tolerance	Stuiver et al. (1992)
Water stress	Shan et al. (2011)

that in *Caenorhabiditis elegans* H_2S confers thermal tolerance and increased longevity (Miller and Roth 2007).

What is worth noting is that if this list is examined with ROS and NO in mind, the same stresses and diseases would be found. It is therefore entirely possibly that a major role of H_2S in signalling is to interact with ROS or NO, or more likely both.

Interactions between ROS and NO have already been reported. Examples here include the production of NO from pollen, which seems to down-regulate the accumulation of H_2O_2 in stigmas (McInnis et al. 2006) presumably allowing the pollen to germinate and for a pollen tube to form. In stomata it has been reported that H_2O_2 is upstream of NO in a signalling pathway (Bright et al. 2006), showing how these two signals may be working together, as can be seen in Fig. 1, ROS and NO are known to react together to form other potential signalling molecules such as peroxynitrite. There is therefore a clear case to include H_2S as a signalling molecule and a need to understand its relationship with ROS and NO pathways and how it may also directly react with ROS and or NO.

Interactions of H_2S with ROS and NO Metabolism

The influence of H_2S on ROS metabolism has been investigated for a long time. One of the major consequences of the addition of H_2S to plants is the increase in glutathione levels (De Kok et al. 1985). Glutathione has two very important roles in cells.

Firstly glutathione is immensely important for the maintenance of the redox status of cells. Its concentration can be mM in cells, and it is the levels of reduced glutathione (GSH), which can maintain the interior of the cell at an appropriately reducing electrical potential. Using the Nernst equation enables the redox status of the cells to be estimated, once GSH and oxidised glutathione (GSSG) have been determined, as discussed previously (Schafer and Buettner 2001; Hancock 2009). Therefore if H_2S increases the level of reduced glutathione then this will enable the cell to maintain its intracellular reduced state, even after potential oxidative stress episodes. Secondly glutathione can have an influence on the thiol status of proteins, through a process of glutathionation (Li and Lancaster 2013; Sun et al. 2013), as discussed further below.

H_2S has been shown to have an influence on other antioxidants too. For example ascorbate is also affected (Shan et al. 2011). However, in a report of the use of H_2S during salt stress in pepper plants it was found that some antioxidants had an increased level after H_2S treatment while others were reduced. However the overall trend seemed to be an increase of the antioxidant capacity of the cells, perhaps hinting that H_2S may have a protective capacity against oxidative stress (Lisjak et al. 2013). Others have reported that antioxidant levels are influential in post-harvest storage of crops, where H_2S modulated antioxidant levels are thought to mediate the protective effects seen (Hu et al. 2012).

Although antioxidants are instrumental in the removal of ROS it is the overall accumulation levels which are important. H_2S can react with ROS directly (Li and Lancaster 2013) so H_2S may remove superoxide anions, H_2O_2, hydroxyl radicals and hypochlorite. It has also been argued that such reactions are not relevant when the levels of antioxidants in cells are also considered (Li and Lancaster 2013). Therefore the enzymes that make ROS need to be considered too. In plants, as in animals, there are NADPH oxidase enzymes, which are able to generate ROS, usually the superoxide anion. However, other enzymes can produce ROS, such as peroxidases and xanthine oxidoreductase. Therefore, the influence of H_2S on these enzymes also needs to be determined. H_2S donors have been shown to affect the expression of NADPH oxidase enzymes in fibroblasts (Pan et al. 2013) and smooth muscle cells (Muzaffar et al. 2008). The effects in plants have yet to be widely explored although ROS generation in roots through the action of glucose-6- dehydrogenase (G6PDH) was increased on H_2S treatment, although these plants were under salt stress so may not exactly reflect normal physiology (Li et al. 2013c).

H_2S has also been shown to influence NO metabolism. In work on stomatal apertures it was shown that treatment with either NaSH or GYY4137, both of which can supply H_2S in solution, reduced the accumulation of NO in plant cells (Lisjak et al. 2010), which would account for the increase in stomatal apertures seen, as NO is known to be a signal which causes stomatal closure (Neill et al. 2002). It has previously been shown that NO and H_2S can react together to form nitrosothiols (Whiteman et al. 2006) thus removing both potential signals from solution. However, the formation of this new compound could potentially form a new signal, which may have different effects in the cell. The chemistry of H_2S is complex, as discussed recently in Li and Lancaster (2013). It has been questioned that the reaction of H_2S with other compounds is in fact as has been previously reported (Li and Lancaster

2013). However, it is unlikely that H_2S cannot have direct reactions with both NO and ROS, such as discussed by Carballal et al. (2011) where the reactivity of H_2S with peroxynitrite was discussed.

However, H_2S may also affect the enzymes that make NO, and it has recently been reported in animals that H_2S can affect the activity of the enzyme NOS (Kubo et al. 2007), albeit in some cases indirectly (Kida et al. 2013), or the up-regulation of a NOS enzyme (Kondo et al. 2013). Care needs to be taken here before such work is immediately transferred to plants, as it is still unknown whether plants contain enzymes that are homologous of the mammalian NOS (Zemojtel et al. 2006). There is considerably debate in this area, with some groups being adamant that plant NOS enzymes exist, but concrete evidence has been hard to come by. What is known is that primitive plants are likely to contain NOS, as reported for the *Ostreococcus* genus, specifically in *O. tauri* and *O. lucimarinus* (Foresi et al. 2010; Correa-Aragunde et al. 2013) but the influence of H_2S has not been determined, and neither has the wider influence on what may be happening in higher plants.

One of the major effects of either ROS or NO is on the status of thiols in proteins. As can be seen in Fig. 4, thiol groups in proteins may be oxidised to the disulfide form, which can have profound effects on both the structure of the protein and its activity. Furthermore, thiols can be oxidised to the sulphenic acid form, which again can have an effect on the protein's activity. For example, the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is inhibited by the presence of H_2O_2, which can be reversed by the application of reducing agents such as dithiothreitol (DTT: Hancock et al. 2005). Similar inhibition of GAPDH can be seen when the enzyme is treated with NO. Interestingly here, once GAPDH has been modified it is seen to move to the nucleus where it can have an influence on transcription, so it not just the

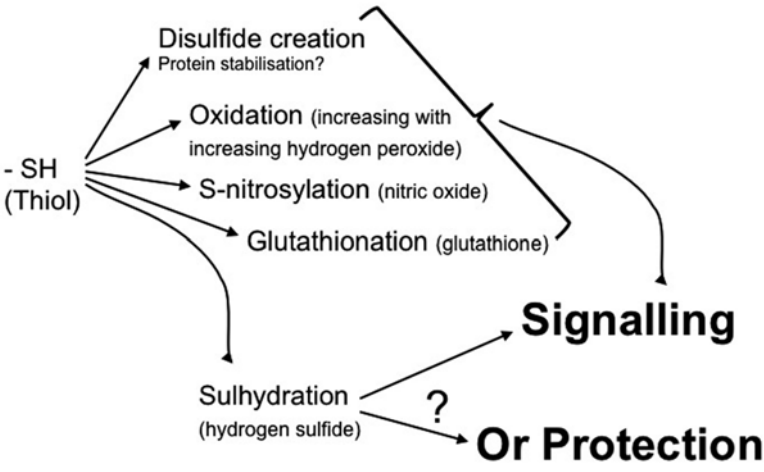


Fig. 4 Reactive oxygen species, nitric oxide, glutathione and hydrogen sulfide can all react with thiols with likely differing outcomes. It most cases it is assumed that this leads to further signalling

removal of GAPDH activity which is of important consideration here (Holtgrede et al. 2008).

One of the ways that GAPDH was identified as being altered by ROS was through the tagging of thiol groups in the absence and presence of ROS (Hancock et al. 2005). This approach also highlighted several other enzymes which could be altered in this way, including SAM synthase and alcohol dehydrogenase. Similar studies have been carried out to determine if NO can modify thiol groups in plant enzymes, mainly using what is referred to as the biotin switch assay (Jaffrey and Snyder 2001). Proteins identified here included those involved in metabolism, signalling and stress responses (Grennan 2007; Lindermayr et al. 2005). This work is of importance to the area of H_2S as recently it has been reported that H_2S can also modify thiol groups, in both animals and plants in a process called S-sulfhydration. In animals one of the proteins found was NF-kB (Sen et al. 2012), which is an important transcription factor, while in plants H_2S modification of proteins has also been reported (Romero et al. 2013).

So How Does H_2S Fit into Signalling Pathways?

The question as to whether H_2S can act as a signal is perhaps easy to answer if the guidelines to what a signal should look like are revisited. H_2S is made by dedicated enzymes, has enzymes and organelles that remove it, and it can both move about and been seen to promote a response. Therefore, as with ROS and NO, the presence of H_2S can be considered to have a signalling role. But it is not that simple as there are many aspects, which need to be considered.

Firstly, sulfide is likely to be present all the time as part of sulphur metabolism (Calderwood and Kopriva 2014). Therefore, just the presence of sulfide cannot be sufficient for the signal to be relayed. The levels of sulfide would need to increase sufficiently to mount a response, but of course could not rise too far to a point where mitochondrial Complex IV is inhibited (Dorman et al. 2002). However, a look at other signals shows that this is not that unusual. NO, ROS and other signalling molecules are often present in low amounts, and the signal comes about by a transient rise in the concentration of that signalling molecule. For example when NO accumulation was recorded by Lisjak et al. (2010) even without any treatment a measurable accumulation of NO could be seen. This increased with the addition of abscisic acid (ABA) and was reduced with the addition of H_2S donors, so it can be assumed that here the signal increased from a low amount where it had no, or little, effect and that the true response came about by a transient rise in the signalling molecule.

However, as already stated, to mount a response the concentration of H_2S needs to rise. This would have to be enough to elicit a response but not too much to inhibit cell function, so there must be a threshold beyond which H_2S has an effect. Obviously this would be different for each interaction that H_2S is involved in, but must all be within a functional range.

Also to be considered is the location of the H_2S within the cell. Compartmentalisation of signals is important and not just between organelles. As seen originally with calcium signalling (for example, Bononi et al. 2012), hotspots of ROS signalling have been reported in the literature (Figueiredo de Rezende et al. 2012), so if H_2S is to react with ROS metabolism it would need to be present in the right area of the cell, and that may mean the right location in the cytoplasm, not just within an organelle. With dedicated enzymes which can make H_2S and dedicated enzymes that can remove it, the location of these enzymes may be instrumental in determining the exact location in the cell where H_2S has an effect. With new probes now available to measure H_2S in cells (Lin et al. 2013) the notion of H_2S hotspots should be able to be determined in plant and animal cells.

As discussed above, H_2S may have a role in the modification of thiol groups, and it is likely that these are the same protein thiols which could be modified by either ROS or NO, or indeed both. Such thiols may also undergo glutathionation (Li and Lancaster 2013; Sun et al. 2013). Therefore, with the presence of NO, ROS or glutathione, the exact modification of any thiol in the cell may be hard to predict, but would be determined by the levels of such compounds in the vicinity of the thiol being modified. H_2S would have a role here too, so it is suggested that a competition exists between these compounds, which will determine the final thiol modification outcome. Clearly assays such as those used to determine if any thiol modification has taken place (Jaffrey and Snyder 2001; Hancock et al. 2005), need to be repeated in the presence of more than one reactive signalling molecule, *i.e.* ROS plus NO, H_2S plus NO, etc., to determine if there is a hierarchy in the modifications that take place on the thiols of any protein, or proteins.

ROS and NO also influence other immensely important signalling molecules. For example, one group of these are the mitogen-activated protein kinases (MAPKs: Kovtun et al. 2000; Wang et al. 2010), which also give a point of convergence of ROS and NO signalling. It is known in plants that ROS and NO have roles in the activation of MAPK3 and MAPK6, so it is tempting to suggest that H_2S may have a similar sphere of influence. In addition, there is now evidence that transcription factors are modified by H_2S directly, so perhaps in some cases the sulfide may have little influence on signalling pathways, but directly influences the end point of the mechanism, such as NF-kB (Sen et al. 2012).

Is H_2S a True Signal, or a Modifying Influence?

In a recent review it was argued that H_2S is not actually working as a true signal (Hancock and Whiteman 2014), that is, being made when needed, being removed when not needed, and having a dedicated response to its generation. It may be that this is not correct, but a review of recent literature does suggest that H_2S is often involved in the relieving of stress or disease states, (as discussed above) and this suggests a role in modifying other signals.

It has already been argued that H_2S must work within concentration limits, where low levels are not influential in signalling and where high levels are detrimental but the same would be true for ROS and NO. Schafer and Buettner (2001) argued, using glutathione as an example, that small effects of the redox status of the cell has little effect, larger perturbation may lead to apoptosis while even larger changes will lead to necrosis, with clear threshold levels for each outcome. Lowering of GSH, or an increase in ROS or NO, will cause such perturbation, leading to a move to the oxidation state of the cell interior. If H_2S can influence ROS metabolism, lowering ROS accumulation or increasing antioxidants may prevent the level of ROS reaching a threshold to partake in signalling. As H_2S and NO can react together perhaps the same may be true for NO metabolism. This would mean a scenario where ROS, NO and H_2S are all at low levels all the time, which seems to be the case. If ROS and NO start to increase then the outcome would be a stress response. However if H_2S acted to damp down this response then the stress response would only occur when ROS and/or NO levels overcame the effect of the H_2S. Such a mechanism would explain much of the literature, especially in the work on animals, where low levels of H_2S appear to dampen down disease states and cell dysfunction, such as in atherosclerosis (Mani et al. 2013), vascular inflammation (Liu et al. 2013) or diabetes (Whiteman et al. 2010). And as argued above, dampening down of NO or ROS accumulation or effects by H_2S may involve a range of mechanisms, including altering the production or scavenging of ROS or NO, or competing for thiol groups (Figs. 1 and 4).

A review of the literature shows clear evidence that H_2S is involved in a wide range of physiological events and responses where ROS and NO are also known to be involved (Table 1). Many of these physiological events are stress responses, where cells need to be able to adapt to the stress where ROS and NO are allowed to function above their signalling thresholds (Schafer and Buettner 2001). In some cases it will be important to allow apoptosis or indeed necrosis to ensue, so the exact influence of H_2S on the mechanisms underlying these responses will be important to determine.

Conclusions

Using the concept of guidelines to define a signalling molecule, when looking at H_2S there seems to be little doubt that it can partake in signalling events, as discussed above. However, its exact influence on those signal transduction pathways has yet to be fully defined. What is clear is that it is unlikely to be working in isolation from ROS and NO, and probably glutathione, the latter which may be directly influenced by H_2S levels. It has been suggested that H_2S may modulate ROS and NO signalling so preventing inappropriate messages (Hancock and Whiteman 2014) and this could account for some of the influences seen following H_2S treatment of both animal cells and plant cells. Certainly the development of tools to work with H_2S, such as new donor molecules (Li et al. 2008) and fluorescent probes (Lin et al.

2013) to determine its presence, will enable future work to determine the exact influence of H_2S on signal transduction pathways, perhaps leading to its use both in the biomedical arena (Zhang et al. 2013) and in crop science (Hu et al. 2012).

References

Ahmed A (2013) Can hydrogen sulfide prevent preeclampsia and fetal growth restriction? Nitric Oxide 31:S17

Al-Magableh M, Ng H, Kemp-Harper B, Miller A, Hart J (2013) Hydrogen sulfide protects endothelial function under conditions of oxidative stress. Nitric Oxide 31:S25

Ali B, Song WJ, Hu WZ, Luo XN, Gill RA, Wang J, Zhou WJ (2014) Hydrogen sulfide alleviates lead-induced photosynthetic and ultrastructural changes in oilseed rape. Ecotoxicol Environ Saf 102:25–33

Alvarez C, Calo L, Romero LC, Garcia I, Gotor C (2010) An *O*-acetylserine(thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis. Plant Physiol 152:656–669

Bloem E, Riemenschneider A, Volker J, Papenbrock J, Schmidt A, Salac I, Haneklaus S, Schnug E (2004) Sulphur supply and infection with *Pyrenopeziza brassica* influence L-cysteine desulfhydrase activity in *Brassica napus* L. J Exp Bot 55:2305–2312

Bloem E, Rubekin K, Haneklaus S, Banfalvi Z, Hesse H, Schnug E (2011) H_2S and COS gas exchange of transgenic potato lines with modified expression levels of enzymes involved in sulphur metabolism. J Agron Crop Sci 197:311–321

Bloem E, Haneklaus S, Kesselmeier J, Schung E (2012) Sulfur fertilization and fungal infections affect the exchange of H_2S and COS from agricultural crops. J Agric Food Chem 60:7588–7596

Bononi A, Missiroli S, Poletti F, Suski JM, Agnoletto C, Bonora M, De Marchi E, Giorgi C, Marchi S, Patergnani S, Rimessi A, Wieckowski MR, Pinton P (2012) Mitochondria-associated membranes (MAMs) as hotspot Ca^{2+} signaling units calcium signaling. Adv Exp Med Biol 740:411–437

Bouillaud F, Ransy C, Andriamihaja M (2013) Sulfide and mitochondrial bioenergetics. Nitric Oxide 31:S15

Bright J, Desikan R, Hancock JT, Neill SJ (2006) ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H_2O_2 synthesis. Plant J 45:113–122

Calderwood A, Kopriva S (2014) Hydrogen sulfide in plants: from dissipation of excess sulfur to signaling molecule. Nitric Oxide 41:72–78

Carballal S, Trujillo M, Cuevasanta E, Bartesaghi S, Möller MN, Folkes LK, García-Bereguiaín MA, Gutiérrez-Merino C, Wardman P, Denicola A, Radi R, Alvarez B (2011) Reactivity of hydrogen sulfide with peroxynitrite and other oxidants of biological interest. Free Radic Biol Med 50:196–205

Chen J, Wang W-H, Wu F-H, You C-Y, Liu T-W, Dong X-J, He J-X, Zheng H-L (2012) Hydrogen sulfide alleviates aluminum toxicity in barley seedlings. Plant Soil 362:301–318

Clarke PH (1953) Hydrogen sulphide production by bacteria. J Gen Microbiol 8:397–407

Correa-Aragunde N, Foresi N, Lamattina L (2013) Structure diversity of nitric oxide synthases (NOS): the emergence of new forms in photosynthetic organisms. Front Plant Sci 4:232

De Kok JL, Bosma W, Maas FM, Kuiper PJC (1985) The effect of short-term H_2S fumigation on water-soluble sulphydryl and glutathione levels in spinach. Plant Cell Environ 8:189–194

Dooley FD, Nair SP, Ward PD (2013) Increased growth and germination success in plants following hydrogen sulfide administration. PLoS One 8:e62048

Dorman DC, Moulin FJ, McManus BE, Mahle KC, James RA, Struve MF (2002) Cytochrome oxidase inhibition induced by acute hydrogen sulfide inhalation: correlation with tissue sulfide concentrations in the rat brain, liver, lung, and nasal epithelium. Toxicol Sci 65:18–25

Fang FC (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat Rev Microbiol 2:820–832

Figueiredo de Rezende F, Lima AM, Niland S, Wittig I, Heide H, Schroder K, Eble JA (2012) Integrin α7β1 is a redox-regulated target of hydrogen peroxide in vascular smooth muscle cell adhesion. Free Radic Biol Med 53:521–531

Foresi N, Correa-Aragunde N, Parisi G, Calo G, Salerno G, Lamattina L (2010) Characterization of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. Plant Cell 22:3816–3830

García-Mata C, Lamattina L (2010) Hydrogen sulfide, a novel gasotransmitter involved in guard cell signalling. New Phytol 188:977–984

García-Mata C, Lamattina L (2013) Gasotransmitters are emerging as new guard cell signaling molecules and regulators of leaf gas exchange. Plant Sci 201(202):66–73

Grennan AK (2007) Protein S-nitrosylation: potential targets and roles in signal transduction. Plant Physiol 144:1237–1239

Gus'kova RA, Ivanov II, Kol'tover VK, Akhobadze VV, Rubin AB (1984) Permeability of bilayer lipid membranes for superoxide (O2−) radicals. Biochim Biophys Acta Biomembr 778:579–585

Hancock JT (2009) The role of redox mechanisms in cell signaling. Mol Biotechnol 43:162–166

Hancock JT (2010) Cell signalling. Oxford University Press, Oxford

Hancock JT, Whiteman M (2014) Hydrogen sulfide and cell signalling: team player or referee? Plant Physiol Biochem 78:37–42

Hancock JT, Henson D, Nyirenda M, Desikan R, Harrison J, Lewis M, Hughes J, Neill SJ (2005) Proteomic identification of glyceraldehyde 3-phosphate dehydrogenase as an inhibitory target of hydrogen peroxide in *Arabidopsis*. Plant Physiol Biochem 43:828–835

Hancock JT, Lisjak M, Teklic T, Wilson ID, Whiteman M (2011) Hydrogen sulfide and signaling in plants. CAB reviews: perspectives in agriculture, veterinary science. Nutr Nat Res 6:1–7

Holtgrede S, Godlke J, Starmann J, Druce S, Klocke S, Altmann B, Wcjtera J, Lindermayr C, Scheibe R (2008) Regulation of plant cytosolic glyceraldehyde 3-phosphate dehydrogenase isoforms by thiol modifications. Physiol Plant 133:211–228

Hu L-Y, Hu S-L, Wu J, Li Y-H, Zheng J-L, Wei Z-J, Liu J, Wang H-L, Liu Y-S, Zhang H (2012) Hydrogen sulfide prolongs postharvest shelf life of strawberry and plays an antioxidative role in fruits. J Agric Food Chem 60:8684–8693

Jaffrey SR, Snyder SH (2001) The biotin switch method for the detection of *S*-nitrosylated proteins. Sci STKE 2001(86):11

Jin ZP, Xue SW, Luo YN, Fang BH, Tian HH, Li H, Pei YX (2013) Hydrogen sulfide interacting with abscisic acid in stomatal regulation responses to drought stress in *Arabidopsis*. Plant Physiol Biochem 62:41–46

Kida M, Sugiyama T, Yoshimoto T, Ogawa Y (2013) Hydrogen sulfide increases nitric oxide production with calcium-dependent activation of endothelial nitric oxide synthase in endothelial cells. Eur J Pharm Sci 48:211–215

Kimura H (2013) Physiological function of hydrogen sulfide and beyond. Nitric Oxide 31:S11

Kolluru GK, Shen X, Bir SC, Kevil CG (2013) Hydrogen sulfide chemical biology: pathophysiological roles. Nitric Oxide 35:5–20

Kondo K, Bhushan S, King AL, Prabhu SD, Hamid T, Koenig S, Murohara T, Predmore BL, Gojon G Sr, Gojon G Jr, Wang R, Karusula N, Nicholson CK, Calvert JW, Lefer DJ (2013) H_2S protects against pressure overload-induced heart failure via upregulation of endothelial nitric oxide synthase. Circulation 127:1116–1127

Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci U S A 97:2940–2945

Kubo S, Kurokawa Y, Doe I, Masuko T, Sekiguchi F, Kawabata A (2007) Hydrogen sulfide inhibits activity of three isoforms of recombinant nitric oxide synthase. Toxicology 241:92–97

Li ZG (2013) Hydrogen sulfide: a multifunctional gaseous molecule in plants. Russ J Plant Physiol 60:733–740

Li Q, Lancaster JR Jr (2013) Chemical foundations of hydrogen sulfide biology. Nitric Oxide 35:21–34

Li L, Whiteman M, Guan YY, Neo KL, Cheng Y, Lee SW, Zhao Y, Baskar R, Tan CH, Moore PK (2008) Characterization of a novel, water-soluble hydrogen sulfide releasing molecule (GYY4137): new insights into the biology of hydrogen sulphide. Circulation 117:2351–2360

Li L, Rose P, Moore PK (2011) Hydrogen sulfide and cell signaling. Annu Rev Pharmacol 51:169–187

Li ZG, Gong M, Liu P (2012a) Hydrogen sulfide is a mediator in H_2O_2-induced seed germination in *Jatropha Curcas*. Acta Physiol Plant 34:2207–2213

Li ZG, Gong M, Xie H, Yang L, Li J (2012b) Hydrogen sulfide donor sodium hydrosulfide-induced heat tolerance in tobacco (*Nicotiana tabacum* L.) suspension cultured cells and involvement of Ca^{2+} and calmodulin. Plant Sci 185(186):185–189

Li L, Wang Y, Shen W (2012c) Roles of hydrogen sulfide and nitric oxide in the alleviation of cadmium-induced oxidative damage in alfalfa seedling roots. Biometals 25:617–631

Li ZG, Ding XJ, Du PF (2013a) Hydrogen sulfide donor sodium hydrosulfide-improved heat tolerance in maize and involvement of proline. J Plant Physiol 170:741–747

Li J, Jia H, Wang J, Cao Q, Wen Z (2013b) Hydrogen sulfide is involved in maintaining ion homeostasis via regulating plasma membrane Na^+/H^+ antiporter system in the hydrogen peroxide-dependent manner in salt-stress *Arabidopsis thaliana* root. Protoplasma 251:899–912

Li ZG, Yang SZ, Long WB, Yang GX, Shen ZZ (2013c) Hydrogen sulfide may be a novel downstream signal molecule in nitric oxide-induced heat tolerance of maize (*Zea mays* L.) seedlings. Plant Cell Environ 36:1564–1572

Lin Y-T, Li M-Y, Cui W-T, Lu W, Shen W-B (2012) Haem oxygenase-1 in involved in hydrogen sulfide induced cucumber adventitious root formation. J Plant Growth Regul 31:519–528

Lin VS, Lippert AR, Chang CJ (2013) Cell-trappable fluorescent probes for endogenous hydrogen sulfide signalling and imaging H_2O_2-dependent H_2S production. Proc Natl Acad Sci U S A 110:7131–7135

Lindermayr C, Sallbach G, Durner J (2005) Proteomic identification of S-nitrosylated proteins in Arabidopsis. Plant Physiol 137:921–930

Lisjak M, Srivastava N, Teklic T, Civale L, Lewandowski K, Wilson I, Wood ME, Whiteman M, Hancock JT (2010) A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation. Plant Physiol Biochem 48:931–935

Lisjak M, Teklic T, Wilson ID, Wood M, Whiteman M, Hancock JT (2011) Hydrogen sulfide effects on stomatal apertures. Plant Signal Behav 6:1444–1446

Lisjak M, Teklic T, Wilson ID, Whiteman M, Hancock JT (2013) Hydrogen sulfide: environmental factor or signaling molecule? Plant Cell Environ 36:1607–1616

Liu J, Hou L-X, Liu G-H, Liu X, Wang X-C (2011) Hydrogen sulfide induced by nitric oxide mediates ethylene-induced stomatal closure of *Arabidopsis thaliana*. Chin Sci Bull 56:3547–3553

Liu Z, Han Y, Li L, Lu H, Meng G, Li X, Shirhan M, Peh MT, Xie L, Zhou S, Wang X, Chen Q, Dai W, Tan CH, Pan S, Moore PK, Ji Y (2013) The hydrogen sulfide donor, GYY4137, exhibits anti-atherosclerotic activity in high fat fed apolipoprotein E(−/−) mice. Br J Pharmacol 169:1795–1809

Mani S, Li H, Untereiner A, Wu L, Yang G, Austin RC, Dickhout JG, Lhoták Š, Meng QH, Wang R (2013) Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. Circulation 127:2523–2534

Martin W, Baross J, Kelley D, Russell MJ (2008) Hydrothermal vents and the origin of life. Nat Rev Microbiol 6:805–814

McInnis SM, Desikan R, Hancock JT, Hiscock SJ (2006) Production of reactive oxygen species and reactive nitrogen species by angiosperm stigmas and pollen: potential signalling crosstalk? New Phytol 172:221–228

Miller DL, Roth MB (2007) Hydrogen sulfide increases thermotolerance and lifespan in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 104:20618–20622
Mustafa AK, Gadalla MM, Snyder SH (2009) Signaling by gasotransmitters. Sci Signal 2:re2
Muzaffar S, Shukla N, Bond M, Newby AC, Angelini GD, Sparatore A, Del Soldato P, Jeremy JY (2008) Exogenous hydrogen sulfide inhibits superoxide formation, NOX-1 expression and Rac1 activity in human vascular smooth muscle cells. J Vasc Res 45:521–528
Nagpure B, Bian J-S (2013) Neuroprotective effect of hydrogen sulfide: regulation of amyloidosis and inflammation in SH-SY5Y neuroblastoma and BV-2 micro glia cells. Nitric Oxide 31:S22
Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. J Exp Bot 53:1237–1247
Palmer RMJ, Ferrige AG, Moncada S (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327:524–526
Pan LL, Liu XH, Shen YQ, Wang NZ, Xu J, Wu D, Xiong QH, Deng HY, Huang GY, Zhu YZ (2013) Inhibition of NADPH oxidase 4-related signaling by sodium hydrosulfide attenuates myocardial fibrotic response. Int J Cardiol 168:3770–3778
Prabhakar NR (2012) Carbon monoxide (CO) & hydrogen sulfide (H_2S) in hypoxic sensing by the carotid body. Respir Physiol Neurobiol 184:165–169
Romero LC, Aroca MA, Serna A, Gotor C (2013) Proteomic analysis of endogenous S-sulfhydration in *Arabidopsis thaliana*. Nitric Oxide 31:S23
Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 30:1191–1212
Sen N, Paul BD, Gadalla MM, Mustafa AK, Sen T, Xu R, Kim S, Snyder SH (2012) Hydrogen sulfide-linked sulfhydration of NF-κB mediates its antiapoptotic actions. Mol Cell 45:13–24
Shan CJ, Zhang SL, Li DF, Zhao YZ, Tian XL, Zhao XL, Wu YX, Wei XY, Liu RQ (2011) Effects of exogenous hydrogen sulfide on the ascorbate and glutathione metabolism in wheat seedlings leaves under water stress. Acta Physiol Plant 33:2533–2540
Stuiver CEE, De Kok LJ, Kuiper PJC (1992) Freezing tolerance and biochemical changes in wheat shoots as affected by H_2S fumigation. Plant Physiol Biochem 30:47–55
Sun C, Shi Z-Z, Zhou X, Chen L, Zhao X-M (2013) Prediction of *S*-glutathionylation sites based on protein sequences. PLoS One 8:e55512
Szinicz L (2005) History of chemical and biological warfare agents. Toxicology 214:167–181
Tai CH, Cook PF (2000) O-acetylserine sulfhydrylase. Adv Enzymol RAMB 74:185–234
Turrens JF (2004) Mitochondrial formation of reactive oxygen species. J Physiol 552:335–344
Wang R (2002) Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? FASEB J 16:1792–1798
Wang R (2003) The gasotransmitter role of hydrogen sulfide. Antioxid Redox Signal 5:493–501
Wang R (2013) A stinky remedy for atherosclerosis. Nitric Oxide 31:S17–S18
Wang P, Du Y, Li Y, Ren D, Song CP (2010) Hydrogen peroxide-mediated activation of MAP kinase 6 modulates nitric oxide biosynthesis and signal transduction in Arabidopsis. Plant Cell 22:2981–2998
Wang Y, Li L, Ciu W, Xu S, Shen W, Wang R (2012) Hydrogen sulfide enhances alfalfa (*Medicago sativa*) tolerance against salinity during seed germination by nitric oxide pathway. Plant Soil 351:107–119
Whiteman M, Li L, Kostetski I, Chu SH, Siau JL, Bhatia M, Moore PK (2006) Evidence for the formation of a novel nitrosothiol from the gaseous mediators nitric oxide and hydrogen sulphide. Biochem Biophys Res Commun 343:303–310
Whiteman M, Gooding KM, Whatmore JL, Ball CI, Mawson D, Skinner K, Tooke JE, Shore AC (2010) Adiposity is a major determinant of plasma levels of the novel vasodilator hydrogen sulphide. Diabetologia 53:1722–1726
Wientjes FB, Segal AW (1995) NADPH oxidase and the respiratory burst. Semin Cell Biol 6:357–365
Wilson ID, Neill SJ, Hancock JT (2008) Nitric oxide synthesis and signalling in plants. Plant Cell Environ 31:622–631
Xu S, Liu Z, Liu P (2014) Targeting hydrogen sulfide as a promising therapeutic strategy for atherosclerosis. Int J Cardiol 172:313–317

Youssefian S, Nakamura M, Sano H (1993) Tobacco plants transformed with the O-acetylserine (thiol) lyase gene of wheat are resistant to toxic levels of hydrogen sulphide gas. Plant J 4:759–769

Zemojtel T, Fröhlich A, Palmieri MC, Kolanczyk M, Mikula I, Wyrwicz LS, Wanker EE, Mundlos S, Vingron M, Martasek P, Durner J (2006) Plant nitric oxide synthase: a never-ending story? Trends Plant Sci 11:524–525

Zhang H, Hu LY, Hu KD, He YD, Wang SH, Luo JP (2008) Hydrogen sulfide promotes wheat seed germination and alleviates the oxidative damage against copper stress. J Integr Plant Biol 50:1518–1529

Zhang H, Tang J, Liu XP, Wang Y, Yu W, Peng WP, Fang F, Ma DF, Wei ZJ, Hu LY (2009a) Hydrogen sulfide promotes root organogenesis in *Ipomoea batatas*, *Salix matsudana and Glycine max*. J Integr Plant Biol 51:1084–1092

Zhang H, Ye YK, Wang SH, Luo JP, Tang J, Ma DF (2009b) Hydrogen sulfide counteracts chlorophyll loss in sweet potato seedling leaves and alleviates oxidative damage against osmotic stress. Plant Growth Regul 58:243–250

Zhang H, Hu LY, Li P, Hu KD, Jiang CX, Luo JP (2010a) Hydrogen sulfide alleviated chromium toxicity in wheat. Biol Plant 54:743–747

Zhang H, Tan ZQ, Hu LY, Wang SH, Luo JP, Jones RL (2010b) Hydrogen sulfide alleviates aluminum toxicity in germinating wheat seedlings. J Integr Plant Biol 52:556–567

Zhang H, Wang MF, Hua LY, Wang SH, Hua KD, Bao LJ, Luo JP (2010c) Hydrogen sulfide promotes wheat seed germination under osmotic stress. Russ J Plant Physiol 57:532–539

Zhang H, Hua SL, Zhang ZJ, Hua LY, Jiang CX, Wei ZJ, Liu J, Wang HL, Jiang ST (2011) Hydrogen sulfide acts as a regulator of flower senescence in plants. Postharv Biol Technol 60:251–257

Zhang Y, Tang Z-H, Ren Z, Qu S-L, Liu M-H, Liu L-S, Jiang Z-S (2013) Hydrogen sulfide, the next potent preventive and therapeutic agent in aging and age-related diseases. Mol Cell Biol 33:1104–1113

Investigation of Protein-Protein Interactions of Ferredoxin and Sulfite Reductase Under Different Sodium Chloride Concentrations by NMR Spectroscopy and Isothermal Titration Calorimetry

Ju Yaen Kim, Takahisa Ikegami, Yuji Goto, Toshiharu Hase, and Young-Ho Lee

Abstract Sulfite reductase (SiR) catalyzes the reduction of sulfite to sulfide by using six electrons transported from ferredoxin (Fd) for eventual sulfur assimilation. As efficient electron flows are ensured by forming a productive Fd:SiR complex, detailed characterization of a Fd:SiR complex in solution is of particular importance. Here, we show that acidic residues of Fd play essential roles in forming an electron transfer complex with SiR by using attractive electrostatic interactions with putative basic residues of SiR. The thermodynamic approach using calorimetry revealed a favorable electrostatic contribution to form the Fd:SiR complex at the molecular level. Solution-state nuclear magnetic resonance (NMR) spectroscopy on ^{15}N-labeled Fd in the presence of SiR showed large perturbations in NMR signals of acidic residues of Fd. The addition of NaCl diminished overall perturbations of NMR signals of SiR-bound Fd which resulted from the decrease in interprotein affinity. However, acidic residues at both termini still showed relatively large peak perturbation. These results at the residue level suggested that intermolecular interactions between Fd and SiR are electrostatic in nature and the electrostatic interaction is a dominant contributor to form the Fd:SiR complex. We suggest that a combination of calorimetry and NMR is a powerful approach to investigating protein-protein interactions.

A variety of life phenomena such as electron transfer, signal transduction, and protein homeostasis, are conducted by intermolecular interactions of multiple proteins. Protein surfaces generally consist of both charged/polar hydrophilic and apolar hydrophobic regions. The intermolecular interactions, achieved by the electrostatic

J.Y. Kim • T. Ikegami • Y. Goto • T. Hase • Y.-H. Lee (✉)
Institute for Protein Research, Osaka University,
3-2 Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: mr0505@protein.osaka-u.ac.jp

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_17

and hydrophobic forces, occur in an energetically favorable way. Several physicochemical studies have been reported on the molecular interaction between electron transfer proteins and their partner enzymes. Cytochrome *c* uses the highly positive electrostatic patches to interact with acidic residues of the subunit II of cytochrome *c* oxidase for electron transfer (Maneg et al. 2004). Similarly, positive residues of cytochrome *f* interact with negative residues of plastocyanin (Gross and Pearson 2003). In chloroplasts, Fd, which possesses clusters of acidic regions, acts as a multiple electron donor for various redox proteins. Biochemical and biophysical studies have suggested that various Fd-dependent enzymes such as nitrite reductase (NiR), sulfite reductase (SiR), and ferredoxin-NADP$^+$ reductase (FNR) mainly interact with Fd using their positive residues on surfaces (Akashi et al. 1999; Nakayama et al. 2000; Kurisu et al. 2001; Saitoh et al. 2006; Lee et al. 2011; Sakakibara et al. 2012).

SiR reduces sulfite to sulfide using six electrons transferred from Fd. Linked sequential metabolic reactions with other enzymes produce amino acid residues such as methionine and cysteine using sulfide. Electrons are transferred from a [2Fe-2S] cluster of Fd to a [4Fe-4S] cluster of SiR and then from a [4Fe-4S] to siroheme intramolecularly. We have reported biochemical analyses of interprotein interactions between Fd and SiR to form the electron transfer complex (Akashi et al. 1999; Nakayama et al. 2000; Saitoh et al. 2006). In this study, we first applied isothermal titration calorimetry (ITC) to investigate interactions between Fd and SiR (Fig. 1). By measuring gradual heat changes attributed to the binding of SiR and Fd under a titration of increasing concentrations of Fd in the presence or absence of 100 mM NaCl, thermodynamic parameters, changes in enthalpy (ΔH), entropy (ΔS) and binding affinity (K_d) with binding stoichiometry (n), were determined using the equations as below.

Observed ΔH for binding and the dissociation constant (K_d) were directly calculated from the integrated heats using the two-sets (Fig. 1a, lower panel) or one-set (Fig. 1b, lower panel) of an independent binding site mode using Eq. 1:

$$Q = \frac{n[P]_t \Delta H V_0}{2}\left[1 + \frac{L_R}{n} + \frac{K_d}{n[P]_t} - \sqrt{\left(1 + \frac{L_R}{n} + \frac{K_d}{n[P]_t}\right)^2 - \frac{4L_R}{n}}\right] \quad (1)$$

where Q is the change in heat, V_0 is the effective volume of the calorimeter cell (~1.43 ml), L_R is the ratio of the total SiR concentration to total Fd concentration ($[P]_t$) at any given point during titrations, and n is the binding stoichiometry of Fd per a binding site on SiR. By using the values of ΔH and K_d, the change in Gibbs free energy (ΔG) and ΔS were calculated using thermodynamic relationships (Eqs. 2 and 3) as follows:

$$\Delta G = RT \ln K_d \quad (2)$$

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

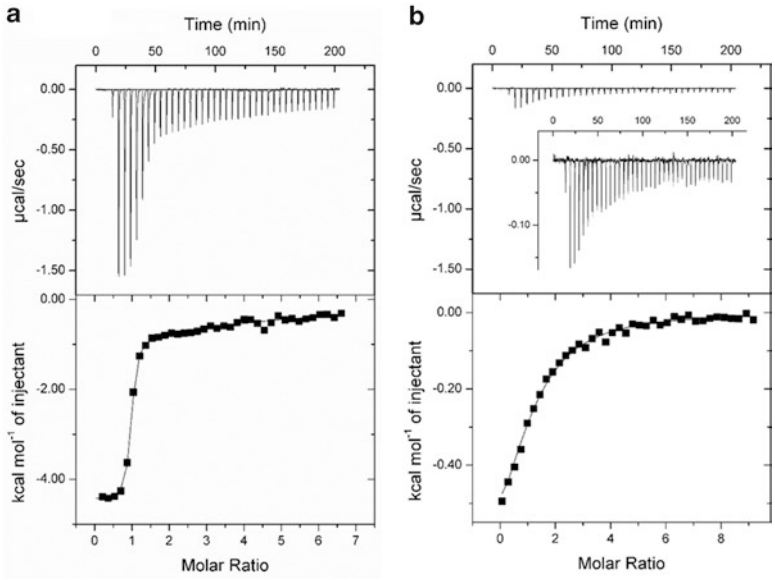


Fig. 1 Isothermal calorimetric titration of Fd with SiR. Binding reactions between Fd and SiR were initiated by adding Fd (1.49 mM) in the syringe to SiR (40 µM) in the reaction cell using VP-ITC instrument (GE-Healthcare, USA) at 30 °C in 50 mM Tris/HCl buffer (pH 7.5) in the absence (**a**) and presence (**b**) of 100 mM NaCl. Titration consisted of 38 injections spaced at intervals of 300 s. The injection volume was 7 µL for each, and the cell was continuously stirred at 307 rpm. The corresponding heat of dilution of Fd titrated to the buffer was used to correct data. Thermograms and binding isotherms are shown in *upper* and *lower panels*, respectively. For clarity, the thermogram with 100 mM was magnified in inset of B. *Solid lines* in *lower panels* are theoretical curves

where R is the gas constant and T is the temperature in Kelvin. Schematic representation of binding states of Fd and SiR are shown in the upper panels.

A large exothermic reaction was observed during a successive titration of Fd to SiR without NaCl and thus the formation of the Fd:SiR complex was assumed to be driven with negative ΔH ($\Delta H < 0$; Fig. 1a). As the molar ratio of Fd/SiR increased, the extent of binding heat decreased and saturation was reached, indicating that all SiR was occupied with Fd. Interestingly, there were two binding phases: a high binding affinity phase at the low molar ratio of Fd/SiR (0 to ~2) was followed by a low binding affinity phase at the high molar ratio (~2 to ~7). The analyses of these isotherms provided that the values of ΔH, K_d, and n for a high affinity binding reaction were −4.4 kcal/mol, 0.2 µM, and ~1, respectively, and, for a low affinity binding reaction, ΔH of several negative kcal/mol, K_d of several hundred µM, and n of several values (~3 to ~5) (Table 1): the value of "n" indicates the number of Fd which binds to SiR. Negative ΔH clearly revealed, regardless of the binding affinity and site, an exothermic nature of the Fd:SiR complex formation, which suggested the favorable contribution of interprotein electrostatic interactions. Furthermore, it was shown that when concentrations of Fd were lower than those of SiR, one Fd

Table 1 Summary of thermodynamic parameters for the formation of the Fd:SiR complex

NaCl (mM)	Binding site	n[a]	K_d (μM)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
0	High affinity[b]	~1	0.2	−4.4	4.9	−9.3
	Low affinity[c]	~5[d]	~330[d]	−~2[d]	~2.8[d]	~−4.8[d]
100	High affinity[b]	~1	23	−0.9	5.6	−6.5
	Low affinity[c]	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]

[a]The number of Fd which binds to a binding site of SiR
[b]High affinity binding site
[c]Low affinity binding site
[d]Several Fds interact with a low affinity binding site of SiR. The gradual increase in ΔH hampered a best fit to a non-linear curve which was based on Eq. 1. Thus, a range of n values were obtained (~3 to ~5). The values of n and ΔG in Table were obtained with a fixed value of $K_a = 3 \times 10^3$ M^{-1} ($K_d = 333.3$ μM) and $\Delta H = -2$ kcal/mol. The total values of ΔH and $T\Delta S$ must be divided by an n value for values of each binding reaction
[e]n.d. indicates "not determined"

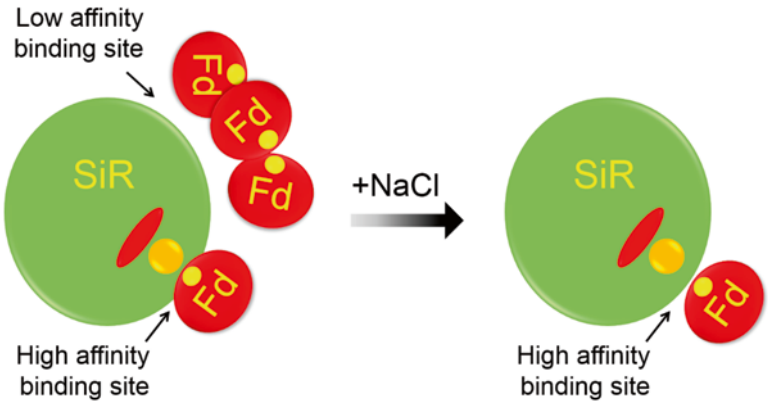


Fig. 2 Schematic representation of the effect of the NaCl addition on interprotein interactions between Fd and SiR. *Green* and *red spheres* represent SiR and Fd, respectively. *Red ellipses* and *orange spheres* indicate the siroheme and the [4Fe-4S] cluster in SiR, respectively. *Yellow spheres* signify the [2Fe-2S] cluster in Fd. In the absence of NaCl, a high affinity binding site of SiR accommodates one Fd as evidenced by the n value (n = ~1; Table 1) with high affinity and specificity. Several Fds (n = ~3 to ~5) interact with SiR at a low affinity binding site in a lesser specific way. In the presence of 100 mM NaCl, SiR holds one Fd just at a high affinity binding site with relatively high affinity and specificity

bound to one SiR (n = ~1) with a strong affinity (K_d = 0.2 μM; Table 1) at a high affinity binding site (Fig. 2). However, as excess amounts of Fd existed, several Fds further bound to SiR (n = ~3 to ~5) with a weak affinity (K_d of several hundred μM) at a low affinity binding site. Considering the difference in the molecular size of small Fd (~11 kDa) and large SiR (~65 kDa), multiple bindings of Fd to SiR are possible.

In order to further reveal characteristics of the interaction between Fd and SiR, ITC measurements were performed in the presence of 100 mM NaCl (Fig. 1b). If electrostatic interactions are dominant, binding affinity should decrease upon addition of NaCl. In contrast, in the case of hydrophobic interactions playing a major role, reinforced hydrophobic interactions by increasing NaCl concentration stabilize the complex. Although exothermic heat was generated from the binding of the two proteins, the extent of heat was found to be much smaller than that without NaCl. Decreases in the degree of exothermic heat were interpreted as dropping of attractive electrostatic interactions between negative charges of Fd and positive charges of SiR due to the screening effects of NaCl and/or direct binding of the counter ions (Na^+ and Cl^-) to charged/polar residues. Accordingly, ΔH decreased in magnitude from −4.4 to −0.9 kcal/mol. K_d and ΔG also increased to 23 μM and −6.5 kcal/mol, respectively, indicative of decreases in affinity between proteins. Intriguingly, adding NaCl apparently abolished weak interprotein interactions even at excess amounts of Fd (Fig. 2), which suggested that the origin of such weak interactions was also electrostatic in nature.

The entropy term ($T\Delta S$) exhibited positive values in the absence (4.9 kcal/mol) and presence (5.6 kcal/mol) of NaCl (Table 1). Large energetic costs (3.5 kcal/mol) coming from the increase in ΔH by adding NaCl were still compensated for by energetic gains from the positive entropy change. These indicated that entropy changes were also an important driving force to form the Fd:SiR complex which may be a result of dehydration of water upon the complex formation: decreasing ΔG by increasing $T\Delta S$ favors the binding reaction (Eq. 3).

Consequently, ITC results showed that intermolecular electrostatic interactions are a main driving force to form the Fd:SiR complex by using enthalpy. However, it is worth noting that a favorable hydrophobic contribution to complexation is also plausible since hydrophobic interactions gain entropy (i.e., $\Delta S > 0$) from dehydration on interfacial packing among hydrophobic residues.

Solution-state NMR spectroscopy is useful to investigate intermolecular interactions at the atomic/residue level. NMR further probes weak and/or dynamic features of intermolecular interactions (Mizushima et al. 2014). We first carried out the measurement of ^{1}H-^{15}N heteronuclear single-quantum coherence correlation (HSQC), using ^{15}N-labeled Fd without SiR and NaCl (Fig. 3a). Clear and sharp NMR signals of free Fd were obtained with good dispersion ranging from 6 to 11 ppm in the transverse axis for proton (^{1}H). One peak in the HSQC spectrum corresponds to one amino acid residue of the backbone in Fd. Based on the previous assignment information (Saitoh et al. 2006), we assigned the NMR peaks to the corresponding residues. The addition of SiR changed the position of several NMR peak signals of Fd (i.e., chemical shift), indicating that a set of Fd residues was involved in SiR binding. The number of assigned peaks (78) was the same as that without SiR, suggestive of a fast exchange regime in terms of the NMR time scale between free and complex states, thereby showing an averaged one peak between these two conformational states. A binding system, which shows a fast exchange regime has suggested (relatively) weak intermolecular interactions over a nanomolar order of K_d. Thus, a system with a fast exchange often shows K_d in a micromolar order. A

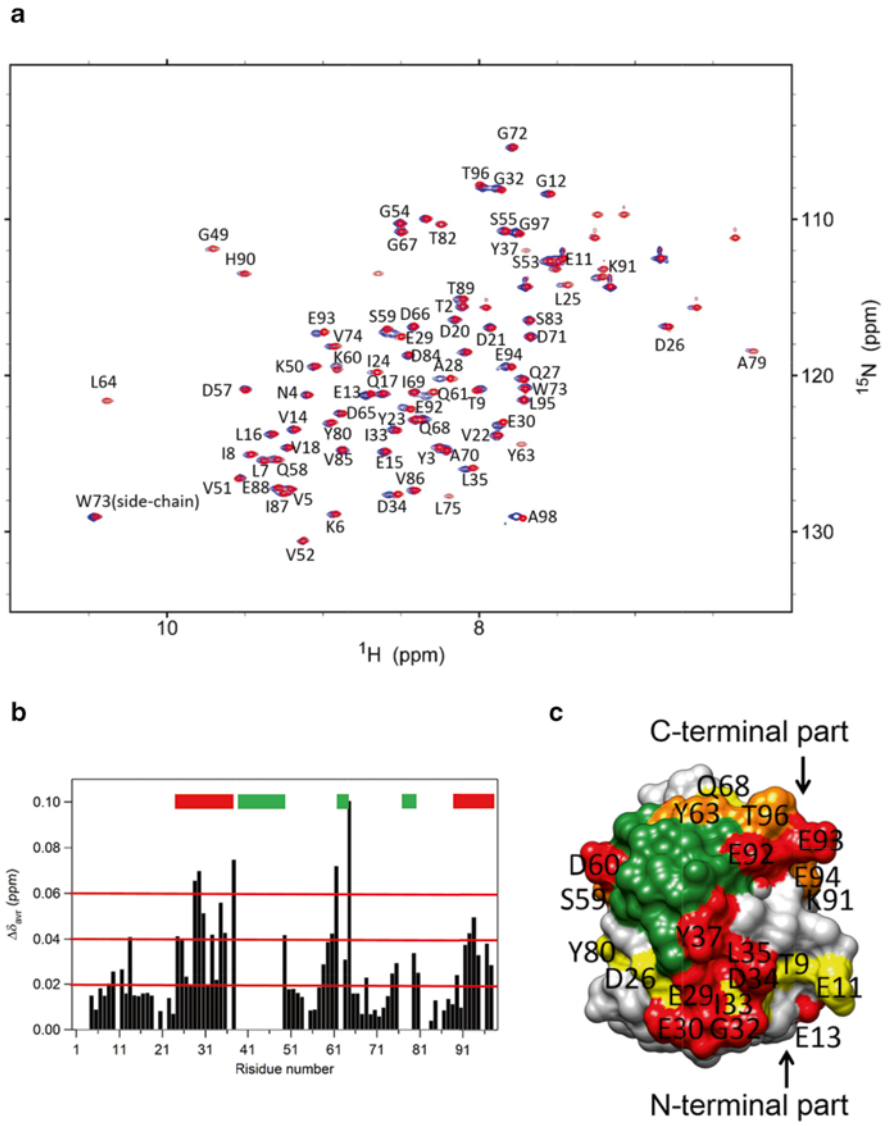


Fig. 3 NMR spectra of SiR-(un)bound Fd without NaCl and binding interfaces of Fd for SiR. (**a**) ^{1}H-^{15}N HSQC spectra of ^{15}N uniformly-labeled Fd without (*red*) and with (*blue*) SiR were obtained using an AVANCE II-800 spectrometer equipped with a cryogenic probe (Bruker, Germany) in 50 mM Tris/HCl buffer (pH 7.4) containing 10 % D_2O at 25 °C. Data were processed by NMRPipe and analyzed by Sparky. Stable isotope-labeled maize leaf Fd with ^{15}N was expressed using *E. coli* cultured in minimal media containing [^{15}N]-NH_4Cl as a nitrogen source and purified as previously described (Kurisu et al. 2001; Saitoh et al. 2006; Sakakibara et al. 2012). Maize leaf SiR was prepared as described in our previous study (Saitoh et al. 2006). The protein concentrations for NMR measurements were 100 μM for Fd and 50 μM for SiR. The assigned peaks are shown with the one letter amino acid code and residue number. The NMR signal stemmed from the side chain of tryptophan is shown (W73). (**b**) The values of chemical shift differences (CSD) were plotted against residue numbers. Mainly acidic clusters are shown by the *red rectangles*. The regions where NMR peaks are invisible due to the paramagnetic relaxation effect (PRE) from iron are displayed with the *green rectangle*. (**c**) Mapping of residues which showed CSD on the crystal structure of Fd (PDB ID: 1GAQ) (Kurisu et al. 2001). The degrees of CSD are shown by the color code: *red* > 0.06 ppm, 0.04 < *orange* < 0.06 ppm, and 0.02 < *yellow* < 0.04 ppm. NMR invisible regions due to PRE are represented with *green colors*. N- and C-terminal parts are indicated

slow exchange regime produces two separated peaks which correspond individually to free and complex states because intermolecular interactions are significantly strong. Typically, K_d in a nanomolar order shows slow exchange regimes. Therefore, the binding affinity of Fd for SiR is judged to be relatively strong based on a fast exchange, consistent with ITC results which showed the submicro molar K_d value.

The degree of shifts in the peak position (i.e., chemical shift difference, CSD) in NMR peaks with and without SiR (Fig. 3a) were calculated by using Eq. 4:

$$\mathrm{CSD}\left(\Delta\delta_{\mathrm{ave}}\right) = \left[\left(\Delta\delta_{\mathrm{HN}}\right)^2 + \left(\Delta\delta_{\mathrm{N}} \times 0.158\right)^2\right]^{0.5} \tag{4}$$

where $\Delta\delta_{HN}$ and $\Delta\delta_N$ are changes in ^{1}H and ^{15}N chemical shifts in ppm, respectively. The weighting factor of 0.158 was used to adjust the relative magnitudes of the amide nitrogen chemical shift range and the amide proton chemical shift range.

By using CSD information, we could identify the important residues of Fd responsible for SiR binding (Fig. 3b). The largely perturbed residues which showed CSD over 0.03 were distributed in the N-terminal region (I24, A28, E29, E30, D34, L35, and Y37), the central region (G49, Q61, and L64), and the C-terminal region (E92, E93, and E94), respectively (Fig. 3b, c). Together with a few hydrophobic residues (I24, L35, and L64), it was obvious that many negatively charged residues were responsible for forming a complex with SiR.

To clarify the effects of salts on Fd:SiR interactions, ^{1}H-^{15}N HSQC spectrum of SiR-unbound Fd at 100 mM NaCl was obtained (Fig. 4a). The spectrum also exhibited sharp peaks with broad dispersion as observed without NaCl and SiR. ^{1}H-^{15}N HSQC spectrum was then obtained with SiR: adding SiR shifted many peak positions with a fast exchange regime but to a lesser extent than those without NaCl (Fig. 4b). The overall direction of peak shifts in the presence of SiR was similar at 0 and 100 mM NaCl, which suggested the similarity of an overall binding mode between Fd and SiR at two distinct salt concentrations. The CSD analysis displayed an overall decrease in CSD of SiR-bound Fd (Fig. 4b) compared to that without NaCl (Fig. 3b). This indicated the decrease in the population of a Fd:SiR complex due mainly to the dropping of interprotein affinity by the disruption of attractive electrostatic interactions by NaCl, in good accordance with our ITC results which showed the increase in K_d values from 0.2 to 22 μM (Table 1).

The analysis further revealed that acidic residues at the N- and C terminal parts and the central part (I24, A28, E29, E30, D34, L35, Y37, G49, Q61, L64, E92, E93, and E94) showed larger CSD values than those in the other parts (Fig. 4b, c). These acidic residues were almost the same as those without NaCl although the degree of change in CSD for each residue was different. Furthermore, considering the fact that acidic residues of Fd in both termini were also used for interactions with FNR (Kurisu et al. 2001; Saitoh et al. 2006) and NiR (Sakakibara et al. 2012), a favorable electrostatic force may be a common feature in interprotein interactions between Fd and its redox partners. Meanwhile, it should be noted that hydrophobic residues, L35 and L64 also exhibited large CSD values, which implied the contribution of hydrophobic interactions to the complex formation.

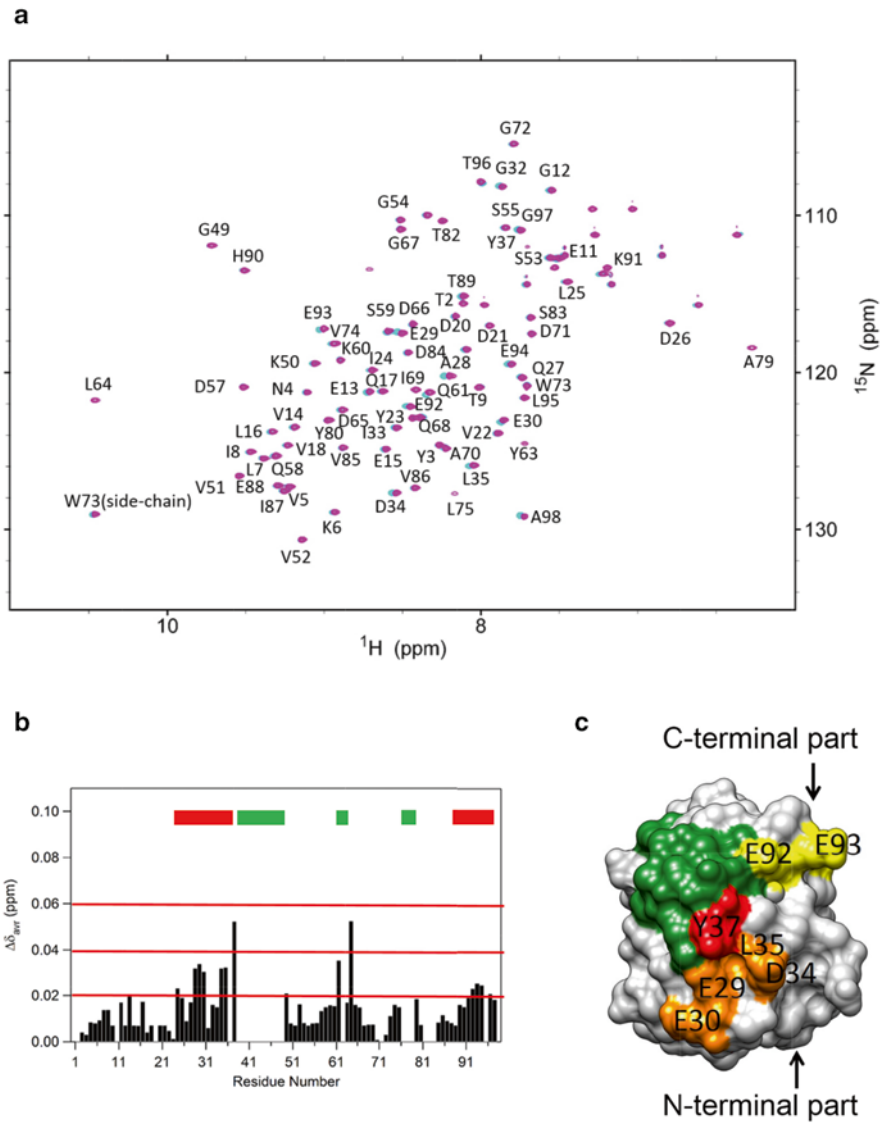


Fig. 4 NMR spectra of SiR-(un)bound Fd with NaCl and binding interfaces of Fd for SiR. (**a**) ^{1}H-^{15}N HSQC spectra of ^{15}N uniformly-labeled Fd with (*cyan*) and without (*magenta*) SiR were obtained using the identical condition and procedure which are described in Fig. 3 except the presence of 100 mM NaCl. (**b**) CSD in NMR peaks with and without SiR was calculated and plotted against residue numbers. Mainly acidic clusters are shown by the *red rectangles*. The regions where NMR peaks are invisible due to PRE are displayed with *green rectangles*. (**c**) Mapping of residues which showed CSD on the crystal structure of Fd (PDB ID: 1GAQ) (Saitoh et al. 2006). The degree of CSD is shown by the color code: *red* > 0.04 ppm, 0.03 < *orange* < 0.04 ppm and 0.02 < *yellow* < 0.03 ppm. NMR invisible regions due to PRE are represented with green colors and both termini are indicated

Taken all together, it was concluded that attractive electrostatic interactions act as a main stabilizer for forming a Fd:SiR complex and NaCl attenuates interprotein interactions between electrically-charged residues. On the one hand, although favorable electrostatic interactions for complexation are evident, hydrophobic contributions are still unclear. Favorable hydrophobic interactions enhanced by adding of 100 mM NaCl might promote the complex formation to a much lesser extent than opposite contribution of electrostatic interactions decreased by 100 mM NaCl. Thus, it appears that only electrostatic contributions may appear as the dominant contributor to complexation. Further detailed study will require the complete understanding of interprotein interactions between Fd and SiR by considering hydrophobic interactions such as the residues detected here (I24, L35, and L64) and the hydrophobic region around the [2Fe-2S] clusters that is NMR-invisible due to PRE. Site-directed mutagenesis combined with SiR activity assays and direct structural studies based on X-ray crystal and solution-state NMR approaches should prove promising in obtaining a clue or solution for this issue. Finally, current results imply that subtle changes in salt concentration, which might reflect conditions in the chloroplast, control electrostatic interaction for the electron transfer complex.

References

Akashi T, Matsumura T, Ideguchi T, Iwakiri KI, Kawakatsu T, Taniguchi I, Hase T (1999) Comparison of the electrostatic binding sites on the surface of ferredoxin for two ferredoxin-dependent enzymes, ferredoxin-NADP$^+$ reductase and sulfite reductase. J Biol Chem 274:29399–29405

Gross EL, Pearson DC (2003) Brownian dynamics simulations of the interaction of chlamydomonas cytochrome f with plastocyanin and cytochrome c6. Biophys J 85:2055–2068

Kurisu G, Kusunoki M, Katoh E, Yamazaki T, Teshima K, Onda Y, Kimata-Ariga Y, Hase T (2001) Structure of the electron transfer complex between ferredoxin and ferredoxin-NADP$^+$ reductase. Nat Struct Biol 8:117–121

Lee YH, Ikegami T, Standley DM, Sakurai K, Hase T, Goto Y (2011) Binding energetics of ferredoxin-NADP$^+$ reductase with ferredoxin and its relation to function. ChemBioChem 12:2062–2070

Maneg O, Malatesta F, Ludwig B, Drosou V (2004) Interaction of cytochrome c with cytochrome oxidase: two different docking scenarios. Biochim Biophys Acta 1655:274–281

Mizushima R, Kim JY, Suetake I, Tanaka H, Takai T, Kamiya N, Takano Y, Mishima Y, Tajima S, Goto Y, Fukui K, Lee YH (2014) NMR characterization of the interaction of the endonuclease domain of MutL with divalent metal ions and ATP. PLoS One 9:e98554

Nakayama M, Akashi T, Hase T (2000) Plant sulfite reductase: molecular structure, catalytic function and interaction with ferredoxin. J Inorg Biochem 82:27–32

Saitoh T, Ikegami T, Nakayama M, Teshima K, Akutsu H, Hase T (2006) NMR study of the electron transfer complex of plant ferredoxin and sulfite reductase. J Biol Chem 281:10482–10488

Sakakibara Y, Kimura H, Iwamura A, Saitoh T, Ikegami T, Kurisu G, Hase T (2012) A new structural insight into differential interaction of cyanobacterial and plant ferredoxins with nitrite reductase as revealed by NMR and X-ray crystallographic studies. J Biochem 151:483–492

Prospective Post-translational Regulation of Plant Sulfite Oxidase

David Kaufholdt, Christin-Kirsty Baillie, Thorsten Wille, Christina Lang, Stephan Hallier, Cornelia Herschbach, Heinz Rennenberg, Ralf Mendel, and Robert Hänsch

Abstract Sulfite oxidase is of vital importance for sulfite homeostasis in plants. Sulfite homeostasis is required, since high amounts of sulfite are toxic for all living organism and, therefore, sessile organisms such as plants have had to develop mechanisms to protect themselves from exogenous sulfite. Sources of SO_2 in the present time largely originate from fossil fuel combustion and manufacturing industries especially in developing countries. Plant sulfite oxidase (pSO) is a molybdenum-containing enzyme that is localized in peroxisomes, uses oxygen as an electron acceptor, and produces hydrogen peroxide. Sulfite oxidase plays an essential role in the detoxification of SO_2 in plants. Overexpression of pSO promotes survival upon high levels of SO_2 fumigation. Furthermore, the activity of pSO is increased in two out of four species grown in Rapolano Terme (Italy) under permanent SO_2 exposure in the range of 10–100 ppb. Experiments conducted with plant extracts taken at different time points over the day as well as at different time points in the lifecycle of *Nicotiana tabacum* plants suggest a hitherto unknown regulation *via* induction/inhibition of pSO. Screening with various inhibitors of phosphorylation did not reveal regulation of pSO *via* phosphorylation unlike its sister enzyme nitrate reductase yet the experiments did show vanadate to be an effective inhibitor for pSO. Western blotting of plant extracts from different tissues pointed to a potential SUMOylation of pSO, but *in vitro* analyses of SUMOylation of pSO were negative. Using in vivo protein-protein interaction assays, however, an interaction between pSO and SUMO1 as well as SUMO3 was demonstrated. These results were confirmed by both the bimolecular fluorescence complementation (BiFC) as well as the floated-leaf luciferase complementation imaging (FLuCI), which are both split reporter protein assays.

D. Kaufholdt • C.-K. Baillie • T. Wille • C. Lang • S. Hallier • R. Mendel • R. Hänsch (✉)
Institut für Pflanzenbiologie, Technische Universität Braunschweig,
Humboldtstraße 1, 38106 Braunschweig, Germany
e-mail: r.haensch@tu-bs.de

C. Herschbach
Fakultät für Umwelt und Natürliche Ressourcen, Institut für Forstwissenschaften, Albert-Ludwigs-Universität Freiburg, Georges-Köhler-Allee 053/054, 79110 Freiburg, Germany

H. Rennenberg
Professur für Baumphysiologie, University of Freiburg, Freiburg, Germany

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_18

Sulfite homoeostasis is of vital importance for living organisms. Surplus of toxic sulfite can originate from endogenous sources such as catabolism of sulfur-containing amino acids in animals (Mudd et al. 1967) or can arise exogenously (Cohen et al. 1973; Rennenberg 1984). In the past, volcanic eruptions or forest fires produced tremendous amounts of SO_2 that is converted into sulfite in the aqueous phase of the apoplastic space or within the plant cell. In the present time, anthropogenic sulfite has a great impact on plant growth and development. Here, burning of coal or oil, steel production and chemical industry produces SO_2 that is emitted into the atmosphere especially in developing countries (Lu et al. 2010). In contrast to animals, plants as sessile organism have no chance to escape air pollution by movement and so have had to evolve effective protection mechanisms. Fast and highly efficient closure of stomata or nearly SO_2-indifussible cuticles avoid the influx of SO_2 (Pfanz et al. 1987). Moreover, there are several mechanisms leading to a reduction of the internal level of sulfite, which are discussed in literature (Rennenberg 1984; Rennenberg and Herschbach 1996).

Low concentrations of sulfite can be used as a nutrient for plants – the threshold of toxicity of sulfite or to what extent sulfite can be useful depends on the sulfur need of the particular plant species. Sulfite or its reduction product H_2S is fed into the assimilation stream of sulfur to produce cysteine (Stuiver and De Kok 2001). Despite the reduction of sulfite, oxidation also takes place inside the plant (Heber and Hüve 1998). Pfanz and colleagues (1990) and Pfanz and Oppermann (1991) described the activity of an apoplastic peroxidase which is able to detoxify sulfite to a still unknown extent. Jolivet and colleagues (1995a, b) described a sulfite oxidase activity associated with the chloroplastic fraction. In 2001, Eilers and colleagues discovered the molybdenum-containing enzyme sulfite oxidase in plants (pSO: AT3G01910), which is a direct sister enzyme of the essential animal counterpart. A lack of animal SO leads to early death of new-born infants. Contrary to the animal enzyme, pSO is localized inside the peroxisome (Nowak et al. 2004); it uses oxygen as an electron acceptor and produces hydrogen peroxide (Hänsch et al. 2006). pSO is of vital importance for plants (Hänsch and Mendel 2005; Lang et al. 2007): Arabidopsis knock-out plants suffer or die under SO_2-fumigation (Hamisch et al. 2012; Randewig et al. 2012); overexpression of the pSO helps plants to survive high levels of SO_2-exposure as shown for poplar (Lang et al. 2007) and tomato (Brychkova et al. 2007).

There are several indications for the regulation of pSO at different levels. Two out of four species grown in Rapolano Terme (Italy) under permanent SO_2 exposure in the range of 10–100 ppb showed increased pSO activity (Lang et al. 2007). Transcriptional regulation was studied using promoter-reporter gene constructs stably integrated into Arabidopsis as well as the RNA deep sequencing approach. Harsh fumigation led to increased reporter gene activity (Lang et al. 2007), mild fumigation increased the *so*-message in KO-plants (Hamisch et al. 2012). However, transcriptional regulation is relatively slow when plants are suddenly exposed to high SO_2-levels. Therefore, we suggested a post-transcriptional or post-translational regulation of pSO.

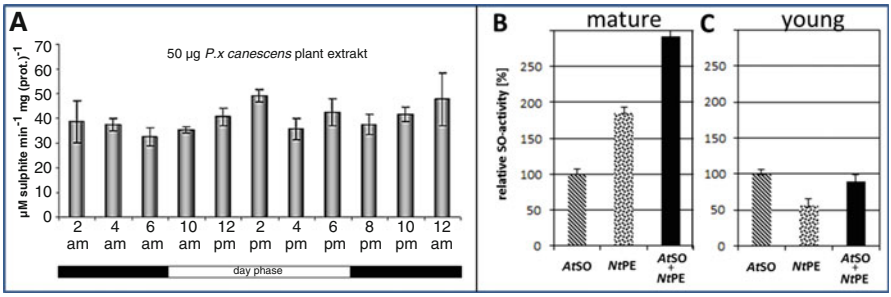


Fig. 1 Time depended activity of plant sulfite oxidase. (**a**) Sulfite oxidase activity of 50 µg (protein) *P.* x *canescens* plant extract (leaves) taken at different time points over the day. (**b**, **c**) Shown is the relative pSO activity of 100 ng recombinant *A. thaliana* SO, being defined as 100 %, 100 µg (protein) of *N. tabacum* plant extract (from leaves) and a mixture of the two with amounts adding up. All solutions were incubated for 2 h at room temperature. Shown are approaches with plant extracts of (**b**) mature leaves and (**c**) young leaves

As a first step to study regulatory mechanisms, plant extracts were prepared from poplar (*Populus x canescens*) leaves at different time points over 1 day and pSO activity was analysed at each time point (Fig. 1a). The activity rose up to the extract taken at 2 pm (with 6 h of exposure to light) showing a maximal turnover of 46 µM sulfite per minute and mg of protein. The following time points showed less activity up to the sample taken at midnight which showed an activity comparable to the maximum at 2 pm. The diurnal fluctuations in pSO activity, however, were relatively small. This suggests that the activity of pSO may be only slightly regulated. Based on these results the extracts of plants from different stages in their lifecycle were analyzed for pSO activity to investigate a possible long-term regulation of the enzyme (Fig. 1b, c).

100 ng of recombinant *Arabidopsis thaliana* pSO (r*At*SO), produced in *Escherichia coli* and purified *via his*-tag was tested for SO activity in a colorimetric assay and the measured activity was defined as 100 %. Leaf extract (100 µg total protein) of mature *Nicotiana tabacum* plants tested in the same assay yielded a relative pSO activity of 186 %. A mixture of 100 ng recombinant r*At*SO added to 100 µg of crude protein of mature leaves led to an activity of 292 % (Fig. 1b). This represents a direct and expected summation of the two activities. Contrary results were obtained with protein extracts of young *N. tabacum* plants which showed only 56 % of pSO activity while the mixture of recombinant *At*SO and young leaf extract led to an activity of only 89 % (Fig. 1c). The expected addition of both would have resulted in about 150 % activity. pSO activity in the mixture was even lower than the activity of r*At*SO alone; apparently the young leaf extract inhibited the activity of r*At*SO in the mixture. These findings suggest a hitherto unknown regulation of pSO *via* inhibition.

However, nitrate reductase (NR), the direct plant sister enzyme of pSO, is known to be highly regulated *via* transient phosphorylation/dephosphorylation. Phosphorylated serine in the hinge I region of NR is used to bind a nitrate reductase

inhibitor protein (Campbell 1999; Kaiser and Huber 2001) and inactivates the enzyme. To test for a possible phosphorylation of pSO, various chemicals inhibiting either phosphorylation or dephosphorylation due to inhibition of phosphatases or kinases were tested in a screening approach with respect to putative phosphorylation sites of pSO. These analyses were performed in parallel to the time-dependent enzyme activity analyses of pSO. Tests included (1) a combination of r*At*SO and plant extract of young *A. thaliana* leaves separately, and (2) a mixed assay with both extracts. The use of EDTA, cantharidin, phenylarsine oxide and natrium fluoride did not lead to changes compared to the untreated control (data not shown). Only the treatment with the tyrosine phosphatase inhibitor sodium orthovanadate (Swarup et al. 1982) showed a reduced activity of pSO in crude plant extract, with higher concentrations of vanadate leading to a stronger inhibition (Fig. 2b). However, it turned out that this reduced activity was dependent on a direct inhibition of the enzyme.

Additional experiments showed that the threshold for inhibition down to 20 % of activity of pSO was reached with a concentration of only 100 µM vanadate. The observed inhibition was partially reversible. After removal of vanadate from the medium with ammonium sulfate precipitation of pSO and subsequent resuspension of the pelleted protein, 60 % of the activity was regained compared to untreated pSO activity (Fig. 2a).

To exclude the possibility of an inhibition based on the loss of function of the prosthetic group molybdenum cofactor (Moco) due to a substitution of molybdenum with vanadium inside the active centre, the Mo-enzymes aldehyde oxidase (AO), xanthine dehydrogenase (XDH) and NR were incubated with vanadate and their activity was analyzed. The mono-oxo Mo-enzymes AO and XDH were treated with up to 50 mM vanadate and showed no loss in activity. NR showed an inhibition

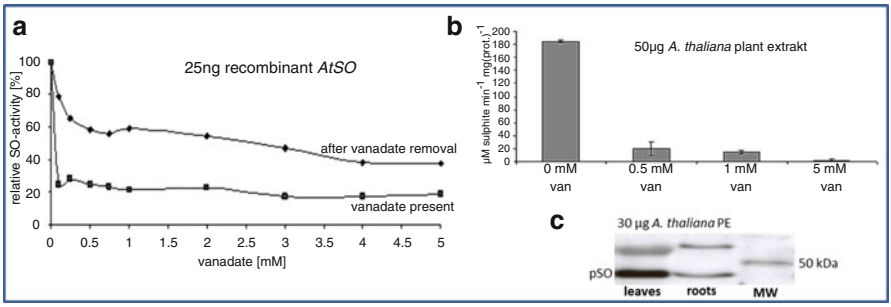


Fig. 2 (**a**) Relative pSO activity of 25 ng recombinant *A. thaliana* SO after incubation with different concentrations of vanadate for 45 min. Shown are the results with vanadate present during the activity measurement and after removal of vanadate *via* precipitation of the protein with ammonium sulfate and dissolving it in a vanadate-free buffer. (**b**) Sulfite oxidase activity of 50 µg (protein) *A. thaliana* plant extract (leaves) incubated with different vanadate concentrations for 45 min. Vanadate is present during activity measurement. (**c**) Western blotting analysis. Samples with 30 µg (protein) of *A. thaliana* plant extract were used taken from leaves and roots, respectively. Additionally, a molecular weight marker MW is shown at 50 kDa height

starting with a concentration of 2 mM vanadate (data not shown), representing a 20-fold higher concentration necessary for inhibition compared to pSO. Vanadate, therefore, represents an effective and specific inhibitor for pSO, but the mechanism of this inhibition needs further analysis.

As a next step, protein extracts from different tissues were used in a Western blot with a polyclonal anti-pSO antibody made in rabbit (Fig. 2c) in order to identify whether different amounts of the enzyme could be detected in different tissues. In all tissues containing RuBisCo, such as leaves, one band running at a height of about 45 kDa was detected with the antibody, which represents the pSO with a size of 43.3 kDa (Eilers et al. 2001). Additionally, a second band between 50 and 55 kDa was stained which seems to originate from a cross-reactivity with the small subunit of the RuBisCo complex. The sample taken from roots, however, reveals a second band larger than 55 kDa which could not originate from cross-reactivity, based on the lack of RuBisCo inside roots. This second band, therefore, seems to represent pSO molecules detected approximately 10 kDa larger than expected. We assume this larger band represents pSO post-translationally modified with a small ubiquitin-like modifier (SUMO) protein bound to the enzyme. The addition of 12 kDa from SUMO to pSO would result in the band shift observed. SUMO is known to be a modifier involved in environmental stress responses in plants (Johnson 2004). Taking these facts into account, the possible SUMOylation of pSO was studied *in vitro* with a SUMOylation kit (Enzo Life Sciences) which is based on the covalent linkage of SUMO-1, -2 or -3 to a target protein and the detection of the complex *via* Western blotting. The pSO showed no SUMOylation *in vitro* with either SUMO-1. -2, or -3 (data not shown). However, the kit used was optimized for animal protein samples, which could lead to false negative results with plant samples. Due to the negative *in vitro* results, the hypothesized SUMOylation was tested with in vivo protein-protein interaction assays. These analyses benefit from the use of the original plant system and, therefore, the native environment for the pSO.

Two in vivo protein-protein-interaction assays were used. Bimolecular fluorescence complementation (BiFC) and floated-leaf luciferase complementation imaging (FLuCI) are split reporter assays which were combined with transient *Agrobacterium tumefaciens* (syn: *Rhizobium radiobacter*) transformation in *N. benthamiana* leaves (Kaufholdt et al. 2013). For both assays a set of Gateway® (GW) destination vectors is available. BiFC was performed with the N-terminal part of the fluorescence protein Venus (pDest-*vyne*-GW; pDest-GW-*vyne*) and the C-terminal counterpart of the fluorescence protein SCFP (pDest-*scyce*-GW; pDest-GW-*scyce*), each fused to one gene of interest which is cloned into the position of the Gateway®-cassette (more detailed vector description in Gehl et al. 2009). The luciferase termini NLuc and CLuc were used as reporters in the FLuCI assay, each fused to one of the proteins of interest. The corresponding destination vectors (pDest-*nluc*-GW, pDest-GW-*nluc*, pDest-*cluc*-GW, pDest-GW-*cluc*) were described by Gehl and colleagues (2011). The desired expression vectors for *Agrobacterium*-mediated transformation were generated in a Gateway® recombination reaction; for this purpose, the stated entry and created expression vectors were used with the genes of interest previously amplified from *Arabidopsis* cDNA.

In *Arabidopsis*, the family of SUMO consists of nine proteins. Four of the SUMO encoding genes are expressed in detectable amounts *via* mRNA analysis (Kurepa et al. 2003). As potential interaction partners for pSO, SUMO1 (AT4G26840) and SUMO3 (AT5G55170) were chosen as representatives for the SUMO proteins present in plants. The interpretation of BiFCs was performed by comparing the fluorescence intensities of the interaction approach with both proteins of interest fused to a reporter protein half, to a negative control approach. The negative control consists of one fused protein of interest and the exchange of the other by a protein, which is known not to interact as it originates from a completely different organism and has no catalytical function of its own. As an additional control, the so-called abundance control was applied, which allows checking for the amount of expressed gene constructs inside the cell.

The BiFCs assay conducted between pSO and SUMO1 and compared with the negative control protein (CLuc), showed a bright interaction. However, the corresponding abundance control using a second negative protein SCFP did not fully confirm this result (data not shown). Taking all viewed pictures and analyzed leaves into account, an interaction between pSO and SUMO1 is indicated, yet not with a strong signal. However, the results for the BiFCs assay between pSO and SUMO3 were clearer. The direct interaction (Fig. 3a) was much stronger fluorescing than the negative control (Fig. 3b). The main part of fluorescence was detected as small spots representing peroxisomal structures. This signifies the ability to detect an interaction between two proteins of interest forming an interacting complex inside these small organelles. The corresponding abundance controls (Fig. 3c, d) showed a comparable fluorescence between $SCFP^{C}$-SUMO3 and $Venus^{N}$-$SCFP^{PTS}$ compared to the negative control protein CLuc-$SCFP^{C}$ and $Venus^{N}$-$SCFP^{PTS}$, underlying the strong interaction of pSO and SUMO3.

Comparable to BiFC, the FluCI assay was performed with a negative control approach as well as an abundance control (Fig. 3e, f). The difference between both approaches lies in the reconstitution of the N-terminal and C-terminal half of luciferase as a reporter protein. This reconstitution is reversible which leads to a dynamic detection of the interaction between the proteins of interest. The split LUC interaction factor shows the difference of luminescence of the interaction compared to the negative control. A value of 1 identifies both sides to be equally strong, which means no interaction. Values above this threshold classify an interaction between the two investigated proteins. The higher the value, the greater is the difference between luminescence intensities, which therefore shows a stronger interaction. The abundance control is also used to calculate a split LUC factor, which displays the amount of proteins inside the cell as described before. The FluCI approach between pSO and SUMO1 (Fig. 3e) led to a calculated split LUC factor of 9.0 which is very high in itself. However, as known from BiFC, the corresponding abundance control reduced the interaction factor to only twofold. The interaction between pSO and SUMO3 (Fig. 3f) resulted in a split LUC factor of 5.0 with a corresponding abundance control factor of 0.9. Thus, the interaction side between pSO and SUMO3 is almost sixfold stronger than the negative control. This result is in line with the BiFCs results, which also showed a better fluorescence between pSO

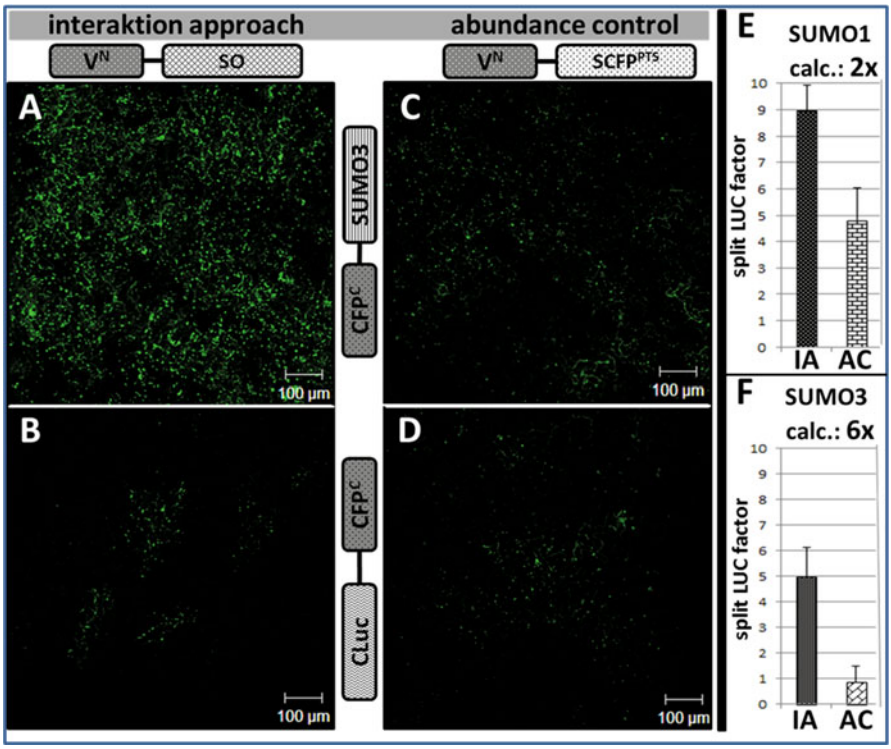


Fig. 3 In vivo interaction studies to analyse the potential regulation of pSO by SUMOylation using bimolecular fluorescence complementation (BiFC: **a**–**d**) and floated-leaf luciferase complementation imaging (FLuCI: E-F). BiFC were analysed by confocal laser scanning microscopy after gene transfer *via* Agrobacterium infiltration in *N. benthamiana*. Used excitation wavelength was 488 nm, emitted photons were detected between 500 and 540 nm. The BiFC complex emits at a maximum of 515 nm. Shown are representative images from eight to ten leaf discs of three to four plants. All images were taken with the same settings. BiFC interaction approaches with the two fusion proteins $Venus^N$-SO/$SCFP^C$-SUMO3 (**a**) and negative control $Venus^N$-SO/$SCFP^C$-CLuc (**b**). Corresponding abundance control to the constructs used in (**c**) and (**d**). FLuCI assays for the interaction approaches (IA) NLuc-SO/CLuc-SUMO1 (**e**) and NLuc-SO/CLuc-SUMO3 (**f**). As negative control, CLuc-StrepII was used in exchange for CLuc-SUMO1 and CLuc-SUMO3, respectively. Also shown is the corresponding abundance control (AC) in which NLuc-CFP was exchanged for NLuc-SO. Depicted is the corresponding Split LUC factor as bars for the interaction of **e** SUMO1 and pSO **f** SUMO3 and pSO. The *depicted bar* for the abundance control AC is used to calculate the corrected Split LUC factor. Ten to 15 leaf discs of four to five plants were analysed. *Error bars* represent the standard error of the mean

and SUMO3 than with SUMO1. Both assays therefore indicate a weak interaction of pSO with SUMO1, but a strong interaction between pSO and SUMO3.

In conclusion, we demonstrated that pSO is not only regulated at the level of gene expression after fumigation with SO_2, as shown by the RNA deep sequencing (Hamisch et al. 2012) and by the *so*-promoter::reporter fusion (Lang et al. 2007), but obviously it is also regulated post-transcriptionally. We observed different pSO

activities between young and mature *N. tabacum* plants, with a much higher activity in mature than in young plants and an effect of these extracts on recombinant pSO activity. A possible phosphorylation as regulating factor, as is known for the sister plant enzyme nitrate reductase, of pSO seems not to be the case. Vanadate, however, was identified as a strong inhibitor. Results from Western blotting and from both the BiFC and FluCI in vivo protein-protein interaction assays point to a putative post-translational regulatory mechanism *via* SUMOylation of pSO, most likely with the SUMO3 protein.

References

Brychkova G, Xia Z, Yang G, Yesbergenova Z, Zhang Z, Davydov O, Fluhr R, Sagi M (2007) Sulfite oxidase protects plants against sulfur dioxide toxicity. Plant J 50:696–709

Campbell WH (1999) Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. Annu Rev Plant Mol Biol 50:277–303

Cohen HJ, Drew RT, Johnson JL, Rajagopalan KV (1973) Molecular basis of the biological function of molybdenum the relationship between sulfite oxidase and the acute toxicity of bisulfite and SO_2. Proc Natl Acad Sci U S A 70:3655–3659

Eilers T, Schwarz G, Brinkmann H, Witt C, Richter T, Nieder J, Koch B, Hill R, Hänsch R, Mendel RR (2001) Identification and biochemical characterization of Arabidopsis thaliana sulfite oxidase A new player in plant sulfur metabolism. J Biol Chem 276:46989–46994

Gehl C, Waadt R, Kudla J, Mendel RR, Hänsch R (2009) New gateway vectors for high throughput analyses of protein-protein interactions bimolecular fluorescence complementation. Mol Plant 2:1051–1058

Gehl C, Kaufholdt D, Hamisch D, Bikker R, Kudla J, Mendel RR, Hänsch R (2011) Quantitative analysis of dynamic protein-protein interactions in planta by floated-leaf luciferase complementation imaging (FluCI) assay using binary Gateway vectors. Plant J 67:542–553

Hamisch D, Randewig D, Schliesky S, Bräutigam A, Weber AP, Geffers R, Herschbach C, Rennenberg H, Mendel RR, Hänsch R (2012) Impact of SO_2 on *Arabidopsis thaliana* transcriptome in wildtype and sulfite oxidase knockout plants analyzed by RNA deep sequencing. New Phytol 196:1074–1085

Hänsch R, Mendel RR (2005) Sulfite oxidation in plant peroxisomes. Photosynth Res 86:337–343

Hänsch R, Lang C, Riebeseel E, Lindigkeit R, Gessler A, Rennenberg H, Mendel RR (2006) Plant sulfite oxidase as novel producer of H_2O_2: combination of enzyme catalysis with a subsequent non-enzymatic reaction step. J Biol Chem 281:6884–6888

Heber U, Hüve K (1998) Action of SO_2 on plants and metabolic detoxification of SO_2. Int Rev Cytochem 177:255–286

Johnson E (2004) Protein modification by SUMO. Annu Rev Biochem 73:355–382

Jolivet P, Bergeron E, Meunier JC (1995a) Evidence for sulfite oxidase activity in spinach leaves. Phytochemistry 40:667–672

Jolivet P, Bergeron E, Zimierski A, Meunier JC (1995b) Metabolism of elemental sulfur and oxidation of sulfite by wheat and spinach chloroplasts. Phytochemistry 38:9–14

Kaiser WM, Huber SC (2001) Post-translational regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers. J Exp Bot 52:1981–1989

Kaufholdt D, Gehl C, Geisler M, Jeske O, Voedisch S, Ratke C, Bollhöner B, Mendel RR, Hänsch R (2013) Visualization and quantification of protein interactions in the biosynthetic pathway of the molybdenum cofactor in *Arabidopsis thaliana*. J Exp Bot 64:2005–2016

Kurepa J, Walker JM, Smalle J, Gosink MM, Davis SJ, Durham TL, Sung DY, Vierstra RD (2003) The small ubiquitin-like modifier (SUMO) protein modification system in *Arabidopsis*. J Biol Chem 278:6862–6872

Lang C, Popko J, Wirtz M, Hell R, Herschbach C, Kreuzwieser J, Rennenberg H, Mendel RR, Hänsch R (2007) Sulfite oxidase as key enzyme for protecting plants against sulfur dioxide. Plant Cell Environ 30:447–455

Lu Z, Streets DG, Zhang Q, Wang S, Carmichael GR, Cheng YF, Wie C, Chin M, Diehl T, Tan Q (2010) Sulfur dioxide emissions in China and sulfur trends in East Asia since 2000. Atmos Chem Phys 10:6311–6331

Mudd SH, Irreverre F, Laster L (1967) Sulfite oxidase deficiency in man: demonstration of the enzymatic defect. Science 156:1599–1602, New Series

Nowak K, Luniak N, Witt C, Wüstefeld Y, Wachter A, Mendel RR, Hänsch R (2004) Peroxisomal localization of sulfite oxidase separates it from chloroplast-based sulfur assimilation. Plant Cell Physiol 45:1889–1894

Pfanz H, Oppmann B (1991) The possible role of apoplastic peroxidases in detoxifying the air pollutant sulfur dioxide. In: Lobarzewski J, Greppin H, Penel C, Gaspar T (eds) Biochemical, molecular and physiological aspects of plant peroxidases. University of Geneva, Geneva, pp 401–417

Pfanz H, Martinoia E, Lange OL, Heber U (1987) Mesophyll resistances to SO_2 fluxes into leaves. Plant Physiol 85:922–927

Pfanz H, Dietz K-J, Weinerth I, Oppmann B (1990) Detoxification of sulfur dioxide by apoplastic peroxidases. In: Rennenberg H, Brunold C, De Kok LJ, Stulen I (eds) Sulfur nutrition and sulfur assimilation in higher plants. SPB Academic Publishing, The Hague, pp 229–233

Randewig D, Hamisch D, Herschbach C, Eiblmeier M, Gehl C, Jurgeleit J, Skerra J, Mendel RR, Rennenberg H, Hänsch R (2012) Sulfite oxidase controls sulfur metabolism under SO2 exposure in Arabidopsis thaliana. Plant Cell Environ 35:100–115

Rennenberg H (1984) The fate of excess sulfur in higher plants. Annu Rev Plant Physiol 35:121–153

Rennenberg H, Herschbach C (1996) Responses of plants to atmospheric sulphur. In: Yunus M, Iqbal M (eds) Plant response to air pollution. Wiley, Chichester, pp 285–294

Stuiver CEE, De Kok LJ (2001) Atmospheric H_2S as sulfur source for *Brassica oleracea*: kinetics of H_2S uptake and activity of O-acetylserine (thiol)lyase as affected by sulfur nutrition. Environ Exp Bot 46:29–36

Swarup G, Cohen S, Garbers DL (1982) Inhibition of membrane phosphotyrosyl-protein phosphatase activity by vanadate. Biochem Biophys Res Commun 107:1104–1109

Identification of the Genes for Intracellular Glutathione Degradation in *Arabidopsis thaliana*

Naoko Ohkama-Ohtsu, Taisuke Kitaiwa, and Tadashi Yokoyama

Abstract To understand the physiological role of glutathione (GSH) degradation and how it contributes to other sulfur metabolites, it is necessary to determine the GSH degradation pathway. In mammals it has long been believed that GSH is degraded outside of the cell by γ-glutamyl transpeptidase (GGT). However most GSH exists inside the cell. In *Arabidopsis* it was suggested that GSH is catabolized by the GGT-independent pathway *via* 5-oxoproline, by γ-glutamyl cyclotransferase (GGCT). This study aims to identify gene(s) that code the degradation of GSH in the cytosol. In *Saccharomyces cerevisiae*, the DUG2-DUG3 complex degrades GSH to Glu and Cys-Gly then DUG1 cleaves the peptide bond of Cys-Gly. None of the *dugΔ* strains are able to grow on the medium where GSH is the sole sulfur source. Transformants of *dugΔ* strains with an *A. thaliana* cDNA library were screened on a medium with GSH as the sole sulfur source. Sequences of inserts in positive clones were searched against *A. thaliana* cDNA database by BLAST. One *A. thaliana* gene complemented *dug1Δ* and four genes complimented *dug2Δ* and *dug3Δ* strains. The same genes complemented both of *dug2Δ* and *dug3Δ* strains, indicating that in *Arabidopsis* GSH is degraded by single proteins, unlike in yeast in which complexed proteins are required. Two pathways were suggested for GSH degradation in *Arabidopsis*, GGCT pathway and AtDUG pathway.

Glutathione (GSH) is a tripeptide composed of Glu, Cly and Gly. The bond between Glu and Cys is a γ-bond which is resistant to normal peptidase unlike an α-bond. Thus GSH is relatively stable and a major storage and transport form of organic sulfur in higher plants. Ohkama-Ohtsu et al. (2008) demonstrated that when GSH synthesis was inhibited, about 80 % of GSH was degraded in 1 day, meaning that turnover of GSH is rapid. After GSH is degraded to the constituent amino acids, Cys

N. Ohkama-Ohtsu (✉) • T. Yokoyama
Institute of Agriculture, Tokyo University of Agriculture and Technology,
Saiwai-cho 3-5-8, Fuchu, Tokyo 183-0054, Japan
e-mail: nohtsu@cc.tuat.ac.jp

T. Kitaiwa
Graduate School of Agriculture, Tokyo University of Agriculture and Technology,
Saiwai-cho 3-5-8, Fuchu, Tokyo 183-0054, Japan

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_19

is incorporated into other sulfur compounds such as proteins and coenzymes, etc. Degradation of GSH should be important for providing Cys to other sulfur compounds; however the degradation pathway has not yet been clearly established. To understand the physiological role of GSH degradation and how it contributes to other sulfur metabolites, it is necessary to determine the GSH degradation pathway. In mammals it has long been believed that GSH is degraded in γ-glutamyl cycle (Meister and Larsson 1995). In this cycle GSH is degraded outside of the cell by γ-glutamyl transpeptidase (GGT). GGT has both hydrolase activity to produce Glu and Cys-Gly from GSH, and transpeptidase activity which transfers γ-Glu moiety to other amino acids to produceγ-Glu amino acids. γ-Glu amino acids are converted to 5-oxoproline by γ-Glu cyclotransferase followed by conversion to Glu by 5-oxoprolinase. Cys-Gly was digested to Cys and Gly by the dipeptidase. The Glu, Cys and Gly produced were again used for GSH synthesis. Knockout mouse of GGT shows Cys deficiency, thus this cycle was found to be responsible for Cys absorption in the kidney (Harding et al. 1997; Kumar et al. 2000; Lieberman et al. 1996). Compared to GGTs in mammals, little is understood about GGTs in plants such as *Arabidopsis*, which was chosen for our study.

There are three GGTgenes in *Arabidopsis* (excluding GGT3 which was not detected by RT-PCR). GGT1 was mainly expressed in leaves (Ohkama-Ohtsu et al. 2007a; Martin et al. 2007). Leaves of the *ggt1* knockout mutant were yellowish and lipid peroxodation of *ggt1* leaves were higher compared to wild type leaves. GGT1 was found in the apoplast because the activity was on the surface of protoplast. In the apoplastic solution, *ggt1* mutant plants accumulated oxidized glutathione, GSSG. These results suggested that GGT1 alleviates oxidative stress by degrading GSSG in the apoplast (Ohkama-Ohtsu et al. 2007a, 2009). To observe expression of GGT1 and GGT2 at tissue level, promoters of GGT1 and GGT2 were fused to the β-glucronidase (GUS) gene and transformed into *A. thaliana* plants. GUS staining was observed in the vascular tissues of various organs including leaves in GGT1 promoter-GUS plants, whereas it was limited in young siliques in promoter GGT2-GUS. Particularly strong staining was observed in funiculus, which is the location for nutrient transport to seeds. These GUS expression patterns suggested that GGT1 and GGT2 may function in transport of GSH from leaves to seeds (Ohkama-Ohtsu et al. 2007a, 2009; Martin et al. 2007). GGT4 was found to be localized to the vacuole as transient expression analysis of GGT4-GFP fusion protein in onion epidermal cells resulted in GFP fluorescence in the vacuoles (Ohkama-Ohtsu et al. 2007b; Grzam et al. 2007). For *in vivo* degradation analysis of GSH-conjugates in the vacuole, monobromobimane (mBB) was applied to plant culture medium which was known to be transported to the vacuole within 1 h as GSH-mBB conjugate (Fricker and Meyer 2001). In *gg4* knockout mutants, GSH-mBB conjugates were not degraded but it was degraded in wild- type and *ggt1* knockout mutants, demonstrating that GGT4 is responsible for the degradation of GSH-conjugates in the vacuole (Ohkama-Ohtsu et al. 2007b, 2009; Grzam et al. 2007).

As mentioned above, GGTs in *Arabidopisis* function in the apoplast or vacuole. However most GSH exists inside the cell. When buthionine sulfoximine (BSO), an inhibitor for γ-GluCys synthesis, was applied to plantlets, GSH was decreased in

the same ratio as in wild-type plants and in *ggt1*/*ggt4* double knockout mutants in which GGT activity was not detectable in plantlets (Ohkama-Ohtsu et al. 2008, 2009). This confirmed that enzymes other than GGTs degrade GSH in the cell.

γ-Glu cyclotransferase (GGCT) was has also been considered as the candidate for the GSH-degrading enzyme in cells. GGCT cyclorizes the γ-Glu residue to produce 5-oxoproline (5OP; Orlowski et al. 1969). Feeding isotope-labeled GSH to cultured tobacco cells demonstrated that 5OP is a degradation product of GSH (Rennenberg et al. 1980). GGCT was studied in tobacco suspension cultures by Steinkamp and Rennenberg (1985, 1987), who suggested that this soluble enzyme is localized in the cytoplasm. The substrate of GGCT was considered to be γ-Glu amino acids. As GSH is a γ-Glu compound, it may possibly be a substrate of GGCT. To confirm this hypothesis, concentrations of GSH and 5OP concentrations were examined for any correlation (Ohkama-Ohtsu et al. 2008, 2009). The *oxp1-1* mutant was used for this experiment because in this mutant metabolism of 5OP was blocked when the 5-oxoprolinase gene was knocked out. When BSO was applied to the *oxp1-1* plantlets, GSH, γ-GluCys and 5OP were decreased. Furthermore the double knockout of *oxp1-1*/*cad2-1* mutant in which both of 5-oxoprolinase and γ-GluCys synthetase genes were mutated, the concentrations of GSH, γ-GluCys and 5OP were decreased to about half of those in the *oxp1-1* single mutant. These result suggested that γ-GluCys or GSH were degraded by GGCT to produce 5OP. Activity of 5-oxoprolinase was higher in plant extract with γ-GluCys as a substrate than with GSH. However, when cytosolic concentrations of GSH and γ-GluCy as 2 mM and 0.1 mM, respectively (Fricker et al. 2000) were applied to the kinetics of GGCT, it was calculated that 5.5 times more 5OP is synthesized from GSH than γ-EC *in vivo*. From these results, it was demonstrated that GSH is catabolized by GGCT *via* 5-oxoproline (Ohkama-Ohtsu et al. 2008, 2009). In addition, degradation of GGT to Glu through 5-oxoproline was suggested to contribute to Glu production in plants because Glu concentrations were significantly lower in the *oxp1-1* knockout mutants.

Although the activity of GGCT was found in plants about 30 years before (Steinkamp and Rennenberg 1985, 1987), the gene encoding this enzyme has only recently been identified. Recently, a GGCT acting specifically on GSH was identified in mice (Kumar et al. 2012). In addition the homologue of GGCT in *Arabidopsis* was shown to confer heavy metal tolerance by recycling Glu and saving energy for *de novo* synthesis for Glu (Paulose et al. 2013). In the present study we examined if the GGCT homologues in *Arabidopsis* have a role in degradation and if there are other genes involved in intracellular GSH degradation. Arabidopsis cDNAs, which complemented yeast mutants defective in cytosolic GSH degradation, were screened.

In *Saccharomyces cerevisiae*, the DUG2-DUG3 complex degrades GSH to Glu and Cys-Gly. DUG1 then cleaves the peptide bond of Cys-Gly. None of the *dugΔ* strains are able to grow on a medium with GSH as the sole sulfur source (Ganguli et al. 2007). Yeast *dug1Δ* (accession number Y05858), *dug2Δ* (Y05729) and *dug3Δ* (Y02021) strains were obtained from EUROSCARF. Transformants of *dugΔ* strains

with an *A. thaliana* cDNA library cloned in expression plasmid pFL61 (Minet et al. 1992) were screened on a solid synthetic dextrose (SD) medium with GSH as the sole sulfur source. Sequences of inserts in positive clones were searched against *A. thaliana* cDNA database by BLAST. Full length ORF of the corresponding genes amplified from *A. thaliana* cDNAs were subcloned into pTEF416 (Mumberg et al. 1995) then transformed into *dugΔ* mutants. The transformants demonstrated the ability to grow on the medium with GSH as the sole sulfur source.

One *A. thaliana* gene complemented *dug1Δ*, and another gene complemented the *dug2Δ* and *dug3Δ* strains. The same gene complemented both *dug2Δ* and *dug3Δ* strains, indicating that in *Arabidopsis* GSH is degraded by a single protein unlike in yeast in which complexed proteins are required for GSH degradation. The gene complemented *dug1Δ* strain was named AtDUG1 and the gene complemented both *dug2Δ* and *dug3Δ* strains was named AtDUG3. Both AtDUG1 and AtDUG3 were annotated as peptidase and predicted to localize to the cytosol in the TAIR database (www.arabidopsis.org). The full length ORF of AtDUG1 was subcloned in pTF416 and introduced into the *dug1Δ* yeast mutant. The *dug1Δ* strain transformed with the full length ORF of AtDUG1 was able to grow on SD medium with GSH as the sole sulfur source, indicating that the product of AtDUG 1 has the capacity to degrade Cys-Gly. The full length ORF of AtDUG3 and three homologues of GGCT (Paulose et al. 2013) were introduced to the *dug2Δ* and *dug3Δ* strains. Both of AtDUG3 and three homologues of GGCT complemented *dug2Δ* and *dug3Δ* strains. These results suggest that there are two pathways for GSH degradation in Arabidopsis, one is through AtDUG3 and the other is through GGCT. Future study will address differences in the roles of these pathways and their functions in sulfur metabolism.

Acknowledgements This work was supported by Grants-in-Aid for Young Scientists (B) (No. 21770057) and Funds for Development of Human Resources in Science and Technology "Supporting positive activities for female researchers", the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) to N, O-O.

References

Fricker MD, Meyer AJ (2001) Confocal imaging of metabolism *in vivo*: pitfalls and possibilities. J Exp Bot 52:631–640

Fricker MD, May M, Meyer AJ, Sheard N, White NS (2000) Measurement of glutathione levels in intact roots of Arabidopsis. J Microsc 198:162–171

Ganguli D, Kumar C, Bachhawat AK (2007) The alternative pathway of glutathione degradation is mediated by a novel protein complex involving three new genes in *Saccharomyces cerevisiae*. Genetics 175:1137–1151

Grzam A, Martin MN, Hell R, Meyer AJ (2007) Gamma-glutamyl transpeptidase GGT4 initiates vacuolar degradation of glutathione *S*-conjugates in Arabidopsis. FEBS Lett 581:3131–3138

Harding CO, Williams P, Wagner E, Chang DS, Wild K, Colwell RE, Wolff JA (1997) Mice with genetic γ-glutamyl transpeptidase deficiency exhibit glutathionuria, severe growth failure, reduced life spans, and infertility. J Biol Chem 272:12560–12567

Kumar TR, Wiseman AL, Kala G, Kala SV, Matzuk MM, Lieberman MW (2000) Reproductive defects in γ-glutamyl transpeptidase-deficient mice. Endocrinology 141:4270–4277

Kumar A, Tikoo S, Maity S, Sengupta S, Sengupta S, Kaur BAK (2012) Mammalian proapoptotic factor ChaC1 and its homologues function as γ-glutamyl cyclotransferases acting specifically on glutathione. EMBO Rep 13:1095–1101

Lieberman MW, Wiseman AL, Shi ZZ, Carter BZ, Barrios R, Ou CN, Chevez-Barrios P, Wang Y, Habib GM, Goodman JC, Huang SL, Lebovitz RM, Matzuk MM (1996) Growth retardation and cysteine deficiency in γ-glutamyl transpeptidase-deficient mice. Proc Natl Acad Sci U S A 93:7923–7926

Martin MN, Saladores PH, Lambert E, Hudson AO, Leustek T (2007) Localization of members of the γ-glutamyl transpeptidase family identifies sites of glutathione and glutathione *S*-conjugate hydrolysis. Plant Physiol 144:1715–1732

Meister A, Larsson A (1995) Glutathione synthetase deficiency and other disorders of the γ-glutamyl cycle. In: Scriver CR, Beaudet al, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, vol 1. McGraw-Hill, New York, pp 1461–1495

Minet M, Dufour M-E, Lacroute F (1992) Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J 2:417–422

Mumberg D, Müller R, Funk M (1995) Yeast vectors for controlled expression of heterologous proteins in different genetic backgrounds. Gene 156:119–122

Ohkama-Ohtsu N, Radwan S, Peterson A, Zhao P, Badr AF, Xiang C, Oliver DJ (2007a) Characterization of the extracellular γ-glutamyl transpeptidases, GGT1 and GGT2, in *Arabidopsis*. Plant J 49:865–877

Ohkama-Ohtsu N, Zhao P, Xiang C, Oliver DJ (2007b) Glutathione conjugates in the vacuole are degraded by γ-glutamyl transpeptidase GGT3 in *Arabidopsis*. Plant J 49:878–888

Ohkama-Ohtsu N, Oikawa A, Zhao P, Xiang C, Saito K, Oliver DJ (2008) A γ-glutamyl transpeptidase-independent pathway of glutathione catabolism to glutamate via 5-oxoproline in *Arabidopsis*. Plant Physiol 148:1603–1613

Ohkama-Ohtsu N, Fukuyama K, Oliver DJ (2009) Roles of γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase in glutathione and glutathione-conjugate metabolism in plants. Adv Bot Res 252:87–113

Orlowski M, Richman P, Meister A (1969) Isolation and properties of γ-L-glutamylcyclotransferase from human brain. Biochemistry 8:1048–1055

Paulose B, Chhikara S, Coomey J, Jung H, Vatamaniuk O, Dhankher OP (2013) A γ-glutamyl cyclotransferase protects *Arabidopsis* plants from heavy metal toxicity by recycling glutamate to maintain glutathione homeostasis. Plant Cell 25:4580–4595

Rennenberg H, Steinkamp R, Polle A (1980) Evidence for the participation of a 5-oxoprolinase in degradation of glutathione in *Nicotiana tabacum*. Z Naturforsch 35c:708–711

Steinkamp R, Rennenberg H (1985) Degradation of glutathione in plant cells: evidence against the participation of a γ-glutamyl transpeptidase. Z Naturforsch 40c:29–33

Steinkamp R, Rennenberg H (1987) γ-Glutamylcyclotransferase in tobacco suspension cultures: catalytic properties and subcellular localization. Physiol Plant 69:499–503

Altered Regulation of *MYB* Genes Changes the Aliphatic Glucosinolate Accumulation Under Long-Term Sulfur Deficiency in *Arabidopsis*

Yimeng Li, Yuji Sawada, Akiko Hirai, Muneo Sato, Ayuko Kuwahara, Xiufeng Yan, and Masami Yokota Hirai

Abstract Aliphatic glucosinolates (AGSLs) constitute an important part of sulfur-containing secondary metabolites in *Arabidopsis*. Biosynthesis of AGSLs is positively regulated by transcription factors MYB28, MYB29, and MYB76. Compared to plants grown under full S conditions (1,500 μM sulfate), in wild type *Arabidopsis*, the AGSL content was reduced to nearly 70 % when grown under 1/10 S conditions (150 μM sulfate) and nearly disappeared in plants grown under 0 S conditions. The expression of *MYB29* and *MYB76* was positively correlated with sulfur concentration, whereas the expression of *MYB28* was slightly elevated in lines grown under 1/10 S conditions, and maintained at basal levels under 0 S conditions. To eliminate the effects of MYB interaction, transgenic lines in which one of these three *MYB* genes was expressed in the *myb28myb29* background, were subjected to sulfur deficiency. In the absence of MYB29 and MYB76, an apparent increase of *MYB28* expression level in Pro_{MYB28}*:MYB28* lines was detected when grown under 1/10 S conditions. Altering the regulation of *MYB* genes allows the plants to allocate the limited sulfur resources between primary and secondary metabolism under sulfur deficiency.

Y. Li • A. Hirai • M.Y. Hirai (✉)
RIKEN Plant Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan
e-mail: masami.hirai@riken.jp

Y. Sawada
RIKEN Center for Sustainable Resource Science,
1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan

M. Sato • A. Kuwahara
JST, CREST, 4-1-8 Honcho, Kawaguchi 332-0012, Japan

X. Yan
Alkali Soil Natural Environmental Science Center, Northeast Forestry University, Key Laboratory of Saline-Alkali Vegetation Ecology Restoration in Oil Field, Ministry of Education, Harbin 15040, China

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_20

Sulfur is a macronutrient essential for plant metabolism and defense. In *Arabidopsis*, aliphatic glucosinolates (AGSLs) constitute an important part of sulfur-containing secondary metabolites. These metabolites are derived from the amino acid methionine, which undergoes chain elongation cycles to form intermediates that are eventually converted into AGSLs with side-chain length ranging from 3C to 8C (Kroymann et al. 2001; Textor et al. 2007; Sawada et al. 2009b). Biosynthesis of AGSLs is positively regulated by transcription factors (TFs) MYB28, MYB29, and MYB76, which belong to the MYB superfamily (Gigolashvili et al. 2007, 2008; Hirai et al. 2007; Sønderby et al. 2007; Malitsky et al. 2008). Most of the AGSL biosynthetic genes are induced by these MYB TFs (Li et al. 2013). Under sulfur deficiency, the content of sulfur-containing metabolites is affected through reduced sulfur assimilation. This study investigated the impact of long-term sulfur deficiency on the AGSL content and *MYB* gene expression.

Arabidopsis was grown on agar-solidified 1/2 Murashige and Skoog medium containing 1,500 μM sulfate (defined as full S conditions). The sulfate concentration was adjusted to 150 μM (1/10 S conditions) and 0 μM (0 S conditions) by replacing $MgSO_4$ with $MgCl_2$. Seeds were plated on the full S medium, and after 1 week of growth, the seedlings were transferred to the 1/10 S and 0 S mediums and exposed to long-term sulfur deficiency. Aerial parts of the 3-week-old plants were sampled and the amount of metabolites and level of gene expression was determined.

Obtained results suggest that, under sulfur deficiency, the biosynthesis of AGSLs is reduced to preserve sulfur resources (Table 1). Total AGSLs in the wild type (Col-0) plants grown under mild 1/10 S conditions were reduced to about 70 % of the level found in plants grown under full S conditions. The degree of reduction in AGSLs biosynthesis differed between the long-chain AGSLs (side-chain length ranging from 6C to 8C) and short-chain AGSLs (side-chain length ranging from 3C to 5C), suggesting that under mild sulfur deficiency, plants will preferentially reduce the synthesis of long-chain AGSLs, which have a more complex structure. The *MYB* gene expression analyses showed that in plants grown under 1/10 S conditions, the expression levels of *MYB29* and *MYB76* were significantly decreased (Fig. 1b, c), whereas *MYB28* expression level was slightly increased (Fig. 1a). The expression of *MYB28*, *MYB29*, and *MYB76* is essential for activation of the AGSL biosynthetic pathway; therefore, the double knockout of *MYB28* and *MYB29* results in the repression of expression in all three *MYB* genes and the absence of AGSL accumulation (Sønderby et al. 2007; Beekwilder et al. 2008). Under mild sulfur deficiency, plants preferentially repress the expression of *MYB29* and *MYB76* to reduce the outflow of the methionine pool to the secondary metabolism branch, while they maintain the expression of *MYB28* to maintain the basal levels of AGSLs accumulation. In plants grown under 0 S conditions, due to the lack of sulfur resources, the contents of both short- and long-chain AGSLs decreased to less than 2 % of those under full S conditions. Similarly, expression of *MYB29* and *MYB76* was reduced to less than 10 % of that under full S conditions, whereas the expression of *MYB28* was maintained at about 40 %. From these results, it can be concluded that the *MYB* genes respond to sulfur deficiency in different ways; *MYB28* showed lower sensitivity to sulfur stress

Table 1 Aliphatic glucosinolates in aerial parts of *Arabidopsis* under altered sulfur conditions

		4MT	4MS	8MS	Short-chain	Long-chain	Total AGSLs
Col-0	Full S	1.180 ± 0.082 a	1.117 ± 0.150 a	0.124 ± 0.030 a	2.563 ± 0.087 a	0.185 ± 0.039 a	2.749 ± 0.106 a
	1/10 S	0.853 ± 0.118 b	0.907 ± 0.111 b	0.054 ± 0.010 b	1.960 ± 0.148 b	0.085 ± 0.017 b	2.045 ± 0.164 b
	0 S	0.039 ± 0.061 c	0.012 ± 0.020 c	0.002 ± 0.001 c	0.054 ± 0.085 c	0.002 ± 0.001 c	0.056 ± 0.085 c
myb28myb29	Full S	0.000 ± 0.000 a	0.000 ± 0.000 a	0.001 ± 0.001 a	0.000 ± 0.000 a	0.002 ± 0.001 a	0.002 ± 0.001 ab
	1/10 S	0.000 ± 0.000 a	0.000 ± 0.000 a	0.001 ± 0.001 a	0.001 ± 0.000 a	0.002 ± 0.001 a	0.003 ± 0.001 a
	0 S	0.000 ± 0.000 a	0.000 ± 0.000 a	0.001 ± 0.001 a	0.000 ± 0.000 a	0.001 ± 0.001 a	0.001 ± 0.001 b
Pro_{MYB28}:*MYB28*	Full S	0.512 ± 0.105 a	0.433 ± 0.089 a	0.004 ± 0.003 a	1.029 ± 0.191 a	0.007 ± 0.006 a	1.036 ± 0.191 a
	1/10 S	0.470 ± 0.133 a	0.325 ± 0.124 b	0.002 ± 0.001 b	0.860 ± 0.220 b	0.004 ± 0.002 b	0.863 ± 0.220 b
	0 S	0.009 ± 0.018 b	0.002 ± 0.004 c	0.002 ± 0.002 b	0.012 ± 0.023 c	0.002 ± 0.002 b	0.014 ± 0.023 c
Pro_{MYB29}:*MYB29*	Full S	0.236 ± 0.104 a	0.158 ± 0.076 a	0.001 ± 0.001 a	0.428 ± 0.190 a	0.002 ± 0.001 a	0.430 ± 0.190 a
	1/10 S	0.238 ± 0.106 a	0.121 ± 0.071 a	0.002 ± 0.001 a	0.387 ± 0.175 a	0.003 ± 0.001 a	0.390 ± 0.175 a
	0 S	0.000 ± 0.000 b	0.000 ± 0.000 b	0.002 ± 0.001 a	0.001 ± 0.001 b	0.002 ± 0.002 a	0.003 ± 0.002 b
Pro_{MYB76}:*MYB76*	Full S	0.006 ± 0.007 a	0.003 ± 0.004 a	0.001 ± 0.001 a	0.009 ± 0.011 a	0.002 ± 0.001 a	0.011 ± 0.011 a
	1/10 S	0.001 ± 0.004 b	0.004 ± 0.010 a	0.001 ± 0.001 a	0.006 ± 0.014 a	0.002 ± 0.001 a	0.008 ± 0.014 a
	0 S	0.000 ± 0.000 b	0.000 ± 0.000 a	0.001 ± 0.001 a	0.001 ± 0.001 a	0.001 ± 0.001 a	0.002 ± 0.001 a

Aerial parts of 3-week-old plants were sampled and subjected to metabolite analyses using ultra performance liquid chromatography-tandem quadrupole mass spectrometry (Sawada et al. 2009a). Content of AGSLs is given as mean ± SD (μmol g^{-1} FW). AGSL contents under three conditions within each line were processed by one-way multiple ANOVA with Duncan's test, and the results were shown as "a", "b", and "c" following mean ± SD. Different letters represent values that are statistically different at $P < 0.05$. 4MT, 4-methylthiobutyl glucosinolate; 4MS, 4-methylsulfinylbutyl glucosinolate; 8MS, 8-methylsulfinyloctyl glucosinolate; Short-chain, total short-chain aliphatic glucosinolates with side-chain length ranging from 3C to 5C; Long-chain, total long-chain aliphatic glucosinolates with side-chain length ranging from 6C to 8C; Total AGSLs, total aliphatic glucosinolates; Full S, normal conditions with 1,500 μM sulfate; 1/10 S, mild sulfur deficiency conditions with 150 μM sulfate; 0 S, extreme sulfur deficiency conditions with 0 μM sulfate

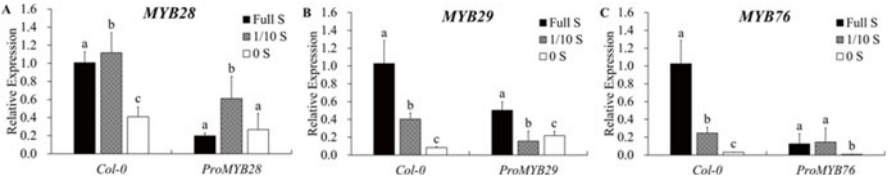


Fig. 1 Relative expression levels of (**a**) *MYB28*, (**b**) *MYB29*, and (**c**) *MYB76*. Expression levels of *MYB28*, *MYB29*, and *MYB76* in aerial parts of 3-week-old plants were analyzed using qRT-PCR. *Black bar*, normal conditions with 1,500 μM sulfate (Full S); *gray bar*, mild sulfur deficiency conditions with 150 μM sulfate (1/10 S); *white bar*, extreme sulfur deficiency conditions with 0 μM sulfate (0 S). Expression of *MYB* genes in wild type, Pro_{MYB28}*:MYB28*, Pro_{MYB29}*:MYB29*, and Pro_{MYB76}*:MYB76* lines under three conditions was processed by one-way multiple ANOVA with Duncan's test. Different letters within a line represent values that are statistically different at $P<0.05$

than *MYB29* or *MYB76*, and even under extreme sulfur deficiency *MYB28* maintained a high expression level.

The complex interaction of MYB TFs (Sønderby et al. 2010) makes it difficult to evaluate the individual role of each MYB under sulfur deficiency. To eliminate the effects of MYB interaction, each *MYB* gene driven by its own promoter was transformed into the *myb28myb29* double knockout mutant in which the expression of *MYB28*, *MYB29*, and *MYB76* was repressed resulting in no AGSL accumulation, to generate Pro_{MYB28}*:MYB28*, Pro_{MYB29}*:MYB29*, and Pro_{MYB76}*:MYB76* lines. Metabolite analyses showed that AGSL content in Pro_{MYB28}*:MYB28* and Pro_{MYB29}*:MYB29* lines had the same declining pattern as the wild type grown under 1/10 and 0 S conditions. Accumulation of AGSLs was not recovered in Pro_{MYB76}*:MYB76* lines (Table 1). In Pro_{MYB28}*:MYB28* lines, a threefold increase of *MYB28* expression level was detected when grown under 1/10 S conditions (Fig. 1a), which was a significant increase compared to the wild type, suggesting that in the absence of MYB29 and/or MYB76, *MYB28* was more susceptible to variations in sulfur content. When grown under 0 S conditions, *MYB28* in Pro_{MYB28}*:MYB28* lines retained basal expression levels.

Methionine not only serves as a protein component and a methyl donor (Droux 2004), but is also a precursor for secondary metabolites of AGSLs, which are involved in plant defense. By regulating the expression of *MYB* genes in various ways, the allocation of limited sulfur resources may be balanced between primary and secondary metabolism under sulfur deficiency.

References

Beekwilder J, van Leeuwen W, van Dam NM, Bertossi M, Grandi V, Mizzi L, Soloviev M, Szabados L, Molthoff JW, Schipper B, Verbocht H, de Vos RCH, Morandini P, Aarts MGM, Bovy A (2008) The impact of the absence of aliphatic glucosinolates on insect herbivory in Arabidopsis. PLoS One 3:e2068

Droux M (2004) Sulfur assimilation and the role of sulfur in plant metabolism: a survey. Photosynth Res 79:331–348

Gigolashvili T, Yatusevich R, Berger B, Muller C, Flugge U (2007) The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in *Arabidopsis thaliana*. Plant J 51:247–261

Gigolashvili T, Engqvist M, Yatusevich R, Muller C, Flugge U (2008) HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana*. New Phytol 177:627–642

Hirai MY, Sugiyama K, Sawada Y, Tohge T, Obayashi T, Suzuki A, Araki R, Sakurai N, Suzuki H, Aoki K, Goda H, Nishizawa OI, Shibata D, Saito K (2007) Omics-based identification of Arabidopsis Myb transcription factors regulating aliphatic glucosinolate biosynthesis. Proc Natl Acad Sci U S A 104:6478–6483

Kroymann J, Textor S, Tokuhisa JG, Falk KL, Bartram S, Gershenzon J, Mitchell-Olds T (2001) A gene controlling variation in Arabidopsis glucosinolate composition is part of the methionine chain elongation pathway. Plant Physiol 127:1077–1088

Li Y, Sawada Y, Hirai A, Sato M, Kuwahara A, Yan X, Hirai MY (2013) Novel insights into the function of Arabidopsis R2R3-MYB transcription factors regulating aliphatic glucosinolate biosynthesis. Plant Cell Physiol 54:1335–1344

Malitsky S, Blum E, Less H, Venger I, Elbaz M, Morin S, Eshed Y, Aharoni A (2008) The transcript and metabolite networks affected by the two clades of Arabidopsis glucosinolate biosynthesis regulators. Plant Physiol 148:2021–2049

Sawada Y, Akiyama K, Sakata A, Kuwahara A, Otsuki H, Sakurai T, Saito K, Hirai MY (2009a) Widely targeted metabolomics based on large-scale MS/MS data for elucidating metabolite accumulation patterns in plants. Plant Cell Physiol 50:37–47

Sawada Y, Kuwahara A, Nagano M, Narisawa T, Sakata A, Saito K, Hirai MY (2009b) Omics-based approaches to methionine side chain elongation in Arabidopsis: characterization of the genes encoding methylthioalkylmalate isomerase and methylthioalkylmalate dehydrogenase. Plant Cell Physiol 50:1181–1190

Sønderby IE, Hansen BG, Bjarnholt N, Halkier BA, Kliebenstein DJ (2007) A systems biology approach identifies a R2R3 MYB gene subfamily with distinct and overlapping functions in regulation of aliphatic glucosinolates. PLoS One 2:e1322

Sønderby IE, Burow M, Rowe HC, Kliebenstein DJ, Halkier BA (2010) A complex interplay of three R2R3 MYB transcription factors determines the profile of aliphatic glucosinolate in Arabidopsis. Plant Physiol 153:348–363

Textor S, de Kraker JW, Hause B, Gershenzon J, Tokuhisa JG (2007) MAM3 catalyzes the formation of all aliphatic glucosinolate chain lengths in Arabidopsis. Plant Physiol 144:60–71

Identification of Genes Potentially Encoding *S*-Oxygenation Enzymes for the Biosynthesis of *S*-Alk(en)yl-L-cysteine Sulfoxides in Onion

Naoko Yoshimoto and Kazuki Saito

Abstract *S*-Alk(en)yl-L-cysteine sulfoxides are sulfur-containing secondary metabolites characteristically found in the genus *Allium*. Upon tissue damage, they are converted to a variety of sulfur-containing compounds that have a range of pharmacological activities. Despite the pharmaceutical importance of *S*-alk(en)yl-l-cysteine sulfoxides, to date very little is known about the molecular details of their biosynthesis. Previous tracer experiments have indicated that *S*-oxygenation reactions to convert biosynthetic intermediate sulfide compounds to their respective sulfoxides are involved in the later stage of the biosynthesis of *S*-alk(en)yl-l-cysteine sulfoxides. In order to obtain molecular insights into the biosynthesis of *S*-alk(en) yl-L-cysteine sulfoxides, we searched for nucleotide sequences homologous to known genes encoding flavin-containing monooxygenases (FMOs) that have *S*-oxygenation activities, and identified two EST clones from onion (*Allium cepa*). The deduced amino acid sequences of these ESTs showed the high sequence similarity to *Arabidopsis* $FMO_{GS\text{-}OX}$ proteins catalyzing *S*-oxygenation of *S*-methylthioalkyl glucosinolates, and contained some sequence motifs typically found in plant FMOs. These observations suggest that the onion ESTs identified in this study were derived from genes encoding functional FMO proteins catalyzing the *S*-oxygenation reactions, which may be required for the biosynthesis of *S*-alk(en) yl-L-cysteine sulfoxides. Future studies aimed at isolating full-length cDNAs corresponding to these ESTs and elucidating the functions of their encoded proteins may provide new insight into the molecular mechanisms underlying the biosynthesis of *S*-alk(en)yl-L-cysteine sulfoxides.

N. Yoshimoto (✉)
Graduate School of Pharmaceutical Sciences, Chiba University,
Chuo-ku, Chiba 260-8675, Japan
e-mail: naokoy@faculty.chiba-u.jp

K. Saito
Metabolomics Research Group, RIKEN Center for Sustainable Resource Science,
Yokohama, Kanagawa, Japan

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_21

The *Allium* family of plants generally contain high concentrations of *S*-alk(en)yl-l-cysteine sulfoxides in the cytosol of their tissues. Tissue damage causes the release of the enzyme alliinase (EC. 4.4.1.4) from the vacuole, which immediately converts *S*-alk(en)yl-L-cysteine sulfoxides to their respective sulfenic acids. As a result of the extremely high chemical reactivity, the sulfenic acids formed in this reaction are quickly and spontaneously converted to a variety of volatile sulfur-containing compounds. These compounds produced from *S*-alk(en)yl-L-cysteine sulfoxides are important from a pharmacological point of view since they have diverse biological activities such as antibacterial, antifungal, antiviral, antioxidant, immunostimulating, anticarcinogenic, antithrombotic, cholesterol and triglyceride-lowering, and hypotensive effects (Jones et al. 2004; Rose et al. 2005; Iciek et al. 2009). To date, four major *S*-alk(en)yl-L-cysteine sulfoxides, *S*-methyl-L-cysteine sulfoxide (methiin), *S*-propyl-L-cysteine sulfoxide (propiin), *S*-allyl-L-cysteine sulfoxide (alliin), and *S-trans*-1-propenyl-L-cysteine sulfoxide (isoalliin), are reported to be contained in *Allium* plants (Fig. 1). The abundance ratio of four *S*-alk(en)yl-l-cysteine sulfoxides is different among species: methiin is present in most *Allium* plants, propiin is a minor component in most *Allium* plants, alliin is characteristic of garlic (*Allium sativum*) and wild leek (*Allium obliquum*), and isoalliin is characteristic of onion (*Allium cepa*) and chive (*Allium schoenoprasum*) (Fritsch and Keusgen 2006; Block 2010).

The hypothetical biosynthetic pathway of *S*-alk(en)yl-L-cysteine sulfoxides has been proposed to involve the *S*-alk(en)ylation of a cysteine residue in glutathione, the removal of glycyl and γ-glutamyl groups, and the *S*-oxygenation of sulfide to sulfoxide, based on the results of pulse-chase experiments with $^{35}SO_4^{2-}$ to onion, garlic and *Allium siculum* (Lancaster and Shaw 1989). However, to date, the enzymes responsible for the biosynthesis of *S*-alk(en)yl-L-cysteine sulfoxides in *Allium* plants are largely unknown and await elucidation. Based on the facts that flavin-containing monooxygenases (FMOs; EC 1.14.13.8) from rabbit and rat are capable of *S*-oxygenating putative alliin biosynthetic intermediate (Ripp et al. 1997; Krause et al. 2002; Novick and Elfarra 2008), and five FMOs from *Arabidopsis thaliana* ($FMO_{GS\text{-}OX1}$, $FMO_{GS\text{-}OX2}$, $FMO_{GS\text{-}OX3}$, $FMO_{GS\text{-}OX4}$, and $FMO_{GS\text{-}OX5}$) are responsible for *S*-oxygenation of *S*-methylthioalkyl glucosinolates to yield *S*-methylsulfinylalkyl glucosinolates in the modification of side-chain structure of aliphatic glucosinolates (Hansen et al. 2007; Li et al. 2008), we hypothesized that *S*-oxygenation reactions in the biosynthesis of *S*-alk(en)yl-L-cysteine sulfoxides would be catalyzed by FMO proteins in *Allium* plants. By BLAST analyses, we found two onion EST clones, EST676846 and EST685423 (GenBank accession

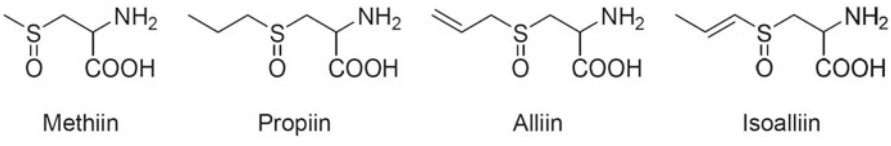


Fig. 1 Structures of four major *S*-alk(en)yl-L-cysteine sulfoxides biosynthesized in *Allium* plants

numbers CF440501 and CF449078, respectively), that share high sequence similarities with *Arabidopsis* $FMO_{GS\text{-}OX}$ proteins in GenBank (http://www.ncbi.nlm.nih.gov/genbank) (Fig. 2). In general, plant FMO proteins have four characteristic sequence motifs: FAD-binding motif (GXGXXG), NADP-binding motif (GXGXXG) that is less well-conserved, TGY motif (TGY), and FMO identifying sequence motif (FXGXXXHXXXY/F) (Schlaich 2007). A multiple sequence alignment revealed that the deduced amino acid sequences of two onion ESTs we identified in this study possess putative NADP-binding motif, TGY motif, and FMO-identifying motif, at the positions equivalent to those of these motifs found in *Arabidopsis* $FMO_{GS\text{-}OX}$ proteins (Fig. 2). These observations suggest that these two EST clones were derived from genes encoding functional FMO proteins with *S*-oxygenation activities in onion. Future efforts in cloning of full-length cDNAs corresponding to these ESTs and in analyzing the enzymatic functions of the encoded proteins both *in vitro* and *in vivo* may shed light on the molecular

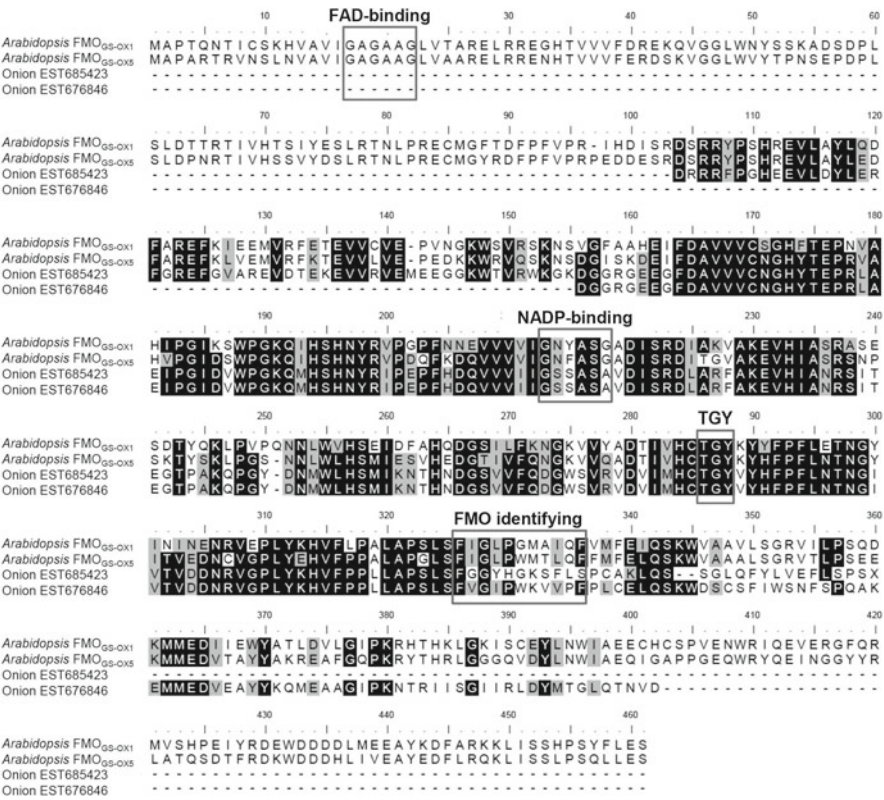


Fig. 2 ClustalW alignment of deduced amino acid sequences of two FMO-like onion ESTs with two *Arabidopsis* FMOs for glucosinolate *S*-oxygenation. Sequence motifs characteristically found in plant FMO proteins are indicated in boxes. The GenBank accession numbers for the sequences are shown in parentheses: *Arabidopsis* $FMO_{GS\text{-}OX1}$ (AEE34434); *Arabidopsis* $FMO_{GS\text{-}OX5}$ (AEE28841); Onion EST685423 (CF449078); Onion EST676846 (CF440501)

mechanisms of *S*-oxygenation reactions in the biosynthesis of *S*-alk(en)yl-L-cysteine sulfoxides in *Allium* plants.

Acknowledgements This study was supported in part by JSPS KAKENHI of Japan.

References

Block E (2010) Garlic and other alliums: the Lore and the science. The Royal Society of Chemistry, Cambridge

Fritsch RM, Keusgen M (2006) Occurrence and taxonomic significance of cysteine sulphoxides in the genus *Allium* L (Alliaceae). Phytochemistry 67:1127–1135

Hansen BG, Kliebenstein DJ, Halkier BA (2007) Identification of a flavin-monooxygenase as the *S*-oxygenating enzyme in aliphatic glucosinolate biosynthesis in Arabidopsis. Plant J 50:902–910

Iciek M, Kwiecień I, Włodek L (2009) Biological properties of garlic and garlic-derived organosulfur compounds. Environ Mol Mutagen 50:247–265

Jones MG, Hughes J, Tregova A, Milne J, Tomsett AB, Collin HA (2004) Biosynthesis of the flavour precursors of onion and garlic. J Exp Bot 55:1903–1918

Krause RJ, Glocke SC, Elfarra AA (2002) Sulfoxides as urinary metabolites of *S*-allyl-L-cysteine in rats: evidence for the involvement of flavin-containing monooxygenases. Drug Metab Dispos 30:1137–1142

Lancaster JE, Shaw ML (1989) γ-Glutamyl peptides in the biosynthesis of *S*-alk(en)yl-L-cysteine sulphoxides (flavour precursors) in *Allium*. Phytochemistry 28:455–460

Li J, Hansen BG, Ober JA, Kliebenstein DJ, Halkier BA (2008) Subclade of flavin-monooxygenases involved in aliphatic glucosinolate biosynthesis. Plant Physiol 148:1721–1733

Novick RM, Elfarra AA (2008) Purification and characterization of flavin-containing monooxygenase isoform 3 from rat kidney microsomes. Drug Metab Dispos 36:2468–2474

Ripp SL, Overby LH, Philpot RM, Elfarra AA (1997) Oxidation of cysteine *S*-conjugates by rabbit liver microsomes and cDNA-expressed flavin-containing mono-oxygenases: studies with *S*-(1,2-dichlorovinyl)-L-cysteine, *S*-(1,2,2-trichlorovinyl)-L-cysteine, *S*-allyl-L-cysteine, and *S*-benzyl-L-cysteine. Mol Pharmacol 51:507–515

Rose P, Whiteman M, Moore PK, Zhu YZ (2005) Bioactive *S*-alk(en)yl cysteine sulfoxide metabolites in the genus *Allium*: the chemistry of potential therapeutic agents. Nat Prod Rep 22:351–368

Schlaich NL (2007) Flavin-containing monooxygenases in plants: looking beyond detox. Trends Plant Sci 12:412–418

Part III
Nutritional and Environmental Aspects

Determining Sulfur-Limiting Conditions for Studies of Seed Composition in Common Bean (*Phaseolus vulgaris*)

Sudhakar Pandurangan and Frédéric Marsolais

Abstract Soil fertility and mineral nutrition play an important role in crop yield and grain quality improvement. Two common bean genotypes, SARC1 and SMARC1N-PN1, differ markedly in seed composition. The lack of the 7S globulin phaseolin and major lectins in SMARC1N-PN1 is compensated by increased levels of several sulfur-rich proteins, including the 11S globulin legumin. Conditions were determined that are limiting sulfur nutrition only at the reproductive stage. These conditions will be used to investigate whether the two genotypes respond differently to sulfur nutrition.

Grain legumes are considered an essential source of nutrients but their protein is low in the sulfur-containing amino acids, methionine and cysteine. Delivery of adequate sulfur to seed tissues is needed to maximize production and improve protein quality (Hawkesford and De Kok 2006). Two related genotypes of common bean differ in storage protein composition (Osborn et al. 2003). SARC1 integrates a lectin, arcelin-1, from a wild *Phaseolus vulgaris* accession. A deficiency in phaseolin and major lectins was introgressed from a *Phaseolus coccineus* accession and Great Northern 1140, respectively, into SMARC1N-PN1. SMARC1N-PN1 compensates for the absence of phaseolin and lectins by increased levels of several sulfur-rich proteins, including the 11S globulin legumin (Marsolais et al. 2010; Yin et al. 2011; Liao et al. 2012). This is associated with increased cysteine concentration, by 70 %, and methionine concentration, by 10 %, as compared with SARC1, mostly at the expense of the non-protein amino acid *S*-methylcysteine (Taylor et al. 2008). To study the sulfur response of these two genotypes, conditions were determined where sulfur nutrition may limit reproductive growth and impact seed composition without affecting vegetative growth. Systematic studies of the effect of sulfur deficiency

S. Pandurangan
Department of Biology, University of Western Ontario,
1151 Richmond St., London, ON N6A 5B7, Canada

F. Marsolais (✉)
Genomics and Biotechnology, Southern Crop Protection and Food Research Centre,
Agriculture and Agri-Food Canada, 1391 Sandford St., London, ON N5V 4T3, Canada
e-mail: frederic.marsolais@agr.gc.ca

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_22

Table 1 Concentration of macro- and micro-nutrients in the nutrient solution with low and high sulfur used in the sulfur treatment trials

Element	Trial 1	Trial 2
	4 mM $Ca(NO_3)_2$	4.5 mM $Ca(NO_3)_2$
N P K	1 mM KNO_3	1.7 mM K_2HPO_4
	1 mM KH_2PO_4	0.2 mM K_2SO_4
	2 mM $MgSO_4$ (HS)	1.8 mM $MgSO_4$ (HS)
Mg	0.2 mM $MgSO_4$ (LS)	1.8 mM $MgCl_2$ (LS)
	1.8 mM $MgCl_2$ (LS)	
Mn	10 µM $MnSO_4$	4 µM $MnSO_4.H_2O$
B	50 µM H_3BO_3	5 µM H_3BO_3
Fe	50 µM Fe-citrate	10 µM Fe-EDTA
Na	50 µM Na_2-EDTA	–
Ca	Comes from $Ca(NO_3)_2$	Comes from $Ca(NO_3)_2$
Cu	1 µM $CuSO_4$	0.25 µM $CuSO_4.5H_2O$
Zn	1 µM $ZnSO_4$	1 µM $ZnSO_4.7H_2O$
Mo	0.5 µM Na_2MoO_4	0.2 µM $Na_2MoO_4.2H_2O$
Co	0.2 µM $CoSO_4$	Not vital for bean

have been performed in the model plants Arabidopsis and *Medicago truncatula* (Hirai et al. 2005; Higashi et al. 2006; Hoefgen and Nikiforova 2008; Zuber et al. 2013). In pea, soybean and lupin, sulfur deficiency decreases the levels of sulfur-rich proteins such as albumins and legumin-type globulins while increasing the levels of sulfur-poor globulins (Blagrove et al. 1976; Chandler et al. 1983, 1984; Gayler and Sykes 1985; Spencer et al. 1990). SARC1 and SMARC1N-PN1 offer a unique system to investigate how related legume genotypes, harboring natural genetic variation in sulfur-rich protein composition, respond to sulfur deficiency.

This study was designed based on previous work with navy bean (Sánchez et al. 2002) and chickpea (Chiaiese et al. 2004). Two trials were performed. In both trials, controlled conditions were used to grow the two genotypes, SARC1 and SMARC1N-PN1, under sulfur-deficient (referred to as low sulfur, LS) and sufficient conditions (referred to as high sulfur; HS) (Table 1). The sulfur-deficient plants were given nutrient solutions similar to the sulfur-sufficient ones except that sulfate salts had been replaced with comparable chloride salts in equal concentration. Nitrogen levels were kept constant. Plants were grown in pots in growth cabinets (Conviron E8H, Controlled Environments, Winnipeg, Manitoba, Canada) with 16 h light (300–400 µmol photons m^{-2} s^{-1}) and 8 h dark, with a temperature cycling between 18 and 24 °C (Pandurangan et al. 2012). Seeds were germinated in coarse vermiculite and transplanted after 2 weeks into a mixture of sand/perlite/vermiculite (2:1:1). The experimental unit comprised a pot with two plants. Plants were fertilized once weekly. In sulfur treatment trial 1, a nutrient solution was applied as described by Chiaiese et al. (2004). In sulfur treatment trial 2, a nutrient solution was prepared based on recommendations by Hall (1991) for common bean. The nutrient solution had reduced manganese, boron, and iron concentrations. In this trial, vermiculite was fertilized once with 20:20:20 (Plant Products, Ancaster, Ontario, Canada) prior to sowing, to facilitate seedling establishment.

Soil fertility is one of the most important yield-limiting factor for common bean (Fageria 2002). Nutrient deficiencies may occur in low fertility soils, and sulfur requirement is increasing due to reduced inputs from atmospheric deposition and other sources (Scherer 2009). On the other hand, nutrient levels in excess of a plant's sufficiency range will cause overall crop growth and health decline due to toxicity, and can cause environmental problems. In sulfur treatment trial 1, nutrient-fed plants showed symptoms of mineral toxicities after a few applications, including leaf scorching, yellowing, necrosis of leaf margins, interveinal chlorosis on young leaves and other symptoms specific to boron (B) and manganese (Mn) toxicity as described by Hall (1991). This could be due to imbalances of micronutrients in the solution (Table 1). In sulfur treatment trial 2, no phenotypic symptoms associated with nutrient toxicity and/or deficiency were noticed when the plants were fertilized. There were no apparent differences in vegetative growth between the LS and HS conditions (Fig. 1). After plants were grown to maturity, dry seeds were collected and analysed for several characteristics.

The effects of genotype and sulfur nutrition (S) on seed composition, number of seeds, seed weight and yield were analyzed by two-way analysis of variance (ANOVA) using SAS version 9.2 (Toronto, Ontario, Canada) (Tables 2 and 3). Among the agronomic parameters, the two genotypes differed in number of seeds and yield regardless of the treatment. SARC1 and SMARC1N-PN1 are genetic

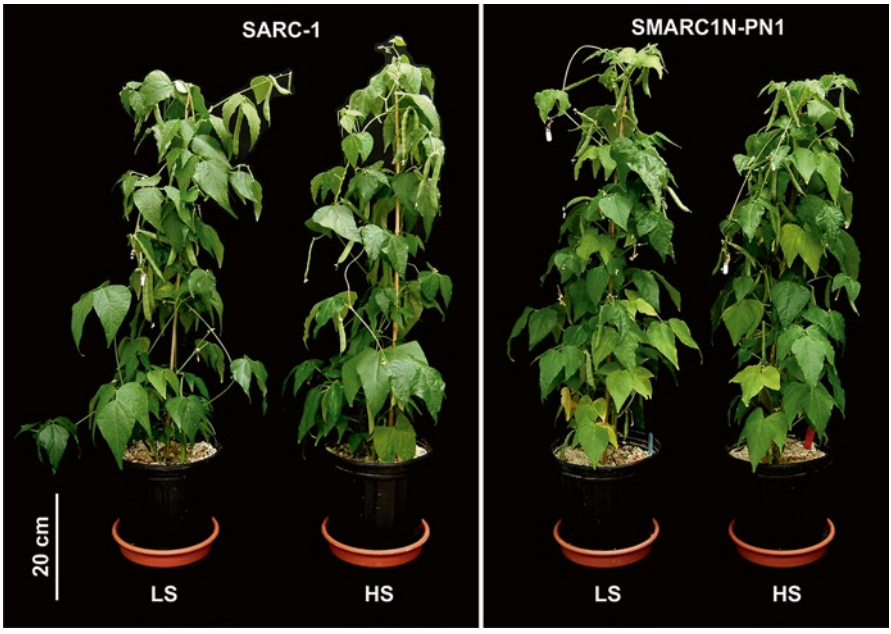


Fig. 1 Plants grown during sulfur treatment trial 2, 60 days after germination and 26 days after flowering. Vegetative growth appeared similar between genotypes. *LS* low sulfur, *HS* high sulfur (see Table 1 for experimental details)

Table 2 Effect of sulfur on number of seeds, seed weight and yield. $n=5$; values are the average ± SD

Genotype	Trt.	Number of seeds	Seed weight (mg)	Yield (g)
SARC1	LS	134 ± 16	215 ± 10	28.6 ± 2.1
	HS	143 ± 9	197 ± 9	28.2 ± 1.5
SMARC1N-PN1	LS	152 ± 12	196 ± 9	29.6 ± 1.2
	HS	157 ± 9	204 ± 12	32.0 ± 0.7
Source of variation	d.f.			
Genotype (G)	1	0.008	n. s.	0.002
Treatment (T)	1	n. s.	n. s.	n. s.
G × T	1	n. s.	0.01	0.05
Error	15			

n. s. not significant

Table 3 Elemental and sulfate concentrations in mature seed. $n=5$; values are the average ± SD

Genotype	Trt.	C (%)	N (%)	S (%)	SO_4^{2-} (nmol mg^{-1})
SARC1	LS	46.4 ± 0.2	3.90 ± 0.11	0.20 ± 0.12	0.18 ± 0.03
	HS	46.4 ± 0.1	3.91 ± 0.17	0.23 ± 0.01	0.21 ± 0.03
SMARC1N-PN1	LS	46.0 ± 0.1	3.63 ± 0.20	0.19 ± 0.01	0.21 ± 0.05
	HS	46.0 ± 0.1	3.71 ± 0.14	0.23 ± 0.01	0.29 ± 0.02
Source of variation	d.f.				
Genotype (G)	1	0.0001	0.006	n. s.	0.002
Treatment (T)	1	n. s.	n. s.	0.0001	0.001
G × T	1	n. s.	n. s.	n. s.	n. s.
Error	15				

stocks and therefore not completely isogenic. They share 87.5 and 83.6 % of the recurrent, Sanilac parental background (Osborn et al. 2003). There was no significant effect of the sulfur treatment alone on the agronomic parameters (Table 2). However, there were significant interactions between factors for seed weight and yield. Whereas the average seed weight decreased for SARC1 under HS, it actually increased for SMARC1N-PN1 (G × T; $p \leq 0.01$). In dry bean, seed weight is an important yield component having a positive correlation with grain yield (Fageria and Santos 2008). Yield increased under HS only in SMARC1N-PN1, by 8 % (G × T; $p \leq 0.05$). The yield response of dry bean to sulfur fertilization is typically low under field conditions (below 10 %) (Malavolta et al. 1987). In general, the yield response of pulse crops to sulfur fertilization is relatively low, as compared with oilseeds or cereals (Khurana et al. 2008).

To evaluate potential effects of the treatment on seed composition, seeds were analyzed for their carbon, nitrogen and sulfur concentration by dry combustion with a LECO CNS-2000 Elemental Analyzer (LECO Instruments ULC, Mississauga, Ontario, Canada), as previously described (Taylor et al. 2008). Sulfate concentration was also determined by chemical suppression ion chromatography and conductivity detection using a Dionex DX-600 Ion Chromatograph (Thermo Fisher Scientific,

Sunnyvale, California, USA), as described by Herschbach et al. (2000), with modifications. Replicate samples (approximately 50 seeds) were ground to a fine powder in a Kleco Ball Mill (Visalia, California, USA) and lyophilized. Approximately 100 mg of tissue was extracted in 0.5 ml of deionized water. The suspension was centrifuged at 16,000 × *g* for 10 min at 4 °C. A 300 µl aliquot of the cleared supernatant was transferred to an ion chromatography vial for testing using an IonPac anion-exchange column (AS14A, 4 mm; Dionex) and eluted with a mixture of 3.5 mM sodium hydrogen carbonate and 1.0 mM sodium carbonate. A 10 µl aliquot of the solution contained in vials was injected into the eluent stream and background conductivity of eluents reduced by a suppressor (Anion Self-Regenerating Suppressor Ultra, 4 mm; Dionex). An AS50 auto sampler equipped with a refrigerated chamber was used to house the vials and Dionex Peaknet 6.0 software was employed to track and analyze data.

There were significant genotypic differences for carbon, nitrogen and sulfate concentration (Table 3). More importantly, the sulfur treatment had a significant effect on sulfur and sulfate concentration in both genotypes. Sulfur concentration was raised by approximately 15–20 % in the HS treatment. Sulfate concentration was raised by 17 % in SARC1 and 38 % in SMARC1N-PN1. These data indicate that the sulfur treatment was effective in modulating the concentration of sulfur and of a sulfur metabolite, sulfate, in seed. These growth conditions are therefore suitable to investigate whether the two genotypes respond differently to sulfur nutrition, in relation with the presence of a higher level of sulfur-rich, and particularly cysteine-rich, proteins in SMARC1N-PN1 as compared with SARC1.

In summary, suitable experimental conditions have been defined to study the effect of sulfur nutrition on seed composition in common bean. In future research, these conditions will be used to investigate potential effects on additional seed characteristics, such as total amino acid profiles, including that of the non-protein amino acid, *S*-methylcysteine, the concentrations of different protein fractions, such as globulins and albumins, and effects on the abundance of specific proteins using polyacrylamide electrophoresis and proteomic identification.

Acknowledgements This research was supported in part by a grant from the Ontario Research Fund – Research Excellence Round IV of the Ontario Ministry of Research and Innovation to F.M. We thank staff at the Southern Crop Protection and Food Research Centre, Ronald Beyaert and Tim McDowell for help with elemental analysis, and Kenneth Conn and Igor Lalin for help with sulfate analysis. Kenneth Conn is also thanked for advice on sulfur treatments.

References

Blagrove RJ, Gillesie JM, Randall PJ (1976) Effect of sulphur supply on the seed globulin composition of *Lupinus angustifolius*. Aust J Plant Physiol 3:173–184

Chandler PM, Higgins TJ, Randall PJ, Spencer D (1983) Regulation of legumin levels in developing pea seeds under conditions of sulfur deficiency: rates of legumin synthesis and levels of legumin mRNA. Plant Physiol 71:47–54

Chandler PM, Spencer D, Randall PJ, Higgins TJ (1984) Influence of sulfur nutrition on developmental patterns of some major pea seed proteins and their mRNAs. Plant Physiol 75:651–657

Chiaiese P, Ohkama-Ohtsu N, Molvig L, Godfree R, Dove H, Hocart C, Fujiwara T, Higgins TJ, Tabe LM (2004) Sulphur and nitrogen nutrition influence the response of chickpea seeds to an added, transgenic sink for organic sulphur. J Exp Bot 55:1889–1901

Fageria NK (2002) Nutrient management for sustainable dry bean production in the tropics. Commun Soil Sci Plant Anal 33:1537–1575

Fageria NK, Santos AB (2008) Yield physiology of dry bean. J Plant Nutr 31:983–1004

Gayler KR, Sykes GE (1985) Effects of nutritional stress on the storage proteins of soybeans. Plant Physiol 78:582–585

Hall R (ed) (1991) Compendium of bean diseases. American Phytopathological Society, St. Paul

Hawkesford MJ, De Kok LJ (2006) Managing sulphur metabolism in plants. Plant Cell Environ 29:382–395

Herschbach C, Van Der Zalm E, Schneider A, Jouanin L, De Kok LJ, Rennenberg H (2000) Regulation of sulfur nutrition in wild-type and transgenic poplar over-expressing g-glutamylcysteine synthetase in the cytosol as affected by atmospheric H_2S. Plant Physiol 124:461–473

Higashi Y, Hirai MY, Fujiwara T, Naito S, Noji M, Saito K (2006) Proteomic and transcriptomic analysis of Arabidopsis seeds: molecular evidence for successive processing of seed proteins and its implication in the stress response to sulfur nutrition. Plant J 48:557–571

Hirai MY, Klein M, Fujikawa Y, Yano M, Goodenowe DB, Yamazaki Y, Kanaya S, Nakamura Y, Kitayama M, Suzuki H, Sakurai N, Shibata D, Tokuhisa J, Reichelt M, Gershenzon J, Papenbrock J, Saito K (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in *Arabidopsis* by integration of metabolomics and transcriptomics. J Biol Chem 280:25590–25595

Hoefgen R, Nikiforova VJ (2008) Metabolomics integrated with transcriptomics: assessing systems response to sulfur-deficiency stress. Physiol Plant 132:190–198

Khurana MPS, Sadana US, Bijay-Singh (2008) Sulfur nutrition of crops in the Indo-Gangetic plains of South Asia. In: Jez J (ed) Sulfur: a missing link between soils, crops and nutrition. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, pp 11–24

Liao D, Pajak A, Karcz SR, Chapman BP, Sharpe AG, Austin RS, Datla R, Dhaubhadel S, Marsolais F (2012) Transcripts of sulphur metabolic genes are co-ordinately regulated in developing seeds of common bean lacking phaseolin and major lectins. J Exp Bot 63:6283–6295

Malavolta E, Vitti GC, Rosolem CA, Fageria NK, Guimarães PTG (1987) Sulfur responses of Brazilian crops. J Plant Nutr 10:2153–2158

Marsolais F, Pajak A, Yin F, Taylor M, Gabriel M, Merino DM, Ma V, Kameka A, Vijayan P, Pham H, Huang S, Rivoal J, Bett K, Hernandez-Sebastia C, Liu Q, Bertrand A, Chapman R (2010) Proteomic analysis of common bean seed with storage protein deficiency reveals up-regulation of sulfur-rich proteins and starch and raffinose metabolic enzymes, down-regulation of the secretory pathway. J Proteomics 73:1587–1600

Osborn TC, Hartweck LM, Harmsen RH, Vogelzang RD, Kmiecik KA, Bliss FA (2003) Registration of *Phaseolus vulgaris* genetic stocks with altered seed protein compositions. Crop Sci 43:1570–1571

Pandurangan S, Pajak A, Molnar SJ, Cober ER, Dhaubhadel S, Hernández-Sebastià C, Nelson RL, Huber SC, Marsolais F (2012) Relationship between asparagine metabolism and protein concentration in soybean seed. J Exp Bot 63:3173–3184

Sánchez E, Ruiz JM, Romero L (2002) Proline metabolism in response to nitrogen toxicity in fruit of French Bean plants (*Phaseolus vulgaris* L. cv Strike). Sci Hortic (Amsterdam) 93:225–233

Scherer WH (2009) Sulfur in soils. J Plant Nutr Soil Sci 172:326–335

Spencer D, Rerie WG, Randall PJ, Higgins TJV (1990) The regulation of pea seed storage protein genes by sulfur stress. Aust J Plant Physiol 17:355–363

Taylor M, Chapman R, Beyaert R, Hernandez-Sebastia C, Marsolais F (2008) Seed storage protein deficiency improves sulfur amino acid content in common bean (*Phaseolus vulgaris* L.): redirection of sulfur from γ-glutamyl-*S*-methyl-cysteine. J Agr Food Chem 56:5647–5654

Yin F, Pajak A, Chapman R, Sharpe A, Huang S, Marsolais F (2011) Analysis of common bean expressed sequence tags identifies sulfur metabolic pathways active in seed and sulfur-rich proteins highly expressed in the absence of phaseolin and major lectins. BMC Genomics 12:268

Zuber H, Poignavent G, Le Signor C, Aime D, Vieren E, Tadla C, Lugan R, Belghazi M, Labas V, Santoni AL, Wipf D, Buitink J, Avice JC, Salon C, Gallardo K (2013) Legume adaptation to sulfur deficiency revealed by comparing nutrient allocation and seed traits in *Medicago truncatula*. Plant J 76:982–996

Effect of an Alfalfa Plant-Derived Biostimulant on Sulfur Nutrition in Tomato Plants

Andrea Ertani, Michela Schiavon, Annarita Trentin, Mario Malagoli, and Serenella Nardi

Abstract The excessive use of chemical fertilizers has affected soil and water quality causing the reduction of organic matter content in soils and the increase of nitrates in waters. Organic products known as "biostimulants" could be used in agricultural practices to promote plant growth and mineral nutrient uptake. Previous studies showed that applications of a *Medicago sativa* L. hydrolysate-based biostimulant (EM) to maize plants stimulated the main metabolic pathways, such as nitrogen assimilation and the tricarboxylic acid cycle, as well as the secondary metabolism associated with the synthesis of phenylpropanoids. In order to evaluate whether EM could also influence sulfur (S) metabolism, the content of S, glutathione (GSH) and the expression of genes involved in S transport were analyzed in tomato plants cv. Micro-Tom treated with the biostimulant. Furthermore, the expression of genes coding for enzymes that use GSH as a substrate in redox reactions (glutathione reductase, GSR2, and glutathione peroxidase, GPX) was assayed.

Plants were cultivated in hydroponics in the presence of EM at the dosages of 0.1 or 1.0 ml l^{-1}. The application of EM to tomato significantly stimulated sulfur accumulation in plants, and in roots the increase was dose-dependent. Interestingly, in roots the level of glutathione concomitantly decreased. qRT-PCR experiments evidenced the up-regulation of genes coding for sulfate transporters (ST1 and ST2) in plants supplied with EM, especially when the biostimulant was furnished at 0.1 ml l^{-1}. The same trend was observed for the GSR2 gene in leaves. The transcript accumulation of GSR2 and GPX in roots was maximal in plants treated with 1 ml l^{-1} EM. The results obtained suggest a positive role of EM on sulfur transport in tomato plants, and are consistent with previous studies where the enhancement of nitrogen metabolism by EM was reported. The concomitant stimulation of S and N nutrition by EM is likely due to the fact that the pathways of these nutrients in plants are highly inter-related. The increase of GSR2 and GPX transcript level suggests a role for EM to supporting plants to overcome stress by inducing antioxidant enzyme activity.

A. Ertani • M. Schiavon • A. Trentin • M. Malagoli (✉) • S. Nardi
Department of Agronomy, Food, Natural Resources, Animal and Environment, University of Padova, Agripolis,Viale dell'Università, 16, 35020 Legnaro, Padova, Italy
e-mail: mario.malagoli@unipd.it

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_23

In recent years many approaches have been evaluated to decrease mineral fertilization and at the same time improve plant nutrient uptake and yield. In this context, the use of biostimulants to improve plant nutrition and diminish the adverse effects of synthetic fertilizers can be exploited (Nardi et al. 2002; Schiavon et al. 2008; Ertani et al. 2009). Biostimulants are currently defined as "substances and materials, with the exception of nutrients and pesticides, which, when applied to plant, seeds or growing substrates in specific formulations, have the capacity to modify physiological processes of plants in a way that provides potential benefits to growth, development and/or stress response". Biostimulants can derive from different sources, including animal, plant and algal sources. The mechanisms they trigger in plants are partially unknown and often difficult to identify, because most of these products can contain different and complex bioactive molecules (Ertani et al. 2011). Their effects are hence the result of many components that may work synergistically. When applied in small amounts, biostimulants can promote plant development, increase yields, and support plants to overcome stress situations by acting directly or indirectly on plant physiology (Ertani et al. 2009).

Previous studies showed that application of a *Medicago sativa* L. plant hydrolysate-based biostimulant (EM) to maize plants strongly stimulated nitrogen primary and secondary metabolism (Ertani et al. 2013). Nitrogen and sulfur are highly inter-related in plants, as both elements are required for the synthesis of proteins. On this account, it is presumable that EM can also influence S nutrition in plants. To test this hypothesis, EM was furnished to tomato (*Solanum lycopersicon*) plants and the content of S and glutathione (GSH), as well as the expression of genes encoding sulfate transporters and enzymes involved in redox reaction where GSH was used as substrate, were determined.

Tomato plants were cultivated in a thoroughly aerated Hoagland modified nutrient solution in 3 l pots (density = six plants per pot). The solution was renewed every three days to ensure a constant supply of macro- and microelements to plants. After 3 weeks from the transplant, EM was added to the nutrient solution at 0.1 or 1 ml l^{-1} for 48 h. A group of plants that was not treated with the biostimulant was used as control. At harvest, plants were carefully washed with distilled water, dried with blotting paper, divided in roots and leaves and weighed. A part of the sample from each plant was immediately frozen with liquid nitrogen and kept at −80 °C for further analyses. Determination of sulfur in foliar and root dry tissues was performed via inductively coupled plasma atomic emission spectroscopy (ICP-AES) as described by Fassel (1986), while GSH in frozen tissues was quantified via HPLC according to Masi et al. (2002). Gene expression analysis was performed via qRT-PCR as described by Schiavon et al. (2012). EM addition to tomato plants positively influenced the growth in terms of fresh weight (data not shown), and increased S accumulation compared to EM-untreated plants (Fig. 1). A linear and positive correlation was found between S concentration in leaves and the dose of EM applied, while in roots maximum values of S accumulation were determined in plants treated with EM 0.1 ml l^{-1}.

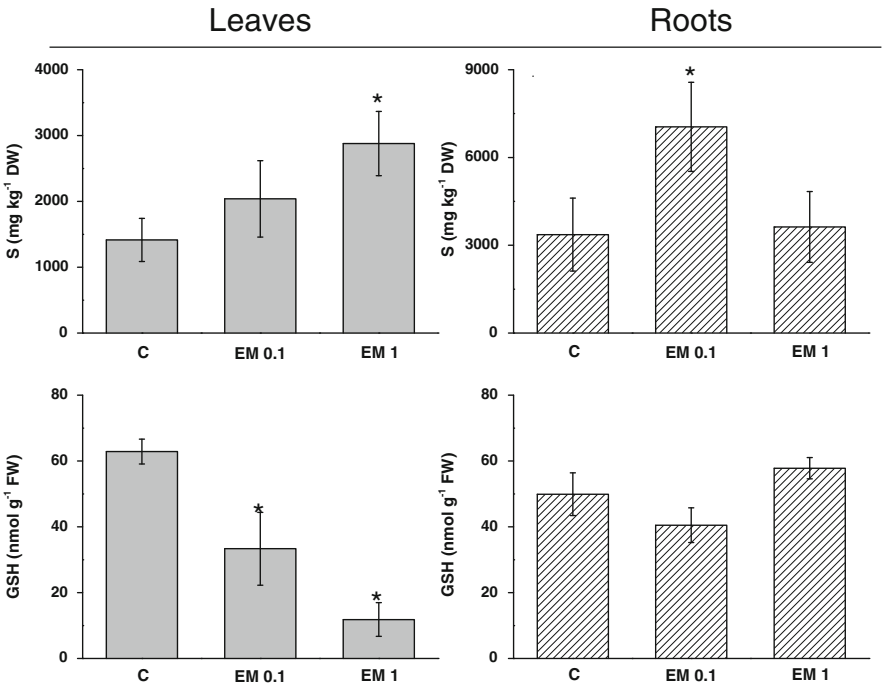


Fig 1 Accumulation of sulfur (S) and glutathione (GSH) in leaves and roots of tomato plants treated or not with EM at different dosages (0.1 and 1 ml l^{-1}). Data represent mean values (n = 3; ± SD). *Asterisks* above *bars* indicate significant differences compared to the control ($p < 0.05$, Student's *t*-test)

The leaf content of glutathione (GSH) was inversely correlated to the concentration of S (Fig. 1). Indeed, it decreased accordingly to the increase of the EM dose. In roots, no significant variation in GSH content was observed, although a weak reduction was observed in plants supplied with EM 0.1 ml l^{-1}, which displayed higher S accumulation. The analysis of gene expression of two high affinity sulfate transporters, ST1 and ST2, evidenced their up-regulation in roots of plants treated with EM compared to the control plants (Fig. 2). In particular, the increase in transcript levels of both ST1 and ST2 was more evident when plants were grown in the presence of the lower EM dosage. In leaves, a similar trend of ST2 transcript accumulation as in roots was observed, while ST1 mRNA abundance increased only in plants treated with EM 0.1 ml l^{-1}. The transcripts of genes coding for GSH-dependent enzymes involved in multiple stress responses in plants, glutathione reductase (GSR2) and glutathione peroxidase (GPX), were significantly up-regulated in roots of tomato, accordingly to the EM dose applied (Fig. 3). In leaves, higher levels of GSR2 in EM-treated plants were observed only at the 0.1 ml l^{-1} dosage, while the transcript level of GPX decreased.

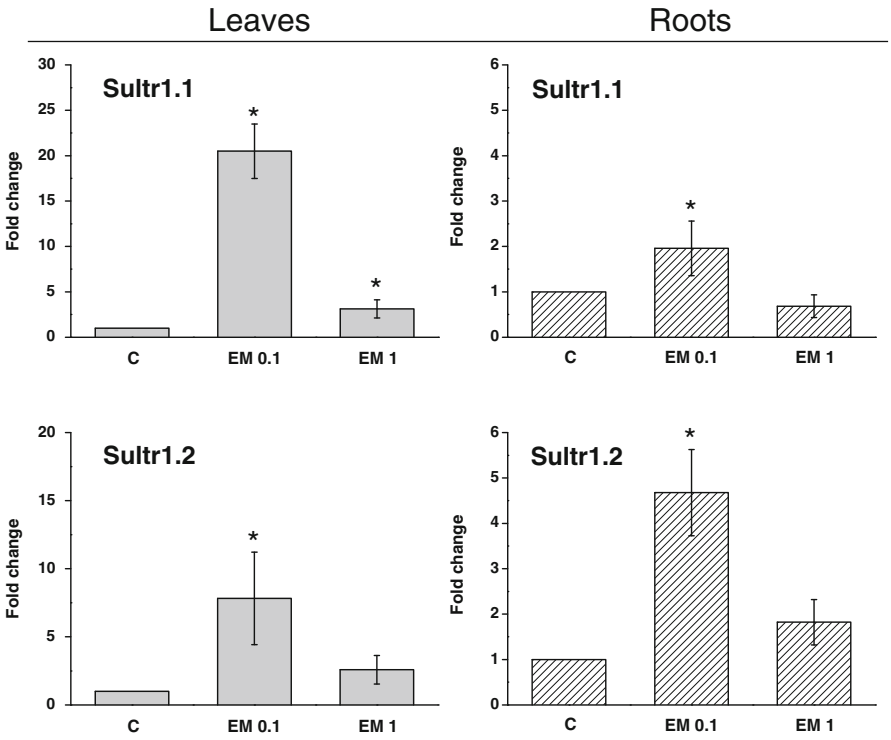


Fig. 2 Transcript accumulation of sulfate transporters, ST1 and ST2, in leaves and roots of tomato plants treated or not with EM at different dosages (0.1 and 1 ml l^{-1}). Data represent mean values (n = 3; ± SD). *Asterisks* above *bars* indicate significant differences compared to the control ($p < 0.05$, Student's *t*-test)

The results obtained suggest a positive role of EM on sulfur uptake in tomato plants. The opposite effects of EM on S and GSH accumulation may suggest that EM can promote S absorption by plants by lowering GSH content. Indeed, high levels of GSH are known to inhibit sulfate uptake and loading into the xylem (Herschbach and Rennenberg 1991). Interestingly, EM could induce the expression of *Sultr1.1* and *Sultr1.2* both in roots and leaves. This is a novelty, as in a previous investigation only *Sultr1.1* was detected in leaves, while *Sultr1.2* was found to be root-specific (Howarth et al. 2003). The stronger effect of EM on the gene expression of both transporters was observed in plants treated with the low EM dosage, and justified the higher S accumulation. In a study by Schiavon et al. (2008) nitrogen assimilation was also found to be more stimulated by EM at 0.1 ml l^{-1} than at 1 ml l^{-1} dose in maize. Therefore, EM seems to induce a concomitant stimulation of S and N acquisition in plants. This was likely because of the pivotal role of these two elements in the synthesis of proteins, amino acids and certain secondary compounds. Although the level of GSH was reduced in plants by EM, the increase

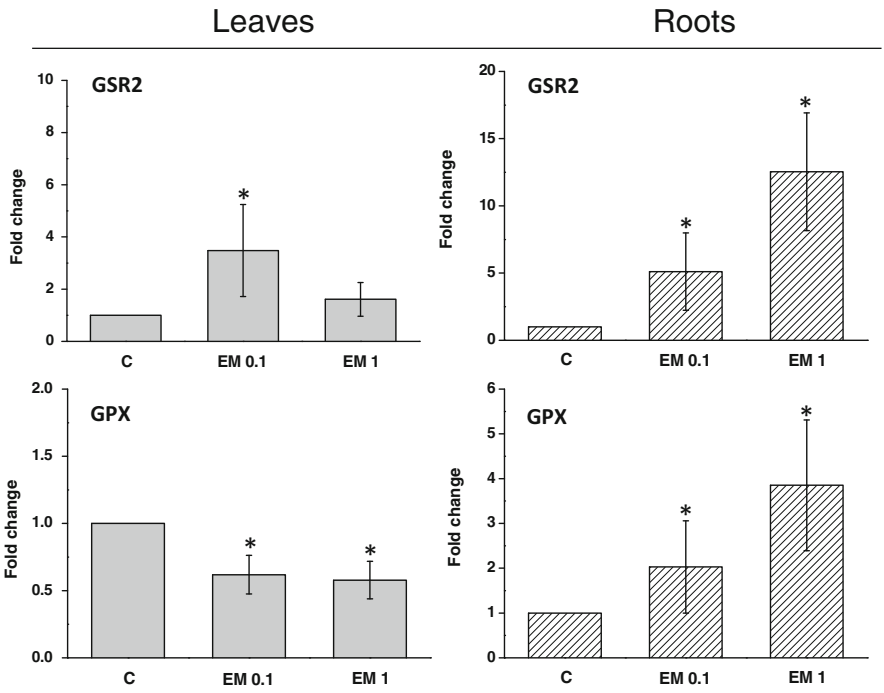


Fig. 3 Transcript accumulation of glutathione reductase (GSR2) and glutathione peroxidase (GPX) in leaves and roots of tomato plants treated or not with EM at different dosages (0.1 and 1 ml l^{-1}). Data represent mean values (n = 3; ± SD). *Asterisks* above *bars* indicate significant differences compared to the control ($p < 0.05$, Student's *t*-test)

of transcript levels of genes encoding GSH-depending enzymes involved in stress-responses indicates a role for EM in inducing antioxidant enzymatic defense systems. The capacity of EM to support plants to overcome stress by inducing antioxidant enzyme activity was previously reported by Ertani et al. (2013) in maize.

References

Ertani A, Cavani L, Pizzeghello D, Brandellero E, Altissimo A, Ciavatta C, Nardi S (2009) Biostimulant activities of two protein hydrolysates on the growth and nitrogen metabolism in maize seedlings. J Plant Nutr Soil Sci 172:237–244

Ertani A, Francioso O, Tugnoli V, Righi V, Nardi S (2011) Effect of commercial lignosulphonate-humate on *Zea mays* L. metabolism. J Agr Food Chem 59:11940–11948

Ertani A, Schiavon M, Muscolo A, Nardi S (2013) Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed *Zea mays* L. plants. Plant Soil 364:145–158

Fassel VA (1986) Analytical inductively coupled plasma spectroscopies: past, present, and future. Fresenius J Anal Chem 324:511–518

Herschbach C, Rennenberg H (1991) Influence of glutathione (GSH) on sulfate influx, xylem loading and exudation in excised tobacco roots. J Exp Bot 42:1021–1029

Howarth JR, Fourcroy P, Davidian JC, Smith FW, Hawkesford MJ (2003) Cloning of two contrasting high-affinity sulfate transporters from tomato induced by low sulfate and infection by the vascular pathogen *Verticillium dahliae*. Planta 218:58–64

Masi A, Ghisi R, Ferretti M (2002) Measuring low-molecular-weight thiols by detecting the fluorescence of their SBD-derivatives: application to studies of diurnal and UV-B induced changes in *Zea mays* L. J Plant Physiol 159:499–507

Nardi S, Pizzeghello D, Muscolo A, Vianello A (2002) Physiological effects of humic substances on higher plants. Soil Biol Biochem 34:1527–1536

Schiavon M, Ertani A, Nardi S (2008) Effects of an alfalfa protein hydrolysate on the gene expression and activity of enzymes of TCA cycle and N metabolism in *Zea mays* L. J Agr Food Chem 56:11800–11808

Schiavon M, Galla G, Wirtz M, Pilon-Smits EAH, Telatin V, Quaggiotti S, Hell R, Barcaccia G, Malagoli M (2012) Transcriptome profiling of genes differentially modulated by sulfur and chromium identifies potential targets for phytoremediation and reveals a complex S Cr interplay on sulfate transport regulation in *B. juncea*. J Hazard Mater 15:192–205

Effect of Sulfur and Nod Factors (LCOs) on Some Physiological Features and Yield of Pea (*Pisum sativum* L.)

Anna Podleśna, Jerzy Wielbo, Janusz Podleśny, and Dominika Kidaj

Abstract The response of pea var. Medal to treatment with Nod factors (LCOs) and mineral sulfur was estimated in a pot experiment with a completely randomized design. Foliar spraying of plants was performed at the 5–6 leaf stage (BBCH 15) at concentrations of 10^{-12} M dm^{-3} and 12 g S dm^{-3} for LCOs and sulfur, respectively. The use of these factors, both individually and in combination, caused an increase in leaf area and "greenness" (SPAD), gas exchange parameters, straw and seed yields and in the root system. The number of nodules and respective nodule dry weight also increased with these treatments. A significant increase in seed yield resulted from the beneficial effects of LCOs and sulfur with an increase in the number of pods and seeds per plant compared to control plants, is clearly significant from the agricultural point of view. Although each factor improved the traits studied, the best results were achieved in the case of plants treated with both LCOs and sulfur.

Leguminous plants have a great importance in agriculture for the production of valuable seeds, which are used as food for human and fodder for animals. However, a relatively low and variable seed yield has led to a decrease in their cultivation (Graham and Vance 2003). Therefore scientists are looking at factors, which may improve the yield of legumes. Some of these studies are focused on the legumes' ability for biological nitrogen fixation (BNF) by forming a relationship with specialized nitrogen-fixing bacteria called rhizobia (van Hameren et al. 2013). The rhizobia convert atmospheric di-nitrogen into forms of nitrogen usable for the plant, whilst being housed in novel root organs – nodules. Optimizing BNF processes, such as nodulation, has the potential to increase crop yields and enhance soil fertility whilst reducing farming costs and harmful environmental impacts (van Hameren

A. Podleśna (✉) • J. Podleśny
Institue of Soil Science and Plant Cultivation, State Research Institute,
Czartoryskich 8, 24-100 Puławy, Poland
e-mail: ap@iung.pulawy.pl

J. Wielbo • D. Kidaj
Maria Curie-Skłodowska University, Akademicka 19, 24-033 Lublin, Poland

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_24

et al. 2013). Research into legume-rhizobia symbioses has identified numerous plant and bacterial metabolites, which are essential for the establishment of symbiosis and development of root nodules (Brewin 2004). This group of metabolites includes bacterial Nod factors (lipochitooligosaccharides-LCOs), which are bacteria-to-plant signals required for the establishment of rhizobia-legume nitrogen fixing symbioses (Cullimore et al. 2001). LCOs induce the formation of root nodules (Geurts et al. 2005; Podleśny et al. 2014a) and improve plant germination, growth and yield (Podleśny et al. 2014b) so they could be used as biofertilizers (Bhardwaj et al. 2014; Kidaj et al. 2012). On the other hand, BNF is particularly sensitive to environmental stresses such as nutrient deficiency (Divito and Sadras 2014). Varin et al. (2010) showed that sulfur has an important role in this process by demonstrating that its deficiency reduces nitrogen fixation in pea (*Pisum sativum* L.) and lucerne (*Medicago sativa* L.). Some *Rhizobium*-legume symbiotic interactions are mediated by Nod factors (LCOs), which can be sulfated (Snoeck et al. 2003). Moreover sulfur, as glutathione or ascorbate-glutathione cycle enzymes, is essential for the establishment of legume-rhizobia symbiosis, regulation of the cell cycle and growth, and for root meristem activity (Groten et al. 2005). However, the amount of sulfur in the soil profile is frequently not sufficient to fulfill the nutritional needs of legumes (Cazzato et al. 2012; Szulc et al. 2014). The aim of the present study was the evaluation of LCOs, mineral sulfur and the combined application of both factors on physiological and agricultural parameters of pea yield.

An experiment was conducted in the greenhouse, in Mitscherlich pots, which contained a mixture of soil (5 kg) and sand (2 kg) and which were planted with pea var. Medal (afila type). The plants were sprayed with: 1, control (distilled water); 2, LCOs/Nod factors (concentration: 10^{-12} M dm^{-3}); 3, sulfur (concentration: 12 g S dm^{-3}); and 4, LCOs and sulfur in the above-mentioned concentrations. Rhizobial Nod factors (LCOs) were isolated from liquid cultures of *Rhizobium leguminosarum* bv. *viciae* GR09 (*Rlv* GR09) strain induced by a plant flavonoid extract (Wielbo et al. 2007). Foliar spraying (25 ml per pot of five plants) was performed in the 5–6 leaf phase of growth (BBCH 15). Plants were harvested at three developmental phases: flowering (BBCH 60), fruit development (BBCH 75) and full maturity (BBCH 89). Dry matter of specific plant organs and seed yield were measured (Fig. 1).

Both LCOs, sulfur, and their combined use had an effect on the parameters measured. Firstly, an increase in leaf area during the flowering and green pod phases of pea growth in comparison to control plants (treated with distilled water) was observed (Table 1). Moreover, these leaves also demonstrated an increased leaf greenness index (SPAD). The application of LCOs and sulfur increased the values of the main gas exchange parameters in the pea leaves (Table 2). It is probable that these changes of photosynthesis (Pn) and transpiration (E) intensity were the result of greater leaf area and greater concentration of chlorophyll in leaves as an effect of plants treated with LCOs, sulfur or both these factors. The best results of studied traits were achieved in plants treated with both LCOs and sulfur, and were lower in plants treated with LCOs (leaf area and SPAD) and with sulfur (Pn, net photosynthesis and E, transpiration intensity). Similar responses of peas to LCOs were observed earlier (Kidaj et al. 2012; Podleśny et al. 2014a, b). The same trends in the effect of

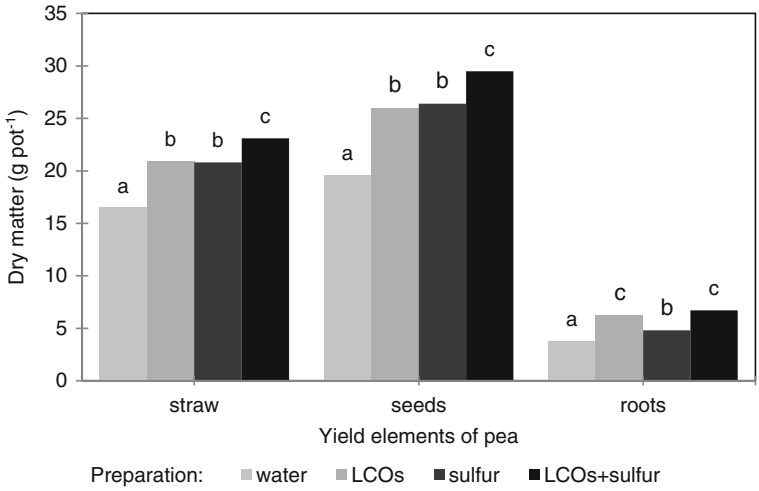


Fig. 1 Impact of LCOs (10^{-12} M dm^{-3}) and sulfur (12 g S dm^{-3}) on yield of pea plants. The weight of straw, seeds and root system was determined upon harvest at full maturity (BBCH 89). Seed yield was calculated for 14 % moisture content and expressed per pot. Roots were rinsing in dense metal sieves, dried and weighed. Different letters indicate significant differences between treatments ($p \leq 0.05$, Tukey's test)

Table 1 Impact of LCOs (10^{-12} M dm^{-3}) and sulfur (12 g S dm^{-3}) on chosen pea leaf indices during growth

Description	Preparation	Developmental phase of pea (BBCH)		
		60	75	Mean
Leaf area	Water	394 ± 8a	457 ± 7a	425 ± 7a
	LCOs	426 ± 6b	471 ± 6b	448 ± 8b
	Sulfur	431 ± 7b	480 ± 5b	455 ± 9b
	LCOs + sulfur	444 ± 6b	494 ± 6c	469 ± 9b
SPAD	Water	501 ± 12a	485 ± 8a	493 ± 6a
	LCOs	511 ± 10a	509 ± 9a	510 ± 7b
	Sulfur	514 ± 12a	508 ± 9a	511 ± 7b
	LCOs + sulfur	518 ± 12a	534 ± 10b	526 ± 9b

Leaf area (cm^2 $plant^{-1}$) was measured with using a Leaf Area Scanner AM 300 (ADC BioScientific Ltd., UK) and SPAD values were determined by using a Minolta chlorophyll meter SPAD – 502. The results were expressed in terms of mean values per four pots. Statistical analysis was performed with Statgraphic ver. 5.1 program. Results of LSD range test are shown. Values followed by similar superscript letters are not significantly different at the 5 % probability level

LCOs on soybean were found by Almaraz et al. (2007), who observed a 13 % increase in photosynthesis over controls which was accompanied by increase in stomatal conductance. Previous studies have suggested that Nod factors sprayed onto shoots stimulate carbon sink strength by increasing early cell division in meristems and this may trigger an increase in photosynthetic rate, based on photosynthetic regulation by carbon sinks. Moreover, the observed increase in stomatal

Table 2 Impact of LCOs (10^{-12} M dm^{-3} of water) and sulfur (12 g S dm^{-3}) on gas exchange parameters of pea leaves

Preparation	Parameter		
	Pn	E	Gs
Water	10.4 ± 0.3a	5.42 ± 0.14a	724 ± 14.3a
LCOs	13.0 ± 0.2c	6.37 ± 0.32b	783 ± 16.8b
Sulfur	12.1 ± 0.3b	6.14 ± 0.30b	792 ± 14.3b
LCOs + sulfur	13.8 ± 0.4d	7.08 ± 0.34c	783 ± 14.7b

Measurements of net photosynthesis intensity (Pn, μmol CO_2 m^{-2} s^{-1}), transpiration intensity (E, mmol H_2O m^{-2} s^{-1}) and stomatal conductance (Gs, mmol H_2O m^{-2} s^{-1}) were performed at flowering (BBCH 60) using a CIRAS −2 device. Radiation intensity was 500 μmol m^{-2} s^{-1} and CO_2 380 ppm. Measurements were performed on the first fully developed leaf counted from the top of a plant. Different letters indicate significant differences between treatments ($p \leq 0.05$, Tukey's test)

Table 3 Impact of LCOs (10^{-12} M dm^{-3} of water) and sulfur (12 g S dm^{-3}) on number and dry matter of root nodules (mg $plant^{-1}$) and dry matter of 1 nodule (mg) during flowering (BBCH 60) and green pod (BBCH 75) stages of pea growth

Description	Preparation	Developmental phase of pea (BBCH)		
		60	75	Mean
Number of root nodules	Water	40.3 ± 2.4a	31.3 ± 2.5a	35.8 ± 3.7a
	LCOs	61.6 ± 2.6b	40.5 ± 2.1b	51.0 ± 4.4b
	Sulfur	60.4 ± 2.5b	42.4 ± 1.9b	51.4 ± 5,2b
	LCOs + sulfur	71.3 ± 2.8c	49.3 ± 2.2c	60.3 ± 4.3c
Dry matter of root nodules	Water	46.4 ± 5.3a	38.1 ± 2.7a	42.2 ± 3.1a
	LCOs	62.5 ± 5.8b	52.4 ± 3.3b	57.4 ± 3.4b
	Sulfur	61.6 ± 6.4b	53.3 ± 2.4b	57.4 ± 3.0b
	LCOs + sulfur	77.0 ± 6.9c	58.2 ± 2.1c	67.6 ± 3.2c
Dry matter of 1 nodule	Water	1.15 ± 0.03b	1.23 ± 0.04a	1.19 ± 0.05a
	LCOs	1.02 ± 0.02a	1.30 ± 0.05a	1.16 ± 0.03a
	Sulfur	1.02 ± 0.02a	1.27 ± 0.03a	1.13 ± 0.03a
	LCOs + sulfur	1.09 ± 0.03b	1.19 ± 0.03a	1.14 ± 0.04a

Nodules were removed from rinsed roots, and the number and dry weight of root nodules were determined. Presented values are the mean from an object. Different letters indicate significant differences between treatments ($p \leq 0, 05$, Tukey's test)

conductance may indicate that Nod factors improved photosynthetic rate by increasing the CO_2 supply for photosynthesis (Almaraz et al. 2007). Applied sulfur also showed a beneficial effect on gas exchange parameters, indicating that this nutrient plays an important role in these processes. According to Mazid et al. (2011) the photosynthetic apparatus is severely affected under S deficiency, mainly by the reduction of chloroplast and Rubisco content. As the largest increase in leaf area, photosynthetic activity and transpiration was observed with combined use of LCOs and sulfur, it may indicate that the use of these both factors increases their beneficial effect. Similarly, observation of the roots showed that the use of LCOs and sulfur, and particularly their use in combination, had a significant effect on the number of root nodules and their total dry matter (Table 3). The results obtained are in

Table 4 Impact of LCOs (10^{-12} M dm^{-3}) and sulfur (12 g S dm^{-3}) on pea yield structure features

Preparation	Pods $plant^{-1}$	Seeds pod^{-1}	Seeds $plant^{-1}$	Weight of 1000 seeds
Water	5.91 ± 0.21a	5.30 ± 0.24a	31.3 ± 1.2a	224 ± 12a
LCOs	6.44 ± 0.24b	5.47 ± 0.28a	35.2 ± 1.4b	220 ± 15a
Sulfur	6.53 ± 0.26b	5.38 ± 0.33a	35.1 ± 1.4b	231 ± 14a
LCOs + sulfur	7.24 ± 0.31c	5.32 ± 0.35a	38.5 ± 1.6c	230 ± 14a

The number of pods and seeds per plant and number of seeds per pod was determined upon harvest at fully ripe stage. Then weight of 1000 seeds (g) was estimated. Different letters indicate significant differences between treatments ($p \leq 0.05$, Tukey's test)

agreement with the findings of Kidaj et al. (2012) and Podleśny et al. (2014a, b) in relation to plants response to LCOs and with observations of Scherer et al. (2006) and Zhao et al. (1999) in relation to sulfur. The earlier studies of Podleśny et al. (2014a, b) found that LCOs slightly accelerated pea growth from the first developmental phases and stimulated the growth of vegetative and generative organs. Scherer et al. (2006) showed an effect of sulfur on the amount of sucrose and glucose in shoots and nodules of pea. According to these authors, when S is limiting, protein synthesis is inhibited resulting in lower yields. Moreover, pea plants fertilized with sulfur fixed more nitrogen than control plants (S0). The analysis of yield structure demonstrated the beneficial effect of LCOs and sulfur in increasing the number of pods and seeds per plant (Table 4). It can be supposed that plants sprayed with LCOs improved nitrogen fixation and additionally sprayed with sulfur more effectively used it as sulfur deficiency decrease nitrogen use efficiency (Fismes et al. 2000).

References

Almaraz JJ, Ahou X, Souleimanov A, Smith D (2007) Gas exchange characteristics and dry matter accumulation of soybean treated with Nod factors. J Plant Physiol 164:1391–1393

Bhardwaj D, Ansari MW, Sahoo RK, Tuteja N (2014) Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. Microb Cell Fact 13:1–10

Brewin NJ (2004) Plant cell wall remodeling in the Rhizobium-legume symbiosis. Crit Rev Plant Sci 23:293–316

Cazzato E, Laudation V, Stellacci AM, Ceci E, Tufarelli V (2012) Influence of sulphur application on protein quality, fatty acid composition and nitrogen fixation of white lupin (*Lupinus albus* L.). Eur Food Res Technol 235:963–969

Cullimore JV, Ranjeva R, Bono J-J (2001) Perception of lipo-chitooligosacchardic Nod factors in legumes. Trends Plant Sci 6:25–30

Divito GA, Sadras VO (2014) How phosphorus, potassium and sulphur affect plant growth and biological nitrogen fixation in crop and pasture legumes? Field Crop Res 156:161–171

Fismes J, Vong PC, Guckert A, Frossard E (2000) Influence of sulfur on apparent N-use efficiency, yield and quality of oilseed rape (*Brassica napus* L.) grown on calcareous soil. Eur J Agron 12:127–141

Geurts R, Fedorova E, Bisseling T (2005) Nod factor signaling genes and their function in the early stages of Rhizobium infection. Curr Opin Plant Biol 8:346–352

Graham PH, Vance CP (2003) Legumes: importance and constrains to greater use. Plant Physiol 131:872–877

Groten K, Vanacker H, Dutilleul C, Bastian F, Bernard S, Carzaniga R, Foyer CH (2005) The roles of redox processes in pea nodule development and senescence. Plant Cell Environ 28:1293–1304

Kidaj D, Wielbo J, Skorupska A (2012) Nod factors stimulate seed germination and promote growth and nodulation of pea and vetch under competitive conditions. Microbiol Res 167:144–150

Mazid M, Khan TA, Mohammad F (2011) Response of crop plants under sulphur stress tolerance: a holistic approach. J Stress Physiol Biochem 7:23–57

Podleśny J, Wielbo J, Podleśna A, Kidaj D (2014a) The responses of two pea genotypes to Nod factors (LCOs) treatment. J Food Agric Environ 12:554–558

Podleśny J, Wielbo J, Podleśna A, Kidaj D (2014b) The pleiotropic effect of extract containing rhizobial Nod factors on pea growth and yield. Cent Eur J Biol 9:396–409

Scherer HW, Pacyna S, Schultz NM (2006) Sulphur supply to peas (*Pisum sativum* L.) influences symbiotic N_2 fixation. Plant Soil Environ 52:72–77

Snoeck C, Verreth C, Hernendes-Lucas I, Martinez-Romero E, Vanderleyden J (2003) Identification of a third sulfate activation system in *Sinorhizobium* sp. strain BR816: the CysDN sulfate activation complex. Appl Environ Microbiol 69:2006–2014

Szulc W, Rutkowska B, Sosulski T, Szara E, Stępień W (2014) Assessment of sulphur demand of crops under permanent fertilization experiment. Plant Soil Environ 60:135–140

Van Hameren B, Hayashi S, Gresshoff PM, Ferguson BJ (2013) Advances in the identification of novel factors required in soybean nodulation, a process critical to sustainable agriculture and food security. J Plant Biol Soil Health 1:1–6

Varin S, Cliquet JB, Personeni E, Avice JC, Lemauviel-Levenant S (2010) How does sulfur availability modify acquisition of white clover (*Trifolium repens* L.)? J Exp Bot 61:225–234

Wielbo J, Marek-Kozaczuk M, Kubik-Komar A, Skorupska A (2007) Increased metabolic potential of Rhizobium spp. is associated with bacterial competitiveness. Can J Microbiol 53:957–967

Zhao FJ, Wood AP, McGrath SP (1999) Effects of sulphur nutrition on growth and nitrogen fixation of pea (*Pisum sativum* L.). Plant Soil 212:209–219

Impact of Sulfate Salinity on the Uptake and Metabolism of Sulfur in Chinese Cabbage

Martin Reich, Tahereh Aghajanzadeh, C. Elisabeth E. Stuiver, Aleksandra Koralewska, and Luit J. De Kok

Abstract Increasing soil salinity is a major threat to crop production in many agricultural areas throughout the world. Although sodium chloride (NaCl) is one of the most abundant salts in soils, others viz. sulfate salts may also be present in high concentrations in some soil types. Sulfate salts, e.g. Na_2SO_4, are still widely underrepresented amongst salt stress studies and the mechanism of its toxicity is poorly understood. Exposure of Chinese cabbage to Na_2SO_4 already reduced growth at levels ≥20 mM, accompanied by an increase in the total sulfur content of both roots and shoots, which in the shoot for a greater part could be ascribed to an accumulation of sulfate. Moreover, there was an increase in the total water-soluble non-protein thiol content (glutathione) in roots and shoots. Enhanced sulfur metabolite levels (sulfate, glutathione) would down-regulate the expression and activity of the sulfate transporters and APS reductase (glutathione). Indeed, Na_2SO_4 exposure resulted in a down-regulation of the sulfate uptake capacity of the roots at ≥5 mM, whereas the transcript level of the sulfate transporters Sultr1;2 and Sultr4;1 and APS reductase in the roots was reduced at ≥20 mM. Apparently in the shoot this regulatory signal transduction pathway was overruled by the toxic effects of Na_2SO_4, since in contrast to the roots, the transcript levels of Sultr4;1 and APS reductase were enhanced in the shoot at ≥30 mM and ≥5 mM Na_2SO_4, respectively.

Salt tolerance of plants and its improvement is one of the most prominent topics in crop research due to both the acuteness of the threats of salinity for agriculture and the complex physiology that underlies salt tolerance in plants (Flowers 2004; Parida and Das 2005; Peleg et al. 2011). Sulfur metabolism may have significance in the tolerance of plants to salinity. For instance, salt stress may result in an enhanced glutathione level, which presumably has adaptive significance in the protection of

M. Reich • T. Aghajanzadeh • C.E.E. Stuiver • A. Koralewska
Laboratory of Plant Physiology, University of Groningen,
P.O. Box 11103, 9700 CC Groningen, The Netherlands

L.J. De Kok (✉)
Laboratory of Plant Physiology, Groningen Institute for Evolutionary Life Sciences,
University of Groningen, Groningen, The Netherlands
e-mail: l.j.de.kok@rug.nl

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_25

plants against reactive oxygen species (Noctor et al. 1998; Mittler 2002; Tausz et al. 2004; Szalai et al. 2009). The production of reactive oxygen species may be increased if Na^+ accumulates in other cell compartments than the vacuole (Zhu et al. 2007). The enhanced glutathione levels appeared to be coupled to increased levels of Na^+ in the cytosol, since an enhanced level of glutathione (and cysteine) was absent in transgenic *Brassica napus* that over-expressed a vacuolar Na^+/H^+ antiporter upon NaCl exposure (Ruiz and Blumwald 2002). However, the most important mechanisms in plants to avoid Na^+ toxicity are the so-called includer/excluder strategies where Na^+ is actively transported either back to the outside of the cell/plant and/or into the vacuole in order to prevent cytosolic Na^+ accumulation (Blumwald 2000; Munns and Tester 2008). These strategies also evolved in halophytes, accompanied by anatomical adaptations such as succulence (increased cell size by salt accumulation in vacuoles) or specialized organs for salt exclusion via the leaves (salt glands). Another crucial factor is the cellular K^+/Na^+ ratio, which needs to be kept high in order to prevent an inhibition of enzymes regulated by K^+ (Maathuis and Amtmann 1999; Tester and Davenport 2003; Chen et al. 2005; Zhu 2007; Cuin et al. 2008). In addition to NaCl plants also may have to deal with Na_2SO_4 salinity (Garcia and Hernandez 1996) and many areas are even dominated by sulfate salts (Chang et al. 1983; Keller et al. 1986). Although salt stress is usually mainly attributed to Na^+ toxicity many studies showed that the accompanying anion might change the severity of the toxicity (e.g. Renault et al. 2001). In many species it had been shown that sulfate salinity might be more toxic than chloride salinity (Eaton 1942; Paek et al. 1988; Bilski et al. 1988; Datta et al. 1995; Renault et al. 2001). The physiological basis of the toxic effects of sulfate salinity has still to be resolved. In this study the impact of Na_2SO_4 salinity on the uptake, distribution and assimilation of sulfate was studied in Chinese cabbage.

Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr. cv. Kasumi F1 (Nickerson-Zwaan, Made, The Netherlands)) was germinated in vermiculite. Ten day-old seedlings were pre-grown on a 10 % Hoagland nutrient solution for 2 days and subsequently grown on a 25 % Hoagland nutrient solution (pH 5.9; for composition see Koralewska et al. 2007) at Na_2SO_4 concentrations of 0.5, 5, 10, 20, 30 and 40 mM in 30 l containers (ten sets per container, three plants per set) which were placed in a climate controlled room for 11 days. Day and night temperatures were 21 and 17 °C (±1 °C), respectively, relative humidity was 60–70 % and the photoperiod was 14 h at a photon flux rate of 230 ± 20 µmol m^{-2} s^{-1} (within the 400–700 nm range) at plant height, supplied by Philips HPI-T (400 W) lamps. After 11 days of exposure plants were harvested and shoot and root fresh weight were determined. For determination of the dry matter content, fresh plant tissue was dried at 80 °C for 24 h. For anion analysis, roots were rinsed in ice-cold de-mineralized water (for 3 × 20 s) to remove sulfate from the free space. Shoots and roots were separated, weighed, frozen in liquid N_2 and stored at −20 °C until further analysis. Anions were extracted from frozen plant material and determined refractometrically after separation by HPLC (Shahbaz et al. 2010). Water-soluble non-protein thiols were extracted from freshly harvested plant tissue (Shahbaz et al. 2010) and the total water-soluble non-protein content was determined colorimetrically according to De Kok et al. (1988). For

determination of the total sulfur and nitrogen contents, oven-dried plant material was pulverized by a Retsch Mixer-Mill (type MM2; Haan, Germany). Total sulfur content was determined with the barium sulfate precipitation method (Koralewska et al. 2008) and total nitrogen content was determined according to a modified Kjeldahl method (Barneix et al. 1988). Sulfate uptake capacity was determined as described by Koralewska et al. (2007). Three sets of plants (three plants per set) per treatment were transferred to 25 % Hoagland solution labeled with ^{35}S-sulfate (2 MBq l^{-1}) and incubated for 30 min at 30 °C, at 0.5 mM Na_2SO_4. Subsequently, plants were removed and roots rinsed in ice-cold non-labeled nutrient solution for 3 × 20 s. Roots and shoots were separated and digested in 1 N HCl at room temperature for 7 days. The extracts were filtered through one layer of Miracloth and 100 µl of the filtrate was mixed with 1 ml Emulsifier Scintillator Plus (Perkin Elmer, Boston, MA, USA). Radioactivity was measured with a liquid scintillation counter (TRI-CARB 2000 CA Liquid Scintillation Analyzer, Perkin Elmer, Waltham, MA, USA). Total RNA from roots and shoots was isolated by a method based on Verwoerd et al. (1989), which involved an additional phenol-chloroform-isoamyl alcohol extraction of the aqueous phase after the first centrifugation, or by using TRI REAGENT™ (SIGMA), a mixture of guanidine thiocyanate and phenol in a mono-phase solution. The final air-dried pellet was dissolved in an appropriate volume of diethyl pyrocarbonate-treated water. The quality of the RNA preparations was checked by electrophoresis of a 2 µg aliquot on a 1 % (w/v) Tris-acetate/agarose gel. The concentration was calculated from the absorbance at 260 nm in water. Determination of the expression of sulfate transporter was carried out according to Church and Gilbert (1984), with pre-hybridization and hybridization at 65 and 60 °C, respectively. Ten µg of total RNA per slot was separated on a 1.2 % (w/v) agarose/formaldehyde gel and blotted onto a positively charged nylon membrane (Hybond-N+). Sequence diversity, especially in the 3′ non-coding region, allowed the use of partial cDNA fragments for gene-specific hybridization to the respective Brassica sulfate transporter mRNA. The cDNA fragments were labeled with 32P-dCTP and used as hybridization probes. After hybridization with probes for sulfate transporters, membranes were washed at 65 °C twice with 2xSSC, 0.1 % SDS for 5 and 30 min, once with 1xSSC, 0.1 % SDS and twice with 0.1xSSC, 0.1 % SDS for at least 30 min each, and exposed to Kodak BioMax MS film or to Cyclone MultiPurpose Phosphor Screen (Perkin Elmer, UK). Statistical analysis was performed with an unpaired Student's t-test.

Although in natural ecosystems Brassicaceae species occur in dry and saline habitats and even in extreme sulfur-enriched gypsum-bearing soils (Ernst 1990; Dixon 2007), some of the modern cultivated hybrids and cultivars appear to be very sensitive to sulfate salinity. The results of the present study showed that the shoot growth of Chinese cabbage was already inhibited at 20 mM Na_2SO_4 (Fig. 1). The shoot growth was slightly more susceptible to salt stress than root growth and the latter was only significantly reduced at 40 mM Na_2SO_4, resulting in a slight decrease in the shoot to root ratio at ≥30 mM Na_2SO_4. Dry matter content of shoots was enhanced at Na_2SO_4 concentrations ≥30 mM, up to twofold at 40 mM, whereas that of roots was only slightly enhanced at 40 mM Na_2SO_4 (Fig. 2).

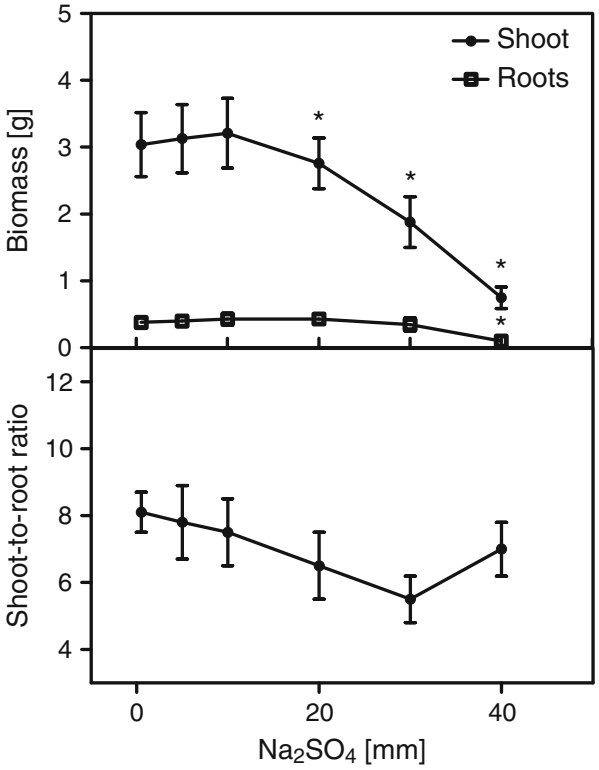


Fig. 1 The impact of Na_2SO_4 salinity on growth and shoot to root ratio of Chinese cabbage. Data represent the mean of two experiments with 15–19 measurements with three plants in each ± SD (* = $p<0.01$; unpaired Student's t-test)

Exposure of plants to Na_2SO_4 salinity resulted in an increase in the total sulfur content of both roots and shoots (Fig. 3), which in the shoot for a greater part could be ascribed to an accumulation of sulfate (Fig. 4). The sulfate content increased gradually with the Na_2SO_4 concentration, but it was strongly enhanced at 40 mM Na_2SO_4 and its content in roots and shoots was increased 1.5-fold and fourfold, respectively. Apparently, the regulatory control of the uptake of sulfate by the roots was overruled at Na_2SO_4 concentrations exceeding 30 mM. In both shoots and roots, total nitrogen and nitrate decreased with the Na_2SO_4 concentration, indicating that the uptake and assimilation of nitrate was negatively affected by sulfate salinity (Figs. 3 and 4). Similar to previous observations there was apparently no direct linkage between the uptake and assimilation of sulfate and nitrate (Stulen and De Kok 2012).

Similar to observations with NaCl (Ruiz and Blumwald 2002), Na_2SO_4 salinity resulted in an increase in the total water-soluble non-protein thiol content (presumably GSH) in roots and shoots at concentrations ≥20 mM (Fig. 5). Evidently this increase remained relatively low and only at toxic Na_2SO_4 concentrations, and at

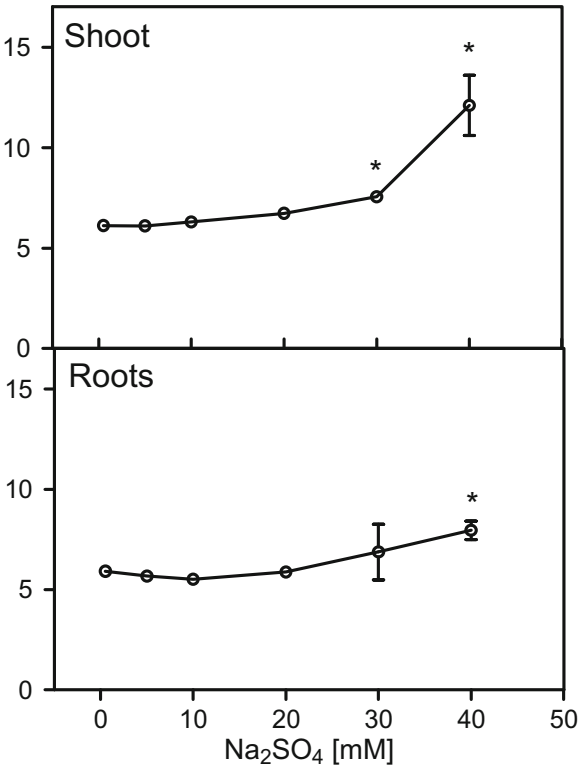


Fig. 2 The impact of Na_2SO_4 salinity on dry matter content of shoot to roots of Chinese cabbage. Data represent the mean of two experiments with three measurements with three plants in each ± SD (* = $p < 0.01$; unpaired Student's t-test)

sulfate levels in shoots fourfold higher than that of the control, a substantial increase of thiol content (twofold) occurred. From the present data, the increased thiol/glutathione level appears to be a consequence of the excessive sulfate accumulation and not an adaptive protective response against salinity.

Sulfate uptake plays a major role in the control of plant sulfur homeostasis (Vauclare et al. 2002). The uptake and distribution of sulfate is mediated by distinct sulfate transporters, which activity may be controlled at a transcriptional, translational and/or post-translational level, and is regulated by the plant sulfur requirement for growth (Hawkesford and De Kok 2006; De Kok et al. 2011). Sulfate salinity had a substantial effect on the expression and activity of the sulfate transporters and the expression of APS reductase of Chinese cabbage (APR; Fig. 6). However, there were considerable differences in response of the different sulfate transporters and APS reductase between roots and shoots of Chinese cabbage. The Group 1 transporters are responsible for the primary uptake of sulfate by the root and in sulfate-sufficient Brassica species only Sultr1;2 is expressed (Hawkesford and De Kok 2006; Koralewska et al. 2007, 2008, 2009; De Kok et al. 2011). Also upon Na_2SO_4 salinity Sultr1;2 was the sole Group 1 sulfate transporter expressed in roots and the transcript levels of Sultr1;1 were negligible (Fig. 6). Na_2SO_4 salinity resulted in a decreased expression of Sultr1;1 in the roots at ≥30 mM, whereas the

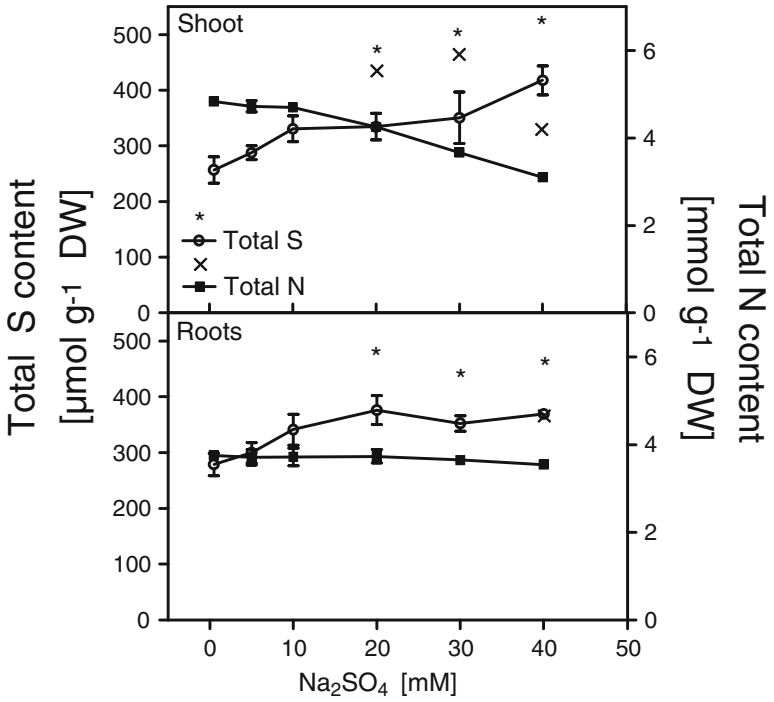


Fig. 3 The impact of Na_2SO_4 salinity on total sulfur and nitrogen content of shoots and roots of Chinese cabbage. Data represent the mean of three measurements with 12 plants in each ± SD (*/x = $p < 0.01$; unpaired Student's t-test)

sulfate uptake capacity was already decreased ≥5 mM. The latter further decreased with the Na_2SO_4 concentration and was reduced more than 2.5-fold at 40 mM (Fig. 6). Apparently, Na_2SO_4 salinity affected the regulation of the sulfate transporters in the roots already at lower concentrations at translational and/or post translational than at transcriptional level. The Group 4 transporters are involved in the vacuolar efflux of sulfate (Hawkesford 2003; Kataoka et al. 2004; Hawkesford and De Kok 2006; De Kok et al. 2011) and there was also a decrease in the transcript level of Sultr4;1 in roots at 40 mM Na_2SO_4, whereas Sultr4;2 was hardly expressed at all. The latter was in agreement with previous observations that Sultr1;1 and Sultr4;2 were only expressed in sulfate-deprived Brassica tissue (Koralewska et al. 2009; De Kok et al. 2012; Shahbaz et al. 2014). The transcript level of APR, the key regulating enzyme in the sulfate reduction pathway (Hawkesford and De Kok 2006; De Kok et al. 2011) was also reduced in the roots at ≥30 mM (Fig. 6). Sultr1;1 was hardly and Sultr4;2 was only slightly expressed in both roots and shoots. Sulfate salinity only resulted in an increased transcript level of Sultr4;1 and Sultr4;2 at 40 mM Na_2SO_4, whereas that of APR was increased at ≥5 mM Na_2SO_4 (Fig. 6). Of the Group 1 and 4 transporters, only Sulftr4;2 was substantially expressed in the shoot of Chinese cabbage (Fig. 5). Contrary to the observations in the root, its

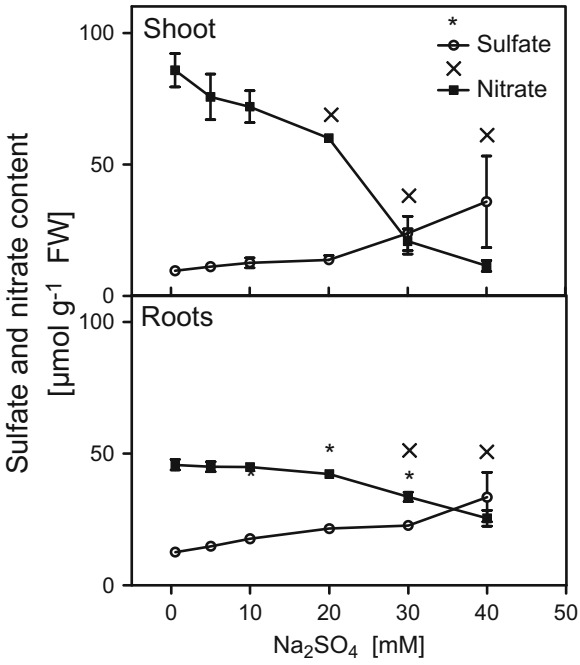


Fig. 4 The impact of Na_2SO_4 salinity on sulfate and nitrate content of shoot and roots of Chinese cabbage. Data represent the mean of three measurements with three plants in each ± SD (*/x = $p<0.01$; unpaired Student's t-test)

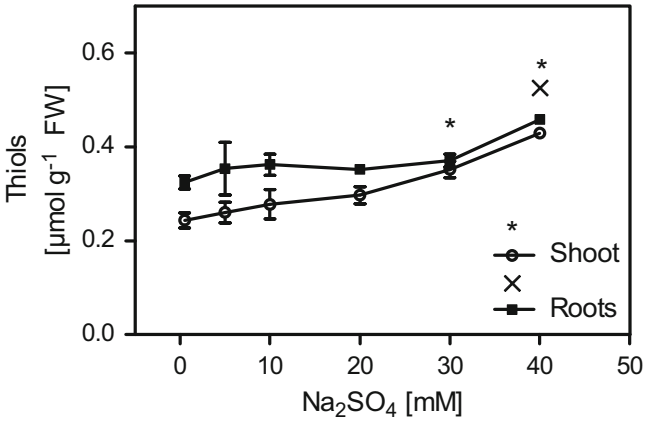


Fig. 5 The impact of Na_2SO_4 salinity on total water-soluble non-protein thiols of shoot and roots of Chinese cabbage. Data represent the mean of three measurements with three plants in each ± SD (x = $p<0.01$; unpaired Student's t-test)

transcript levels increased at ≥30 mM Na_2SO_4, together with that of APS reductase (Fig. 6). The latter needs to be further investigated. It has been suggested that sulfate itself, or reduced sulfur compounds, may have a role in the regulation of expression and activity of the sulfate transporters and APS reductase (Hawkesford and De Kok 2006; De Kok et al. 2011). For instance, high tissue levels of these compounds

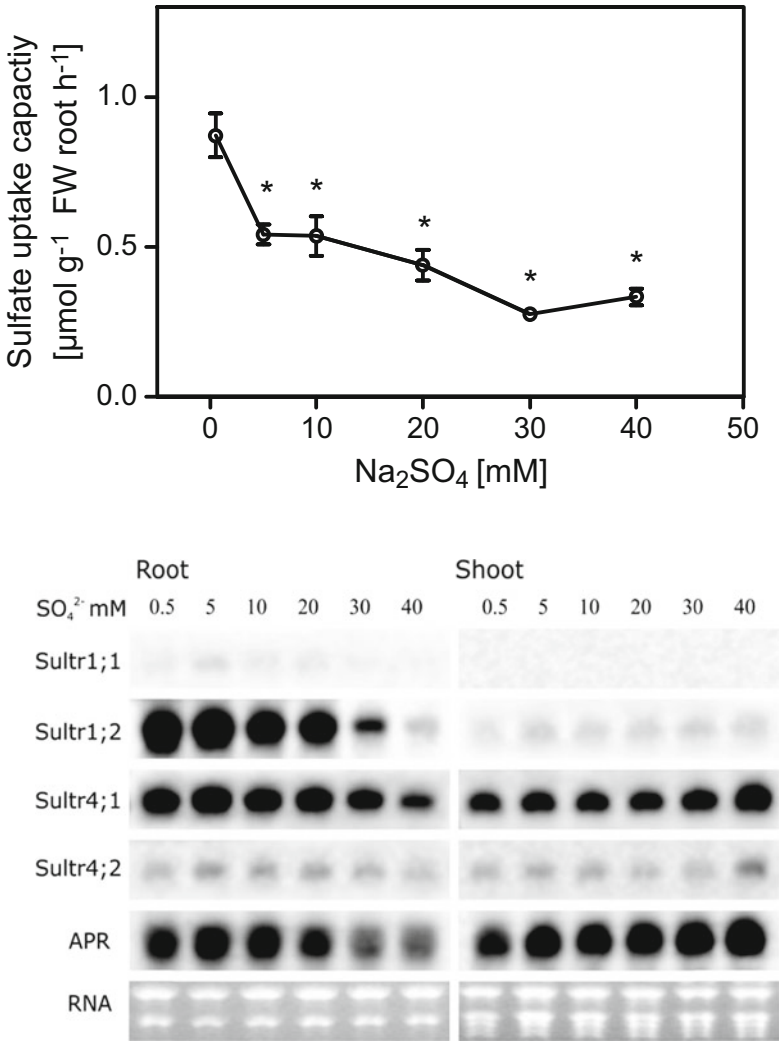


Fig. 6 The impact of Na_2SO_4 salinity on sulfate uptake capacity and gene expression of sulfate transporters (Sultr) and APS reductase (APR; Northern blot analysis) of shoot and roots of Chinese cabbage. Equal RNA loading was determined by ethidium bromide staining of gels (shown in the *bottom* panels). Data on sulfate uptake capacity represent the mean of three measurements with three plants in each ±SD (* = $p < 0.01$; unpaired Student's t-test)

would down-regulate the expression and activity of the sulfate transporters (sulfate, glutathione) and APS reductase (glutathione). Indeed, in the roots this relationship between the content of these sulfur compounds and the expression and activity of the sulfate transporters and expression of APS reductase does exist although in the shoot the toxic effects of Na_2SO_4 salinity apparently overruled this regulatory signal transduction pathway.

Sulfate salinity was described as having a greater inhibitory effect on growth than chloride salinity in wheat (Datta et al. 1995), sugar beet and tomato (Eaton 1942), wild potato (Bilski et al. 1988), pepper (Navarro et al. 2003) and on germination in barley (Huang and Redmann 1995), alfalfa (Redmann 1974) and wheat (Hampson and Simpson 1990). Comparative studies within *Brassica* species are still very scarce. Additionally, the results of a study by Paek et al. (1988) on calli of *B. campestris* revealed that Na_2SO_4 had a stronger negative impact on biomass. The authors of the study also noted that sulfate accumulated much less under Na_2SO_4 than chloride under NaCl salinity. This unequal uptake of Na^+ and its anion under sulfate salinity could explain the increased inhibitory effect on growth (also concluded by Meiri et al. 1971; Navarro et al. 2003). Another observation from older studies is that excess sulfate inhibits calcium uptake (Hayward and Wadleigh 1949) but this also holds true for NaCl and the application of additional calcium usually leads to an amelioration of salt stress (Cramer 2002; Kaya et al. 2002; Shabala et al. 2006). However, Johansen and Loneragan (1975) observed that Na_2SO_4 reduced 75 % of K^+ uptake compared to the absence of Na^+ while the same concentration of NaCl reduced it only by 50 %. A link to cation homeostasis therefore seems likely. Interestingly, Na^+ accumulated mainly in shoots when jack pines were exposed to NaCl whereas it mainly accumulated in the roots under Na_2SO_4 salinity (Apostol et al. 2002). This is another hint that the translocation to the shoot and its control might be a crucial process under salt stress. Besides promoting the toxicity of Na^+, sulfate could also have direct toxic effects (Visscher et al. 2010).

Salt tolerance is known to be a physiological complex and trait, which challenges attempts at improvement. As *Brassica* is a diverse genus with high agricultural importance, many efforts have been made to identify and develop salt tolerant cultivars. Salt tolerance in *B. napus*, for example, could be increased tremendously in transgenic plants with an enhanced Na^+ accumulation in the vacuole (Zhang et al. 2001). In another transgenic approach, plants of *B. juncea* with an introduced bacterial pathway for the synthesis of glycine-betaine showed increased germination rates and seedling growth (Prasad et al. 2000) but the occurrence of sulfate salinity and its increased toxicity compared with chloride salinity also suggests correlations of salt tolerance with sulfate uptake, assimilation and whole plant distribution. More research needs to be carried out on the toxicity of excessive sulfate and its possible role in exacerbating Na^+ toxicity.

References

Apostol KG, Zwiazek JJ, MacKinnon MD (2002) NaCl and Na_2SO_4 alter responses of jack pine (*Pinus banksiana*) seedlings to boron. Plant Soil 240:321–329

Barneix AJ, Cooper HD, Stulen I, Lambers H (1988) Metabolism and translocation of nitrogen in two Lolium perenne populations with contrasting rates of mature leaf respiration and yield. Physiol Plant 72:631–636

Bilski JJ, Nelson DC, Conlon RL (1988) Response of six wild potato species to chloride and sulfate salinity. Am Potato J 65:605–612

Blumwald E (2000) Sodium transport and salt tolerance in plants. Curr Opin Cell Biol 12:431–434
Chang C, Sommerfeldt TG, Carefoot JM, Schaalje GB (1983) Relationships of electrical conductivity with total dissolved salts and cation concentration of sulfate-dominant soil extracts. Can J Soil Sci 63:79–86
Chen Z, Newman I, Zhou M, Mendham N, Zhang G, Shabala S (2005) Screening plants for salt tolerance by measuring K^+ flux: a case study for barley. Plant Cell Environ 28:1230–1246
Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci U S A 81:1991–1995
Cramer GR (2002) Sodium-calcium interactions under salinity stress. In: Läuchli A, Lüttge U (eds) Salinity: environment-plants-molecules. Springer, Dordrecht, pp 205–227
Cuin TA, Betts SA, Chalmandrier R, Shabala S (2008) A root's ability to retain K^+ correlates with salt tolerance in wheat. J Exp Bot 59:2697–2706
Datta KS, Kumar A, Varma SK, Angrish R (1995) Differentiation of chloride and sulphate salinity on the basis of ionic distribution in genetically diverse cultivars of wheat. J Plant Nutr 18:2199–2212
De Kok LJ, Buwalda F, Bosma W (1988) Determination of cysteine and its accumulation in spinach leaf tissue upon exposure to excess sulfur. J Plant Physiol 133:502–505
De Kok LJ, Stulen I, Hawkesford MJ (2011) Sulfur nutrition in crop plants. In: Hawkesford MJ, Barraclough P (eds) The molecular basis of nutrient use efficiency in crops. Wiley-Blackwell, Oxford, pp 295–309
De Kok LJ, Stuiver CEE, Shahbaz M, Koralewska A (2012) Regulation of expression of sulfate transporters and APS reductase in leaf tissue of Chinese cabbage (*Brassica pekinensis*). In: De Kok LJ, Tausz M, Hawkesford MJ, Hoefgen R, McManus MT, Norton RM, Rennenberg H, Saito K, Schnug E, Tabe L (eds) Sulfur metabolism in plants: mechanisms and application to food security, and responses to climate change. Springer, Dordrecht, pp 47–52
Dixon GR (2007) Vegetable brassicas and related crucifers (No. 14). CABI, Cambridge, MA
Eaton FM (1942) Toxicity and accumulation of chloride and sulfate salts in plants. J Agr Res 64:357–399
Ernst WHO (1990) Ecological aspects of sulfur metabolism. In: Rennenberg H, Brunold C, De Kok LJ, Stulen I (eds) Sulfur nutrition and sulfur assimilation in higher plants – fundamental environmental and agricultural aspects. SPB Academic Publishing, The Hague, pp 131–144
Flowers TJ (2004) Improving crop salt tolerance. J Exp Bot 55:307–319
Garcia C, Hernandez T (1996) Influence of salinity on the biological and biochemical activity of a calciorthird soil. Plant Soil 178:255–263
Hampson CR, Simpson GM (1990) Effects of temperature, salt, and osmotic potential on early growth of wheat (Triticum aestivum). I. Germination. Can J Bot 68:524–528
Hawkesford MJ (2003) Transporter gene families in plants: the sulphate transporter gene family – redundancy or specialization? Physiol Plant 117:155–163
Hawkesford MJ, De Kok LJ (2006) Managing sulfur metabolism in plants. Plant Cell Environ 29:382–395
Hayward HE, Wadleigh CH (1949) Plant growth on saline and alkali soils. Adv Agron 1:1–38
Huang J, Redmann RE (1995) Salt tolerance of *Hordeum* and *Brassica* species during germination and early seedling growth. Can J Plant Sci 75:815–819
Johansen C, Loneragan JF (1975) Effects of anions and cations on potassium absorption by plants of high potassium chloride content. Funct Plant Biol 2:75–78
Kataoka T, Watanabe-Takahashi A, Hayashi N, Onishi M, Mimura T, Buchner P, Hawkesford MJ, Yamaya T, Takahashi H (2004) Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in Arabidopsis. Plant Cell 16:2693–2704
Kaya C, Kirnak H, Higgs D, Saltali K (2002) Supplementary calcium enhances plant growth and fruit yield in strawberry cultivars grown at high (NaCl) salinity. Sci Hortic 93:65–74
Keller LP, McCarthy GJ, Richardson JL (1986) Mineralogy and stability of soil evaporites in North Dakota. Soil Sci Soc Am J 50:1069–1071

Koralewska A, Posthumus FS, Stuiver CEE, Buchner P, Hawkesford MJ, De Kok LJ (2007) The characteristic high sulfate content in *Brassica oleracea* is controlled by the expression and activity of sulfate transporters. Plant Biol 9:654–661

Koralewska A, Stuiver CEE, Posthumus FS, Kopriva S, Hawkesford MJ, De Kok LJ (2008) Regulation of sulfate uptake, expression of the sulfate transporters Sultr1;1 and Sultr1;2, and APS reductase in Chinese cabbage (*Brassica pekinensis*) as affected by atmospheric H_2S nutrition and sulfate deprivation. Funct Plant Biol 35:318–327

Koralewska A, Buchner P, Stuiver CEE, Posthumus FS, Kopriva S, Hawkesford MJ, De Kok LJ (2009) Expression and activity of sulfate transporters and APS reductase in curly kale in response to sulfate deprivation and re-supply. J Plant Physiol 166:168–179

Maathuis FJ, Amtmann A (1999) K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. Ann Bot 84:123–133

Meiri A, Kamburoff J, Poljakoff-Mayber A (1971) Response of bean plants to sodium chloride and sodium sulphate salinization. Ann Bot 35:837–847

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681

Navarro JM, Garrido C, Martínez V, Carvajal M (2003) Water relations and xylem transport of nutrients in pepper plants grown under two different salts stress regimes. Plant Growth Regul 41:237–245

Noctor G, Arisi ACM, Jouanin L, Kunert KJ, Rennenberg H, Foyer CH (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J Exp Bot 49:623–647

Paek KY, Chandler SF, Thorpe TA (1988) Physiological effects of Na_2SO_4 and NaCl on callus cultures of *Brassica campestris* (Chinese cabbage). Physiol Plant 72:160–166

Parida AK, Das AB (2005) Salt tolerance and salinity effects on plants: a review. Ecotoxicol Environ Saf 60:324–349

Peleg Z, Apse MP, Blumwald E (2011) Engineering salinity and water-stress tolerance in crop plants: getting closer to the field. Adv Bot Res 57:405–443

Prasad KVSK, Sharmila P, Kumar PA, Saradhi PP (2000) Transformation of *Brassica juncea* (L.) Czern with bacterial codA gene enhances its tolerance to salt stress. Mol Breeding 6:489–499

Redmann RE (1974) Osmotic and specific ion effects on the germination of alfalfa. Can J Bot 52:803–808

Renault S, Croser C, Franklin JA, Zwiazek JJ (2001) Effects of NaCl and Na2SO4 on red-osier dogwood (*Cornus stolonifera* Michx) seedlings. Plant Soil 233:261–268

Ruiz J, Blumwald E (2002) Salinity-induced glutathione synthesis in *Brassica napus*. Planta 214:965–969

Shabala S, Demidchik V, Shabala L, Cuin TA, Smith SJ, Miller AJ, Davies JM, Newman IA (2006) Extracellular Ca^{2+} ameliorates NaCl-induced K^+ loss from Arabidopsis root and leaf cells by controlling plasma membrane K^+-permeable channels. Plant Physiol 141:1653–1665

Shahbaz M, Hwei Tseng M, Stuiver CEE, Koralewska A, Posthumus FS, Venema JH, Parmar S, Schat H, Hawkesford MJ, De Kok LJ (2010) Copper exposure interferes with the regulation of the uptake, distribution and metabolism of sulfate in Chinese cabbage. J Plant Physiol 167:438–446

Shahbaz M, Stuiver CEE, Posthumus FS, Parmar S, Hawkesford MJ, De Kok LJ (2014) Copper toxicity in Chinese cabbage is not influenced by plant sulfur status, but interferes with correlations between sulfur metabolism-related gene expression and the suggested regulatory metabolites. Plant Biol 16:68–78

Stulen I, De Kok LJ (2012) Exploring interactions between sulfate and nitrate uptake at a whole plant level. In: De Kok LJ, Tausz M, Hawkesford MJ, Hoefgen R, McManus MT, Norton RM, Rennenberg H, Saito K, Schnug E, Tabe L (eds) Sulfur metabolism in plants: mechanisms and application to food security, and responses to climate change. Springer, Dordrecht, pp 1–8

Szalai G, Kellős T, Galiba G, Kocsy G (2009) Glutathione as an antioxidant and regulatory molecule in plants under abiotic stress conditions. J Plant Growth Regul 28:66–80

Tausz M, Šircelj H, Grill D (2004) The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid? J Exp Bot 55:1955–1962

Tester M, Davenport R (2003) Na^+ tolerance and Na^+ transport in higher plants. Ann Bot 91:503–527

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, Von Ballmoos P, Krähenbühl U, Den Camp RO, Brunold C (2002) Flux control of sulphate assimilation in *Arabidopsis thaliana*: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. Plant J 32:729–740

Verwoerd TC, Dekker BM, Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res 17:2362

Visscher AM, Paul AL, Kirst M, Guy CL, Schuerger AC, Ferl RJ (2010) Growth performance and root transcriptome remodeling of Arabidopsis in response to Mars-like levels of magnesium sulfate. PLoS One 5:e12348

Zhang HX, Hodson JN, Williams JP, Blumwald E (2001) Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proc Natl Acad Sci U S A 98:12832–12836

Zhu J, Fu X, Koo YD, Zhu JK, Jenney FE, Adams MW, Zhu Y, Shi H, Yun DY, Hasegawa PM, Bressan RA (2007) An enhancer mutant of Arabidopsis salt overly sensitive 3 mediates both ion homeostasis and the oxidative stress response. Mol Cell Biol 27:5214–5224

Zhu JK (2007) Plant salt stress. In: Encyclopedia of life sciences. Wiley, Chichester

Identification and Distribution of Selenium-Containing Glucosinolate Analogues in Tissues of Three Brassicaceae Species

Marian J. McKenzie, Adam J. Matich, Ronan K.-Y. Chen, Ross E. Lill, Tony K. McGhie, and Daryl D. Rowan

Abstract The Brassicaceae are known for their capacity to produce and accumulate the sulfur (S)-rich glucosinolates, which have appreciable human health benefits. Selenium (Se) is chemically very similar to S and many plant enzymes appear unable to distinguish between these two elements. Thus Se may be metabolized through many of the S uptake and assimilation pathways. We were interested in the effect of Se-fertilization on the production of glucosinolate compounds in Brassicaceae. We fertilized broccoli, cauliflower and forage rape with Na_2SeO_4 and examined the glucosinolates produced in four tissues (tap root, stem, leaf and floret) of these plants using liquid chromatography–mass spectrometry (LC-MS). Several Se-containing glucosinolates were identified and measured. In each case, the Se atom substituted for the S atom normally found in the methylthioalkyl moiety of the glucosinolate and was presumably donated by selenomethionine. The highest concentration of these new Se-containing glucosinolates was in broccoli florets and forage rape roots. In forage rape leaves the majority of the methylthio class of glucosinolates was selenized. Se fertilization also appeared to increase the concentration of the non-selenized methylthioglucosinolates in the shoot tissues of these *Brassica* species. Our results show that Se and S metabolism of *Brassica* tissues vary in their responses to Se fertilization and that several enzymes of the glucosinolate biosynthetic and metabolism pathways can use Se in place of S to generate Se-containing glucosinolates.

Selenium (Se) is an essential micronutrient for human health, needed for the production of approximately 25 selenoenzymes that require Se at their catalytic site for activity (Kryukov et al. 2003). However, the recommended daily intake (RDI) for Se can be difficult to achieve in countries where soil Se concentrations are low, such as in New Zealand, the UK, Finland and Germany (Broadley et al. 2006; Thomson 2004).

M.J. McKenzie (✉) • A.J. Matich • R.K.-Y. Chen • R.E. Lill • T.K. McGhie • D.D. Rowan
The New Zealand Institute for Plant & Food Research Limited,
Private Bag 11600, Palmerston North 4442, New Zealand
e-mail: marian.mckenzie@plantandfood.co.nz

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_26

The Brassicaceae have an active sulfur (S) biochemistry, being well known for the production of the S-rich glucosinolates which are deterrents to herbivores and whose breakdown products have demonstrated cancer prevention benefits in mammals (Stoner and Morse 1997). Selenium is chemically similar to S, being immediately below it in the chalcogen group of the Periodic Table, and Se is taken up and metabolized by the enzymes of the S-uptake and assimilatory pathway, which are apparently unable to distinguish between these two elements (Sors et al. 2005). The Brassicaceae are able to produce Se-containing compounds such as methylselenocysteine, which confer additional health benefits (Ip et al. 1991) and there is increasing interest in the use of *Brassica* species as simultaneous sources of both S- and Se-containing health-enhancing compounds.

Recently, selenosulforaphane and a range of isoselenocyanates, Se-containing analogues of natural glucosinolate breakdown products, have been chemically synthesized and demonstrated to have a greater bioactivity than their S-analogues in *in vitro* cancer assays (Emmert et al. 2010; Sharma et al. 2009). In nature, these synthetic compounds would correspond to the breakdown products of glucosinolates where Se had been incorporated into the glucose bridge (position 1, Fig. 1). We have also identified several naturally occurring Se-containing glucosinolates and their breakdown products from broccoli and cauliflower floret material, and forage rape tap roots (Matich et al. 2012). Characterization of these compounds indicated that Se was only ever present in the position normally occupied by S in the glucosinolate side chain and derived biosynthetically from methionine (position 3, Fig. 1). As the Se has not been incorporated into the glucose bridge (position 1, Fig. 1), these plant-produced glucosinolates cannot breakdown to produce the reported synthetic selenosulforaphane and isoselenocyanates and may show different bioactivities.

We were interested in further investigating (i) the concentration and distribution of the Se-glucosinolates in several tissues (floret, leaf, stem and tap root) of these three *Brassica* species; (ii) the effect of Se fertilization on the production of the S-containing glucosinolates; and (iii) if there was any evidence of *in planta* production of Se-glucosinolates containing the Se atom on the glucose bridge. Our previous work indicated that the Se-glucosinolates were all methylthioglucosinolate

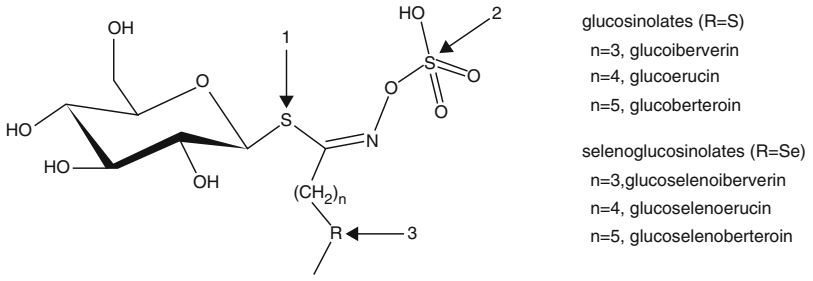


Fig. 1 Structure of the methylthio- and methylseleno- glucosinolates, with possible positions for selenium (Se) substitution indicated by numbered *arrows*. *R* denotes the position of the amino acid methionine

analogues (Matich et al. 2012) and therefore we focused on these compounds, which constitute approximately 10 % of the total glucosinolate pool in the Brassicaceae.

Broccoli (*Brassica oleracea* L. var. *italica* 'Booster'), cauliflower (*Brassica oleracea* L. var. *botrytis* 'Liberty') and forage rape (*Brassica napus* 'Maxima') plants were grown and fertilized with Se as previously described (Matich et al. 2012). Each plant received 20 mL of 5 mM sodium selenate twice weekly for 4 weeks prior to harvest, which was at commercial maturity for broccoli and cauliflower. Samples of floret, leaf, stem and tap root material were harvested from broccoli and cauliflower plants; and leaf, stem and tap root material from forage rape plants. Three replicates of each sample were taken, each replicate comprising material from two individual plants. All samples were immediately frozen prior to liquid chromatography–mass spectrometry (LC-MS) analysis as previously described (Matich et al. 2012).

Analysis of the methylthioglucosinolates confirmed our previous discovery of three Se-containing analogues, glucoselenoiberverin, glucoselenoerucin and glucoselenoberteroin (Fig. 1). These compounds vary from each other in the length of the carbon side chain, having one carbon atom difference between the compounds (n = 3–5) and with the Se atom always found in the side chain (position 3, Fig. 1). In cauliflower, glucoiberverin was the major methylthioglucosinolate detected and was found in the tap root, stem and floret tissue (Table 1). Selenium fertilization reduced the concentration of this compound, particularly in the tap root, and possibly in the stem. Conversely, Se fertilization appeared to double the glucoiberverin concentration in the floret tissue (Table 1); however, the size of the SEM values (reflecting the variability in the data) for this compound should not be ignored. Glucoerucin, and particularly glucoberteroin, were minor components of the methylthioglucosinolate group. Selenium fertilization also reduced the concentration of glucoerucin in tap root and stem tissue, and increased its concentration in the floret material.

Table 1 Mean concentrations (mg kg^{-1} FW as glucoerucin equivalents) of methylthiol- and methylseleno-glucosinoates measured by LC-MS in tissue of 'Liberty' cauliflower with and without fertilization with 5 mM sodium selenate

	Tap root		Stem		Leaf		Floret	
	−Se	+Se	−Se	+Se	−Se	+Se	−Se	+Se
Glucoiberverin	29 ± 19	1.8 ± 3.1	34 ± 2	23 ± 37	nd	nd	20 ± 4	45 ± 43
Glucoerucin	1.0 ± 0.6	0.9 ± 0.3	11 ± 8	5 ± 4	nd	nd	1.3 ± 0.3	2.4 ± 0.7
Glucoberteroin	nd	nd	0.3[a]	nd	nd	nd	0.05[a]	nd
Glucoselenoiberverin	nd	nd	nd	0.6	nd	nd	nd	2.5[a]
Glucoselenoerucin	nd	nd	nd	nd	nd	nd	nd	nd
Glucoselenoberteroin	nd	nd	nd	nd	nd	nd	nd	nd

Concentrations are the average ± SEM of three biological replicates
nd not detected
[a]Detected in only one replicate

The production of the methylselenoglucosinolates in cauliflower was very limited, with glucoselenoiberverin being the only methylselenoglucosinolate detected (Table 1). The majority of this compound was found in the floret tissue, but at concentrations substantially lower than those of its S-analogue.

Broccoli produced a much greater complement of the methylthioglucosinolates than cauliflower, with glucoerucin being the major methylthioglucosinolate detected, at concentrations of up to 500 mg kg^{-1} FW in the tap root material, followed by half that in the stem and 70-fold less in the floret tissue (Table 2). Se fertilization did not appear to affect the concentration of the methylthioglucosinolates in tap root or stem tissues markedly, although a small decrease was noted. In the floret material, a four- to six-fold increase was observed in the content of all three methylthioglucosinolates following Se fertilization (Table 2), and a five-fold increase in glucoerucin also occurred in the leaves.

Glucoselenoerucin was the major methylselenoglucosinolate detected in broccoli. This compound was most abundant in the floret tissue, and was found at a similar concentration to that of its S-analogue in this tissue (Table 2). The two minor methylselenoglucosinolates, glucoselenoiberverin and glucoselenoberteroin, were also found predominantly in the floret tissue of broccoli and also at similar concentrations to those of their S-analogues.

Forage rape produced the highest concentration of methylthioglucosinolates of the three species tested. The predominant compound was glucoberteroin, and this was present at up to 880 mg kg^{-1} FW in the tap root, and about half that concentration in the stems (Table 3). Glucoerucin, the second most abundant methylthioglucosinolate produced by forage rape, was found at a similar concentration to that in broccoli in the tap root and at a slightly lower concentration in the stem tissue. Se fertilization resulted in a small decrease in glucoberteroin and glucerucin content in the tap root and stem tissue, but a three- to five-fold increase of these same compounds in the leaf tissue (Table 3). Glucoselenoerucin was the predominant methylselenoglucosinolate detected in forage rape following Se fertilization, and was found mainly in the tap root, but was also present in the stem and leaves (Table 3). In general, the concentrations of methylselenoglucosinolates in forage rape were many-fold less than those of their S-analogues. However, in the leaves glucoselenoerucin was 53-fold higher than that of its S-analogue, so that 98 % of the erucin type compounds in the leaf tissue were selenized (Table 3).

Of the three *Brassica* species investigated, forage rape contained the highest concentration of methyl(thio/seleno)glucosinolates, followed by broccoli and cauliflower. These compounds were most abundant in the tap root and stem tissues of the three species. In contrast, the methylselenoglucosinolates were found at the highest concentrations in the floret tissue of broccoli and cauliflower and, in proportion to their S-analogues, in the leaf tissue of forage rape (which did not produce floret material at its apical meristem). That is, they were most concentrated in the sink tissues of all the plants investigated. In broccoli, the concentrations of the methylselenoglucosinolates in the floret tissue were equivalent to those of the S-analogues (Table 2), and in forage rape leaf, the total concentration of the methylselenoglucosinolate exceeded that of the S-analogues, with the bulk of erucin being selenized

Table 2 Mean concentrations (mg kg^{-1} FW as glucoerucin equivalents) of methylthiol- and methylseleno-glucosinoates measured by LC-MS in tissue of 'Booster' broccoli with and without fertilization with 5 mM sodium selenate

	Tap root		Stem		Leaf		Floret	
	−Se	+Se	−Se	+Se	−Se	+Se	−Se	+Se
Glucoiberverin	27 ± 4	19 ± 5	6.2 ± 0.4	5.3 ± 4.3	nd	0.54 ± 0.49	0.4 ± 0.04	1.8 ± 0.3
Glucoerucin	500 ± 28	453 ± 170	254 ± 86	197 ± 86	0.24 ± 0.1	1.1 ± 0.3	7 ± 1	27 ± 2
Glucoberteroin	12 ± 1	11.9 ± 5.9	3.1 ± 1.3	2.5 ± 0.5	nd	nd	0.09 ± 0.02	0.56 ± 0.11
Glucoselenoiberverin	nd	0.54 ± 0.25	nd	0.3 ± 0.3	nd	0.3[a]	nd	1.9 ± 0.5
Glucoselenoerucin	nd	4.1 ± 0.9	nd	1.4 ± 0.4	nd	3.1 ± 2.4	nd	30 ± 4
Glucoselenoberteroin	nd	nd	nd	nd	nd	nd	nd	0.13 ± 0.02

Concentrations are the average ± SEM of three biological replicates
nd not detected
[a]Detected in only one replicate

Table 3 Mean concentrations (mg kg^{-1} FW as glucoerucin equivalents) of methylthiol- and methylseleno-glucosinoates measured by LC-MS in tissue of 'Maxima' forage rape with and without fertilization with 5 mM sodium selenate

	Tap root		Stem		Leaf	
	−Se	+Se	−Se	+Se	−Se	+Se
Glucoiberverin	5.2 ± 6.6	1 ± 1	0.1[a]	nd	nd	nd
Glucoerucin	470 ± 330	360 ± 52	192 ± 128	127 ± 68	0.05 [a]	0.17 ± 0.07
Glucoberteroin	880 ± 403	780 ± 184	448 ± 103	354 ± 186	1.8 [a]	8.1 ± 6.7
Glucoselenoiberverin	nd	0.4 ± 0.5	nd	nd	nd	0.09
Glucoselenoerucin	nd	28 ± 11	nd	8.4 ± 4.6	nd	8.6 ± 5
Glucoselenoberteroin	nd	7 ± 3	nd	2.4 ± 1.3	nd	3.8 ± 1.8

Concentrations are the average ± SEM of three biological replicates unless otherwise stated
nd not detected
[a]Detected in only one replicate

(Table 3). These results suggest that in the floret and leaf tissues of *Brassica*, Se-containing substrates may be preferentially used for the production of the methylthio/selenoglucosinolates. There are few examples of proteins that specifically target Se-containing over S-containing substrates in plants. Selenocysteine methyltransferase specifically methylates selenocysteine instead of cysteine (Neuhierl and Bock 1996), and there is recent evidence that one of the S-transporters from *Stanleya pinnata* may transport Se in preference to S (Harris et al. 2014). In humans, selenocysteine lyase has been found to act only on selenocysteine and this specificity is provided by a single crucial amino acid residue, Asp146 (Collins et al. 2012). Further investigation is necessary to determine if similar enzymatic mechanisms are responsible for the apparent accumulation of the methylselenoglucosinolates in the sink tissues of the Brassicaceae, or if this is due to increased translocation of Se compared with S to the sink tissues, as occurs in some Se-hyperaccumulating plants (Galeas et al. 2007).

The effect of Se fertilization on the production of the methylthioglucosinolates was also investigated in the four tissues of the *Brassica* species. Previous studies focusing on glucosinolate production in Se-fertilized Brassicaceae showed that when Se is applied at high rates, glucosinolate production is adversely affected (Charron et al. 2001; Robbins et al. 2005; Toler et al. 2007). However, if applied at lower rates, such as those suitable for human consumption of a food *Brassica*, glucosinolate production does not decline (Hsu et al. 2011). In this research, different tissues responded differently to the same rate of Se application, with the sink tissues (floret and leaf) apparently accumulating methylthioglucosinolates, while concentrations in the tap root and stem tended to decline. However, our research targeted the methylthioglucosinolates, which are not the major glucosinolates in any of the Brassicaceae investigated. It would be of interest to determine if this finding also applied to other classes of glucosinolates.

The Se atom in the methylselenoglucosinolates measured in this work was always present in the carbon side chain (position 3, Fig. 1). This confirms our previous findings (Matich et al. 2012), and suggests the production of glucosinolate com-

pounds with the Se atom in the glucose bridge position (Emmert et al. 2010; Sharma et al. 2009) does not readily occur *in planta*. This is disappointing considering the increased bioactivity of the synthetic Se-containing compounds in animal cancer model systems. However, further analysis of the dominant glucosinolates: glucoraphanin in broccoli, glucobrassicin in cauliflower and 2-phenylethylglucosinolate in forage rape, following Se-fertilization, may identify the presence of such compounds.

In the research presented here, we have identified and quantified three Se-containing methylglucosinolates in three *Brassica* species, and determined the distribution of these compounds across four tissue types. We also investigated the effect of Se fertilization on the production and distribution of the non-selenated S-analogues of these compounds. It is apparent that Se fertilization at the rate used here allows the production of the methylselenoglucosinolates, with minimal negative effect on the S-analogues, particularly in the sink tissues, where increased contents were observed. It remains to be determined if the breakdown products of those methylselenoglucosinolates measured *in planta* also exhibit the interesting bioactivities shown by the synthetically produced selenosulforaphane and isoselenocyanates.

References

Broadley MR, White PJ, Bryson RJ, Meacham MC, Bowen HC, Johnson SE, Hawkesford MJ, McGrath SP, Zhao FJ, Breward N, Harriman M, Tucker M (2006) Biofortification of UK food crops with selenium. Proc Nutr Soc 65:169–181

Charron CS, Kopsell DA, Randle WM, Sams CE (2001) Sodium selenate fertilisation increases selenium accumulation and decreases glucosinolate concentration in rapid-cycling *Brassica oleracea*. J Sci Food Agric 81:962–966

Collins R, Johansson A-L, Karlberg T, Markova N, van den Berg S, Olesen K, Hammarstrom M, Flores A, Schuler H, Schiavone LH, Brzezinski P, Arner ESJ, Hogbom M (2012) Biochemical discrimination between selenium and sulfur 1: a single residue provides selenium specificity to human selenocysteine lyase. PLoS One 7(1):e30581

Emmert SW, Desai D, Amin S, Richie JP (2010) Enhanced Nrf2-dependent induction of glutathione in mouse embryonic fibroblasts by isoselenocyanate analog of sulforaphane. Bioorg Med Chem Lett 20:2675–2679

Galeas ML, Zhang LH, Freeman JL, Wegner M, Pilon-Smits EAH (2007) Seasonal fluctuations of selenium and sulfur accumulation in selenium hyperaccumulators and related nonaccumulators. New Phytol 173:517–525

Harris J, Schneberg KA, Pilon-Smits EAH (2014) Sulfur-selenium-molybdenum interactions distinguish selenium hyperaccumulator *Stanleya pinnata* from non-hyperaccumulator *Brassica juncea* (Brassicaceae). Planta 239:479–491

Hsu FC, Wirtz M, Heppel SC, Bogs J, Kramer U, Khan MS, Bub A, Hell R, Rausch T (2011) Generation of Se-fortified broccoli as functional food: impact of Se fertilization on S metabolism. Plant Cell Environ 34:192–207

Ip C, Hayes C, Budnick RM, Ganther HE (1991) Chemical form of selenium, critical metabolites, and cancer prevention. Cancer Res 51:595–600

Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigo R, Gladyshev VN (2003) Characterization of mammalian selenoproteomes. Science 300:1439–1443

Matich AJ, McKenzie MJ, Lill RE, Brummell DA, McGhie TK, Chen RKY, Rowan DD (2012) Selenoglucosinolates and their metabolites produced in Brassica spp. fertilised with sodium selenate. Phytochemistry 75:140–152

Neuhierl B, Bock A (1996) On the mechanism of selenium tolerance in selenium-accumulating plants – purification and characterization of a specific selenocysteine methyltransferase from cultured cells of *Astragalus bisculatus*. Eur J Biochem 239:235–238

Robbins RJ, Keck AS, Banuelos G, Finley JW (2005) Cultivation conditions and selenium fertilization alter the phenolic profile, glucosinolate, and sulforaphane content of broccoli. J Med Food 8:204–214

Sharma A, Sharma AK, Madhunapantula SV, Desai D, Huh SJ, Mosca P, Amin S, Robertson GP (2009) Targeting Akt3 signaling in malignant melanoma using isoselenocyanates. Clin Cancer Res 15:1674–1685

Sors TG, Ellis DR, Salt DE (2005) Selenium uptake, translocation, assimilation and metabolic fate in plants. Photosynth Res 86:373–389

Stoner GD, Morse MA (1997) Isothiocyanates and plant polyphenols as inhibitors of lung and esophageal cancer. Cancer Lett 114:113–119

Thomson CD (2004) Selenium and iodine intakes and status in New Zealand and Australia. Br J Nutr 91:661–672

Toler HD, Charron CS, Sams CE, Randle WR (2007) Selenium increases sulfur uptake and regulates glucosinolate metabolism in rapid-cycling *Brassica oleracea*. J Am Soc Hortic Sci 132:14–19

Selenate Differentially Alters the Content of Glucosinolates in *Eruca sativa* and *Diplotaxis tenuifolia* Grown in Soil

Michela Schiavon, Stefano Dall'acqua, Chiara Berto, and Mario Malagoli

Abstract Selenium (Se) is a fundamental microelement for several organisms, including humans. Higher plants represent the main dietary source of Se and their enrichment in Se may influence sulfur (S) accumulation and consequently, the synthesis of health promoting S-containing compounds. In this study, the effect of selenate foliar fertilization on the production of total and specific glucosinolates (GSL) was investigated in two rocket species, *Eruca sativa* and *Diplotaxis tenuifolia*. Se accumulated in the leaves of the two plant species at a similar rate, but exerted an opposite effect on S content when supplied at high dose. Specifically, selenate fertilization with 10 mg Se per plant increased the leaf level of sulfur in *E. sativa* and reduced it in *D. tenuifolia*. The trend of leaf GSL accumulation in both plant species was strongly consistent with the variation in S content. We conclude that Se at high dosage affects the production of GSL in rocket plants, the increase or reduction depending on the plant species, while low doses of selenate do not significantly influence S and GSL contents in edible tissues. Furthermore, variations in S content mediated by Se are indicative of changes in the level of GSL.

Selenium (Se) is an essential nutrient for humans, animals and certain microorganisms, because it enters in the composition of crucial enzymes involved in major metabolic pathways (Rayman 2012). Furthermore, some organic Se compounds such as methyl-selenocysteine (MSeC), are well-known anti-carcinogens (Ellis and Salt 2003). In higher plants, a role for Se is still not clear and little is known on how it affects their nutritional qualities (Pilon-Smits et al. 2009). Se in the form of selenate is taken up by plants via sulfate transporters and assimilated through the sulfur

M. Schiavon • M. Malagoli (✉)
Department of Agronomy, Food, Natural Resources, Animal and Environment, University of Padova, Agripolis, Viale dell'Università, 16, 35020 Legnaro, Padova, Italy
e-mail: mario.malagoli@unipd.it

S. Dall'acqua • C. Berto
Department of Pharmaceutical and Pharmacological Sciences, University of Padova, 35131 Padova, Italy

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*, Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_27

(S) assimilation pathway in the S analogues amino acids Se-cysteine (SeCys) and Se-methionine (SeMet; Terry et al. 2000). Se can alter S uptake depending on the plant species and the dose of Se applied (White et al. 2004; Schiavon et al. 2012). As a result, the content of some S-containing compounds, such as glucosinolates (GSL), may be affected. GSL are synthesized by plants as a defense mechanism against insect and herbivore predators (Rask et al. 2000; Mithöfer and Boland 2012). Within the plant cells, GSLs are hydrolyzed by the enzyme myrosinase to produce indoles and isothiocyanates (ITC), which act as cancer-preventive compounds in mammals (Rask et al. 2000; Dinkova-Kostova 2013). GSLs are exclusively found in members of the *Brassicaceae* family, including *Eruca Sativa* (*rocket*) and *Diplotaxis tenuifolia* (*wild rocket*) (Finley et al. 2005). These two plant species differ in antioxidant profile (Pasini et al. 2011) and might display different responses to Se in terms of GSL production. Since information is scarce about the effects of Se on S-secondary metabolites, in this study we evaluated the effect of foliar Se application on the biosynthesis of GSL in these.

Plants were cultivated in pots containing peat, soil and perlite in the ratio 60:30:10 with a density of one plant per pot, and divided in four groups with five plants each. After 1 month a unique foliar application of selenate (Na_2SeO_4) to three of the plant groups was performed at dosages of 2.5, 5 or 10 mg per plant. One group of plants was sprayed with an equal volume of water and served as control. Se and S in leaves and roots of rocket plants were determined using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES), while the content of GSL was measured via HPLC-MS. The experimental design for seedling growth was randomized and the entire experiment was replicated two times.

Selenate fertilization resulted in a similar leaf accumulation of Se in the two plant species (Fig. 1). The trend of Se accumulation was exponential and dose-dependent, but *D. tenuifolia* displayed a lower capacity to translocate Se to the roots, especially when exposed to 10 mg Se per plant. *D. tenuifolia* accumulated more S than *E. sativa* in both leaves and roots. However, Se fertilization caused a significant decrease of S in roots of *D. tenuifolia* (Fig. 2). The reduction of S concentration was evident in this plant species and also in leaves sprayed with 10 mg Se per plant. Conversely, *E. sativa* leaves accumulated more S when exposed to the same Se dosage. Consistent with changes of S level in plants, selenate at high doses stimulated the leaf synthesis of GSL in *E. sativa* and repressed it in *D. tenuifolia* (Fig. 3). Glucosativin, DMB-GSL and glucoraphanin, whose levels were the highest among the identified GSL (Table 1), were influenced by selenate, and their variation in content accounted for the different trend in GSL accumulation observed between *E. sativa* and *D. tenuifolia* (Fig. 3).

We conclude that *E. sativa* and *D. tenuifolia* are both good accumulators of Se. Differences in GSL accumulation between the two cultivars reflected the variation in S content due to selenate fertilization. This meant that the impact of foliar Se application to plants of S-containing products with known health benefits was not only dependent upon the dose of selenate, but it was also species-specific. The use of Se biofortification strategies in members of the *Brassicaceae* family to obtain

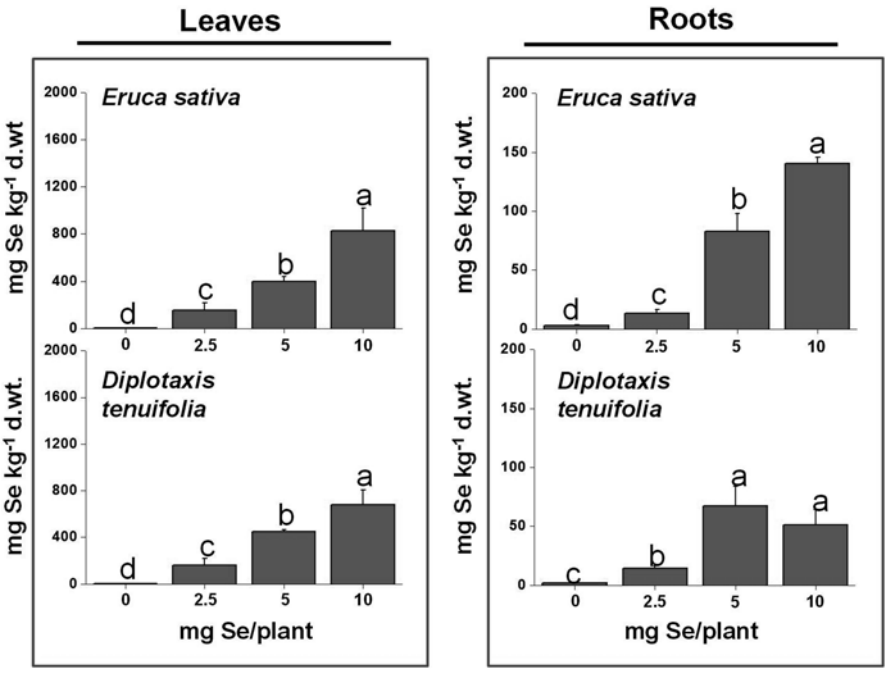


Fig. 1 Selenium accumulation in leaves and roots of *E. sativa* and *D. tenuifolia* plants cultivated in pots for 1 month and then sprayed with selenate dosages ranging from 0 (control) to 10 mg Se plant^{-1}. Plants were harvested after 1 week from Se treatment. Data represent mean values (n = 5; ± SD). Different *letters* above *bars* indicate significant differences between treatments ($p < 0.05$, Student's *t*-test)

functional food should evaluate the secondary effects of Se accumulation in the synthesis of pivotal S compounds. In particular, the peculiarities of the plant species to be fortified must be considered, as Se can differentially affect the content of GSL compounds. Lower accumulation of GSL in edible plant tissues will result in a reduction of food health promoting properties.

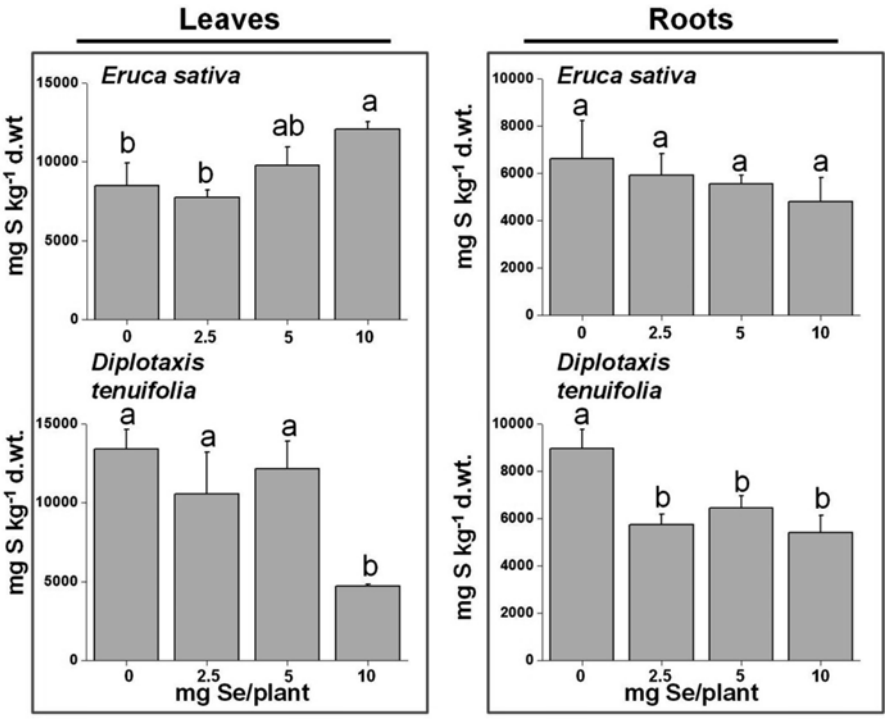


Fig. 2 Impact of selenate fertilization on leaf and root sulfur accumulation in *E. sativa* and *D. tenuifolia*. Plants were cultivated in pots for 1 month and then sprayed with selenate dosages ranging from 0 (control) to 10 mg Se plant^{-1}. Plant harvest was after 1 week from Se treatment. Data represent mean values (n = 5; ± SD). Different *letters* above *bars* indicate significant differences between treatments ($p < 0.05$, Student's *t*-test)

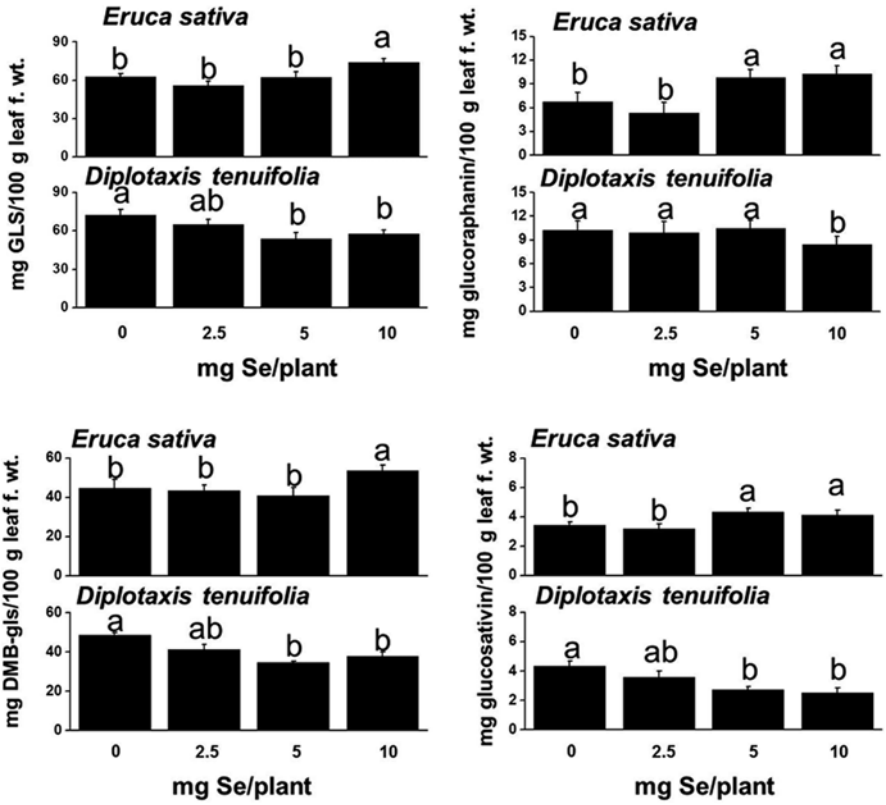


Fig. 3 Impact of selenate fertilization on the content of total glucosinolates, glucoraphanin, DMB-GSL (dimeric-4 mercaptobutyl) and glucosativin in leaves of *E. sativa* and *D. tenuifolia*. Plants were cultivated in pots for 1 month and then sprayed with selenate dosages ranging from 0 (control) to 10 mg Se plant^{-1}. Plant harvest was after 1 week from Se treatment. Data represent mean values (n = 5; ± SD). Different *letters* above *bars* indicate significant differences between treatments ($p < 0.05$, Student's *t*-test)

Table 1 Glucosinolates identified in leaves of *E. sativa* and *D. tenuifolia* plants cultivated in soil

Ions $[M_{DS}+H]^+$	Ions $[M_{DS}+Na]^+$	Ions $[M_{DS}+K]^+$	Ions $[2M_{DS}+Na]^+$	Compound
	364			Glucoerucin
	380			Glucoraphanin
	675			DMB-GSL
	394			Glucoalissin
399		437		Metoglucobrassicin
328				Glucosativin
			791	4-hydroxyglucobrassicin
	421			Neoglucobrassicin

References

Dinkova-Kostova AT (2013) Chemoprotection against cancer by isothiocyanates: a focus on the animal models and the protective mechanisms. Top Curr Chem 329:179–201

Ellis DR, Salt DE (2003) Plant, selenium and human health. Curr Opin Plant Biol 6:273–279

Finley JW, Sigrid-Keck A, Robbins RJ, Hintze KJ (2005) Selenium enrichment of broccoli: interactions between selenium and secondary plant compounds. J Nutr 135:1236–1238

Mithöfer A, Boland W (2012) Plant defense against herbivores: chemical aspects. Annu Rev Plant Biol 63:431–450

Pasini F, Verardo V, Cerretani L, Caboni MF, D'Antuono LF (2011) Rocket salad (*Diplotaxis* and *Eruca* spp.) sensory analysis and relation with glucosinolate and phenolic content. J Sci Food Agric 91:2858–2864

Pilon-Smits EAH, Quinn CF, Tapken W, Malagoli M, Schiavon M (2009) Physiological functions of beneficial elements. Curr Opin Plant Biol 12:267–274

Rask L, Andréasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J (2000) Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol 42:93–113

Rayman MP (2012) Selenium and human health. Lancet 379:1256–1268

Schiavon M, Pittarello M, Pilon-Smits EAH, Wirtz M, Hell R, Malagoli M (2012) Selenate and molybdate alter sulfate transport and assimilation in *Brassica juncea* L. Czern.: Implications for phytoremediation. Environ Exp Bot 75:41–51

Terry N, Zayed AM, de Souza MP, Tarun AS (2000) Selenium in higher plants. Annu Rev Plant Physiol Mol Biol 51:401–432

White PJ, Bowen HC, Parmaguru P, Fritz M, Spracklen WP, Spiby RE, Meacham MC, Mead A, Harriman M, Trueman LJ, Smith BM, Thomas B, Broadley MR (2004) Interactions between selenium and sulphur nutrition in *Arabidopsis thaliana*. J Exp Bot 55:1927–1937

Effects of Glutathione Concentration in the Root Zone and Glutathione Treatment Period on Cadmium Partitioning in Oilseed Rape Plants

Shin-ichi Nakamura, Hikari Kondo, Nobuo Suzui, Yong-Gen Yin, Satomi Ishii, Naoki Kawachi, Hiroki Rai, Hiroyuki Hattori, and Shu Fujimaki

Abstract Glutathione is a sulfur-containing peptide involved in various aspects of plant metabolism. Glutathione is also known to have effects on heavy metal responses in plants. In our previous work, we have found glutathione, applied to roots site-specifically, inhibited cadmium (Cd) translocation from roots to shoots and Cd accumulation in shoots in oilseed rape plants. In addition, we succeeded in visualizing inhibition of root-to-shoot translocation of Cd by using a positron-emitting tracer imaging system (PETIS). In this work, the effects of glutathione concentration in the root zone (hydroponic solution) and the glutathione treatment period on Cd partitioning in oilseed rape plants were investigated. Our experimental results demonstrated that glutathione, exceeding a certain concentration in the root zone, is needed to trigger inhibition of Cd translocation, and that treatment time from the start of glutathione application had different effects on Cd partitioning in oilseed rape plants.

Cd is one of the toxic heavy metals, which can cause serious problems to humans when it enters the food chain (Obata and Umebayashi 1997). Various efforts have been made in order to establish a new method of reducing Cd from crop plants. However, a highly effective method has yet to be developed and put to practical use. To develop a novel method, it is necessary to investigate the behaviour of Cd in plants and what controls it. Glutathione is known to act as a metal chelator and as a substrate for synthesis of phytochelatins (Clemens 2001). These physiological

S.-i. Nakamura (✉) • H. Kondo • H. Rai • H. Hattori
Department of Biological Production, Faculty of Bioresource Sciences, Akita Prefectural University, 241-438 Kaidobata-Nishi, 010-0195 Shimoshinjo-Nakano, Akita, Akita, Japan
e-mail: sinnaka@akita-pu.ac.jp

N. Suzui • Y.-G. Yin • S. Ishii • N. Kawachi • S. Fujimaki
Radiotracer Imaging Group, Quantum Beam Science Center, Japan Atomic Energy Agency, 1233 Watanuki, 370-1292 Takasaki, Gunma, Japan

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*, Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5_28

functions of glutathione suggest that glutathione is related to heavy metal behavior in plant bodies. Glutathione concentration in sieve elements increased in response to Cd treatment (Nakamura et al. 2005), which suggests that glutathione might be playing important roles in controlling Cd behavior in plants. Our experiments focused on the effects of glutathione on Cd behavior in plant bodies. Previously we found that glutathione, applied to roots site-specifically, inhibited Cd translocation from roots to shoots and Cd accumulation in shoots in oilseed rape plants (Nakamura et al. 2013). We also revealed that one of the causes of the phenomenon is activation of Cd efflux from roots by glutathione. Application of this phenomenon to a novel cultivation method enables us to reduce Cd accumulation in crop plants. It is required to establish an optical application of glutathione to plants in order to maximize the inhibitory effect of glutathione, applied to roots, on Cd translocation from roots to shoots. In this work, we investigated the effects of glutathione concentration in the root zone and glutathione treatment period on Cd behavior in oilseed rape plants.

Oilseed rape plants (*Brassica napus* L. cv. Nourin No. 16) were grown in a growth chamber under controlled growth conditions, following methods described in Nakamura et al. (2008). In each experiment, plants were exposed to 10 μM Cd ($CdCl_2$). When the effects of glutathione concentration in the root zone on Cd behavior was investigated, 0.001, 0.01, 0.1 and 1 mM glutathione (GSH, redox form of glutathione) were added to nutrient solutions. After 2 days of treatment, each plant was harvested. When the effects of the glutathione treatment period on Cd behaviour was tested, plants were treated with Cd and GSH for 1, 2, 3, 6, 9 and 14 days. After each treatment, the plants were harvested. In each harvest, the Cd content of the shoots and roots were measured, following methods described in Nakamura et al. (2013). The Cd translocation ratio was calculated from the Cd content of the shoots and roots and their dry weights. PETIS experiments using 2-week old seedlings were performed, following the methods of Fujimaki et al. (2010) and Ishikawa et al. (2011) with a small modification. Imaging data obtained from the PETIS experiments were analyzed in detail to investigate the effects of glutathione on the behavior of Cd in plants.

To examine its effects, glutathione was applied to roots at different concentrations and the Cd content of oilseed rape plants were measured after 2 days of exposure. In the control plants, the Cd content of the shoots was about 0.4 μmol g^{-1} dry weight (DW) (Fig. 1a). Cd contents were roughly threefold lower in plants exposed to 1 mM GSH in the root zone (Fig. 1a). Inhibitory effects were still seen in plants exposed to 0.1 mM GSH (Fig. 1a). However, these effects were lost when plants were exposed to 0.01 mM GSH (Fig. 1a). The Cd translocation ratio decreased significantly when plants were treated with 1 mM GSH (Fig. 1a). These experimental results suggested that glutathione, exceeding a certain concentration in the root zone (hydroponic solution), is needed to inhibit Cd translocation from roots to shoots.

The effects of the glutathione treatment period on Cd accumulation in shoots of oilseed rape plants were investigated. In the control plants, Cd content of shoots reached saturation after 9 days of Cd exposure (Fig. 2a). After 2 weeks of treatment, the Cd content of shoots from control plants were about 1.5 μmol g^{-1} DW (Fig. 2a).

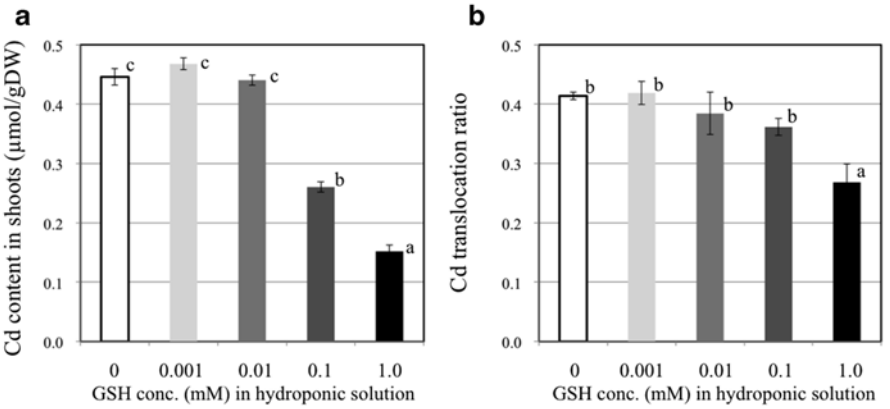


Fig. 1 (**a**) Cd content of shoots of oilseed rape plants harvested after treatment. Each plant was treated with 10 μM Cd for 2 days. GSH concentration in a hydroponic solution is indicated in *horizontal axis* of the graph. (**b**) Cd translocation ratio of oilseed rape plants, calculated from Cd content of shoots and roots and their dry weights. Data are means ± SE (n > 3). Means labeled with different letters are significantly different according to Student's *t*-test ($P < 0.05$)

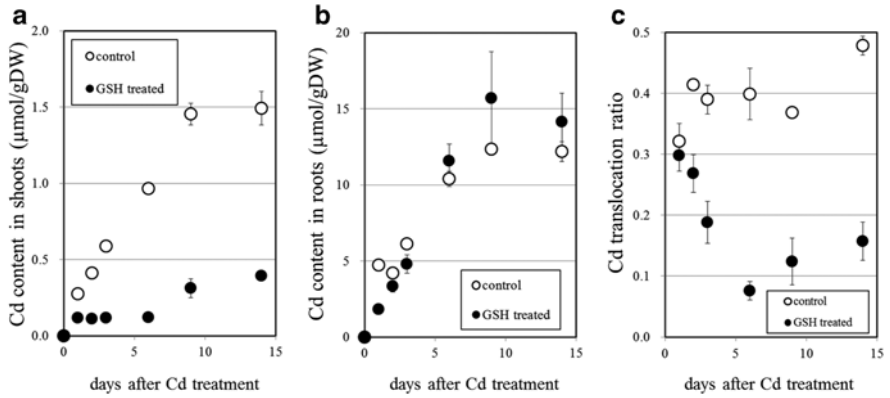


Fig. 2 Cd content of shoots (**a**) and roots (**b**) of oilseed rape plants harvested after treatment. Test plants were treated with 10 μM Cd for 1, 2, 3, 6, 9 or 14 days. In GSH treated plants, GSH concentration in the hydroponic solution was 1 mM. **c** Cd translocation ratio of oilseed rape plants, calculated from Cd content of shoots and roots and their dry weights. Data are means ± SE (n > 3)

The Cd content of shoots of glutathione treated plants increased gradually for 2 weeks. It can be seen from these experimental results that Cd accumulation capacity in shoots of these oilseed rape plants is about 1.5 μmol/g DW. After 2 weeks of GSH treatment, the Cd content of shoots of these plants was about 0.4 μmol g^{-1} DW (Fig. 2a). The Cd content of shoots of GSH treated plants continued to increase over the 2 weeks of the experiment (Fig. 2a). The Cd content of roots of both control plants and GSH treated plants reached saturation after 9 days of Cd exposure (Fig. 2b). After 2 weeks of treatment, the Cd content of the roots of the control

plants and the GSH treated plants was about 12 and 15 μmol g^{-1} DW, respectively (Fig. 2b). The Cd content of the roots of the control plants and GSH treated plants increased in the same way until several days after the start of treatment. The difference in the Cd content of the roots increased gradually when the GSH treatment period became longer (Fig. 2b). These experimental results suggest that application of GSH, exceeding a certain period, activated the ability to accumulate Cd in the roots with differences in the Cd translocation ratio due to changes in ability to accumulate Cd in the roots (Fig. 2c). These experimental results demonstrated that GSH, applied to roots, plays an important role in activating Cd accumulation in roots in addition to activation of Cd efflux from roots.

Figure 3 contains autoradiographs of plants at the end of the PETIS experiments and shows inhibition of root-to-shoot translocation of Cd by glutathione (Fig. 3). The difference in Cd accumulation occurred particularly at the shoot base and leaf. Imaging data from the PETIS experiments were analyzed in detail.

Figure 4 indicated changes in the relative Cd distribution in oilseed rape plants. Cd absorption was suppressed by glutathione treatment (Fig. 4). GSH and

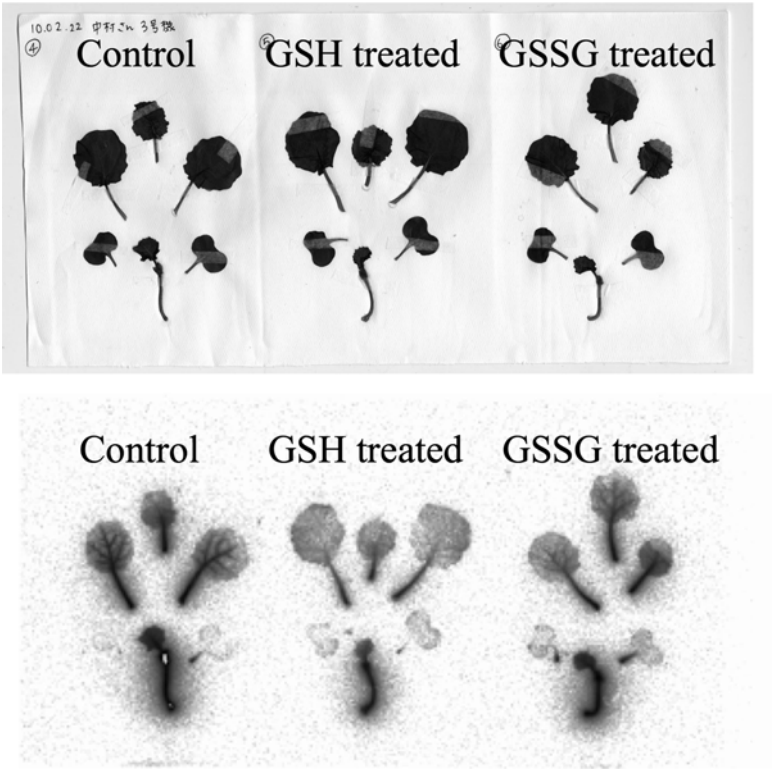


Fig. 3 Autoradiography of plants at the end of PETIS experiment. Images of Cd accumulation in each plant are shown as an autoradiograph (*bottom*). The corresponding optical observations of each plant are also shown (*upper*)

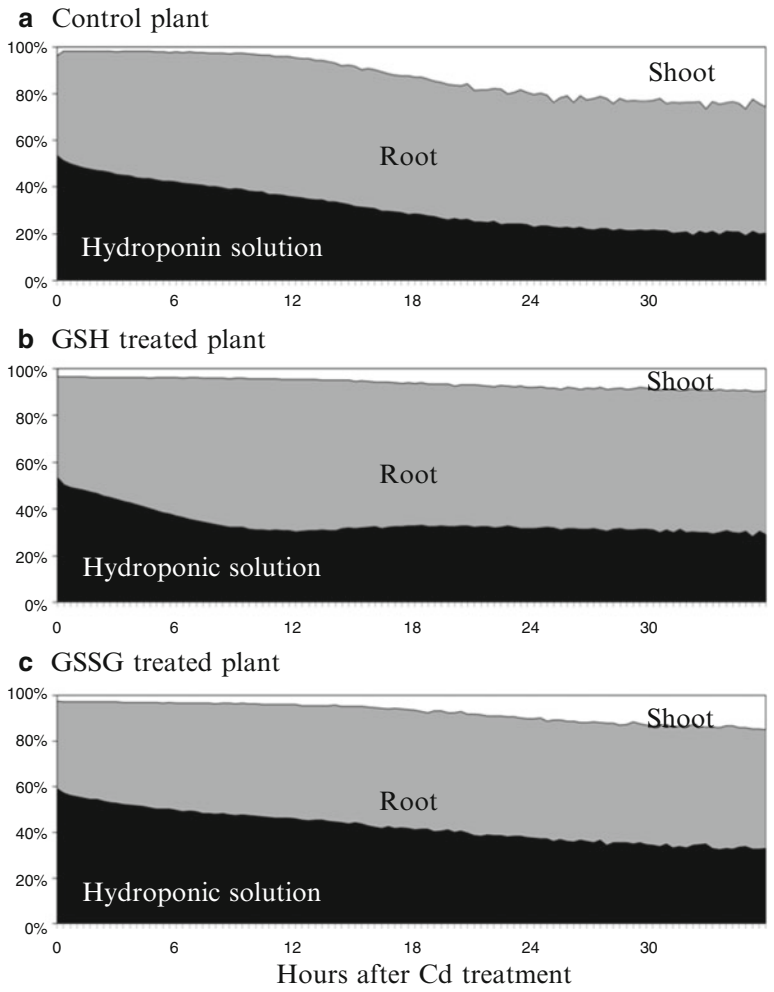


Fig. 4 Changes in the relative Cd distribution in oilseed rape plants. Time course of Cd accumulation in these plants is re-plotted on a relative scale. Total Cd amount, applied to each experiment, is plotted as 100 % from the imaging data. (**a**) Control plant, (**b**) GSH treated plant, (**c**) GSSG treated plant

glutathione oxidized form (GSSG) had similar effects with respect to suppression of Cd absorption (Fig. 4). However, the difference occurred in Cd distribution in the shoots of GSH treated plants and GSSG treated plants. In previous work, it was shown that Cd distribution was similar in those plants harvested after 2 weeks of treatment (Nakamura et al. 2013). GSH and GSSG can be changed to each form in plant bodies. It is reported that the GSH/GSSG ratio functions as a signal (Noctor et al. 2002). Determination of GSH/GSSG ratio in plant bodies is the topic of our further study.

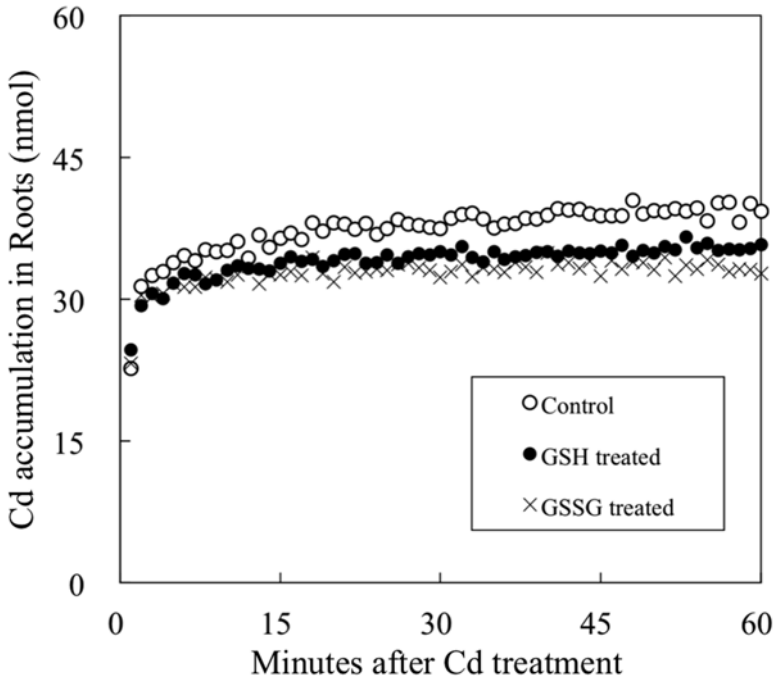


Fig. 5 Close-up of the first hour of Cd accumulation in the roots of oilseed rape plants. Time course of Cd accumulation in these plants is re-plotted from the imaging data

Figure 5 shows a close-up of the first hour of Cd accumulation in the roots of oilseed rape plants. It is reported that the capacity of plant roots to accumulate Cd involves both a physical process and a physiological process. The physical process contributes to Cd accumulation in the early stages of Cd absorption (Yoshihara et al. 2013). There were no differences in the pattern of Cd accumulation in the control plants and the glutathione (GSH and GSSG) treated plants. Reese et al. (1988) found that the binding of Cd and GSH may occur in a weak acidic solution. However, these experimental results suggest GSH in a hydroponic solution has no effects on the physical process of Cd accumulation in plant roots.

This study showed that glutathione concentration in the root zone and the glutathione treatment period had significant impacts on Cd distribution in oilseed rape plants. Further research is needed to establish the best application conditions for suppressing Cd accumulation in crop plants. Glutathione is thought to decompose easily in soils and a method to keep the presence of glutathione in soils is needed. In addition, research is required into the molecular mechanisms involved in the inhibition of Cd translocation from roots to shoots by glutathione. These experimental results enable us to establish a novel cultivation method for suppressing Cd accumulation in crop plants more effectively.

Acknowledgments We gratefully thank Dr. Ken'ichi Ogawa (Research Institute for Biological Sciences Okayama) for providing us with GSSG and Mr. H. Suto (Tokyo Nuclear Services Co., Ltd) for his technical assistance in producing ^{107}Cd by ion beam irradiation. This study was supported in part by the Grant-in-Aid for Scientific Research (no. 23380194, 26292183 to S.N. and no. 17380194, 23380155 to S.F.).

References

Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212:475–486

Fujimaki S, Suzui N, Ishioka NS, Kawachi N, Ito S, Chino M, Nakamura S (2010) Tracing cadmium from culture to spikelet: non-invasive imaging and quantitative characterization of absorption, transport and accumulation of cadmium in an intact rice plant. Plant Physiol 152:1796–1806

Ishikawa S, Suzui N, Ito-Tanabata S, Ishii S, Igura M, Abe T, Kuramata M, Kawachi N, Fujimaki S (2011) Real-time imaging and analysis of differences in cadmium dynamics in rice cultivars (*Oryza sativa*) using positron-emitting^{107}Cd tracer. BMC Plant Biol 11:172

Nakamura S, Maruyama K, Watanabe A, Hattori H, Chino M (2005) Response of glutathione in the sieve tube of *Brassica napus* L. to cadmium treatment. In: Saito K, De Kok LJ, Stulen I, Hawkesford MJ, Schnug E (eds) Sulfur transport and assimilation in plants in the post genomic era. Backhuys Publishers, Leiden, pp 229–232

Nakamura S, Akiyama C, Sasaki T, Hattori H, Chino M (2008) Effect of cadmium on the chemical composition of xylem exudate from oilseed rape plants (*Brassica napus* L.). Soil Sci Plant Nutr 54:118–127

Nakamura S, Suzui N, Nagasaka T, Komatsu F, Ishioka NS, Ito-Tanabata S, Kawachi N, Rai H, Hattori H, Chino M, Fujimaki S (2013) Application of glutathione to roots selectively inhibits cadmium transport from roots to shoots in oilseed rape. J Exp Bot 64:1073–1081

Noctor G, Gomez L, Vanacker H, Foyer CH (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signaling. J Exp Bot 53:1283–1304

Obata H, Umebayashi M (1997) Effects of cadmium on mineral nutrient concentration in plants differing in tolerance for cadmium. J Plant Nutr 20:97–105

Reese RN, Mehra RK, Tarbet E, Winge DR (1988) Studies on the γ-glutamyl Cu-binding peptide from *Schizosaccharomyces pombe*. J Biol Chem 263:4186–4192

Yoshihara T, Suzui N, Ishii S, Kitazaki M, Yamazaki H, Kitazaki K, Kawachi N, Yin Y-G, Ito-Tanabata S, Hashida S, Shoji K, Shimada H, Goto F, Fujimaki S (2013) A kinetic analysis of cadmium accumulation in a Cd hyper-accumulator fern, *Athyrium yokoscense* and tobacco plants. Plant Cell Environ 37:1086–1096

Index

L.J. De Kok et al. (eds.), *Molecular Physiology and Ecophysiology of Sulfur*,
Proceedings of the International Plant Sulfur Workshop,
DOI 10.1007/978-3-319-20137-5