

Review

Sulfur signaling and starvation response in *Arabidopsis*Daniela Ristova^{1,*} and Stanislav Kopriva¹

SUMMARY

As sessile organisms, plants have developed sophisticated mechanism to sense and utilize nutrients from the environment, and modulate their growth and development according to the nutrient availability. Research in the past two decades revealed that nutrient assimilation is not occurring spontaneously, but nutrient signaling networks are complexly regulated and integrate sensing and signaling, gene expression, and metabolism to ensure homeostasis and coordination with plant energy conversion and other processes. Here, we review the importance of the macronutrient sulfur (S) and compare the knowledge of S signaling with other important macronutrients, such as nitrogen (N) and phosphorus (P). We focus on key advances in understanding sulfur sensing and signaling, uptake and assimilation, and we provide new analysis of published literature, to identify core genes regulated by the key transcriptional factor in S starvation response, SLIM1/EIL3, and compare the impact on other nutrient deficiency and stresses on S-related genes.

INTRODUCTION

One of the fundamental questions in biology is how organisms respond to the availability of nutrients and how multifaceted reprogramming occurs at the cellular level, in order to integrate nutrient response with intrinsic regulators to adapt their growth and development. Contradictory to older opinions that nutrient feeding into cellular mechanisms and growth occurs spontaneously, recent research shows that nutrient signaling mechanisms and regulatory networks are specifically tailored in different cell types, and have crucial impact in all organisms (Li et al., 2021).

Plants as sessile organisms are highly efficient in the nutrient acquisition, and play a vital role in conversion of inorganic elements to bioorganic molecules. For instance, carbon (C) as atmospheric CO₂ is converted to sugars in the plant photosynthetic machinery; while plant root systems take up mineral elements like nitrogen (N), phosphorus (P), and sulfur (S) from the soil and synthesize nucleic acids, amino acids, lipids, vitamins, adenosine triphosphate (ATP), sulfatides, vitamins, coenzymes, and prosthetic groups, many of these essential for animal and human nutrition (Li et al., 2020, 2021). In this review, focusing on S sensing and signaling and starvation response, we survey molecular mechanisms of S uptake, signaling, assimilation, and metabolism, and compare it with other key elements including N and P, generally in *Arabidopsis thaliana*, uncovering similarities, differences, and open questions.

NITROGEN AND PHOSPHORUS SENSING AND SIGNALING

Nitrogen and phosphorus are the two most common limiting nutrients for plant productivity. N is taken up by plants mainly as the inorganic anion nitrate. However, nitrate is not only a nutrient but also an important signal in plants (Wang et al., 2004). Nitrate perception starts at the dual affinity transporter and sensor (transceptor), NRT1.1 (Ho et al., 2009). The nitrate signaling cascade (or the primary nitrate response, PNR) is activated in plants that are grown on ammonium and then supplied with nitrate (Alvarez et al., 2014; Guan et al., 2014; Krouk et al., 2010; Maeda et al., 2018; Ristova et al., 2016; Vidal et al., 2014; Wang et al., 2010; Xu et al., 2016). Intensive research in the past years identified details of regulation of NRT1.1 and how NRT1.1 activates multiple downstream mechanisms of nitrate sensing and signaling (for detailed recent review see (Maghiaoui et al., 2020)). For example, phosphorylation of NRT1.1 is a key regulatory step between low and high-affinity function. In *Arabidopsis*, under low nitrate, the kinase CIPK23 (CBL-Interacting Protein Kinase 23) interacts with CBL9/1 (Calcineurin B-Like Protein9 or 1) and

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phosphorylates NRT1.1 at Thr-101 that changes the function of the transporter from low to high affinity (Ho et al., 2009). ABI2 (ABA-INSENSITIVE 2), a protein phosphatase 2C involved in negative regulation of abscisic acid signaling, can dephosphorylate CBL1 and CPK23 and prevent their function (Leran et al., 2015), suggesting that under stress conditions, increased ABA can repress ABI2 function and nitrate uptake (Vidal et al., 2020). NRT1.1 is further connected with calcium signaling. Namely, phosphorylated NRT1.1 induces the phospholipase C (PLC) leading to increase in inositol triphosphate (IP₃) that further induces the calcium waves leading to translocation of group III CALCIUM-DEPENDENT PKs (CPKs) CPK10/30/32 into the nucleus, where they phosphorylate NLP7, which then activates nitrate target genes (Liu et al., 2017). Recent discovery showed that the interaction of NRT1.1 and the cyclic nucleotide-gated channel (CNGC) protein 15 (CNGC15) is part of a molecular switch that controls the calcium influx depending on the nitrate levels (Wang et al., 2021a). In rice, OsNRT1; 1B recruits the RING-type E3 ubiquitin ligase, NBIP1, for 26S proteasome-dependent degradation of the cytosolic OsSPX4 protein in high nitrate, which releases OsNLP3 (functionally similar to NLP7 in *Arabidopsis*) to translocate from cytosol to the nucleus and induce transcription of nitrate-responsive genes (Hu et al., 2019).

In contrast, most of phosphate (Pi) signaling research focusses on the response of plants to P deficiency. In *Arabidopsis*, primary Pi uptake in the roots is mainly facilitated by the PHOSPHATE TRANSPORTER 1 (PHT1) protein, which in sufficient Pi condition is phosphorylated and retained at the ER and recycled by ubiquitination-mediated degradation through receptor-mediated targeting to the vacuole (Cardona-Lopez et al., 2015; Chen et al., 2015). Whether PHT1 acts as a plasma membrane (PM) sensor of external Pi remains unresolved. Under Pi-deficient conditions, PHT1 is dephosphorylated, and PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (PHF1) is mediating the delivery of PHT1 to the PM, which increases Pi uptake (Chen et al., 2015). At the transcription level, PHOSPHATE STARVATION RESPONSE 1 (PHR1) and its closest paralog PHR1-LIKE1 (PHL1) regulate the expression of the Pi starvation-induced (PSI) genes and downstream developmental and metabolic modifications (Bustos et al., 2010; Rubio et al., 2001). In *Arabidopsis* and rice, SPX (Syg1, Pho81, and Xpr1) domains are found in multiple proteins with Pi sensing function. In *Arabidopsis*, SPX domain proteins repress the activity of Pi transporters (Wege et al., 2016; Wild et al., 2016) or change transcription of phosphate starvation response (PSR) genes (Puga et al., 2014). In *Arabidopsis* and rice, SPX single-domain proteins are upregulated during Pi-sufficient conditions, and interact with AtPHR1/OsPHR2 (Puga et al., 2014; Wang et al., 2009) preventing their entry into the nucleus. However, under deficient Pi conditions, SPX proteins undergo degradation and PHRs are released, enter the nucleus, and orchestrate transcriptome reprogramming of PSI genes (Ruan et al., 2019). As mentioned earlier, in rice, SPXs are acting as integrators of Pi and nitrate signaling, because as well as with OsPHR2, SPX4 interacts with one of the nitrate master transcriptional regulators OsNLP3 (analogical to AtNLP7) under nitrate-deficient conditions, and therefore represses the PSI and nitrate responses (Hu et al., 2019). SPX domain proteins in yeast and plants were shown to bind inositol pyrophosphate (InsPP) with higher affinity than Pi. Under Pi-sufficient conditions, InsP₈ accumulates and triggers the formation of an SPX–InsP₈–PHR complex. Under Pi starvation, InsP₈ levels drop and the complex dissociates, PHRs are released and upregulate PSI genes (Dong et al., 2019). Thus, Pi in plants is sensed in the form of InsPP.

Comparing the knowledge of N and Pi signaling to S signaling (Figure 1), it is evident that S signaling is quite unclear. At the uptake level, the mechanisms of regulation of NRT1.1 and PHT1 are clearly described, involving phosphorylation as a switch between low- and high-affinity actions of the transporter NRT1.1 (Ho et al., 2009), and phosphorylation and recycling of PHT1, depending on the available nitrate and phosphate in the environment. However, for S, signaling is not certain whether SULTR1.2 can sense sulfate (Zhang et al., 2014), similar to PHT1 (Chen et al., 2015). The C-terminal STAS domain of SULTR1;2 can interact with OAS-TL A, which results in the reduction of the transporter activity (Shibagaki and Grossman, 2010), but how this is relevant *in vivo* remains unclear. The downstream transcriptional cascade activation is described in detail for nitrate (Vidal et al., 2020), and phosphate signaling (Wang et al., 2021b). However, for S starvation, only one transcription factor SLIM1/EIL3 is known to regulate S starvation response genes (Kawashima et al., 2011; Maruyama-Nakashita et al., 2006; Wawrzynska and Sirko, 2014). Therefore, many open questions remain unresolved.

SULFUR SENSING AND SIGNALING

Sulfur (S) is another essential element for all organisms, especially for humans and animals, since they cannot assimilate inorganic S and synthesize cysteine and therefore must obtain S-containing amino acids

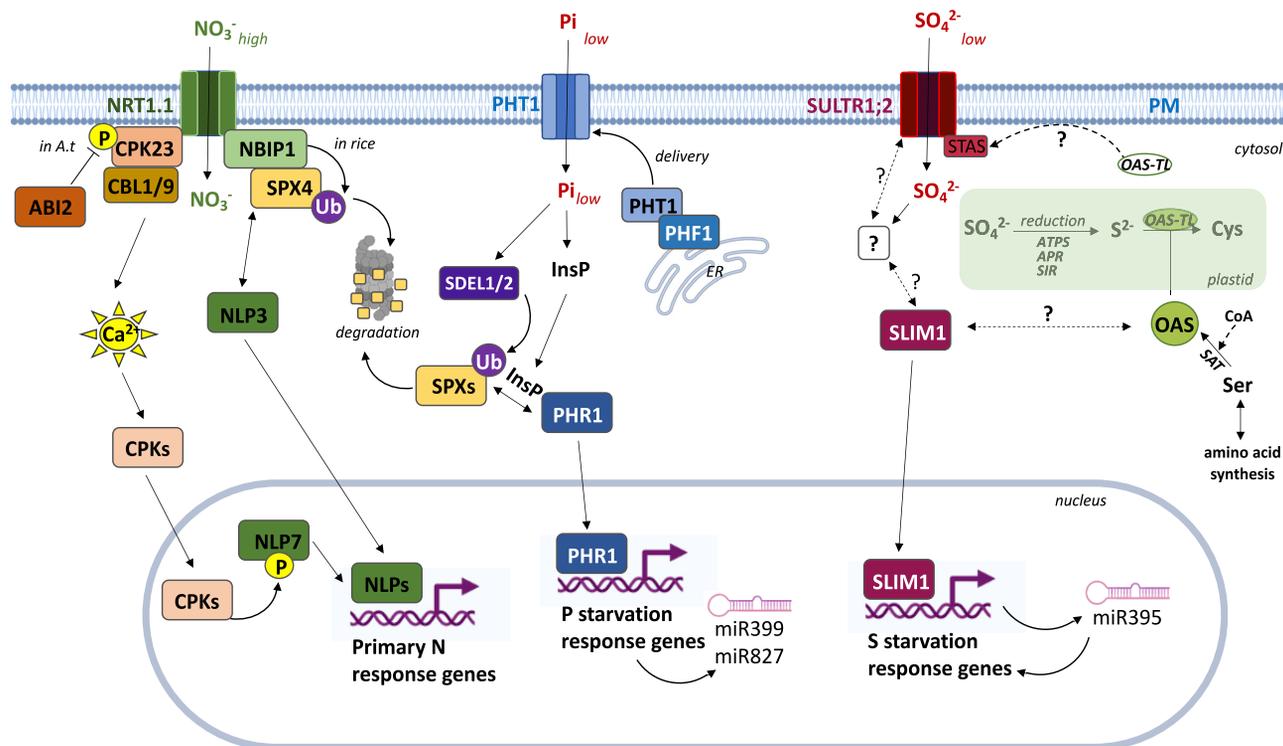


Figure 1. Transport and signaling under nitrate (NO_3^-) supply, phosphate (Pi), and sulfate (SO_4^{2-}) deficiency

In *Arabidopsis thaliana* (A.t.), the NRT1.1, a dual-affinity nitrate transporter and receptor (transceptor), is phosphorylated at low NO_3^- by CPK23 interacting with CBL1/9, while ABI2 can dephosphorylate both and regulate NRT1.1 switch. Under sufficient nitrate conditions, AtNRT1.1 is dephosphorylated, which rapidly increases cytosolic calcium signaling involving subgroup III CPKs which then phosphorylate NLP7, leading to activation of PRN genes. In rice, under sufficient nitrate conditions, OsNRT1;1B interacts with the NBIP1 that targets SPX4 for degradation, thus releasing NLP3 that activates nitrate-responsive genes. Under Pi limitation, the Pi transporter PHT1 is rapidly being delivered to the plasma membrane, and the SPX–InsP–PHR complex dissociates, which targets the SPXs for degradation, and releases the PHRs to drive Pi starvation response. Sulfate is transported by the SULTR transporters (SULTR1;1 and SULTR1;2). S starvation is sensed by an unknown receptor, possibly involving cytosolic OAS and/or OAS-TL, and/or the STAS domain of the SULTR1;2 transporter, but not the SULTR1;1. The key transcriptional regulator of S-deficiency response is SLIM1/EIL3. Possible independent or dependent interactors of SLIM1/EIL3 are unknown. Confirmed regulation and interactions are shown with solid lines. Possible interactions are shown with dashed lines and question mark.

and proteins from dietary sources (Maruyama-Nakashita, 2017). Therefore, S-cycle between plants and the environment has a pronounced impact on human nutrition and health (Maruyama-Nakashita, 2017; Nakai and Maruyama-Nakashita, 2020). S is a constituent of cysteine and methionine and forms an intrinsic regulating mechanism for protein structures, as well as component of chloroplasts, sulfatides, vitamins, coenzymes, and prosthetic groups (iron–S clusters, lipoic acid, thiamine, coenzyme A, etc.) (Günal et al., 2019; Maruyama-Nakashita, 2017; Nakai and Maruyama-Nakashita, 2020). Plant roots mainly take S up in the form of sulfate (SO_4^{2-}), but leaves are also able to absorb gaseous SO_2 and H_2S (Koprivova and Kopriva, 2016). After uptake, SO_4^{2-} is transported to the shoot via the xylem, where it is assimilated to organic S in the chloroplasts, and the excess is stored in the vacuole. In the chloroplasts, SO_4^{2-} is reduced to sulfite (SO_3^{2-}), then to sulfide (S^{2-}), and finally incorporated into cysteine (Cys). Cys is further included in proteins and the tripeptide glutathione (GSH), metabolized to methionine, or it can serve as a donor for other S-containing molecules, such as various coenzymes and specialized metabolites (Kopriva et al., 2012). Despite the importance of S assimilation for plants and humans alike, the molecular mechanisms controlling S sensing and signaling are so far not fully elucidated. This is particularly obvious in comparison with two other major nutrients, nitrogen and phosphorus.

Sulfate uptake

High-affinity sulfate transporters SULTR1;1 and SULTR1;2, are the main components responsible for sulfate uptake in *A. thaliana* (Takahashi et al., 2000; Yoshimoto et al., 2002). Both transporters are expressed in root

hairs, epidermis, and cortex cell layers of roots and mediate active sulfate influx (Yoshimoto et al., 2002). However, the existence and mechanism of sulfate efflux remain unclear, and possibly, SULTR1;1 and SULTR1;2 also function to prevent leakage of sulfate from the epidermal and cortical cells in the roots (Takahashi, 2019). SULTR1;1 and SULTR1;2 have redundant function, but it seems that SULTR1;2 is the major component of the constitutive sulfate uptake system, while SULTR1;1 is becoming important mostly under S starvation environment (Rouached et al., 2008; Shibagaki et al., 2002; Takahashi et al., 2000; Yoshimoto et al., 2002). Under S-deficiency conditions, SULTR1;1 and SULTR1;2 are increased at both transcriptional and protein levels, but the mechanism behind this upregulation is not completely clear, except being at least partly regulated by the transcription factors SULFUR LIMITATION1 (SLIM1) and ETHYLENE-INSENSITIVE3-LIKE1 (EIL1) (Dietzen et al., 2020; Yoshimoto et al., 2007). On the other hand, the activity of sulfate transporter SULTR1;2 and its trafficking to the plasma membrane is dependent of a domain located at the C-terminus, named sulfate transporter and anti-sigma factor antagonist (STAS) domain (Rouached et al., 2005; Shibagaki and Grossman, 2004, 2006). Interestingly, in analogy to NRT1.1, a potential “transceptor” function for sulfate transporters was also proposed (Zhang et al., 2014; Zheng et al., 2014) (Kankipati et al., 2015). In yeast, the SUL1 and SUL2 are involved in sensing extracellular sulfate, triggering a signaling cascade which allows the cells to exit from S-starvation-caused growth arrest. In plants, however, such sensing of external sulfate is highly doubtful, because mutants with low internal sulfate concentration show S starvation response even at high external sulfate levels (Lee et al., 2012). However, two mutant alleles of *SULTR1;2* were isolated, which showed slightly higher transcript levels of S-deficiency marker genes at high external sulfate and a lower sensitivity to feedback inhibition by thiols (Zhang et al., 2014). The downstream mechanisms have not been elucidated and given the minor effect at very high sulfate concentration, the contribution of the *SULTR1;2* to overall sulfur sensing seems rather minor, therefore, the search for S sensor is still on.

Sulfate assimilation and Cys synthesis

Sulfate assimilation occurs in the plastids, mainly in the leaves, and begins with activation of SO_4^{2-} to adenosine 5'-phosphosulfate (APS) using ATP sulfurylase (ATPS). In *Arabidopsis*, four ATPS isoforms have been described, of which ATPS2 occurs in two forms, one cytosolic and one imported to the plastids, whereas for the other three isoforms (ATPS1, ATPS3, and ATPS4), proteins are localized only in the plastids (Murillo and Leustek, 1995). APS can then undergo alternative pathways: primary sulfur assimilation proceeds by reduction to sulfite (SO_3^{2-}) catalyzed by APS reductase (APR) (Rotte and Leustek, 2000), while in secondary sulfur assimilation APS is phosphorylated to 3-phosphoadenosine 5-phosphosulfate (PAPS) by APS kinase (APK) (Kopriva et al., 2012). APR has three isoforms, all of them localized exclusively in plastids with APR2 responsible for approximately 75% of the total activity. Similarly, to ATPS, there are four APK genes (APK1-4), of which APK3 localizes to the cytosol, and the rest are localized in the plastids (Mugford et al., 2009). In the primary pathway, sulfite is further reduced to sulfide (S^{2-}) by sulfite reductase (SiR) by using ferredoxin as a reductant (Kopriva et al., 2012). Interestingly, *SiR* has only single copy in *Arabidopsis* (Bork et al., 1998), suggesting that *SiR* might be a limiting step in the primary sulfate assimilation pathway (Khan et al., 2010; Yarmolinsky et al., 2014). Whereas plant roots mainly take up S in the form of sulfate (SO_4^{2-}), leaves are also able to absorb gaseous SO_2 and H_2S and assimilate them through the reductive pathway (Koprivova and Kopriva, 2016).

The Cys synthesis occurs in two steps. First, serine (Ser) is activated by serine acetyltransferase (SAT; *Serat*) by transferring an acetyl moiety from acetyl-coenzyme A to form O-acetylserine (OAS). Next, the acetyl group of OAS is replaced with sulfide by OAS (thiol) lyase (OAS-TL) to form cysteine (Cys) (Hell and Wirtz, 2011), as the first product of primary sulfate assimilation. Cys synthesis occurs in plastids, mitochondria, and the cytoplasmic matrix (Hell and Wirtz, 2011). In *Arabidopsis*, there are five genes encoding *SERAT* enzymes: *SERAT2;1* and *SERAT2;2* are localized in plastids and mitochondria respectively, while *SERAT1;1*, *SERAT3;1*, and *SERAT3;2* are cytosolic (Krueger et al., 2009). *SERAT* activity might indirectly influence the synthesis of Cys, as a limiting factor for formation of OAS (Maruyama-Nakashita, 2017). *SERAT* and OAS-TL form the hetero-oligomeric cysteine synthase complex (CSC), which is stabilized by sulfide and dissociated by OAS and contributes so to the control of Cys synthesis (Hell and Wirtz, 2011). In the CSC, *SERAT* is activated, whereas OAS-TL is inactive and only serves as a regulatory subunit for the *SERAT*, and therefore produced OAS exits the complex and is further converted to Cys by a free OAS-TL (Feldman-Salit et al., 2009). However, under S-deficiency, sulfide availability is decreasing, that leads to accumulation of OAS, which destabilizes the CSC (Feldman-Salit et al., 2009). Interestingly, the activity of OAS-TL A dominates by 90% in the cytosol, while *SERAT* activity of the mitochondrial isoform (*SERAT2;2*) contributes

ca. 80%, which together with exclusive production of sulfide in plastids leads to an interplay of all three major compartments in Cys synthesis. Although the interaction of the mitochondrial SERAT2;2 and OAS-TL C is occurring spontaneously *in vitro* (Wirtz et al., 2010) and *in vivo* (Wirtz et al., 2012), it remains unknown whether the same regulatory model applies to cytosolic and plastidic CSCs because of the diverse Cys feedback inhibition of SERATs (Noji et al., 1998; Wirtz et al., 2012). Moreover, SERAT and OAS-TL are not altered at transcriptional nor protein level by sulfur deficiency, but are induced upon stress (Kawashima et al., 2005; Lehmann et al., 2009), suggesting complex integration of CSC components in plant metabolism.

Mechanisms of regulation of S uptake and assimilation

In contrast to N and P, it is not known how plants sense S and the research on molecular mechanisms that regulate the S homeostasis and deficiency response is lagging behind (Li et al., 2020). Several signaling molecules that affect transcript levels or function of genes involved in sulfate uptake and assimilation have been described, but the knowledge on the mechanisms of their function is only very patchy. Similar to P, the most knowledge on regulation of S metabolism derived from experiments with S starvation. These and other experiments showed the importance of sulfate uptake and the reduction of activated sulfate to sulfite by adenosine 5'-phosphosulfate reductase (APR) to be the key steps controlling the pathway, although depending on conditions, other processes may participate in the control (Feldman-Salit et al., 2019; Vaublanc et al., 2002). Most studies of regulatory mechanisms addressed the transcriptional responses to long-term S deprivation, and were conducted in *Arabidopsis*, and few other species including oilseed rape, common wheat, cabbage, barley, and tomato (Aarabi et al., 2020). These studies identified some components of the response, but do not allow yet to draw a conclusive general model of the information flow from sulfate sensing to the transcriptional response.

SURE

Transcriptional upregulation of the *SULTR1;1* and *SULTR1;2* transporters under S-deficiency was the first molecular evidence of acclimation to S deprivation (Maruyama-Nakashita et al., 2004). A 16-base *cis*-acting element, named sulfur-responsive element (SURE) was identified in the *SULTR1;1* promoter (about 2.7kb upstream of translational start) and shown to be necessary for S-deficiency response in *Arabidopsis* (Maruyama-Nakashita et al., 2005). However, the promoter of the second transporter, *SULTR1;2*, did not have the SURE element (Maruyama-Nakashita et al., 2005), suggesting that the transcriptional regulation of the two transporters depends on different regulatory networks (Rouached et al., 2008). Consequently, *SULTR1.1* is locally regulated in response to sulfate deprivation, while *SULTR1.2* transcripts appeared to be mainly related to the overall metabolic demand (Rouached et al., 2008). Interestingly, of the 15 reported genes that have the SURE element in their promoter (Maruyama-Nakashita et al., 2005), 13 genes are dependent on SLIM1/EIL3 under S-deficiency (Dietzen et al., 2020) (see below), suggesting that SLIM1/EIL3 is a strong candidate to bind the SURE element.

The core region of the SURE element GAGAC is actually part of the auxin response factor (ARF) binding sequence (GAGACA), responsible for positive auxin signaling (Lancot and Nemhauser, 2020), but SURE was found not to be responsive to the synthetic auxin compound naphthalene acetic acid (NAA) (Maruyama-Nakashita et al., 2005). However, whether auxin signaling is implicated in S starvation response remains elusive as well as the identity of the transcription factor binding SURE.

SLIM1/EIL3 and EIL1

Using a fluorescent reporter gene under control of *SULTR1;2* promoter, a mutant named *sulfur limitation 1* (*slim1*) was unable to induce the expression of the reporter, as well as endogenous gene under S deficiency (Maruyama-Nakashita et al., 2006). The identified transcription factor (TF) belonged to the EIL-family of transcription factors, SULFUR LIMITATION1 (SLIM1)/ETHYLENE-INSENSITIVE3-LIKE3 (EIL3) (Maruyama-Nakashita et al., 2006). SLIM1/EIL3 is a homolog of ETHYLENE-INSENSITIVE3 (EIN3), but it did not seem to regulate ethylene responses (Maruyama-Nakashita et al., 2006). SLIM1 controlled the induction of sulfate transport and activation of sulfate acquisition, and degradation of glucosinolates under S deficiency, but did not require the SURE element for upregulation of the transporters (Maruyama-Nakashita et al., 2006), and its transcript and protein levels were not changed by S deficiency (Maruyama-Nakashita et al., 2006) leaving the question of its mode of function unsolved.

SLIM1 has a significant role in activation of sulfate uptake, and the mutants showed about 60% reduction in high-affinity sulfate uptake under low sulfate conditions (Maruyama-Nakashita et al., 2006). Initial transcriptome analysis using microarrays revealed 79 genes regulated by SLIM1 and S-deficiency (Maruyama-Nakashita et al., 2006). Several sulfate transporters including *SULTR1;1*, *SULTR1;2*, *SULTR3;4*, and *SULTR4;2* that were highly upregulated showed attenuated response in the *slim1* mutant background (Maruyama-Nakashita et al., 2006). Similar trend of response was seen in other genes later implicated in S-deficiency response, including genes from the OAS cluster (*LSU1*, *SDI1*, and *SHM7*, see below), key gene involved in hydrolytic degradation of glucosinolates (*BGLU28*) and one isoform of Ser acetyltransferase (*SERAT3;1*) (Maruyama-Nakashita et al., 2006). Opposite trend of significant downregulation under S deficiency, but showing less reduction in the *slim1* background, was shown by genes involved in glucosinolate synthesis, such as *MAM1*, *CYP79B2*, *CYP79B3*, *CYP83B1*, and *ATR1* (Maruyama-Nakashita et al., 2006). Interestingly, only one gene involved in sulfate reduction ATP sulfurylase (*APS4*) was similarly regulated, suggesting SLIM1/EIL3-independent regulation of sulfate reduction and particularly the key enzyme APR.

None of the six genes encoding EIL family proteins (*EIN3*, *EIL1*, *EIL2*, *EIL4*, and *EIL5*) (Guo and Ecker, 2004) were able to complement the *slim1* mutation and restore the upregulation of the marker gene (Maruyama-Nakashita et al., 2006). EIL proteins bind to PERE (primary ethylene response element) DNA motif found in promoters of various ethylene (ET)-responsive genes as a primary component of ET signaling (Solano et al., 1998) or a similar TEBS motif described in tobacco (Kosugi and Ohashi, 2000). *In vitro* surface plasmon resonance assay showed that SLIM1/EIL3 was able to transiently bind TEBS, although SLIM1 cannot take over the function of *EIN3* and *EIL1* in ET signaling (Yamasaki et al., 2005). Using a promoter region of *UP9C*, a tobacco gene that is strongly induced by S deficiency, it was shown that SLIM1 directly binds as a homodimer to a 20-nt sequence named the UPE-box (Wawrzynska et al., 2010; Wawrzynska and Sirko, 2016). The UPE-box contains two TEBS motifs, and is found in several S-deficiency-induced genes, including the LSU family and OAS cluster genes (Lewandowska et al., 2010; Wawrzynska et al., 2010) (see below). Interestingly, SLIM1/EIL3 also can heterodimerize with *EIN3* which impairs the DNA binding of SLIM1/EIL3 (Wawrzynska and Sirko, 2016). Recently, a comprehensive transcriptome study (RNA-Seq) showed that *EIL1* has supporting role to SLIM1/EIL3 in regulating sulfur-deficiency response, since the lower induction of some S-deficiency genes was stronger in the double mutant of *eil1eil3* than in *eil3*, but both genes showed also specific functions (Dietzen et al., 2020).

To pin-point the core genes regulated by SLIM1/EIL3 under S deficiency, we used the two published transcriptomics studies, including microarrays (Maruyama-Nakashita et al., 2006) and RNA-Seq (Dietzen et al., 2020) and searched for genes that overlap between them. We identified 38 core genes regulated by SLIM1/EIL3 under S deficiency (Figure 2). Hierarchical clustering grouped expression in four distinct clusters (Figure 2B). GO enrichment analysis for molecular function revealed expected overrepresentations of sulfur-related categories, as well as terms involving sugar transporters (Figure 2C), suggesting that SLIM1/EIL3 might be a link in to regulating sugar homeostasis during S-deficiency.

It is very likely that SLIM1 requires post-transcriptional regulation under S-deficiency conditions, and possibly interacts with other regulatory proteins (Wawrzynska and Sirko, 2014). Only one confirmed interacting protein partner of SLIM1 was described; the MYB72 protein, identified in a two-hybrid assay, and involved in induced systemic resistance (Van der Ent et al., 2008). MYB72 is additionally important for metal homeostasis and together with MYB10 required for plant survival in alkaline soil under iron (Fe)-deficiency conditions (Palmer et al., 2013). MYB72 is also a direct target of FER-LIKE IRON DEFICIENCY INDUCED (FIT), a TF that controls the root iron uptake (Sivitz et al., 2012). Whether SLIM1-MYB72 interact in planta and whether this module plays a regulatory role during S-deficiency remains to be determined, especially in the light of the regulatory connections between S and Fe nutrition (Kumar et al., 2021).

SLIM1 has been considered the central regulator of S-deficiency response; however, it clearly has also other functions. For example, it plays a role in the response to arsenic and cadmium, possibly through modification of redox status (Jobe et al., 2021). The *eil3* mutant showed significant changes in expression of genes for response to hypoxia or wounding even at normal S levels (Dietzen et al., 2020). Whether some of these processes are connected to S sensing and signaling is not clear and the function of SLIM1 will certainly be a focus of further investigations. There, the finding that SLIM1 protein can be rapidly degraded by

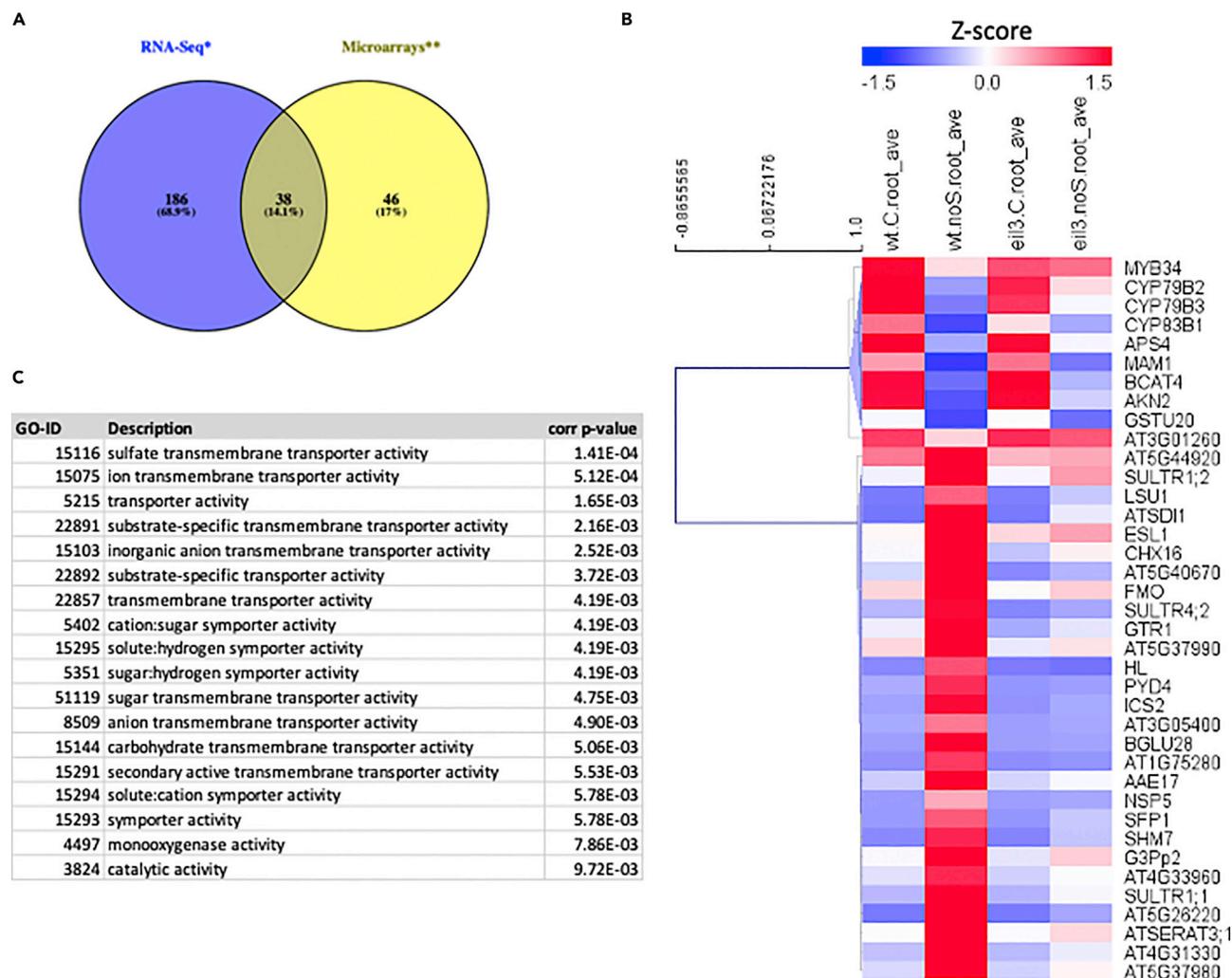


Figure 2. Core genes regulated by SLIM1/EIL3 under S-deficiency

(A) DEGs with interaction term of genotype-condition (*eil3-lowS**) (Dietzen et al., 2020) and DEGs with interaction term of genotype-condition (*slim1-lowS***) (Maruyama-Nakashita et al., 2006) were intersected to find the “core” genes regulated by SLIM1/EIL3 in low S.

(B) Hierarchical average clustering of normalized counts from Dietzen et al. (2020) are transformed to z-scores, for the 38 core genes.

(C) GO analysis of molecular function of the 38 genes (BINGO (Maere et al., 2005)).

proteasome (Wawrzynska and Sirko, 2020) might represent an important step to finally understanding its mechanism of function.

OAS and OAS cluster genes

OAS has long been considered an important signal of S-deficiency and possibly the sensor of S status. OAS accumulates during S-deficiency and its addition induces genes for sulfate uptake and assimilation. However, it is also rapidly metabolized to cysteine and changes in OAS levels are mostly accompanied by changes in S-containing metabolites, making the conclusions on its signaling role uncertain. However, in a systems biology approach, a group of six genes, termed “OAS cluster” genes showed high correlation with OAS independent from S-containing metabolites (Hubberten et al., 2012). The OAS cluster comprises of genes encoding adenosine 5'-phosphosulfate reductase (APR3), two tetratricopeptide repeat (TPR)-like proteins sulfur-deficiency-induced 1 (SDI1) and SDI2 that interact with MYB28 to repress the synthesis of glucosinolates during S-deficiency (Aarabi et al., 2016), serine hydroxymethyltransferase (SHM7), also named More Sulfur Accumulation (MSA1) that is regulating S-adenosylmethionine (SAM) biosynthesis and maintaining S homeostasis epigenetically via DNA methylation (Huang et al., 2016), GGCT2;1 that

functions as γ -glutamyl cyclotransferase involved in glutathione homeostasis during abiotic stress by recycling Glu (Paulose et al., 2013), and LOW SULFUR UPREGULATED 1 (LSU1) that is network hub and coordinates plant responses across many abiotic and biotic stresses (Garcia-Molina et al., 2017). Interestingly, all six genes are significantly upregulated under S starvation, mostly in a SLIM1-dependent manner (except APR3), but also after exogenous application of OAS to not S-starved plants, suggesting that OAS might indeed have a signaling function independent of the sulfur status in the plant (Hubberten et al., 2012). Correspondingly, all OAS cluster genes have SLIM1/EIL3-binding site in their promoter (Ran et al., 2020). Notably, most of the OAS cluster genes seem to function as signal multipliers: the SDI1 and SDI2 through binding to transcription factors, SHM7 through DNA methylation, and LSU1 through protein-protein interactions.

OAS has, however, also a function in modifying stability of protein complexes. As mentioned above, OAS was shown to be critical for stability of the CSC (Wirtz et al., 2012). In addition, OAS enhances the interaction of a C-terminal STAS domain of SULTR1;2, but not SULTR1;1, with cytosolic OAS-TL A that leads to decreased transporter activity (Shibagaki and Grossman, 2010). However, it remains unclear whether and how OAS accumulation under S-deficiency can enhance the OAS-TL-SULTR1;2 interaction and modulate sulfate uptake.

SDI1 and SDI2

SDI1 gene, belonging to the OAS cluster, is one of the most upregulated genes under sulfate limitation (Dietzen et al., 2020; Maruyama-Nakashita et al., 2006). Together with *SDI2*, they often represent marker genes for sulfur deficiency but their function seems to be regulation of GSL biosynthesis in *Arabidopsis* (Aarabi et al., 2016). It is thus possible to speculate that the SDI proteins may play a similar role to the SPX proteins in P signaling. Indeed, we noticed similar genes differentially expressed in separate RNAseq datasets involving *eil3* and *sdi1/sdi2* mutations (Aarabi et al., 2016; Dietzen et al., 2020). To identify common transcriptional patterns, we re-analyzed the data and identified genes regulated by *eil3* under S-deficiency and genes regulated by *sdi1/sdi2* under S-deficiency (Aarabi et al., 2016; Dietzen et al., 2020). We identified 43 genes intersecting the two lists (Table 1). Interestingly, in the *eil3* background under S-deficiency, *SDI1* is about 3-fold less highly upregulated than in WT Col-0, and about 4-fold less induced than WT in the *eil1/eil3*, suggesting that *SDI1* activation is depended on both EIL3 and EIL1, but also on another unknown regulator (Dietzen et al., 2020). Another interesting observation is that three genes involved in reproductive development, including *SWEET13*, *SWEET14*, and *EXLB1*, are similarly regulated in both *eil3* and *sdi1/sdi2*, suggesting a possible link between S-deficiency and reproduction (Table 1). Another set of genes show opposite trend of regulation in the two backgrounds, suggesting independent roles of EIL3 and SDI/SDI2. However, the exact role and position of SDI1 and SDI2 in S signaling needs to be established (Aarabi et al., 2020).

TOR

Target of rapamycin (TOR) kinase has a central role integrating multiple pathways, including nutrient, energy, hormone, growth factors, and environmental factors in plants and animals (for detail review see (Shi et al., 2018)). Additionally, it was suggested that TOR is involved in mediating sulfur signaling, by downregulation of TOR in response to downregulation of glucose metabolism under sulfur limitation (Dong et al., 2017). By comparing transcriptome and phosphorylation state of the sulfite reductase (SiR) mutant *sir1-1*, involved in the final reduction step of sulfite to sulfide, and the triple knockout (*serat tko*) that lacks the major SERAT isoforms, it was suggested that the availability of cysteine precursors (OAS and sulfide), rather than cysteine itself, is sensed by plants (Dong et al., 2017). Moreover, in the *sir1-1* mutant TOR activity assayed by S6K (ribosomal S6 kinase p70, p70S6K) phosphorylation and EdU staining was reduced in both roots and shoots, and resulted in common downstream TOR effects, like growth defects, decreased rRNA, decreased translation, and depletion of TCA cycle intermediates (Dong et al., 2017). This study suggests that plants are able to distinguish between limitations of carbon/nitrogen (C/N) vs. limitation of sulfur (S), in order to coordinate their amino acid biosynthesis and growth (Dong et al., 2017). Recently, another link connecting glucose, TOR kinase, and ethylene-insensitive protein 2 (EIN2) was shown to mediate cell proliferation and growth and various sulfate secondary metabolic pathways (Fu et al., 2021).

Hydrogen sulfide (H₂S)

Emerging evidence in recent years suggest that hydrogen sulfide (HS⁻ and S²⁻) plays a signaling role in different organisms and diverse stress responses in plants, including drought, salinity, hypoxia, as well

Table 1. Intersect of genes between *eil3* and *sdi1sdi2* under S-deficiency

Symbol	log2FC (<i>eil3</i> -noS)	padj (<i>eil3</i> -noS)	log2FC (<i>sdi1sdi2</i> -noS)	padj (<i>sdi1sdi2</i> -noS)
ATSDI1	-2.77	2.0×10^{-18}	-7.70	5.8×10^{-6}
AT4G31330	-2.50	4.6×10^{-15}	-1.02	1.1×10^{-2}
SULTR1;1	-2.26	1.3×10^{-8}	-0.67	1.5×10^{-2}
SWEET14	-2.10	1.2×10^{-6}	-1.99	5.4×10^{-3}
SWEET13	-1.03	1.8×10^{-2}	-1.14	1.2×10^{-2}
EXLB1	-0.83	7.3×10^{-3}	-0.87	2.5×10^{-2}
CYP79B3	2.25	3.2×10^{-29}	2.79	1.8×10^{-3}
MAM1	1.92	4.1×10^{-27}	6.67	4.8×10^{-7}
BCAT4	2.09	7.0×10^{-24}	7.15	1.3×10^{-5}
CYP79B2	1.47	5.8×10^{-18}	2.27	2.6×10^{-4}
GSTF9	0.61	2.9×10^{-6}	0.91	3.8×10^{-3}
CYP83A1	0.69	5.8×10^{-5}	4.32	5.8×10^{-6}
AKN2	0.59	1.7×10^{-4}	2.68	1.9×10^{-5}
GSTU20	0.58	2.6×10^{-4}	3.85	9.7×10^{-6}
CYP712A1	1.09	1.7×10^{-3}	1.96	6.0×10^{-3}
GLY14	1.09	4.9×10^{-3}	1.27	1.4×10^{-3}
ACR3	0.41	6.9×10^{-3}	0.62	4.8×10^{-2}
DOT1	0.70	7.8×10^{-3}	0.96	3.4×10^{-2}
HMT3	0.76	1.2×10^{-2}	3.43	1.4×10^{-5}
MOT2	0.70	1.8×10^{-2}	0.80	9.0×10^{-3}
IMS2	0.46	3.9×10^{-2}	4.27	3.0×10^{-5}
PFK4	0.43	4.6×10^{-2}	1.08	1.3×10^{-3}
J8	0.42	5.6×10^{-3}	1.53	1.6×10^{-3}
AT3G14280	0.70	1.4×10^{-3}	1.27	4.8×10^{-3}
AT2G25450	0.82	4.0×10^{-4}	2.76	1.1×10^{-5}
AT5G04750	0.48	1.9×10^{-3}	0.84	1.6×10^{-2}
AT2G43550	0.78	6.9×10^{-3}	2.01	2.1×10^{-3}
AT2G44130	10.25	7.4×10^{-3}	1.15	4.9×10^{-3}
AT1G27150	0.36	4.9×10^{-2}	0.66	4.2×10^{-2}
AT1G23390	1.12	2.5×10^{-8}	0.73	1.9×10^{-2}
AT3G20390	0.46	1.0×10^{-7}	0.81	1.6×10^{-2}
AT5G26600	0.52	4.2×10^{-6}	0.78	2.0×10^{-2}
AT5G14650	1.45	2.2×10^{-5}	1.11	5.7×10^{-3}
ATSERAT3;1	-1.15	7.7×10^{-14}	0.64	2.3×10^{-2}
GGP1	-0.64	3.7×10^{-13}	0.86	3.8×10^{-3}
AT1G12030	-4.23	1.4×10^{-9}	0.72	4.3×10^{-2}
APK	-0.51	1.8×10^{-5}	1.47	1.2×10^{-3}
AT5G50930	-1.49	7.8×10^{-4}	1.51	1.3×10^{-3}
APS1	-0.39	1.7×10^{-3}	1.06	6.1×10^{-3}
AtAPR2	-0.39	5.7×10^{-3}	0.60	4.4×10^{-2}
AT1G76520	-0.65	3.7×10^{-2}	0.98	2.0×10^{-3}
AT1G60750	-0.78	4.9×10^{-2}	1.59	5.5×10^{-3}
AT1G66760	-2.08	3.1×10^{-19}	0.92	4.2×10^{-2}

DEGs with interaction term of genotype-condition (*eil3*-lowS) (Dietzen et al., 2020) and DEGs with interaction term of genotype-condition (*sdi1sdi2*-lowS) (Aarabi et al., 2016) were intersected using the Genesect tool (<http://virtualplant.bio.nyu.edu/>) and obtained significant Z score (24) for 43 genes. First RNA-Seq dataset was re-analyzed using interaction term in DESeq2 package (Love et al., 2014) in R, while the second microarray dataset was re-analyzed using interaction term in limma package (Ritchie et al., 2015) in R, and cutoff of adjusted p value (padj) of 0.05 for both analysis.

as developmental processes such as seed germination, root development, and autophagy, as well as hormone signaling (for recent review see (Gotor et al., 2019)). Protein persulfidation or S-sulfhydration refers to the oxidative posttranslational modification of cysteine residues caused by H₂S where the thiolate group (-SH) is modified to a persulfide group (-SSH). This modification may have multiple consequences for the modified protein, and can affect their stability, localization, and function (Gotor et al., 2019). Recent study identified that about 1500 proteins showed differential levels of persulfidation under short-term nitrogen starvation in *Arabidopsis*, suggesting that persulfidation is important mechanism of protein modification in response to environmental changes (Jurado-Flores et al., 2021). Among the identified proteins, those involved in protein degradation were highly overrepresented, including the TOR kinase. In animal system, H₂S inhibits TOR activity (Talaie et al., 2013); therefore, the susceptibility of plant TOR to persulfidation suggests that persulfidation could be a possible mechanism mediating nutrient starvation responses (Jurado-Flores et al., 2021).

miRNAs

Among the genes regulated by S-deficiency in SLIM1-dependent manner are genes for a micro RNA miR395 that because of its mobility might serve as a downstream long-distance signal. Indeed, in the past decade, increasing number of studies showed that small non-coding RNAs (ncRNAs), such as micro RNAs (miRNAs), are important regulators of diverse plant stress responses, including nutrient homeostasis and deficiency response. For instance, many miRNAs are differentially regulated upon nutrient deficiency, including induction of expression of miR156, miR399, miR778, miR827, and miR2111, and repression of miR169, miR395, and miR398 in Pi-deficient root (Hsieh et al., 2009). Under N-deficiency, induction was observed for miR160, miR780, miR826, miR842, and miR846, whereas repression for miR169, miR171, miR395, miR397, miR398, miR399, miR408, miR827, and miR857 (Liang et al., 2012). Interestingly, under S-deficiency conditions, we detected upregulation of miR157b, miR159b, miR395b, miR395c, miR396, miR775, and miR864, and downregulation of miR319a and miR398c in the shoot, but only upregulation of miR395b in the root (Dietzen et al., 2020) (when considering main -S effect and cutoff of ± 1 -fold change, and adjusted p-value of 0.05). This suggests that the regulatory actions of miRNAs under S starvation are distinctive in the shoot and in the root.

Under S starvation conditions in *Arabidopsis*, miR395 was previously shown to be critical for translocation of sulfate to the shoots and maintaining S homeostasis, by targeting three out of four isoforms of ATP sulfurylase, and the main low affinity sulfate transporter, *SULTR2;1* (Kawashima et al., 2011). MiR395 is regulated by SLIM1 and together they shape the regulatory circuit of sulfate assimilation under S-deficiency (Kawashima et al., 2009, 2011). Interestingly, miR395 is downregulated under N and Pi starvation (Hsieh et al., 2009; Liang et al., 2012), signifying the involvement of common but oppositely regulated miRNAs to modulate nutrient homeostasis. MiRNAs seems to also orchestrate the crosstalk of the starvation response of multiple macronutrients and the overall stress response. For instance, miRNA398 is significantly downregulated under Pi-, N-, and S-deficiency, and shows dynamic regulation under salt and abscisic acid (ABA)-induced stress (Dietzen et al., 2020; Hsieh et al., 2009; Jia et al., 2009; Liang et al., 2012).

Another miRNA that connects mineral nutrition with development and stress is miR399. MiR399 was discovered as an important component of Pi starvation response and molecular mechanism to maintain P homeostasis (Hsieh et al., 2009). Namely, under Pi deficiency, PHR1 induces the transcription of miR399 in the shoot and the mmir399 is translocated to the roots, where it specifically represses the transcript of its target gene, *PHOSPHATE2* (*PH O 2*) (Bari et al., 2006; Lin et al., 2008). *PH O 2* encodes a ubiquitin-conjugating E2 enzyme and mediates degradation of various phosphate transporter proteins (PHT1 and PHO1). Reduction in PHO2 levels thus promotes Pi uptake and translocation from the root to shoot, in order to maintain Pi homeostasis in the shoots (Bari et al., 2006; Berkowitz et al., 2013; Huang et al., 2013). In addition, the miR399/PHO2 expression module was connected with early flowering in response to temperature fluctuation, as well as with salt stress (Kim et al., 2011; Pegler et al., 2020). Interestingly, most miRNAs conservatively target homologous genes across species (Lin et al., 2018). However, miRNA827 is upregulated by Pi starvation in both *Arabidopsis* and rice, but it has different target genes. In *Arabidopsis*, miRNA827 target is *NITROGEN LIMITATION ADAPTATION* (*NLA*) which encodes a plasma-membrane-associated RING-type ubiquitin E3 ligase with an N-terminal SPX domain, and is involved in adaptive responses to low-nitrogen conditions (Kant et al., 2011). *NLA* is involved in posttranslational regulation of membrane-localized Pi transporters (PHT1s) (Lin et al., 2013), similarly as PHO2-mediated degradation

(Berkowitz et al., 2013; Huang et al., 2013). In rice, miRNA827 targets two transporter proteins of the PHT5 family, OsSPX-MFS1 and OsSPX-MFS2, which have N-terminal SPX domain, and a C-terminal MFS (major facilitator superfamily) transporter domain (Lin et al., 2010). MFS1–MFS3 are shown to function as vacuolar Pi transporters and mediate Pi efflux from the vacuole into cytosol, and are important for Pi remobilization (Wang et al., 2015).

INTERACTION OF SULFATE SIGNALING WITH OTHER NUTRIENT PATHWAYS

During their life cycle, plants experience fluctuations of many environmental factors, and availability of mineral nutrition and water are often limiting for proper development and growth. Therefore, plants have evolved complex signaling mechanisms to be able to cope with these fluctuations and maintain homeostasis (for recent review see (Fan et al., 2021)).

Several links between S signaling and other macro- and microelements have been reported. For instance, Pi deficiency shifts the sulfate content, decreasing it in shoots, while increasing it in the roots (Rouached et al., 2011). Sulfate transporters *SULTR1;3* and *SULTR3;4* showed upregulation under Pi limitation, and the *SULTR1;3* upregulation was dependent on *PHR1* (Rouached et al., 2011). On metabolic level it was reported that under S-deficiency rapid replacement of sulfolipids by phospholipids occurs, and vice versa (Yu et al., 2002). This metabolic switch is possibly regulated by two genes *SQD1* and *SQD2*, involved in sulfolipids biosynthesis and upregulated by Pi deficiency, and in the *phr1* mutant background (Bustos et al., 2010). Interestingly, *PHR1* was found to be interacting with other nutrients as well, including N (Maeda et al., 2018), Zn (Khan et al., 2014), and Fe (Bournier et al., 2013), suggesting that *PHR1* is a strong general integrator of mineral homeostasis. Interestingly, possible interaction between S and N signaling remains unknown, except of the case of interactions between S metabolism and N₂ fixation in legume nodules (Courbet et al., 2019).

Transcriptomics analysis of S and iron (Fe) deficiency suggested co-regulation between S and Fe metabolic pathways. However, deficiency in Fe regulated diverse set of genes of the sulfur assimilation pathway, when compared to S-deficiency, suggesting that specific molecular modules modulate the cross-regulation (Forieri et al., 2017). The same study further identified that potassium (K) deficiency causes increase of reduced S-containing metabolites, without identification of direct mechanisms of action (Forieri et al., 2017). Several studies have also showed interaction of S signaling and homeostasis with micronutrients (for recent review see (Courbet et al., 2019)).

To identify transcriptional patterns of S-related genes under different stresses, we compared 50 genes involved in S transport, assimilation, OAS cluster genes, and key TFs under S-(Dietzen et al., 2020), N-(Luo et al., 2020), Pi-(Lan et al., 2013), Fe-(Li and Lan, 2015) deficiency, and oxidative (He et al., 2021), salt (Suzuki et al., 2016), and heat stress (Suzuki et al., 2016) (Figure 3). Interestingly, N-, Pi-, and Fe-deficiency regulated several sulfate transporters, genes involved in sulfate assimilation and some TFs. However, none of the OAS cluster genes is regulated by these three macroelements limitation. All OAS cluster genes show highest upregulation under S-deficiency and oxidative stress, while under heat stress *SDI1* and *SDI2* are upregulated, but *APR3* and *GGCT2;1* are downregulated, suggesting that OAS cluster genes might play a role in mediating homeostasis between S-deficiency response and stress response, rather than integrations with other nutrient deficiencies (Figure 3). *APS1* and *APR1* were differentially regulated in all conditions, except salt stress, indicating that the first step of sulfate reduction is sensitive to other stresses in the plant. The high overlap of regulation by S starvation and oxidative stress points to possible involvement of the same signaling components and redox regulation being at least partly responsible for S signaling.

FINAL REMARKS AND OPEN QUESTIONS

In plants, research in S sensing and signaling is lagging compared to other macronutrients, predominately because S-deficiency in agriculture does not cause that significant losses, as other macronutrients (e.g. N and Pi) (Bouain et al., 2019). There remain many open questions in the field of S sensing and signaling, in particular in comparison with N and P. The prime question is what is the sensor for S? Is it sulfate, and does sulfate acts as signal similar to nitrate? Is it a downstream S metabolite similar to InsPP? Or is it OAS as suggested by several experiments and the OAS cluster? What is the mechanism of action of *SLIM1* and what TFs control the regulation of *SLIM1*-independent genes, such as *APR2*, by S-deficiency.

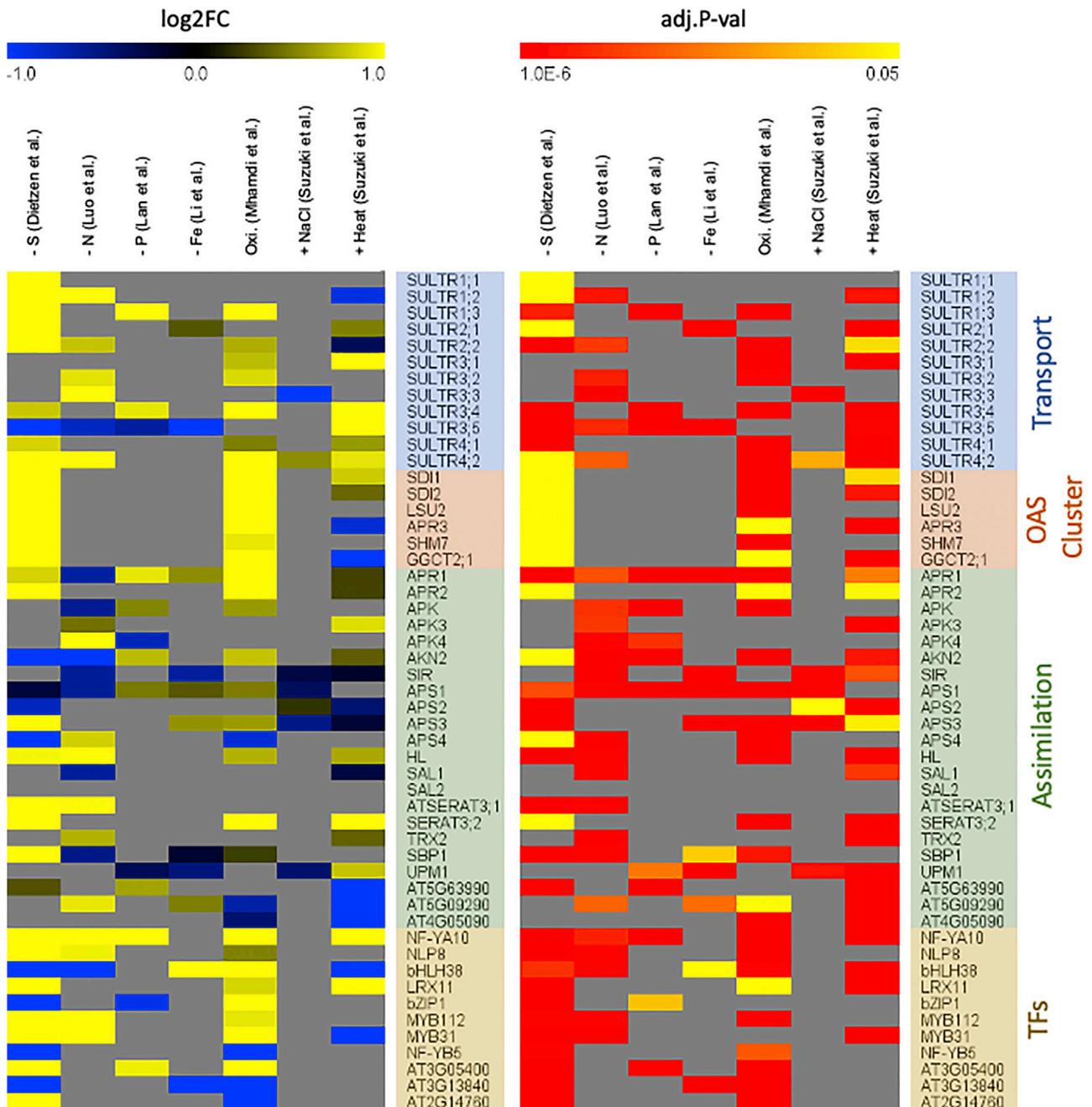


Figure 3. Heatmap of log₂ fold changes and adjusted p values of S-related genes under different nutrient deficiency and stresses
S-related genes implicated in S transport, assimilation, OAS cluster, and key transcription factors (TFs) are shown. S-deficiency results (-S) as in the study by (Dietzen et al., 2020). For the remaining six datasets: nitrogen deficiency (-N) (Luo et al., 2020), phosphorous deficiency (-Pi) (Lan et al., 2013), iron deficiency (-Fe) (Li and Lan, 2015), oxidative stress (Oxi.) (He et al., 2021), salt stress (NaCl) (Suzuki et al., 2016), and heat stress (Heat) (Suzuki et al., 2016), raw files were downloaded and re-analyzed using the limma package (Ritchie et al., 2015) in R.

How is the S signal transmitted to the transcriptional response? Given the involvement of TOR in regulation of S homeostasis, which cellular processes are directly connected with S homeostasis?

Future research should thus be focused on understanding S sensor and components of signaling networks. In particular, it is important to find out how S-containing metabolites integrate into other nutrient signaling

networks and developmental processes, to modulate and maintain homeostasis in the plant. Omics-approaches, high-throughput phenotyping, and live imaging will have great impact in near future to discover unknown S sensors, transcriptional cascades and their targets, cell-type specific actions, and molecular modes of action and interactions.

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AUTHOR CONTRIBUTIONS

Daniela Ristova: Conceptualization; Investigation; Writing-original draft; Writing-review & editing; Project administration. Stanislav Kopriva: Conceptualization; Writing-review & editing; Project administration.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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