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Review

Recent advances in understanding plant response to sulfur-deficiency stress

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Sulfur is an essential macronutrient for all living organisms. Plants are able to assimilate inorganic sulfur and incorporate it into organic compounds, while animals rely entirely on organic sources of sulfur. In the last decades sulfate availability in soils has become the major limiting factor for plant production in many countries due to significant reduction of anthropogenic sulfur emission forced by introducing stringent environmental legislation. The sulfur flux after transferring plants from optimal conditions to sulfur deficiency is regulated on multiple levels including transcription, translation and activity of enzymes needed for sulfate assimilation and synthesis of sulfur-containing metabolites. Most of these regulatory steps are not yet fully characterized. Plant responses to sulfur limitation are complex and can be divided into phases depending on the degree of sulfur shortage. The initial responses are limited to adaptations within sulfur metabolic pathway, while multiple metabolic pathways and developmental process are affected when sulfur shortage becomes more severe. The major aim of this work is a comprehensive review of recent progress in understanding the regulation of plant adaptations to sulfur deficit.

Keywords: cysteine, glutathione, nutrient availability, stress response, sulfate assimilation, sulfur metabolism

GLOBAL SULFUR CYCLE, VARIETY OF SULFUR COMPOUNDS AND IMPORTANCE OF SUFFICIENT SULFUR NUTRITION

Sulfur occurs in the environment in a variety of oxidative states that range from –2 in its most reduced form (sulfide — S^{-2}) to +6 in its most oxidized form (sulfate — SO_4^{2+}). In the aerobic atmosphere of the Earth inorganic sulfur occurs predominantly in the form of sulfate. The main reserve of sulfur are oceans (Giordano *et al.*, 2005; Norici *et al.*, 2005), where sulfur exists primarily in the form of inorganic sulfate, while in the earth sulfur can be found mostly as sulfate minerals, such as gypsum, or sulfide minerals, such as pyrite (Scherer, 2001). Sulfur dioxide (SO_2), mainly, and hydrogen sulfide (H_2S) are emitted to the atmosphere as a result of volcanic activity, decomposition of biological tissues and anthropogenic activities. All sulfur compounds are in constant flux (termed global sulfur cycle) between oxidized and reduced states through the action of living organisms and chemical processes.

Sulfur is necessary for proper growth and development of living organisms, however, it is attributed rather catalytic and regulatory than structural functions because it is much less abundant than other macroelements. For example, there is on average about 30-fold more nitrogen, 8-fold more potassium and 2-fold more phosphorus than sulfur in plant shoot dry matter (Marshner, 2005). The plant biomass consumed as food and feed serves as the main source of organic sulfur for animals and humans (Komarnisky *et al.*, 2003). Plants, bacteria and fungi, contrary to animals, are able to assimilate inorganic sulfur and incorporate it into organic compounds. Inorganic sulfur must follow a cascade of reactions

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Abbreviations: ATPS, ATP sulfurylase; APR, ATS reductase; APS, adenosine 5′-phosphosulfate; CS, cysteine synthase; GSH, glutathione; OAS, *O*-acetyl-L-serine; OAS-TL, *O*-acetylserine (thiol)lyase; SAM, *S*-adenosylmethionine; SAT, serine acetyltransferase

to be changed into organic sulfur compounds. It can be either directly incorporated in a reaction termed sulfation or used as a substrate for the synthesis of cysteine after a multistep reduction to sulfide. The sulfate assimilation pathway was first resolved in enteric bacteria, *Escherichia coli* and *Salmonella typhimurium* (for review see Kredich, 1996) and it was subsequently characterized in plants.

Plants utilize sulfate for the synthesis of diverse primary and secondary metabolites (Fig. 1). The first organic compound synthesized in the sulfate assimilatory pathway is cysteine (Cys). It is an important amino acid incorporated into various proteins, and a precursor of numerous essential compounds such as methionine, *S*-adenosylmethionine (SAM), *S*-methylmethionine, [Fe/S] clusters, hormones, vitamins and enzyme cofactors. Disulfide bonds formed in proteins between the thiol groups of Cys residues play crucial roles in forming and maintaining the tertiary structures of proteins. Some Cys-containing metabolites, including glutathione (GSH), phytochelatins and thionins function in response against environmental stresses. Organic compounds containing sulfur are also responsible for the specific taste and smell of onion, garlic and other valuable vegetables and herbs used in the kitchen or in traditional medicine.

Over the last decades it has become obvious that sulfur availability is limiting for farming in some parts of the world. Paradoxically, it is a result of a positive phenomenon, namely, a strong reduction of atmospheric pollution in industrialized areas of developed countries. The local trends to reduce sulfur dioxide (SO_2) emission all over the world vary from one region to another and are the results of environmental legislations imposed by local governments. In the majority of European countries, including Poland, emission has decreased by more than 60% in the years 1990–2004 (Vestreng *et al.*, 2007), while in Asia these trends are still reversed comparing to Europe and USA. Both the reduction of sulfur emission to the atmosphere resulting in a decrease of atmospheric sulfur deposition onto agricultural land and the use of sulfur-free (but rich in nitrogen and phosphorus) fertilizers have led to insufficient sulfur supply to a variety of crops, especially those with high sulfur requirements (e.g., oilseed rape). Insufficient sulfate nutrition reduces plant growth, vigor and resistance to abiotic and biotic stresses (Scherer, 2001; Knop *et al.*, 2007; Kruse *et al.*, 2007). Sulfur deficit influences not only the crop yield but also food quality. For example, certain sulfur-rich proteins in wheat determine the backing quality of flour (Zhao *et al.*, 1999; Granvogl *et al.*, 2007) and malting quality of barley (Zhao *et al.*, 2006). A decreased sulfur content in wheat may increase the level of cancerogenic acrylamide in processed food (Muttucumaru *et al.*, 2006). Additionally, a sufficient metabolic supply of sulfur amino acids from diet and tissue protein breakdown is necessary for the normal functioning of animals, including the mammalian immune system (Grimble, 1994; Hunter & Grimble, 1997).

Sulfur starvation which decreases the level of sulfur-containing defense compounds, such as elemental sulfur, H_2S, glutathione, phytochelatins, various secondary metabolites and sulfur-rich proteins, is apparently associated with a decreased resistance of plants, while sulfur fertilization of plants increases their resistance to pathogens and stresses. This process was termed sulfur-induced resistance — SIR (Rausch & Wachter, 2005; Kruse *et al.*, 2007). Conversely, environmental stresses resulting in increased formation of reactive oxygen species and oxidative stress have an influence on sulfur metabolism.

The biochemistry of sulfur assimilation is well characterized, however, many questions remain unsolved concerning regulation of sulfur metabolism in response to both the availability of sulfur in the environment and the increased demand of plants for sulfur metabolites in certain environmental conditions. Keeping in mind the common occurrence of sulfur deficiency in soils it is extremely important to understand the molecular mechanisms of plant response to the changing conditions of sulfur nutrition. The recent progress in the understanding of the complexity of plant adaptation to sulfur deficit was possible due to application of a variety of approaches, including systems biology methods, by multiple research groups interested in the field.

SULFATE UPTAKE AND SULFUR METABOLIC PATHWAY

The *Arabidopsis thaliana* genes encoding proteins involved in sulfate uptake, its translocation within plants and selected steps of sulfur metabolism are listed in Table 1. Most of the enzymes of the pathway are encoded by multigene families. The redundancy and multi-compartment location of the enzymes responsible for most of the steps are, on one hand, an indication of the importance of adequate control of sulfur flux and, on the other hand, illustrate the complexity of the system. A general scheme of sulfate assimilation and sulfur metabolism in plants is shown in Fig. 1. Provided below is a short outline of the pathway, which is necessary for understanding the regulatory aspects discussed in the next chapters, and brief descriptions of the problems not covered in earlier reviews. The readers interested in details of the pathway biochemistry are referred to the numer-

Table 1. Proteins from *Arabidopsis thaliana* involved in the major steps of sulfur metabolism compiled according to TAIR database (http://www.arabidopsis.org/index.jsp)

Protein function	Genome locus	Protein name; alternative names
Sulfate transporter (ST)	At4g08620	SULTR1;1
	At1g78000	SULTR1;2
	At1g22150	SULTR1;3
	At5g10180	SULTR2;1
	At1g77990	SULTR2;2
	At3g51895	SULTR3;1
	At4g02700	SULTR3;2
	At1g23090	SULTR3;3
	At3g15990	SULTR3;4
	At5g19600	SULTR3;5
	At5g13550	SULTR4;1
	At3g12520	SULTR4;2
	At1g80310	SULTR5;1
	At2g25680	SULTR5;2
ATP sulfurylase (ATPS); EC 2.7.7.4	At3g22890	APS1; ATP sulfurylase 3
	At1g19920	APS2; ATP sulfurylase 1
	At4g14680	APS3; ATP sulfurylase 2
	At5g43780	APS4
Adenylsulfate kinase (APSK); EC 2.7.1.25	At2g14750	AKN1
	At4g39940	AKN2
	At3g03900	APS kinase, putative
	At5g67520	APS kinase, putative
APS reductase (APR); EC 1.8.99.2	At4g04610	APR1
	At1g62180	APR2
	At4g21990	APR3
Sulfite reductase (SiR); EC 1.8.99.1	At5g04590	SIR
O-Acetylserine (thiol)-lyase (OAS-TL); EC 2.5.1.47	At4g14880	OASA1; ATCYS-3A
	At2g43750	OASB; ACS1; ATCS-B
	At3g59760	OASC; ATCS-C
	At3g61440	ATCYSC1; BSAS3;1
	At3g04940	ATCYSD1
	At5g28020	ATCYSD2
	At3g03630	CS26
	At3g22460	OAS-TL, putative
	At5g28030	OAS-TL, putative
Serine acetyltransferase (SAT); EC 2.3.1.30	At1g55920	SAT1; SAT B; SAT5 AtSerat2;1
	At2g17640	SAT2; SAT-106; AtSerat3;1
	At3g13110	SAT3; SAT A; SAT-1; AtSerat2;2
	At4g35640	SAT4; AtSerat3;2
	At5g56760	SAT5; SAT C;SAT-52; AtSerat1;1
Glutamino-cysteine lyase (GCL); EC 6.3.2.2 /γ-glutamylcysteine synthetase (γ-ECS)	At4g23100	GSH1; RML1; CAD2; PAD2
Glutatione synthetase (GSHS); EC 6.3.2.3	At5g27380	GSH2; GSHB
Sulfite oxidase (SOX); EC 1.8.3.1	At3g01910	SOX
Cystathionine γ-synthase (CGS); EC 2.5.1.48	At3g01120	CGS; CGS1; MTO1
	At1g33320	Similar to MTO1
Cystathionine β-lyase (CBL); EC 4.4.1.8	At3g57050	CBL
Methionine synthase (MS); EC 2.1.1.14	At5g17920	ATMS1
	At3g03780	ATMS2
	At5g20980	ATMS3

ous excellent reviews on the subject (Hell & Hillebrand, 2001; Leustek, 2002; Kopriva & Koprivova, 2003; Saito, 2004; Hawkesford & De Kok, 2006; Kopriva, 2006; Kopriva *et al.*, 2007).

Uptake and redistribution of sulfate

Inorganic sulfate is the primary source of sulfur used by plants. Plants have evolved a network

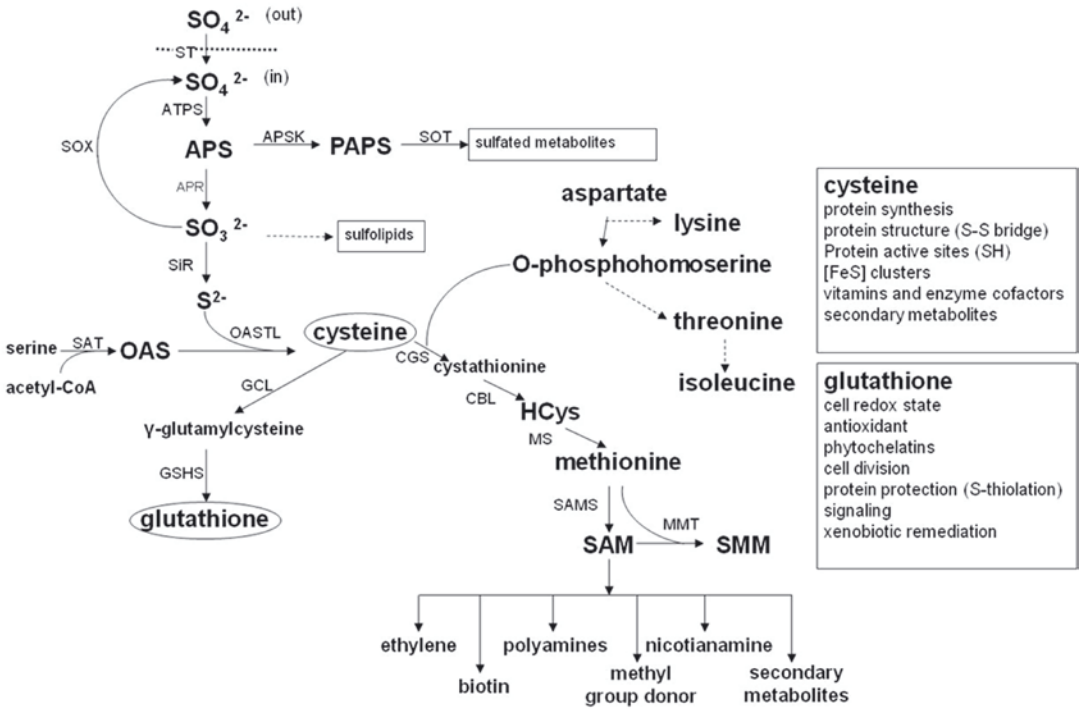

Figure 1. Outline of sulfate assimilation and cysteine metabolism in plants.
Enzymes involved in sulfate and sulfite reduction are present only in plastids, while SAT and OAS-TL are present in plastids, mitochondria and cytosol. Enzymes involved in GSH synthesis are present in chloroplasts and in extrachloroplast fractions. The first two steps of methionine synthesis proceed only in plastids, while the third step only in cytosol because of strictly cytosolic location of MS. APSK, APS kinase; APR, APS reductase; APS, adenosine 5′-phosphosulfate; ATPS, ATP sulfurylase; CBL, cystathionine β-lyase; CGS, cystathionine γ-synthase; GCL, glutamino-cysteine lyase; GSHS, glutathione synthetase; HCys, homocysteine; MMT, *S*-adenosylmethionine:L-methionine *S*-methyltransferase; MS, methionine synthase; OAS, *O*-acetylserine; OAS-TL, *O*-acetylserine (thiol)-lyase; PAPS, adenosine 3′-phosohate 5′-phosphosulfate; SAM, *S*-adenosylmethionine; SAMS, SAM synthetase; SAT, serine acetyltransferase; SMM, *S*-methylmethionine; SiR, sulfite reductase; SOX, sulfite oxidase; SOT, sulfotransferase; ST, sulfate transporter.

of sulfate transporters with different affinity, localization and regulation enabling efficient uptake and distribution of sulfur from root cells into sink organs according to the availability of sulfur and the plant's requirements. Plant sulfate transporters are classified into five groups depending on their protein sequences and characteristics (for review and references see: Hawkesford, 2003). In *A. thaliana*, the family of sulfate transporters (encoded by 14 genes) is larger than that of transporters for nitrate (7 genes), inorganic phosphate (9 genes) or ammonium (6 genes). The best characterized are sulfate transporters from the first group, AtSULTR1;1 and AtSULTR1;2, which are expressed in root hairs and root epidermal and cortical cells (Takahashi *et al.*, 2000; Vidmar *et al.*, 2000; Yoshimoto *et al.*, 2002; 2007; El Kassis *et al.*, 2007). These high-affinity transporters function in the uptake of sulfate from soil. AtSULTR2;1, AtSULTR2;2 (both from group 2) and AtSULTR1;3 (from group 1) are lower-affinity transporters localized in xylem parenchyma and phloem cells of roots and take part in long-distance transport during the source-to-sink translocation. AtSULTR4;1 and AtSULTR4;2, belonging to group 4, are localized in tonoplasts of pericycle and xylem parenchyma cells of roots and hypocotyls and are responsible for sulfate efflux from the vacuole. The role of transporters from group 3 (AtSULTR3;1–AtSULTR3;5) and group 5 (AtSULTR5;1 and AtSULTR5;2) is still not fully clarified.

Sulfate assimilation and cysteine formation

After uptake, sulfate can be either stored in the vacuole within the cell or further metabolized in a series of steps which occur in plastids and comprise (i) activation by adenylation to adenosine 5′-phosphosulfate (APS) in the reaction catalyzed by ATP sulfurylase (ATPS), (ii) reduction of APS to sulfite in the reaction catalyzed by APS reductase (APR) and (iii) sulfite reduction to sulfide in the reaction catalyzed by sulfite reductase (SiR).

Alternatively, APS can be phosphorylated by APS kinase to adenosine 3′-phosphate 5′-phosphosulfate (PAPS), which in turn can be used for the synthesis of sulfated compounds, such as coumarins, glucosinolates, flavonoids, gibberellic acids, hydroxyjasmonates, phenolic acids, steroids or sulfate esters

by the multiprotein family of sulfotransferases (Klein & Papenbrock, 2004). PAPS was also proposed to be used as a reservoir for APS, although a convincing proof for the existence of the enzyme capable of converting PAPS into APS in plants is missing (Droux, 2004). In lower plants, like in bacteria, PAPS can be reduced to sulfite by PAPS reductase (Koprivova *et al.*, 2002).

Sulfite reductase (SiR) contains siroheme and iron-sulfur cluster that are necessary for its activity and catalyzes the reduction of sulfite using electrons donated from ferredoxin. In addition to its catalytic function, SiR plays probably another role in plant chloroplasts. It has been reported that SiR from pea and maize have DNA-binding properties and some results suggest that SiR is essential for proper compacting of nucleoids in plastids, which influences the transcriptional activity of chloroplast DNA (Sekine *et al.*, 2007). The enzymatic activity of SiR from pea was not affected by binding to DNA, indicating that ferredoxin and sulfite are accessible to SiR molecules within the nucleoids. It is possible that SiR could act as a sensor of the redox state of the chloroplast, which is connected with regulation of some chloroplast genes by the redox state. Besides, the extent of SiR association with chloroplast nucleoids varies among plant species.

Interestingly, a molybdoenzyme sulfite oxidase able to produce sulfate from sulfite has been identified recently in plant peroxisomes. This enzyme probably functions as a "safety valve" for detoxifying excess of harmful sulfite and protecting the cells from sulfitolysis (Hansch *et al.*, 2007; Lang *et al.*, 2007).

Two enzymes able to form a complex of cysteine synthase (CS), namely serine acetyltransferase (SAT) and OAS (thiol)-lyase (OAS-TL) are responsible for the next two steps of the pathway. SAT catalyzes synthesis of *O*-acetylserine (OAS) from L-serine and acetyl-CoA, while OAS-TL is responsible for incorporation of sulfur into OAS to synthesize Cys. SAT is a rate-limiting enzyme for biosynthesis of cysteine. Overexpression of SAT in tobacco and potato resulted in increased contents of cysteine and GSH (Blaszczyk *et al.*, 1999; Harms *et al.*, 2000), while overexpression of OAS-TL in Arabidopsis and tobacco caused only moderate increases of Cys and GSH levels (for review see Sirko *et al.*, 2004). In fact, the low ratio of SAT to OAS-TL might be critical for maintaining the strict control of Cys synthesis because also a reduction of OAS-TL activity in transgenic potato resulted in significant elevation of Cys level (Riemenschneider *et al.*, 2005a). On the other hand, these results suggest that OAS-TL could be responsible for both synthesis and breakdown of Cys. The regulatory functions of the CS complex in controlling the sulfur flux are discussed below.

Degradation of Cys is catalysed by cysteine desulfhydrase and results in pyruvate, ammonia, and H_2S production (Papenbrock *et al.*, 2007). The released H_2S might play a role in plant defense upon pathogen attack (Rausch & Wachter, 2005). Several candidates for Cys desulfhydrase, such as NifS-like proteins can be found in *A. thaliana* (Riemenschneider *et al.*, 2005a; 2005b).

Further metabolism of cysteine

Cysteine serves not only for protein production, synthesis of methionine (Met) and Met derivatives such as *S*-adenosylmethionine (SAM) and *S*-methylmethionine (SMM), but it is also a branching point for the synthesis of many other sulfur-containing compounds such as glutathione (GSH) or *S*-methylcysteine, *S*-alkylcysteine, glucosinolates, and phytoalexins (Leustek, 2002).

SAM is a key substrate for numerous enzymes (Fontecave *et al.*, 2004; Roje, 2006). It serves as a donor of methyl group and a source of methylene groups for the synthesis of lipids, pectins, alkaloids, phytosterols, osmoprotectants, precursors of lignins, lignans, suberins, hydroxycinnamic acids, flavonoids, stilbens, various aromatic and volatile fragrance and aroma compounds. The methyl group can be transferred from SAM to a variety of acceptors, including amino-acid residues in proteins, nucleic acids and other molecules. SAM is also a source of reactive 5′-deoxyadenosyl radicals used by numerous enzymes. The amino group of SAM is used in the synthesis of biotin. SAM is also a donor of the aminoisopropyl group in the synthesis of polyamines and a precursor of ethylene, nicotianamine and phytosiderophores.

Glutathione (GSH) is the main form of reduced sulfur transported (through phloem) and stored in plants (Rennenberg *et al.*, 1979). For a long time it has been postulated that GSH acts as an interorgan signal for the sulfur status from shoots to roots (Lappartient *et al.*, 1999; Herschbach *et al.*, 2000). The concentration of GSH is much higher than of free Cys in the cell, what indicates that, being less-reactive than Cys, it is used for storage of reduced sulfur. GSH plays not only an important role in sulfur metabolism, but is also involved in regulation of growth and development of the plant by modulation of such processes as mitosis, cell elongation, senescence, cell death, resistance to environmental stresses, detoxification and maintaining the redox homeostasis. Fundamental to the GSH role is not only its concentration, but also the ratio of its reduced form to the oxidized one. Changes in the GSH pool provide information on the redox state of the cell, which

might influence expression of genes important in defense against environmental stresses. Increases of the GSH pool have been observed in response to many environmental stresses including pathogen attack or treatment with heavy metals (Foyer & Noctor, 2005; Noctor, 2006; Wawrzynski *et al.*, 2006), whereas plants with decreased amounts of GSH were more sensitive to a range of environmental stresses, such as heavy metal treatment or oxidative stresses (Xiang & Oliver, 1998). The regulatory role of GSH in the metabolic control of the sulfur pathway will be discussed below.

S-Methylmethionine (SMM) may be used in cereals for long distance transport of reduced sulfur since it was found to be about 1.5-fold more abundant than GSH in phloem sap of wheat (Bourgis *et al.*, 1999).

MULTIPLE LEVELS OF REGULATION OF SULFATE ASSIMILATION PATHWAY

Current understanding of metabolic control by thiols and OAS

During sulfur limitation the activities of sulfate transporters, ATP sulfurylase (ATPS) and APS reductase (APR) are higher than those observed under optimal sulfur supply. This process is regulated mainly, but not exclusively, at the level of transcription of respective genes (Takahashi *et al.*, 1997; Yamahuchi *et al.*, 1997). It has been known for several decades that GSH, the major organic thiol-containing metabolite, has an important role in regulating sulfur homeostasis. Feeding of plants with thiols, such as Cys and GSH, results in decreased sulfate uptake and reduction as well as reduced expression of genes encoding proteins of the pathway (Smith *et al.*, 1997; Vidmar *et al.*, 1999; Vauclare *et al.*, 2002). Since Cys can be promptly converted in plants into GSH, it is unclear which of these two metabolites takes part in the control of sulfate uptake and assimilation. Experiments conducted in *Brassica napus* prove that GSH rather than Cys is responsible for such control (Lappartient & Touraine, 1996; Lappartient *et al.*, 1999), while in maize Cys influences expression of *ATPS* without the need for conversion to GSH (Bolchi *et al.*, 1999). These contradicting results emphasize that not all models of regulation are common among plant species and they pinpoint the need for investigation of sulfur metabolism in various plants. So far, there is no evidence for a direct negative role of thiols in regulation of expression of the genes involved in sulfate transport and assimilation. The lack of a documented relationship between increased internal levels of GSH and accumulation of transcripts of sulfate transporters argues against GSH as a direct systemic regulator of sulfur metabolism (Buchner *et al.*, 2004; Nocito *et al.*, 2006; Rouached *et al.*, 2008).

O-Acetylserine (OAS) is a direct precursor of Cys. In bacteria, OAS not only serves as a carbon/nitrogen skeleton for Cys synthesis, but it is also a positive regulatory molecule that directly binds to the CysB protein (a member of LysR family of transcription regulators), which activates transcription of genes that belong to the cysteine regulon (Kredich, 1992; 1996). Similarly, a strong influence of externally added OAS might suggest that this molecule serves as a positive factor of sulfur assimilation also in plants (Neuenschwander *et al.*, 1991; Smith *et al.*, 1997; Koprivova *et al.*, 2000). It is also possible that OAS, which in normal conditions is the limiting substrate for cysteine synthesis, might function in plants as a sensor for an imbalance between sulfur and nitrogen metabolism (Hawkesford & De Kok, 2006) and, as such, it might be rather a part of a regulatory network signaling a metabolic demand for sulfur-containing compounds (for example in the conditions of environmental stresses requiring increased level of GSH) than an element of a cascade signaling sulfur shortage in the soil. Nevertheless, a direct regulatory role of OAS is not as obvious in plants as it is in bacteria. Results of some experiments suggest that an increased level of OAS is rather a consequence of sulfur depletion than an early regulatory signal of sulfur shortage (Hopkins *et al.*, 2005). On the other hand, a comparison of transcript profiles of *A. thaliana* in response to sulfur starvation or after OAS treatment revealed a large number of coregulated genes by both factors in either leaves or roots (Hirai *et al.*, 2003). Interestingly, the same studies indicated a significant lack of coregulation of gene expression between leaves and roots of sulfur-deficient plants or between leaves and roots of plants grown in the presence of OAS. However, further analysis of gene expression using metabolite-to-gene network analysis indicated no apparent correlation of OAS accumulation with expression of genes known to be induced by sulfur deficit (Hirai *et al.*, 2005). This further suggests that OAS may be only one of many signals (if not consequences) of sulfur deficiency in plants and, as mentioned above, that it is rather an element signaling the metabolic demand for sulfur than the status of sulfur nutrition available to the plant.

The mechanisms regulating gene expression in plants in response to sulfur shortage are not yet explained. It is not obvious why the external application of either GSH or OAS results in the changes of gene expression described above,

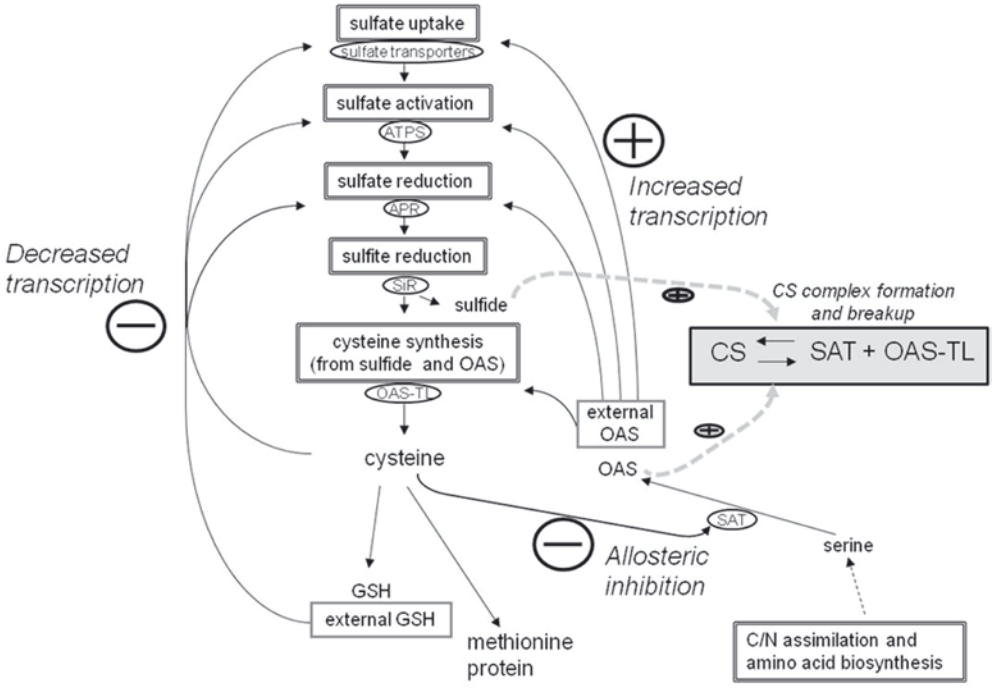

Figure 2. Metabolic control of sulfate uptake and assimilation by thiol-containing commpounds and OAS — modified from Hawkesford (2000).
A series of feedback loops repress or activate expression of genes encoding enzymes catalyzing some steps in the pathway. In addition, there is also feedback allosteric inhibition of SAT by cysteine and regulation of sulfur flux at the level of cysteine synthase (CS) complex. Sulfide stimulates complex formation, while OAS stimulates its dissociation; OAS-TL is inactive in the complex, while SAT is active only in the CS complex. ATPS, ATP sulfurylase; APR, APS reductase; SiR, sulfite reductase; OAS-TL, OAS (thiol)-lyase; SAT, serine acetyltransferase; GSH, glutathione; OAS, *O*-acetylserine. External GSH and external OAS refer to the compounds added to growth medium (see also explanations in the text).

while changes of the endogenous levels of these metabolites do not correlate with changes of the transcript levels. It is known that sulfate transport, reduction and further metabolism of sulfur in plants are controlled not only by availability of sulfate but also by metabolic demand for sulfur-containing metabolites.

It has been proposed recently that these two partly overlapping regulatory mechanisms might be responsible for controlling gene expression, depending on whether the sulfur metabolism is stimulated by sulfur deficiency stress or by an internal metabolic demand (Rouached *et al*., 2008).

A scheme explaining the current understanding of the roles of OAS and thiol-containing compounds in the regulation of sulfur uptake and assimilation is shown in Fig. 2.

Transcriptional control of plant responses to sulfur deficit

Sequences named sulfur-responsive elements (SURE) containing 5-bp core sequences (GAGAC or GTCTC), which are very similar to the auxin response factor (ARF) binding sites, were identified in the promoter of *SULTR1;1* gene of *A. thaliana* (Maruyama-Nakashita *et al*., 2005) and occur in the promoter regions of many sulfur-responsive genes. The SURE element present in the promoter region of *SULTR1;1* is in charge of transcriptional response to sulfur shortage but not to OAS and, what is very important, not to auxin (Maruyama-Nakashita *et al*., 2005). Nevertheless, it is possible that a not-yet-identified member of the ARF family of transcription factors (Guilfoyle & Hagen, 2007) is capable of binding to SURE sequences and inducing sulfur deficiency-dependent expression. The SURE sequences can also be found in promoters of genes not regulated by sulfur supply, therefore, additional regulatory factors must determine the specificity of the response to sulfur status.

The only so far identified *trans*-acting factor specifically regulating transcription of the genes involved in sulfate uptake and assimilation is SLIM1 (sulfur limitation 1), which was found during screening for mutation influencing the activity of the *AtSULTR1;2* promoter in *A. thaliana.* SLIM1 appears to be identical to the transcription factor EIL3 (ethylene-insensitive3-like), a member of the EIL family (Maruyama-Nakashita *et al.*, 2006). SLIM1/EIL3 plays an important role in response to sulfur starvation since its inactivation results in 60%

limitation (in comparison to the wild type plants) of sulfate uptake under the conditions of sulfur deficit. Overexpression of *SLIM1* in *slim1* mutants restores the ability to respond to sulfur starvation. No other member of the EIL family of transcription factors is capable of reverting the *slim1* phenotype, which indicates that SLIM1/EIL3 is specialized for sulfur response. SLIM1 influences expression of many genes regulated by sulfur starvation, especially genes encoding sulfate transporters from groups 1, 3 and 4, and enzymes from the glucosinolate metabolism pathway. Interestingly, the expression of some sulfur starvation-regulated genes, for example *APR2* and *APR3*, is unaffected in the *slim1* mutant. In *slim1* mutants the levels of OAS and GSH are changed (increased and decreased, respectively) comparing to the wild type lines during sulfur starvation, but in the case of GSH only in the upper parts of the plants. The putative SLIM1 binding sites (AYGWAYCT) are not present in all sulfur starvation-induced genes with expression influenced by SLIM1. The information available so far suggests that (i) SLIM1 is located downstream in the regulatory cascade to the regulation of plant sulfur metabolism by OAS and GSH, (ii) SLIM1 influences only a part of the plant response to sulfur deprivation, (iii) SLIM1 is rather not the single *trans*-acting element of sulfur metabolism in plants and is probably an element of a complex signaling cascade.

Glucosinolates are sulfur-rich plant metabolites found almost exclusively in the *Brassicaceae* family containing well-known species such as *Brassica oleracea* (cabbage, cauliflower), *B. rapa* (turnip, Chinese cabbage), *B. napus* (rapeseed), *Raphanus sativus* (common radish), *Armoracia rusticana* (horseradish), *A. thaliana*, and many others. Degradation of glucosinolates is an important aspect of the sulfur limitation response in *Brassicaceae* since sulfur released from glucosinolates can be re-used in primary metabolism. SLIM1 co-regulates this sulfur recycling process by induction of genes encoding enzymes involved in glucosinolate degradation, and down-regulation of genes encoding enzymes needed for glucosinolate synthesis. Furthermore, it is known that sulfur deficiency represses the expression of three Myb transcription factors necessary for glucosinolate biosynthesis, *PMG1/Myb28* and *PMG1/Myb29* that are expressed preferentially in leaves and regulate biosynthesis of aliphatic glucosinolates, and *ATR1/Myb34* expressed preferentially in roots and regulating biosynthesis of indole glucosinolates. The effect of SLIM1 on glucosinolate metabolism during sulfur shortage may be partially explained by its negative influence on *ATR1/Myb34* expression in roots (Maruyama-Nakashita *et al*., 2006; Yan & Chen, 2007). The role of SLIM1 in regulation of *PMG1/Myb28* and *PMG1/Myb29* gene expression is unclear (Hirai *et al*., 2007).

BIG is another gene from Arabidopsis reported as involved in the regulation of gene transcription under sulfur limitation (Kasajima *et al*., 2007). The *BIG* gene encodes a calossin-like protein necessary for the polar transport of auxin and it is, apparently, not a transcription factor. The BIG protein consists of 5098 amino acids and it has probably numerous functions. *big* mutants exhibit pleiotropic phenotypes suggesting defects in multiple processes such as sensing and/or signaling of hormones (e.g., auxin, cytokinin, giberellin, ethylene), light, and alterations of root architecture induced by phosphate deficiency. The significant increase of *APR1* expression in optimal and –sulfur deficient conditions, and the slight increase of *SULTR2;2* expression in optimal conditions in a *big* mutant in comparison to the control line suggest that BIG negatively influences transcription of these genes. The sulfate concentration and GSH level in this mutant seem not to be affected, while the mutation results in a decreased level of OAS in optimal sulfate supply (but not during sulfur deficit). These observations suggest that either BIG has an influence on the signal transduction pathway independently from the OAS/GSH regulation or it is downstream of OAS/GSH in the signal cascade. Experiments with plants treated with auxin or with a polar auxin transport inhibitor imply that the induction of sulfur deficit-responding genes in the *big* mutant is rather independent of auxin (Kasajima *et al*., 2007).

Recently, the influence of three sulfur starvation-responsive transcription factors, IAA13, IAA28 and ARF-2, on the sulfate assimilation pathway in *A. thaliana* has been studied (Falkenberg *et al*., 2008). The authors investigated both overexpressing and knock-down lines under normal and sulfate deficiency conditions and they observed pleiotropic effects of these mutations. The main conclusion of the reported work was that the studied factors serve as coordinators of the metabolic shift driving sulfur homeostasis rather than as direct effectors of the sulfate assimilation pathway.

Regulation by miRNA

Controlling the level of gene expression by miRNA-initiated cleavage of cognate complementary mRNAs has significant regulatory function. miRNAs are not only involved in developmental processes but also in response to biotic and abiotic stress, including oxidative stresses and deprivation of nutrients, such as phosphate or sulfate (Sunkar *et al*., 2007). miRNA395, represented by six loci arranged in two clusters, is induced by sulfur limitation in *A. thaliana*. Targets for this miRNA are genes encoding

ATP sulfurylases (*APS1, APS3, APS4*), sulfate transporter (*AtSULTR2;1*) implicated in the internal translocation of sulfate from roots to shoots, and possibly other genes (Jones-Rhoades & Bartel, 2004; Adai *et al.*, 2005; Allen *et al.*, 2005). Influence on two gene families (*APR*s and *SULTR*s) encoding proteins that function coordinately in the same metabolic pathway is an unusual example for a function of plant miRNA. Despite the fact that an important role for miRNA395 in regulating sulfate homeostasis seems to be obvious, the functional significance of this regulation still needs to be investigated (Sunkar *et al.*, 2007).

Post-translational regulation and involvement of protein kinases

Some high affinity sulfate transporters are apparently regulated on the post-translational level (Yoshimoto *et al.*, 2007). They contain a conserved region named STAS (sulfate transporter and anti-sigma factor antagonist) domain at their C-terminus. The STAS domain shares significant similarity with the *Bacillus* sp. anti-sigma factor antagonist SpoIIAA, and is suggested to function as a domain for protein–protein interactions, which may play a role in regulating the activity and/or stability of sulfate transporters. Experiments performed with SULTR1;1 and SULTR1;2 have revealed that this domain is key for the activity and stability of the transporters, while a linking region containing several amino acids is critical for functioning of the protein. Also phosphorylation of STAS domain regulates the activity of SULTR1;2 (Shibagaki & Grossman, 2004; 2006; Rouached *et al.*, 2005).

Two enzymes involved in sulfur metabolism, APS reductase and glutamate-cysteine ligase (γ-glutamylcysteine synthase), can be activated by oxidation resulting in the formation of intramolecular disulfide bonds, which influences assimilation of sulfate and GSH level, respectively, in response to the redox status of the cell (Bick *et al.*, 2001; Hicks *et al.*, 2007).

The SAT enzyme can be subject to feedback allosteric regulation by cysteine. Different SAT isoforms have different sensitivity towards such feedback regulation: the isoforms localized in organelles in *Arabidopsis* and watermelon are feedback-insensitive, while the cytosolic ones are feedback-sensitive (Noji *et al.*, 1998). Moreover, in *Glycine max* the activity of at least one SAT isoform (GmSerat2;1) can be modified by calcium-dependent protein kinase (Liu *et al.*, 2006). The phosphorylation of GmSerat2;1 occurring under oxidative stress converts this SAT to a form insensitive to feedback inhibition by cysteine. This allows to increase cysteine production and to further support production of glutathione that is important in response to stresses. However, analysis of plant SAT sequences available in data bases for the presence of potential phosphorylation sites does not suggest that phosphorylation of SAT may be a universal mechanism (Liu *et al.*, 2006).

Recently, it has been shown that a member of the plant-specific SNRK2 kinase family acts as a key regulator in the signaling cascade of the sulfur deprivation response in *Chlamydomonas reihardtii* (Gonzalez-Ballester *et al.*, 2008). Some limited data exist that allow one to speculate that the SNRK2 type kinases are involved in regulation of the sulfur metabolism pathway also in higher plants. For example, mutation of the *SNRK2.3* gene of *A. thaliana* resulted in reduced induction of *SULTR2;2* during sulfur deficit, while the accumulation of OAS was higher than that in wild type plants grown in the same conditions (Kimura *et al.*, 2006). However, an unchanged expression of other sulfur nutrition-responsive genes (*APR* and *SAT*) in the *snrk2.3* mutant suggests rather a moderate role of this kinase in regulation of the sulfur pathway.

Control of sulfur flux by formation of the cysteine synthase complex affecting SAT and OAS-TL activities

The last steps of cysteine biosynthesis are catalyzed by a bi-enzyme complex, called cysteine synthase (CS), which is composed of a dimer of homotrimers of serine acetyltransferase (SAT) and two homodimers of *O*-acetylserine (thiol)-lyase (OAS-TL). Formation of the CS complex plays an important role in the regulation of both enzymatic activities. OASTL is active only in a free form, while SAT activity is dependent on the association with OAS-TL (Droux *et al.*, 1998; Wirtz & Hell, 2007). Sulfide stimulates the formation of the complex, while OAS stimulates its dissociation (Fig. 2). The tertiary structures of bacterial SAT and of OAS-TL from bacteria and plants are known (Burkhard *et al.*, 1998; Tai *et al.*, 2001; Hindson & Shaw, 2003; Gorman & Shapiro, 2004; Olsen *et al.*, 2004; Pye *et al.*, 2004; Bonner *et al.*, 2005). No crystal structure of a full CS complex is available yet, however, on the basis of the recently determined tertiary structure of OAS-TL with the C-terminal decapeptide of SAT (Huang *et al.*, 2005; Francois *et al.*, 2006) and the fluorescence spectroscopy data for the OAS-TL-SAT mixture (Campanini *et al.*, 2003) it is assumed that SAT binds to OAS-TL from its catalytic cavity side. The substrate, OAS, binds to OAS-TL also at the catalytic cavity, thus the interaction of OAS-TL with SAT appears to effectively inhibit the activity of the former enzyme.

The formation of the CS complexes appears to control cysteine synthesis including the flux through the entire assimilatory pathway. In plants the situ-

ation is additionally complicated by the subcellular compartmentalization of isoforms of both enzymes and the complexes, which may serve different purposes and may be differentially regulated in each compartment. It was recently demonstrated that the disruption of CS complex formation due to production of enzymatically inactive SAT (but capable of interaction with OAS-TL) in the cytosol of transgenic tobacco plants resulted in deregulation of not only the cytosol CS complex but also of the organellar CS complexes and strongly increased accumulation of Cys and GSH (Wirtz & Hell, 2007). Furthermore, analysis of knock-out mutants of the major OAS-TL isoforms in *A. thaliana* has shown that, surprisingly, mitochondria play the most important role for cysteine synthesis in this plant despite that both sulfide and cysteine can be efficiently exchanged between cytosol and organelles (Heeg *et al.*, 2008).

For examples of the effects of modification of SAT and OAS-TL levels in plants and for a model explaining the significance of CS complex formation the reader is referred to other reviews (Hell & Hillebrand, 2001; Sirko *et al.*, 2004; Wirtz & Droux, 2005).

SULFUR FLUX AS PART OF GENERAL PLANT METABOLISM — ELUCIDATION OF PLANT RESPONSE TO SULFUR DEFICIT USING SYSTEMS BIOLOGY APPROACH

Sulfur metabolism cannot be separated from the general plant metabolism, and it is strongly influenced by such factors as availability of other nutrients (nitrogen, phosphorus), carbohydrate metabolism, and light. Expression of many genes of the pathway, including APS reductase (APR), sulfite reductase and chloroplastic isoforms of OAS-TL undergo diurnal rhythm. Besides, it has been demonstrated that addition of sucrose to the medium after treatment with darkness induces APR activity in roots (Koprivova *et al.*, 2000; Kopriva & Koprivova, 2003). The reductive assimilation pathway of nitrate and sulfate are linked through the availability of electrons and pools of carbohydrates, which fluctuate diurnally. Both metabolic pathways are very well coordinated and depletion of one element represses the other pathway (Koprivova *et al.*, 2000; Migge *et al.*, 2000; Prosser *et al.*, 2001; Hesse *et al.*, 2004).

Taking into account the multiple interactions between sulfur metabolism and other metabolic pathways it should not be surprising that numerous transcriptomic and metabolomic changes are induced in plants by sulfur deprivation. It has been shown in tobacco that even a short-term sulfur deficit influences expression of numerous genes from different functional categories, including those involved in stress- and pathogen response, formation of cell-wall structure, protein degradation, photosynthesis, carbon metabolism, translation and, of course, sulfur metabolism (Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003; Lewandowska *et al.*, 2005; Wawrzynska *et al.*, 2005). The possibility of using macro- and microarrays of *A. thaliana* covering almost the complete genome has facilitated the analysis of transcriptional changes under sulfur starvation in this model plant (Hirai *et al.*, 2003). The molecular and metabolic changes in plants that encounter sulfur shortage were recently arranged in an interesting schema depicting the sequence of events (Hawkesford & De Kok, 2006). It has been proposed that plant responses to sulfur starvation can be divided into three major phases depending on the degree and duration of sulfur deficit (Fig. 3A). During the initial phase, changes in expression concern predominantly genes from the sulfur assimilation pathway, sulfate uptake and remobilization of inorganic sulfur reserves from the vacuole. After this phase, when sulfur is still a limiting factor, changes involve multiple metabolic pathways. Plants intensify the turnover of organic sulfur, initiate stress defense response and down-regulate genes responsible for the uptake and assimilation of nitrogen. This phase is followed by changes in developmental processes such as increase of root-to-shoot mass ratio, induction of senescence and, at the end, growth deprivation of both shoots and roots and starting of earlier reproduction to save the sulfur resources for production of vital seeds. Long-term sulfur starvation causes a decrease in the level of total proteins, chlorophyll, RNA and biomass. One of the visual symptoms of sulfur shortage is chlorosis occurring mainly in young leaves which produce insufficient amounts of chlorophyll and lipids which in turn leads to reduction of photosynthetic activity and an overall decline of metabolism.

Integration of data from transcriptomic and metabolomic studies from various periods of sulfur deprivation has revealed a complexity of the plant response to a gradually progressing reduction of sulfur availability (Hirai & Saito, 2004; Nikiforova *et al.*, 2004; 2005a; 2005b; Noctor, 2006). A compiled model proposing the existence of two states, the state of sulfur limitation (short-term response) and the state of sulfur deficiency (long-term response) has been proposed recently (Hoefgen & Nikiforova, 2008). According to this model, which is schematically shown in Fig. 3B, auxin is implicated in the physiological changes during short-term response. Plant metabolism is directed towards intensification of sulfate uptake and assimilation, and sulfur mobilization from various resources. Although no evidence from direct

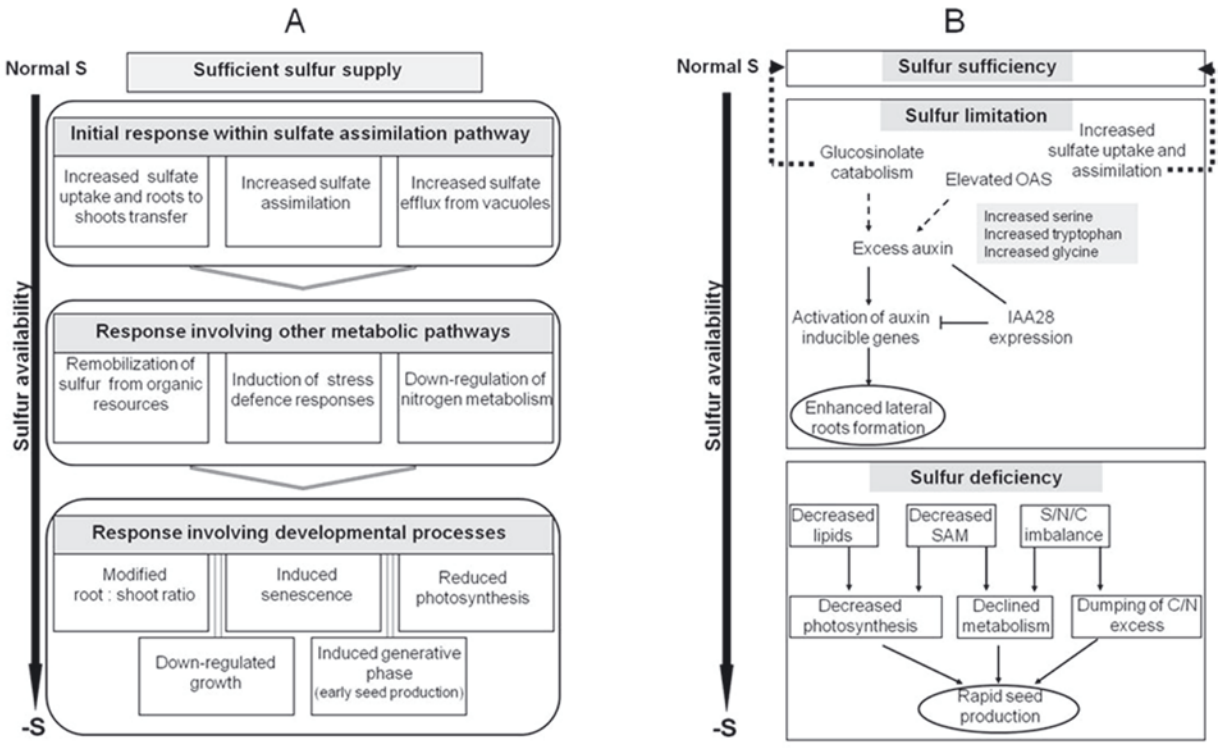

Figure 3. Models of the sequence of events in plants grown at insufficient sulfur supply (–S).
The model A is adapted from Hawkesford and De Kok (2006) and the model B is adapted from Hoefgen & Nikiforova (2008).

measurements of auxin level exist, the presumably elevated auxin level triggers enhanced production of lateral roots (in search of sulfur resources). In the case of a prolonged period of sulfur starvation, the next state defined as sulfur deficiency is observed in plants. The factors influencing the transition between these two states are poorly understood, however, it is proposed that the feedback inhibition of auxin-induced genes by transcriptional regulator IAA28 (see also above) might be responsible for such a switch. Decreased SAM and lipid levels, reduced photosynthesis and misbalanced sulfur/nitrogen/carbon metabolism lead toward an overall decline of metabolism, growth inhibition, rescue reprogramming of the life cycle and premature seed production.

In conclusion, despite our knowledge of the regulatory mechanisms responsible for the plant response to sulfur availability having advanced significantly, mostly due to application of modern high-throughput and systems biology approaches, full understanding of the process requires numerous questions to be answered.

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