Control of sulfur partitioning between primary and secondary metabolism in Arabidopsis

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Sulfur is an essential nutrient for all organisms. Plants are able to take up inorganic sulfate and assimilate it into a range of bio-organic molecules either after reduction to sulfide or activation to 3′-phosphoadenosine 5′-phosphosulfate. While the regulation of the reducing 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 the genes and enzymes activating sulfate and transferring it onto suitable acceptors have been investigated in detail with emphasis on understanding the diversity of the sulfotransferase gene family and the control of partitioning of sulfur between the two branches of sulfate assimilation. Here, the recent progress in our understanding of these processes will be summarized.

Keywords: sulfate assimilation, cysteine, glucosinolates, adenosine 5′-phosphosulfate, sulfotransferase, sulfated metabolites

INTRODUCTION

Sulfur is essential for life as a component of proteins in the amino acids cysteine and methionine, a large number of co-enzymes and prosthetic groups as well as in many natural products of the secondary metabolism (Takahashi et al., 2011). The particu-

ar characteristic of sulfur, leading to its frequent occurrence in various compounds, is its ability to readily change its oxidation state. In nature, the major form of sulfur is the oxidized inorganic sulfate, however, most of the bio-organic compounds of primary metabolism contain the reduced form of sulfur as organic sul-

fide or thiol. Thus, the sulfate entering living organisms has to be assimilated, i.e., reduced and incorporated into organic matter. Not all organisms are able to cover their needs by assimilating sulfate, most notably all metazoans and most microorganisms adopting a parasitic lifestyle, in which sulfate reduction seems to be one of the first pathways being lost (Patron et al., 2008). Thus, plants (and algae) together with fungi and bacteria, which are capable of sulfate assimilation, play a crucial role in the food chain and in the biogeochemical cycle of sulfur.

Plant sulfur metabolism starts with taking up inorganic sulfate (Figure 1). The uptake is facilitated by sulfate transporters present in plasma membranes. Different cells possess different complement of individual sulfate transporters depending on the tissue and developmental stage (Buchner et al., 2004; Takahashi et al., 2011). Sulfate entering root cells can be rapidly moved through the cortex into the xylem and transported into the above ground plant organs or it can be directly utilized in the roots. Inside the cell it can be transported into the vacuole for storage or used directly for assimilation. Because sulfate is very sta-

ble, before assimilation it has to be activated. This is achieved in a reaction with ATP sulfurylase, in which sulfate replaces pyrophosphate in the ATP molecule. The resulting adenosine 5′-phosphosulfate (APS) is a branching point in primary and sec-

ondary sulfate assimilation. APS can either be reduced by APS reductase to sulfite in the primary sulfate assimilation pathway, or it can be phosphorylated by APS kinase to 3′-phosphoadenosine 5′-phosphosulfate (PAPS). PAPS is the active sulfate donor for the incorporation of sulfate into a variety of secondary prod-

ucts. Sulfite is reduced by sulfite reductase to sulfide, which is the form of reduced sulfur incorporated into the amino acid skeleton of O-acetylserine to form cysteine, the first product of primary sulfate assimilation (Figure 1). Cysteine can be used for protein and peptide synthesis or as a reduced sulfur donor for biosynthesis of methionine and a large range of co-enzymes and co-
factors.

Sulfate assimilation is an essential process in plants: reduced expression of genes for several steps of the pathway lead to strong growth phenotypes, e.g., sulfate reductase (Khan et al., 2010), APS kinase (Mugford et al., 2009), or serine acetyltransferase (Haas et al., 2008), complete knock-outs are lethal (Watanabe et al., 2008;
Whereas sulfur in primary metabolites, such as cysteine, methio-

iodothyronines, as well as to the detoxification of xenobiotics

endogenous chemicals, such as catecholamines, steroids, and

the homeostasis and regulation of numerous biologically potent

ferases (SOT). In mammals, sulfation is a major contributor to

hydroxylated substrates, i.e., sulfation, is catalyzed by sulfotrans-

identified to date. The transfer of the functional sulfo group to

known function is in sharp contrast to the importance of those

stress defense and also for human diet and health. The increas-

rating oxidized sulfur. Such sulfated compounds form a diverse

plants synthesize a number of secondary metabolites incorpo-

nosteroids (Marsolais et al., 2007) and/or salicylate (Baek et al.,

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AtSOT16, AtSOT17, and AtSOT18 isoforms are responsi-

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for sulfation of d-sulfo-glucosinolates (Piotrowski et al., 2004; Hirai et al., 2005) with a broad substrate specificity, but clear pref-

AtSOT15 was shown to catalyze the sulfation of 11- and

12-hydroxyjasmonate, but the closely related (based on sequence

identity) AtSOT14 was inactive with this substrate (Gidda et al.,

2003). AtSOT10 and AtSOT12 are involved in sulfation of brassi-

nosteroids (Maroslas et al., 2007) and/or salicylate (Baek et al.,

2010), while the preferred substrates of AtSOT3 are flavonoids

(Gidda and Varin, 2006). The remaining SOTs are of unknown

function. However, all evidence on SOT substrate specificity was

obtained in vitro in studies with recombinant proteins, and may

not reflect the situation in vivo. Similarly, not much is known

about regulation of SOTs, except that in agreement with the

potential role of sulfated compounds in plant defense, the mRNA

levels of AtSOT12, AtSOT13, AtSOT16, and AtSOT17 signifi-

cantly increased upon treatment with jasmonate (Gidda et al.,

2003). AtSOT12 mRNA was induced also by salicylic acid and

by interaction with bacterial pathogens and elicitors, whereas

AtSOT16 mRNA level responded to coronatine, an analog of

octadecanoid signaling molecules, and to ACC, the precursor of

ethylene (Lacomme and Roby, 1996). No SOTs are found in

the moss Physcomitrella patens or green alga Chlamydomonas

reinhardtii revealing a late evolutionary origin of SOT.

Mugford et al., 2010). The pathway is strongly regulated accord-

ing to the demand for reduced sulfur and availability of various

sulfur sources (reviewed in Takahashi et al., 2011). The regulation

of the reductive part of sulfate assimilation has been extensively

studied in the last two decades leading to a very good under-

standing of the responses of individual genes and enzymes to

various environmental conditions and changes in metabolite lev-

els. The availability of genetic resources in Arabidopsis allowed

more precise defining of functions of individual members of gene

families encoding various steps of sulfate assimilation to cysteine

(reviewed in Kopriva et al., 2009). On the other hand, much less

attention was paid to the PAPS branch of sulfate assimilation

and synthesis of sulfated compounds. The partitioning of sulfur

into the primary (reductive) and secondary (sulfated) assimila-

tion represents an important step controlling the availability of

this nutrient for synthesis of numerous compounds and has been

addressed very recently (Mugford et al., 2009, 2011). Here, we

will summarize and discuss new findings concerning the sec-

ondary branch of sulfate assimilation, particularly the role of APS

kinase and APS reductase in control of sulfur flux through the two

branches of sulfate assimilation and a possible mechanism of such

control.

SULFATED COMPOUNDS AND SULFOTRANSFERASES

Whereas sulfur in primary metabolites, such as cysteine, methio-
nine, glutathione, and most co-enzymes, is in its reduced form, plants synthesize a number of secondary metabolites incorpo-

rating oxidized sulfur. Such sulfated compounds form a diverse

group of secondary metabolites important for crop fitness and

stress defense and also for human diet and health. The increas-

ing, but still limited number of plant sulfated compounds with

known function is in sharp contrast to the importance of those

identified to date. The transfer of the functional sulfo group to

hydroxylated substrates, i.e., sulfation, is catalyzed by sulfotrans-

ferases (SOT). In mammals, sulfation is a major contributor to

the homeostasis and regulation of numerous biologically potent

endogenous chemicals, such as catecholamines, steroids, and

iodothyronines, as well as to the detoxification of xenobiotics

(Coughtrie et al., 1998). In bacteria, sulfation is essential for the

signaling of rhizobial nod factors to the plant (Truchet et al., 1991).

In plants, a large proportion of the known sulfated metabolites

play various roles in plant defense against biotic and abiotic stress.

A well-studied example of such compounds is the glucosinolates,

which participate in defense against herbivores and pathogens in

Brassicaceae (Halakier and Gershenzon, 2006). They are responsi-

ble for taste and flavor of cruciferous vegetables and possess an

anti-cancer activity (Fabey et al., 2001; Mathen et al., 2003). Sul-

fation is the last step in synthesis of the glucosinolate core and is

essential for their biological activity, as it enables formation of the

reactive volatile products upon reaction with myrosinase. Another

large group of medically important sulfated compounds are sul-

fated flavonoids, present in more than 250 species of 32 families

(Barron et al., 1988), where they are involved in detoxification of

reactive oxygen species and regulation of plant growth (Varin et al.,

1997). Several other sulfated compounds were shown to directly

participate in plant defense against pathogens: a sulfated derivative

of jasmonic acid identified in Arabidopsis (Gidda et al., 2003) or

sulfated β-1,3-glucan oligosaccharides (Ménard et al., 2004) that

induce salicylic acid defense signaling. Small sulfated peptides,

such as phytoalexins, PSY1, and ROG are important regulators

of plant growth (Matsuhayashi and Sakagami, 1996; Amano et al.,

2007; Matsuzaki et al., 2010).

The SOT-catalyzed sulfation requires PAPS as the sulfate donor

and a compound with a free hydroxyl group as an acceptor. Mul-
tiple SOT isoforms are found in higher Eukaryotes because of

the structural diversity of the biological acceptors of the sulfate

group. The SOT family in Arabidopsis consists of 18 members

divided into seven groups according to sequence similarity (Klein

and Papebrock, 2004). Only about half of these isoforms have

been assigned a substrate specificity and/or physiological function.

The AtSOT16, AtSOT17, and AtSOT18 isoforms are responsi-

ble for sulfation of d-sulfo-glucosinolates (Piotrowski et al., 2004; Hirai et al., 2003) with a broad substrate specificity, but clear pref-

erence of AtSOT16 for aromatic precursors (Klein, et al., 2006).

The AtSOT15 was shown to catalyze the sulfation of 11- and

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identity) AtSOT14 was inactive with this substrate (Gidda et al.,

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reinhardtii revealing a late evolutionary origin of SOT.
Adenosine 5′-phosphosulfate kinase catalyzes the phosphorylation of APS to form PAPS. This enzyme is an essential component of primary sulfate assimilation of yeast, fungi, and some bacteria (Marzuf, 1997), which require this second activation of sulfate to enable its reduction by a PAPS reductase (Kopriva and Koprivaova, 2004). While the cDNA for APS kinase were reported at the same time as those of other genes of sulfate assimilation (Axt et al., 1994; Jain and Leustek, 1994), the enzyme and its regulation was studied much less frequently compared to, e.g., ATP sulfurylase or APS reductase. A high affinity for APS (ca. 1–18 μM) was reported for APS kinase from Arabidopsis alongside a strong substrate inhibition (Lee and Leustek, 1998; Lillig et al., 2001). Only after the Arabidopsis genome sequence became available, the full family of four genes encoding APS kinase has been identified. All four genes encode functional enzymes with similarly high affinity for APS (Mugford et al., 2009). Three isoforms are localized in plastids and one, APK3, is cytosolic. APK2 and APK2 transcript levels in leaves are higher than those of APK3 and APK4 (Mugford et al., 2009).

To find the biological functions of individual APS kinase isoforms, Mugford et al. (2009) systematically analyzed T-DNA lines disrupting the corresponding genes and their combinations. Unsurprisingly, disruptions of single genes had no effects on plant growth or contents of a major class of sulfated metabolites, glucosinolates. Among all six combinations of double mutants, only the apk1 apk2 combination resulted in smaller plants in which glucosinolates reached ca. 15% of wild type levels (Mugford et al., 2009). Further crossing revealed that APS kinase isoforms, APK1, APK3, and APK4 alone are capable to sustain plant growth, albeit with great difference in performance (Mugford et al., 2010). Plants possessing APK1 as the only isoform of APS kinase were undistinguishable from wild type plants showing that this isoform contributes most to total enzyme activity. Plants possessing APK3 or APK4 only were affected in growth to a greater degree than apk1 apk2 plants but were still capable to finish their life cycle and produce viable seeds. On the other hand, mutants with APK2 transcript in pollen, where the other three isoforms are highly expressed, of particular interest is the high and very specific expression of APK1 and APK2 in fusicocarp and APK3 and APK4 in seed radical (Mugford et al., 2009). Analysis of available microarray data in eFP browser confirmed low expression levels of APS kinase isoforms and predominant expression of APK2 in vegetative tissues (Wester et al., 2007). It also revealed that APK1 and APK2 are induced by methyl jasmonate, which agrees with up-regulation of these two genes by wounding (S. G. Mugford, unpublished). Interestingly, APK1 transcript levels increased in imbibed seeds compared to dry controls, whereas APK3 and APK4 were down-regulated by this treatment (Nakabayashi et al., 2005). Thus, the differential tissue-specific expression of APS kinase isoforms together with the different growth characteristics of triple mutants indicates strongly that each isoform has a specific role in plant sulfur metabolism. However, to assess how APS kinase affects the general sulfur metabolism, it is necessary to consider the overall control of the flux through sulfate assimilation.

APS REDUCTASE AS A KEY CONTROL STEP OF SULFUR METABOLISM

Adenosine 5′-phosphosulfate reductase has been studied extensively as the key enzyme controlling flux through reductive sulfate assimilation. The enzyme and the corresponding genes are highly regulated according to demand for reduced sulfur and sulfate availability. In particular, the enzyme is feedback inhibited by reduced sulfur compounds such as cysteine and glutathione (Vaudaire et al., 2002). Since in the experiments with Arabidopsis root cultures this inhibition was specific to APS reductase and other enzymes of the pathway were not affected, the contribution of individual enzymes to total control of flux could be calculated. When internal sulfate was considered as the beginning of the pathway, APS reductase was responsible for 91% of control of the flux, when sulfate transport was also taken into account, the control was equally shared between transport and the enzyme (Vaudaire et al., 2002). Using flux measurements with different transgenic poplars, APS reductase was again shown to possess a high control over the pathway, however not as strong as in case of Arabidopsis, contribution of other components of the pathway was clearly detectable (Schreier et al., 2010).

The data from control flux analysis showing importance of APS reductase in control of sulfate assimilation were corroborated by a very different experimental approach. To understand
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FIGURE 2 | Effects of manipulation of APS reductase and APS kinase on levels of S-containing compounds and flux through the pathway in Arabidopsis. Red and green colors symbolize increased and decreased enzyme activity, metabolite concentration, or flux through the pathway. The following genotypes were analyzed: (A) PpAPR-B expressed in plastids, (B) apr2, (C) EcAPK targeted to plastids, and (D) apr1 apr2.

the control of sulfate accumulation in plants, analysis of Bay-0 × Shahdara recombinant inbred lines identified a major QTL on chromosome 1 (Loudet et al., 2007). The QTL was cloned as a gene encoding the APR2 isoform of APS reductase. In Shahdara, a single-nucleotide polymorphism results in exchange of an alanine in the proximity of active center into glutamate, leading to a strongly diminished affinity of the enzyme for the reductant and thus highly reduced reaction velocity. Since APR2 is the major isoform of the enzyme contributing about 75% of total leaf APS reductase activity in Shahdara leaves and as a consequence, accumulation of sulfate (Loudet et al., 2007). This result was confirmed by analysis of Arabidopsis T-DNA line in which APR2 gene was disrupted, as also this apr2 mutant accumulated sulfate. Surprisingly, neither in Shahdara, nor in Col-0, the disruption of APR2 and thus strongly reduced APS reductase activity affected the levels of reduced sulfur compounds, cysteine and glutathione (Loudet et al., 2007). This lack of effect, however, may be dependent on growth conditions, as the same apr2 T-DNA line was reported to possess slightly but significantly lower glutathione level when grown under different conditions (Grant et al., 2011). Disruption of APR2 may not affect glutathione levels, but the rate of its synthesis. Indeed, the flux through sulfate assimilation was diminished in the apr2 mutants (Figure 2) (Mugford et al., 2011).
APS reductase overexpressing fur compounds sulfite and thiosulfate (T sakraklides et al., 2002). a strong accumulation of thiols, but also reduced inorganic sul-
of bacterial APS reductase in Arabidopsis and maize resulted in a strong accumulation of thiols, but also reduced inorganic sulfur compounds sulfite and thiosulfite (Tsakraklides et al., 2002). In maize, this metabolic imbalance led to leaf necrosis (Martin et al., 2005). Clearly, increased capacity for APS reduction results in increased flux of sulfur through reductive assimilation, but the accumulation of inorganic sulfur compounds shows that under these conditions the assimilation is limited by the availability of carbon acceptors of reduced sulfur. Indeed, feeding OAS to the APS reductase overexpressing Arabidopsis resulted in much higher accumulation of thiol (Tsakraklides et al., 2002). However, when the increase of APS reductase is only moderate, the effects on plant sulfur metabolism are much milder. This was shown with poplars overexpressing APS reductase from Lemma minor, which despite an increase in activity did not increase flux through the pathway. On the other hand, expression of APR-B form of APS reductase from P. patens, which does not possess iron sulfur cluster and is thus less catalytically efficient (Kopriva et al., 2007), has only marginal effect on the total enzyme activity but even this small rise is sufficient to enhance the flux through the pathway and even increase metabolite accumulation (Figure 2) (Mugford et al., 2011). This suggests that the increase in APS reductase activity by APR-B is uncoupled from the usual regulatory network and able to produce enough product surplus that the thiols accumulate. The effects of both reduced and increased APS reductase activities on flux through primary sulfate assimilation thus confirm the important role this enzyme has in the control of the pathway.

APS KINASE AND SULFUR METABOLISM

As discussed previously, plants possess a range of sulfated compounds produced by secondary metabolism. Particularly in Arabidopsis and other Brassicaceae, the glucosinolates contribute significantly to sulfur pools in the plant and thus it can be expected that manipulation of APS kinase will have a general effect on sulfur metabolism. Indeed, the Arabidopsis apk1 apk2 mutants with low glucosinolate levels showed remarkable increase in cysteine and glutathione (Mugford et al., 2009). Apparently, the block in the PAPS branch of sulfate assimilation caused a redirection of sulfur flow into the primary reductive pathway. Indeed, when the flux was quantified using incorporation of [35S]sulfate, the labeling of reduced sulfur compounds was higher in the mutant than in wild type (Figure 2) (Mugford et al., 2011). Surprisingly, the increased flux cannot be attributed to changes in APS reductase activity as this enzyme was not affected in the mutant. On the other hand, ATP sulfurylase activity was about twofold higher in the mutants, which resulted in accumulation of sulfate (Mugford et al., 2009, 2011). The reduction in APS kinase activity in apk1 apk2 had consequences for other parts of sulfur metabolism as well. Microarray and qPCR analysis revealed that genes of the glucosinolate biosynthesis were highly and coordinately induced in the mutant (Mugford et al., 2009). Correspondingly, the mutants accumulated high levels of the desulfo-glucosinolate precursors. Similarly, the apk1 apk2 plants possessed less sulfo-jasmonate and increased levels of the hydroxyl-jasmonate precursors. The genes encoding precursors of the sulfate peptides phytosulfokines and PSY1 were also more highly expressed in the mutants than in wild type (Mugford et al., 2009). The diminished availability of PAPS in the mutants thus affects all classes of sulfated products and leads to accumulation of the precursors.

As reduction in APS kinase affects both primary and secondary sulfur metabolism, what are the effects of increased PAPS production? While the levels of glucosinolates were not affected in plants overexpressing bacterial APS kinase, the manipulation of the enzyme level did not remain without consequences. Irrespective of localization of the additional APS kinase in cytosol or plastids, APS reductase activity was induced and consequently the flux through primary sulfate assimilation was increased (Mugford et al., 2011). Interestingly, the increase in sulfate reduction rate did not result in alteration of thiol accumulation (Figure 2). As with the apk2 mutant, it seems that the levels of glutathione and cysteine are highly regulated within a narrow range by adjusting the synthesis and turnover rates. The increase in flux through primary sulfate assimilation and not the secondary branch in the APS kinase overexpressing plants seems rather counterintuitive. The most probable explanation is that increased APS kinase activity reduced the accumulation of APS and thus increased efficiency of ATP sulfurylase which is notorious for its low rate of forward (APS synthesis) reaction. The higher APS production then probably caused induction of APS reductase as the demand for the enzyme was higher and, consequently, the flux increased (Mugford et al., 2011).

FURTHER LINKS BETWEEN PRIMARY AND SECONDARY SULFATE ASSIMILATION

An exciting new link between the two branches of sulfate assimilation has been uncovered very recently. The microarray analysis of apk1 apk2 mutants indicated that a FIERY1 gene, encoding a 2′,3′-5′-diphosphoadenosine (PAP) phosphatase, may be part of the glucosinolate synthesis network, because PAP is the product of SOT reactions (Figure 1) and the transcript for FIERY1 was induced in apk1 apk2 as the transcripts of other glucosi-

nolate biosynthetic genes. Indeed, in leaves of a fosl allele of fiery1 (fry1) mutant (Rodriguez et al., 2010) glucosinolate content was reduced and desulfo-glucosinolates accumulated (Lee et al., 2012). Apparently, the PAP produced during glucosinolate synthesis accumulated in the fosl mutant and inhibited either the transport of PAPS between chloroplasts and cytosol or directly the SOT activity resulting in accumulation of the desulfo-precursors similar to apk1 apk2. However, the extent of reduction of glucosinolate levels and accumulation of desulfo-glucosinolates in fosl was much milder than in apk1 apk2 mutants and the expression of genes of the glucosinolate synthesis network was only mildly affected (Lee et al., 2012). Analysis of apk3 fosl double mutants,
in which the only cytosolic APS kinase was disrupted, revealed that it is not the PAPS transport causing the low glucosinolate phenotype, as their levels were identical to fout plants. Thus, it seems that the accumulation of PAP in the fout mutant inhibits SOTs and leads to reduced efficiency of desulfo-glucosinolate sulfation (Lee et al., 2012).

**fiery1** is a rather enigmatic gene, it has been identified in numerous genetic screens for different phenotypes, such as screens for plant genes increasing Li" tolerance of yeast (Quintero et al., 1996), affecting abscisic acid and stress signaling (Xiong et al., 2001), cold signaling (Xiong et al., 2004), RNA silencing suppressors (Oy et al., 2007), elevated expression of ascorbate peroxidase 2 (Wilson et al., 2009), venation patterning (Robles et al., 2010), deregulation of fatty acid oxygenation rate (Rodriguez et al., 2010), and for mutations affecting expression of a phosphate transporter (Hirsch et al., 2011). In fiery1 mutants PAP accumulates, causing large alterations in gene expression and inhibition of euersorinoclesases (Estavillo et al., 2011). PAP levels are also highly induced by drought or high light stress acting as retrograde stress signals, from chloroplast to the nucleus to induce expression of stress-responsive genes. Indeed, fiery1 mutants are resistant to drought stress (Estavillo et al., 2011). The analysis of sulfur metabolism in fout allele of fiery1 added another phenotype to the list, reduced accumulation of sulfate. The expression pattern of genes of primary sulfate assimilation in fout is similar to that in plants under sulfate starvation: increased mRNA levels for APS reductase and reduced levels of ATP54 isoform of ATP sulfurylase (Lee et al., 2012). However, the expression pattern was not caused by disturbance in signaling, as the foliar sulfate and glutathione levels were significantly lower in fout than in wild type. The mechanism by which sulfate levels are affected in fout is not known, as the sulfate uptake rate to the roots is not affected (Lee et al., 2012). The strong correlation of sulfate starvation-like gene expression and low sulfate accumulation, however, indicate that the signal for induction of sulfate starvation response is reduction in internal rather than external sulfate content (Lee et al., 2012). Thus **fiery1** or rather its substrate PAP accumulating in the fout mutant represents another link between primary and secondary sulfate assimilation.

**REGULATORY MECHANISMS**

The accumulation of desulfo-glucosinolates and coordinated induction of glucosinolate synthesis genes in the apk1 apk2 mutant revealed that glucosinolate accumulation is under control of a feedback regulatory loop. The trigger for the regulation can be either the desulfo-glucosinolates, which are almost undetectable in wild type plants, or a decrease in glucosinolate(s) levels below a certain threshold. Since in the desulfo-glucosinolate accumulating fout mutant the glucosinolate synthesis genes are not affected to the same degree as in apk1 apk2, it seems that Arabidopsis plants possess a mechanism reacting to low levels of one or more glucosinolates as a signal for induction of their synthesis. It is possible to speculate that binding of a certain glucosinolate, or its degradation product, to a transcription factor might prevent its binding to DNA. When the levels of such a signaling metabolite(s) are low, free transcription factors might be able to activate the gene expression of the glucosinolate synthesis network. An alternative signaling molecules might be PAP, which accumulates in nucleus during stress (Estavillo et al., 2011) or APS, which in bacteria binds to Chi regulator and prevents Chi-dependent transcription of genes for utilization of organic sulfur compounds (Bykowski et al., 2002). While the signal and its mechanism of action are unknown so far, transcription factors controlling the network are well-established.

Glucosinolate synthesis is under control of two families of R2R3-MYB transcription factors (Gigolashvili et al., 2007a,b, 2008; Hirai et al., 2007; Sønderby et al., 2007). The first clade (MYB28, MYB76, and MYB29) is specifically involved in the control of synthesis of aliphatic glucosinolates (Gigolashvili et al., 2007b, 2008; Hirai et al., 2007; Sønderby et al., 2007) while MYB31, MYB122, and MYB34 regulate synthesis of indolic glucosinolates (Coelman et al., 2005; Gigolashvili et al., 2007a; Malitsky et al., 2008). Transcript levels of all these MYB factors are elevated in apk1 apk2, and the coordinated accumulation of mRNAs for the glucosinolate biosynthesis genes is in agreement with experiments in which these factors were overexpressed (Gigolashvili et al., 2007a,b, 2008; Mugford et al., 2009).

The MYB factors, however, function beyond regulation of genes of the core glucosinolate synthesis. The expression analysis of apk1 apk2 plants revealed increased transcript levels of *ATPS1* and *ATPS3*. This prompted investigation of the ability of the MYB factors to control expression of genes of primary sulfate assimilation using in vitro transactivation assays (Berger et al., 2007). Given the dependence of glucosinolate synthesis on PAPS it is not surprising that *ATPS1*, *ATPS3*, *APK1*, and *APK2* are directly regulated by these transcription factors (Tatushevich et al., 2010). This has been confirmed by expression analysis of plants overexpressing these MYB factors. While both groups of the MYB factors controlled *APK1* and *APK2* to the same extent, the factors associated with aliphatic GSs, MYB28, MYB76, and MYB29, induced a stronger reaction with *ATPS5* than with *ATPS3*, while the opposite was true for all three indolic glucosinolate transcription factors (Tatushevich et al., 2010). Unexpectedly, the MYB factors appeared to regulate also genes of the reductive part of sulfate assimilation, APR and sulfite reductase. The regulation of APR by the MYB factors, however, is more complex. While *ATPS1* and *ATPS3* transcripts were induced in plants overexpressing the MYB factors and not affected in corresponding knock-out mutants, mRNA levels for APR were induced also in mutants in the MYB factors of the indolic glucosinolate group. It is possible that reduction of indolic glucosinolates in these mutants triggers synthesis of alternative defense compounds requiring reduced sulfur. Both disruption and overexpression of MYB28 and MYB31 had significant consequences for primary sulfate assimilation, including accumulation of glutathione and increased flux through the pathway (Tatushevich et al., 2010). Indeed, microarray analysis of plants overexpressing MYB28, MYB29, and MYB76 showed a similar regulation of genes of primary sulfate assimilation (Sønderby et al., 2007). It is thus clear that primary and secondary sulfate assimilation are interconnected and coordinated by these six MYB transcription factors.

Another factor regulating both primary and secondary sulfate assimilation is SULFUR LIMITATION 1 (SLIM1). SLIM1 has been identified in a screen for mutants in response to sulfate
Therefore, despite some progress in understanding of the control of partitioning of sulfur between primary and secondary metabolism, the coordination is achieved on several levels, transcriptional regulation through a common set of MYB transcription factors and post-translation of the biosynthesis of sulfur-containing compounds. The process in understanding of the control of partitioning has been admirable, so clearly from the exploitation of these findings in praxis to manipulate the synthesis of sulfur-containing compounds in plants by genetic engineering. This will certainly be a target of further studies, as demonstrated, e.g., by the ability to engineer glucosinolate synthesis to tobacco (Geu-Flores et al., 2009). The time for designer crops with manipulated contents of specific sulfur-containing metabolites is coming!

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However, there appears to be an additional mechanism regulating the partitioning of sulfur beyond the transcriptional regulation. It has been long known that APS reductase is reductively regulated, the enzyme is activated during oxidative stress (Bick et al., 2001) and inactivated by incubation with excess reductant (Kopriva and Kopriva, 2004). Very recently it has been shown that also APS kinase is susceptible to reducton regulation (Ravilious et al., 2012). Resolving the protein structure of APK1 revealed that the enzyme possesses a disulfide bond between conserved cysteines. Reduction of the disulfide either chemically or via site-directed mutagenesis resulted in increased activity and lower substrate inhibition. It seems therefore, that changes in redox environment in the plastids may change the flow of sulfur to more reduced products when oxidized and to more secondary assimilation when sufficient reduction equivalents are available (Ravilious et al., 2012). Such a mechanism ensures that the redox connection with production of reactive oxygen species and more oxidizing conditions, stimulates sulfate reduction through activation of APR and inhibition of APS kinase.

In conclusion, it is evident that both APS reductase and APS kinase are capable of regulating the flux through sulfate assimilation and that it is the coordination of these two activities that is responsible for control of partitioning of sulfur between primary and secondary metabolism. The coordination is achieved on several levels, transcriptional regulation through a common set of MYB transcription factors and post-translation of the biosynthesis of sulfur-containing compounds. This will certainly be a target of further studies, as demonstrated, e.g., by the ability to engineer glucosinolate synthesis to tobacco (Geu-Flores et al., 2009). The time for designer crops with manipulated contents of specific sulfur-containing metabolites is coming!

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