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

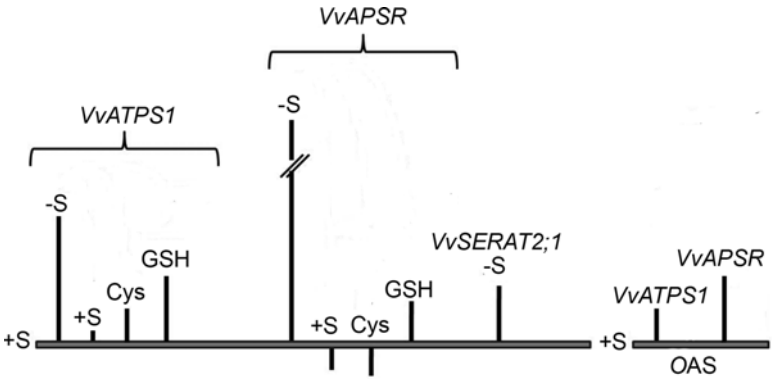

Fig. 1 Relative expression analyzed by RT- qPCR of *VvATPS1*, *VvAPSR* in *V. vinifera* S-depleted cells transferred to +S (1.5 mM) or receiving 1 mM GSH or 1 mM cysteine for 24 h; and in +S cells supplemented with 0.5 mM OAS. The relative expression of *VvSERAT2;1* in S-depleted cells is also shown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

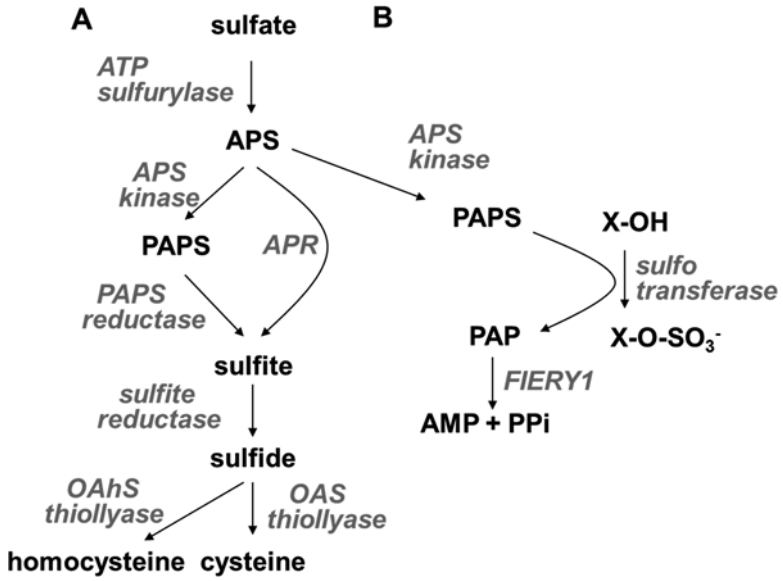

Fig. 1 Scheme of primary (**A**) and secondary (**B**) sulfate metabolism

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.