Quo Vadis Sulfur Investigation?: 25 Years of Research into Plant Sulfate Reduction

Stanislav Kopriva

Abstract Sulfate assimilation is an essential pathway of plant primary metabolism providing cysteine for methionine and protein synthesis and as a source of reduced sulfur for synthesis of numerous other essential metabolites. The control of the pathway has long been a focus of plant sulfur research. Significant progress in understanding the pathway and its regulation has been made since the first Sulfur Workshop in 1989. All enzymatic steps have been identified, and the corresponding genes cloned. The investigations of sulfate assimilation were always quickly adopting newly developed approaches and the introduction of molecular biology led to a rapid switch to Arabidopsis as the main model for sulfur research. We learned a lot about the regulation of the pathway and identified genes affected by various environmental conditions or chemical signals. Sulfur research was one of the first areas of plant science to make use of systems biology with several seminal studies of major importance. The increased use of genetic approaches resulted in identification of new regulatory factors and most recently in finding genes responsible for genetic variation of sulfur-related traits in natural populations. Nevertheless, many questions remain open. This overview of the different approaches to study sulfur metabolism will highlight the success stories. The major progress since the first workshop will be summarised, and a new set of open questions will focus on how plant sulfur research will develop over the next 25 years.

Introduction

Plants take up the essential nutrient sulfur in the oxidized form of sulfate, reduce and incorporate it into various metabolites and cellular components (Kopriva 2006; Takahashi et al. 2011). The assimilation of sulfate into cysteine is therefore an essential pathway of plant primary metabolism, with the reductive part being key not just for the pathway but for life. Therefore sulfate reduction has been in the focus of plant sulfur research for many years, including being a major topic from

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

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the beginning of the Plant Sulfur Workshops (Brunold 1990). The final pathway (Fig. 1), however, has been formulated quite recently, after years of controversy (Kopriva and Koprivova 2004). Sulfate is firstly activated by adenylation catalyzed by ATP sulfurylase to adenosine 5′-phosphosulfate (APS). The activated sulfate is reduced in two steps, first through a two electron reduction to sulfite and subsequently by six more electrons to sulfide. Sulfide is then incorporated into O-acetylserine to form cysteine (Takahashi et al. 2011). The pathway in plants is thus very similar to sulfate assimilation in bacteria and fungi, the original models for dissection of the pathway (Kopriva 2015), which was one of the main reasons for the controversy and discussions regarding the plant pathway. As the model microorganisms *Escherichia coli* and *Saccharomyces cerevisiae* require a second activation step for the sulfate to be reduced (phosphorylation of APS to 3′-phosphoadenosine 5′-phosphosulfate (PAPS)), it was believed that plant sulfate assimilation proceeds in the same way with PAPS as an intermediate (Thompson 1967). However, in the beginning of the 1970s it was shown that APS is used directly in plants and algae and the corresponding enzyme was named APS sulfotransferase (Schmidt 1972). The reduction of APS was shown to result in a carrier-bound intermediate and a thiosulfonate reductase was described as a component of plant sulfate assimilation (Schmidt 1973). In the following years, APS sulfotransferase was shown to be highly regulated and a key enzyme for the control of the pathway, however, PAPS sulfotransferases have also been part of the literature (Schmidt and Jäger 1992; Brunold and Suter 1984). Thus, at the time of the first Plant Sulfur Workshop in Groningen, in 1989, many fundamental questions about sulfate reduction remained open (Brunold 1990) which are summarized in the following:

1. the physiological significance of the enzyme reactions detected *in vitro*, especially of the APS sulfotransferase versus the PAPS sulfotransferase pathway and of the organic thiosulfate reductase versus the sulfite reductase mechanism,
2. the detailed characterization of APS sulfotransferase and PAPS sulfotransferase,
3. the molecular basis of the regulatory phenomena observed,
4. the contribution of the root system to assimilatory sulfate reduction.

In this review the progress in answering these questions will be summarized, an overview of the different approaches to study sulfur metabolism presented, and new set of open questions formulated on how plant sulfur research will develop in the next 25 years.

Current Understanding of the Sulfate Assimilation Pathway

As evident from the first question, at the time of the first Sulfur workshop, two alternative enzymes for the first step of sulfate reduction were discussed, APS sulfotransferase and PAPS sulfotransferase (Brunold 1990). The situation however

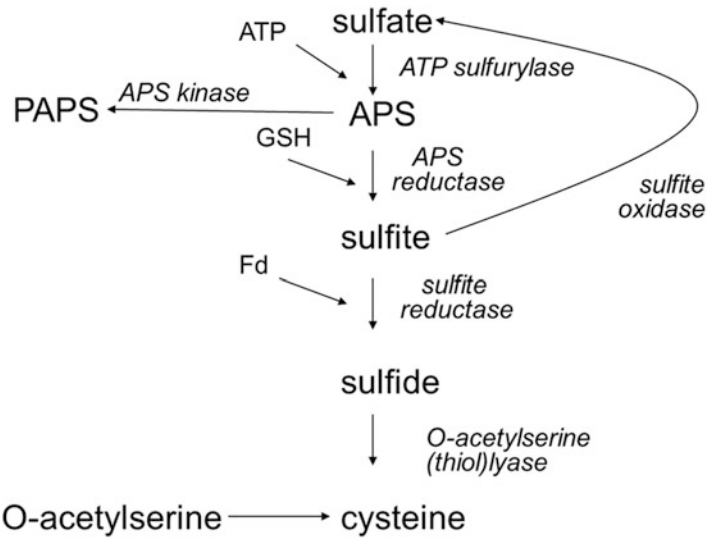

Fig. 1 Current understanding of plant sulfate assimilation

became even more complicated after the cloning of an Arabidopsis enzyme, which produced sulfite from APS and was named APS reductase (Gutierrez-Marcos et al. 1996; Setya et al. 1996). Three possible routes of sulfate to sulfide have been proposed, APS dependent bound-sulfite way (through APS sulfotransferase and thiosulfonate reductase), APS dependent reduction (APS reductase, sulfite reductase) or PAPS-dependent pathway (Fig. 2). The controversy has been clarified when the enzyme characterized as APS sulfotransferase was purified from *Lemna minor*, its N-terminal sequence was determined and the corresponding cDNA cloned (Suter et al. 2000). The sequence of the APS sulfotransferase was highly similar to the sequence of APS reductase, which together with identification of free sulfite as reaction product resulted in postulating APS reductase as the component of sulfate assimilation. In addition, experiments in oxygen free atmosphere showed that the bound sulfite is the result of reaction of sulfite with oxidized thiols, and therefore probably irrelevant for the sulfate reduction pathway, which proceeds through APS reductase and sulfite reductase (Suter et al. 2000). However, the question of PAPS reductase/sulfotransferase in plants has not been completely resolved. Even though everything suggests that APS reductase is the sole reducing enzyme, it is impossible to completely rule out the existence of PAPS-dependent enzyme. The crucial evidence, which would show that disruption of all APS reductase isoforms is lethal, is not available and thus the plant PAPS reductase is still possibly out there.

Although the question of the first reductive step in sulfate assimilation was resolved, the pathway has changed further. A new enzyme was added to the pathway, which had not been known in plants, sulfite oxidase (Eilers et al. 2001). Sulfite oxidase acts against the flow of sulfur in sulfate assimilation, therefore, to

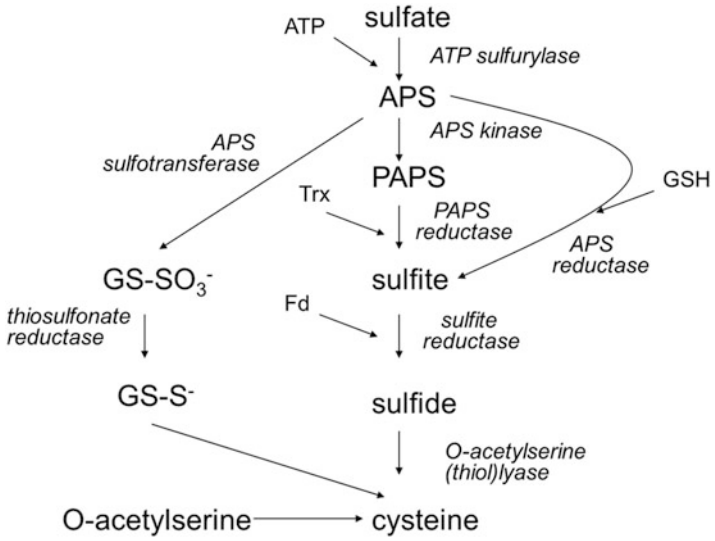

Fig. 2 Three possible routes of sulfate reduction in plants as discussed 1996–2000

prevent a futile cycle it is spatially separated from the reducing enzymes in peroxisomes. The enzyme protects plants against elevated sulfur dioxide and appears to be important for sulfite homeostasis, but whether this is its main physiological function is still under discussion (Lang et al. 2007; Brychkova et al. 2007, 2013).

In addition, in the last decade it became apparent that a second non-reductive path of sulfate assimilation, into PAPS and further into sulfated compounds, is essential also in plants (Mugford et al. 2010). APS kinase, producing PAPS from APS, has been shown to be important for synthesis of glucosinolates, a group of sulfated secondary compounds with plethora of functions in plant defence (Mugford et al. 2009). However, while the loss of glucosinolates does not compromise Arabidopsis in the absence of pathogens, a mutant disrupted in *APK1* and *APK2* isoforms of APS kinase showed a clear semi-dwarf phenotype (Mugford et al. 2009). These experiments showed the importance of PAPS for plant performance, however, the exact nature of the essential metabolites is not known.

In conclusion, the first question concerning the pathway of sulfate assimilation now seems to be largely answered (Fig. 1).

Biochemistry of APS Reductase

The second question, on the detailed characterization of APS and PAPS sulfotransferases, has naturally been limited to the APS dependent enzyme. The analysis of purified APS sulfotransferase revealed that it is identical to recombinant

APS reductase, identified by molecular cloning (Suter et al. 2000). The enzyme uses APS as a substrate with affinity of 10–20 μM, and glutathione or other thiols as reductant. Sequence analysis has shown that the enzyme is composed from three domains, a plastid targeting peptide, a reductase domain similar to bacterial PAPS reductases, and a C-terminal thioredoxin-like domain (Setya et al. 1996; Gutierrez-Marcos et al. 1996). The enzyme can be split into these two domains and the activity can be reconstituted (Bick et al. 1998). This makes sense because bacterial PAPS reduction is also dependent on thioredoxin or glutaredoxin. The two domains have distinct functions: the reductase domain is responsible for the interaction with APS and the formation of sulfite, whereas the C-terminal domain interacts with the reductant and resumes the function of glutaredoxin (Bick et al. 1998). APS reductase contains an iron-sulfur cluster, which was shown to be diamagnetic and asymmetric, as in the enzyme isoform studied (APR2 from *Arabidopsis thaliana*) it is most probably bound only by three cysteine residues in the reductase domain (Kopriva et al. 2001). When the enzyme is incubated with APS in the absence of reductants, a stable reaction intermediate can be detected with sulfite covalently bound to a cysteine residue in the reductase domain (Weber et al. 2000). The sulfite can be released by treatment with free thiols or sulfite, or by addition of the recombinant C-domain, confirming that the two domains have different functions in the catalysis, but raising a question on the function of the iron sulfur cluster.

Genes for the $[Fe_4S_4]$ cluster containing APS reductase have been found in all seed plants, basal plants, and green algae. However, this is not the only form of this enzyme (Fig. 3). Comparison of plant APS reductase with its bacterial counterparts revealed that the bacteria possess two classes of enzymes, similar to the reductase domain. The "classical" PAPS reductases from *E. coli* and *Salmonella* (and also yeast and fungi) differ from the plant enzyme mainly due to the absence of two conserved cysteine pairs, which are responsible for binding of the FeS cluster (Kopriva et al. 2002). Correspondingly, the cofactor has never been reported for PAPS reductases. However, another form of bacterial enzyme has been identified, more similar in sequence to the plant APS reductase, including the two cysteine pairs (Bick et al. 2000; Williams et al. 2002). These bacterial enzymes use APS rather than PAPS and contain the FeS cluster (Kopriva et al. 2002). Due to their similarities, the APS and PAPS reductases have been both referred to as sulfonucleotide reductases and shown to have a very similar reaction mechanism (Carroll et al. 2005). The specificity for APS or PAPS seems to be linked to the presence or absence of the cluster, respectively (Bhave et al. 2012). Incidentally this is also true for the dissimilatory APS reductase in sulfur oxidizing bacteria, which, although having a completely different primary structure, possess FeS centre (Frigaard and Dahl 2009).

The variation in APS reductase forms is even greater. Another isoform of APS reductase has been found in the moss *Physcomitrella patens*, which in contrast to the "classical" plant APS reductase does not bind the FeS cluster, and does not possess the thioredoxin-like domain but still reduces APS and not PAPS (Kopriva et al. 2007). This challenges the simple link between FeS cluster and APS reduction, which seems to be valid only in prokaryotes, and reinforces the question

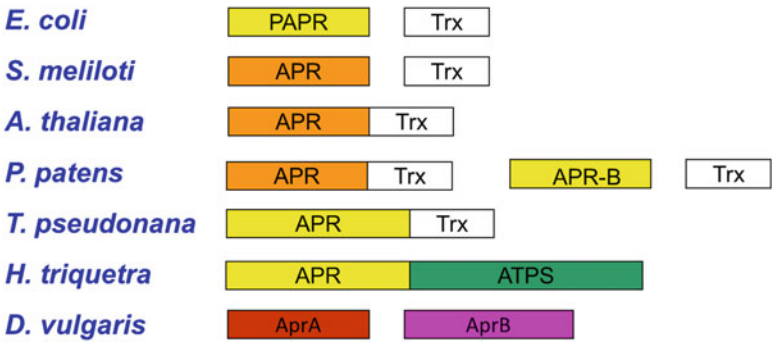

Fig. 3 Diversity of APS reductase forms in different organisms. *Yellow marks* PAPS reductases or APS reductases type B without FeS cluster, the presence of the cluster is marked by *orange colour*. The AprA and AprB isoforms of dissimilatory reductase from *Desulfovibrio vulgaris* are shown for comparison

regarding the function of the FeS cluster. This isoform of APS reductase, named APR-B, is present in several other basal plants, including *Selaginella* and *Marchantia* (Kopriva et al. 2007). A highly similar enzyme is dominating in the Eukaryotic microalgae, such as diatoms and haptophytes. These organisms, e.g. *Thalassiosira pseudonana* or *Emiliania huxleyi* possess genes for APS reductase of the APR-B type, and with the thioredoxin-like domain (Fig. 3) (Patron et al. 2008). Interestingly, the APS reductase activity in the microalgae is approximately two orders of magnitude higher than in higher plants (Bochenek et al. 2013). Thus, although a lot of progress in understanding the biochemistry of APS reductase has been made, the reaction mechanism is not yet completely resolved, since structure is available from the bacterial enzymes, or the APR-B, but not the full plant enzyme with both domains. It is however remarkable, that the same reaction can be catalyzed by enzymes with a similar primary structure but with, and without, the FeS cofactor.

Molecular Mechanisms of Regulation

At the time of the first Sulfur Workshop, the regulation of sulfate assimilation, particularly the reductive part, was well described at the physiological level. It was shown that sulfate reduction is induced by limiting sulfur availability and cadmium, and decreased by reduced sulfur, both by thiols and atmospheric SO_2 (Brunold 1990). Regulatory interactions with nitrogen nutrition had been described: a decrease of sulfate reduction capacity at low N and induction by ammonium (Brunold and Suter 1984). The developmental regulation of the pathway was described, showing an increase in activity from etiolated seedlings to green tissues and in the development of leaves until maturity was reached (Brunold 1990). In addition, feedback inhibition of individual enzymes of the pathway was described,

such as APS inhibiting ATP sulfurylase and AMP acting negatively on APS sulfotransferase. Generally, it was believed that the regulation was mainly at the level of ATP sulfurylase, with many conditions affecting additionally APS reductase.

The progress in understanding the regulation of sulfate assimilation has been significant over the past 25 years. It is impossible to describe all new findings on compounds and conditions affecting the pathway, the mechanisms, signalling pathways, transcription factors, etc.; these have been described in many reviews (Davidian and Kopriva 2010; Kopriva 2006; Koprivova and Kopriva 2014; Takahashi et al. 2011; Leustek et al. 2000; Hell 1997; Rausch and Wachter 2005; Chan et al. 2013). Instead, a personal selection of the major findings will be given, not only showing the major concepts but also the variety of links between sulfate assimilation and plant metabolism. The progress in uncovering the mechanisms of regulation is highly interconnected with the variety of methods and approaches used and with a shift in focus of the studies towards APS reductase.

The classical physiological studies led to formulation of the major concept in regulation of sulfate assimilation, the demand driven control, which explained most of the known regulatory events (Lappartient et al. 1999; Lappartient and Touraine 1996). The demand driven regulation is an overarching concept that still helps to explain observed regulation of sulfate assimilation by many environmental conditions. Another major impact on studies of the control of the pathway was the identification of signalling function of O-acetylserine (Neuenschwander et al. 1991), leading to some controversies but also to formulation of function on different levels (Hubberten et al. 2012b; Wirtz and Hell 2006). Other signals have been found with a more or less clear contribution to the regulation, such as sugars and phytohormones, particularly jasmonate and cytokinins (Jost et al. 2005; Maruyama-Nakashita et al. 2004). Most important was the finding of sulfur starvation inducible microRNA miR395, which targets three isoforms of ATP sulfurylase and the sulfate transporter SULTR2;1 and contributes strongly not only to response to sulfate starvation but also to regulation of sulfate partitioning (Kawashima et al. 2009, 2011; Liang et al. 2010).

The first major step in understanding the mechanisms of regulation was the introduction of molecular biology methods to complement the physiological and biochemical studies. These showed in the beginning that regulation of the pathway, e.g. by sulfate starvation, light, nitrogen sources, or reduced sulfur compounds, is mostly on the transcriptional level (Kopriva et al. 1999; Koprivova et al. 2000; Vauclare et al. 2002; Takahashi et al. 1997). APS reductase was more highly regulated than other components of the pathway, suggesting its central role in the control of the pathway. The key role of this enzyme was confirmed using flux control analysis, at least for the feedback regulation by thiols (Vauclare et al. 2002). These studies were followed by numerous reports showing transcriptional regulation of one or multiple genes of sulfate assimilation by a large variety of metabolites or environmental conditions (reviewed e.g. in Davidian and Kopriva 2010; Kopriva and Rennenberg 2004).

Numerous expression analyses showed that multiple genes are regulated by a single treatment. The availability of Arabidopsis genome then enabled the design of the first global transcriptomics studies and with three seminal papers on response to sulfate starvation sulfur research entered the systems biology era (Maruyama-Nakashita et al. 2003; Nikiforova et al. 2003; Hirai et al. 2003). The sulfate starvation response is one of the best studied environmental conditions by systems biology approaches, pioneering the combination of metabolite-gene networks and producing some of the best examples of mechanistic understanding derived from –omics data (Nikiforova et al. 2005; Hirai et al. 2005; Hubberten et al. 2012b). These studies also delivered first hints to the connection between sulfate assimilation and synthesis of glucosinolates (Hirai et al. 2005; Malitsky et al. 2008). Altogether these studies revealed a core cluster of genes highly regulated by sulfate starvation, some belonging to the pathway, e.g. sulfate transporters *SULTR1;1*, *1;2*, *4;1*, *4;2* or the APS reductase and some with unknown function, which is now only starting to be understood. They showed that sulfate starvation response includes modulation of phytohormone synthesis, particularly jasmonate and auxin but, while they identified potential regulatory hubs and factors, the progress in deciphering mechanisms of regulation has been rather limited.

Comparing results of expression analyses with activity measurements or metabolite concentrations it became soon apparent that not all regulation can be explained by transcriptional control and that various post-transcriptional mechanisms contribute to control of sulfate assimilation (Takahashi et al. 2011). Obviously, the miR395 is one of these mechanisms, restricting expression of the SULTR2;1 transporter to the xylem during sulfate deficiency and so increasing efficiency of sulfate root to shoot translocation (Kawashima et al. 2009). Another regulatory mechanism is the fluid dissociation and association of the cysteine synthase complex by changes in concentration of sulfide and O-acetylserine, which is probably an important player in sensing sulfur availability (Wirtz and Hell 2006). However, the most common and fundamental post-transcriptional mechanism of regulation of sulfur metabolism is redox. The first enzyme of the pathway shown to be redox regulated was, not surprisingly, APS reductase. This regulatory mechanism was initially based on *in vitro* observations of activation of recombinant APS reductase by oxidative agents and inactivation by reduction and corroborated *in vivo* by observed discrepancy between response of APS reductase protein and enzyme activity to oxidative stress (Bick et al. 2001; Kopriva and Koprivova 2004). The next target of redox regulation is the γ-glutamylcysteine synthetase (γECS), the first of two enzymes catalyzing glutathione synthesis. The inhibition of this enzyme by glutathione has been shown to be caused by redox regulation rather than product inhibition (Hothorn et al. 2006). The latest acquisition in the list of redox regulating enzymes of sulfur metabolism is APS kinase (Ravilious et al. 2012). Interestingly, the redox regulation has been recognized only after the structure of the enzyme has been solved, whereas previous biochemical characterizations had not found any hints. In contrast to APS reductase, APS kinase is activated in its reduced form and inactivated by oxidation (Ravilious et al. 2012). Since APS kinase is important for control of sulfur partitioning between primary reductive assimilation and PAPS and

secondary products (Mugford et al. 2011), the redox regulation may be important for the mechanism, but its biological relevance needs to be shown *in planta*.

The increasing availability of molecular and genetic resources in Arabidopsis allowed the use of genetic tools to further dissect the regulation of the sulfate assimilation pathway. Reverse genetic approaches helped, e.g., to understand the differences in functions of individual isoforms of the pathway components. The main contributions if this approach were linking the two branches of sulfate assimilation with glucosinolates synthesis through analysis of mutants in APS kinase (Mugford et al. 2009) and evidence that also other components of sulfate assimilation pathway than APS reductase may represent a bottleneck, namely sulfite reductase and serine acetyltransferase (Haas et al. 2008; Khan et al. 2010). To find new regulatory factors, however, other genetic methods have been applied. The biggest success in unravelling the mechanisms of regulation has been the identification of SULFATE LIMITATION1 (SLIM1), a transcription factor of the Ethylene-Insensitive-3-Like family (EIL3), a central regulator of the response to sulfate starvation (Maruyama-Nakashita et al. 2006). SLIM1 is responsible for the induction of transcripts for sulfate transporters, miR395, and many other genes by sulfate deficiency (Maruyama-Nakashita et al. 2006; Wawrzynska and Sirko 2014). The mechanism of its action is unknown yet, as *SLIM1* mRNA is not affected by sulfate starvation, and it is also not the only factor responsible for the response, since upregulation of transcripts for APS reductase is SLIM1 independent (Wawrzynska and Sirko 2014). Other transcription factors have been shown to regulate sulfate assimilation, e.g. the LONG HYPOCOTYL 5 (HY5) (Lee et al. 2011), but the understanding of the transcriptional machinery controlling sulfate assimilation is limited. This is in contrast to the regulation of glucosinolates synthesis, where a complex interplay of six MYB factors and three bHLH (MYC) factors has been described (Schweizer et al. 2013; Sonderby et al. 2010; Frerigmann and Gigolashvili 2014). Interestingly, these MYB factors also regulate genes of primary sulfate assimilation, showing that the glucosinolates biosynthesis should be considered within the core sulfur metabolism, at least in Arabidopsis and other Brassicaceae (Yatusevich et al. 2010).

All the approaches mentioned above gave some insights into the regulation of sulfur metabolism. The next set of experiments, exploiting quantitative genetics, identified further genes that are responsible for the natural variation of sulfur related traits and that can be potentially used for their modification in crops. These approaches profited from the availability of well defined Arabidopsis ecotypes and the progress in their genotyping. Two areas of sulfur research have been focus of the natural variation studies, glucosinolates and sulfate homeostasis. The glucosinolates diversity in Arabidopsis covers both quantitative and qualitative differences, particularly among the aliphatic, methionine derived glucosinolates. Five loci were identified that explain most of the qualitative variation among multiple accessions, primarily in the chain length, and one of them controls up to 75% of the quantitative differences (Kliebenstein et al. 2001b). In a more refined QTL study 20 loci were found to control the variation in glucosinolates between Ler and Cvi accessions (Kliebenstein et al. 2001a). Among the most important loci is

the GS-ELONG locus, containing multiple methylthioalkylmalate synthase genes, which plays a key role in the methionine elongation step of glucosinolate synthesis (Kroymann et al. 2001). The second major QTL is the GS-AOP, which is formed by two genes for 2-oxoglutarate–dependent dioxygenases, responsible for chain modification of the aliphatic glucosinolates and contributing greatly to the diversity (Kliebenstein et al. 2001c). The same loci were identified in a genome wide association study (GWAS), along with other, so far unknown ones (Chan et al. 2011). Therefore, it can be expected that the current view of control of glucosinolates synthesis remains incomplete.

The second theme approached by quantitative genetics is the accumulation of sulfate and sulfur. A QTL analysis of sulfate content in leaves of recombinant inbred lines from Bay-0 and Shahdara ecotypes revealed a non-synonymous single nucleotide polymorphism (SNP) in gene for APR2 isoform of APS reductase, resulting in almost complete inactivation of the corresponding enzyme (Loudet et al. 2007). Loss of APR2 leads to diminishing of total enzyme activity by 75%, consequently the flux through sulfate assimilation is reduced and sulfate accumulates. Interestingly, a second QTL from the same screen has been identified as ATPS1 isoform of ATP sulfurylase, additive to the effect of APR2 (Koprivova et al. 2013). The phenotype is caused by variation in mRNA accumulation, which correlates with a deletion in the first intron of *ATPS1* genes, leading to lower enzyme activity, reduced flux, and accumulation of sulfate. The QTL analysis, however, can only reveal variation in the two parent ecotypes, whereas much larger variation exists among the different accessions. When a wider variation in sulfur homeostasis has been assessed in 350 accessions, particularly high total sulfur content was detected in the Hod ecotype. The high sulfur content was caused mainly by high accumulation of sulfate, and exactly as in the Bay-0 × Shahdara population the causal gene was shown to be *APR2* (Chao et al. 2014). A further analysis of links between high sulfate/sulfur accumulation and APR revealed another small group of ecotypes related to Lov-1, with naturally inactive APR2. All three accessions (Shahdara, Hod, and Lov-1) possess different amino acid substitutions that lead to at least 1000-fold reduction of enzyme activity (Chao et al. 2014). Thus, in the Arabidopsis population, APR2 was at least three times independently inactivated, with slightly different consequences for accumulation of sulfate and sulfur.

Three more findings are considered potentially important for understanding the regulation of sulfate assimilation and its integration within general metabolic networks. Firstly, the unexpected finding of coordinated enrichment of expression of sulfate assimilation and glucosinolates synthesis in bundle sheath cells in Arabidopsis (Aubry et al. 2014). This resembles the localization of sulfate assimilation in plants with C4 photosynthesis, which is one of the unsolved questions of sulfur research. Secondly, several reports indicated an important role of the interplay between sulfite reductase and sulfite oxidase in "sulfite network" for sulfite homeostasis and regulation of sulfate assimilation (Brychkova et al. 2013). Finally, linking glucosinolates into core sulfur metabolism brought a connection to an enigmatic enzyme, 3′(2′),5′-bisphosphate nucleotidase, known as SAL1 or Fiery1,

which has been found in many genetic screens targeting mutants in stress response or leaf morphology (Robles et al. 2010). The enzyme metabolizes phosphoadenosine phosphate (PAP), which is a by-product of sulfation reactions, e.g., in synthesis of glucosinolates. PAP accumulates during drought stress and was proposed as a retrograde signal of stress from plastids to the nucleus (Estavillo et al. 2011). The loss of FRY1 also has a direct effect on sulfur assimilation, the glucosinolate content is lower in the mutants and the plants accumulate desulfoglucosinolate precursors (Lee et al. 2012). In addition, *fry1* mutants show lower accumulation of sulfate and total sulfur, making the enzyme and PAP interesting targets for further investigations.

Overall, many mechanisms of regulation have been discovered, but since every step in the pathway can become limiting, the control of sulfur metabolism is far from being fully understood.

Sulfate Assimilation in Roots

The fourth question formulated at the first Sulfur Workshop is one with the least definite answer. Root can reduce sufficient sulfate for their own demand, as shown clearly in root cultures growing in sulfate as the sole sulfur source (Vauclare et al. 2002). However, sulfate reduction in roots cannot complement the reduced APS reductase activity in the shoots, as demonstrated by reciprocal grafting of Col-0 and Hod accessions (Chao et al. 2014). In a split root system, the regulation of sulfate transport seems to be dependent on local sulfate availability and sulfate is translocated to shoots but not to a sulfate deficient part of the root system (Hubberten et al. 2012a). Roots contribute to sulfate deficiency response by increased uptake and translocation of sulfate, and there seems to be a communication between shoots and roots regarding the sulfur status (Hubberten et al. 2012a). The contribution of roots to overall sulfur homeostasis, sensing, and signalling, however, needs to be systematically investigated, e.g., using more grafting experiments with various mutants in sulfate assimilation and signalling pathways.

Sulfur Research in the Next 25 Years

The progress in understanding the pathway of sulfur assimilation and the regulation has been overwhelming. However, there are still many open questions, some reformulating the "old" concepts, some derived from the emerged new insights. Thus, in analogy with the first Sulfur Workshop and the most intriguing open questions formulated there, the following questions can be considered key for the next 25 years of plant sulfur research:

How is the sulfur flux controlled? This question has not changed much in the past 25 years, but the concept has changed dramatically. Whilst searching for the rate

limiting enzyme, it became obvious that control is shared and may lie on every step of the pathway, dependent on the actual metabolic and developmental status of the plant. Understanding the flux control will require consideration of sulfur fluxes in the context of the whole plant metabolism and employment of advanced mathematical tools and models (Calderwood et al. 2014). Solving this question will obtain a fundamental understanding about the control of the pathway and its integration with other pathways and finding the corresponding mechanisms – transcription factors and signals, and help with applying the knowledge generated in the course of plant sulfur research. Many traits are connected with sulfur, many sulfur compounds are important for various applications, e.g. the health promoting glucosinolates. The knowledge about control of sulfur fluxes will enable smart genetic engineering to create plants with tailored contents of diverse sulfur compounds.

What are the molecular mechanisms of the regulation? In the course of past investigations, much has been learned about the regulation of sulfur metabolism, and this trend will certainly continue. The number of known transcription factors, signalling compounds, and other regulatory mechanisms is limited, and it is crucial to find the full complement of transcription factors regulating the pathway, and to understand their functions. The bigger picture of transcriptional regulation has also to be considered, such as epigenetics and the role of transcriptional complexes, e.g. the Mediator. The redox regulation of APS kinase and APS reductase and possibly other enzymes has to be assessed in plants and the contribution to the control of sulfur fluxes quantified. We expect that further use of quantitative genetics will identify new genes and alleles controlling the variation in sulfur related traits in natural populations.

What are the biochemical properties of the new enzyme isoforms from algae? The new forms of ATP sulfurylase and APS reductase and their numerous fusions await characterization and determination of their biochemical properties. This will enable better understanding of sulfur metabolism in these organisms, that have often very high activity of these enzymes, but also generate sources of new more efficient enzymes for engineering of sulfur assimilation in plants or synthetic microbes.

What is the identity of the unknown sulfur compounds? In an untargeted metabolomics approach, a large number of unknown sulfur containing compounds have been detected in Arabidopsis (Glaser et al. 2014). This shows that the catalogue of sulfur compounds is far from complete even in model plants, and therefore new enzymes have to be connected to the sulfur network, e.g., a large number of sulfotransferase isoforms have unknown substrate specificity. Identification of these compounds will enable a better assessment of sulfur pools and may lead to discovery of new signalling and regulatory compounds.

How is sulfur metabolism integrated in the whole plant metabolism? Although the connections between sulfur and nitrogen nutrition, or sulfur and carbohydrates have frequently been described, the mechanisms and signals are unknown. The sensing of sulfur status and its transduction to the general response is part of this

question and is also completely unclear, again a large contribution of mathematical tools will be necessary to answer this question.

What is the role of sulfur metabolism in evolution and adaptation of plants? Large variation in many sulfur related traits have been observed within Arabidopsis or Brassica species. There are also differences in localization of the pathway in C3 and C4 plants, which highlight the links between C4 photosynthesis and sulfur reduction. So, how conserved are the regulatory mechanisms in different plant families? With reduced costs for sequencing and genotyping, this may be answered for some plant species. However, research shows that variation in glucosinolates has a large role in underpinning variation of Arabidopsis accessions in resistance to insects (Kliebenstein et al. 2002). Therefore, the ecological significance of varied sulfate and sulfur levels is of immense interest.

How can we apply the knowledge on sulfur metabolism for improvement of plants and/or added value? Adequate sulfur nutrition is essential for high yields and quality of crops. Due to reduced atmospheric sulfur depositions yields can be sustained only through sulfur fertilization. Therefore, understanding sulfur homeostasis will underpin approaches to breed low input crop varieties allowing reduction of the environmental costs of intensive agriculture. In addition, sulfur compounds are often linked with resistance to pests or abiotic stress, so the improvement of synthesis of these compounds might be beneficial. Many sulfur compounds, however, are important beyond the plant; the best example is the glucosinolate from broccoli, glucoraphanine, which is a precursor of sulforaphane, a plant derived metabolite with a plethora of functions in cancer prevention (Clarke et al. 2008). Broccoli varieties with increased content of glucoraphanine have been created and shown to possess an increased capacity to prevent prostate cancer (Traka and Mithen 2009). A better knowledge of the control of plant sulfur metabolism will enable the generation of plants accumulating other beneficial compounds.

Altogether, we can surely look forward to many new exciting stories about sulfur and hope that the next 25 years of plant sulfur research will be as successful as the past 25 years.

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