Journal of Experimental Botany, Vol. 74, No. 1 pp. 308–320, 2023
https://doi.org/10.1093/jxb/erac402 Advance Access Publication 12 October 2022

RESEARCH PAPER

Unique features of regulation of sulfate assimilation in monocots

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Received 13 June 2022; Editorial decision 3 October 2022; Accepted 10 October 2022

Editor: Karl-Josef Dietz, Bielefeld University, Germany

Abstract

Sulfate assimilation is an essential pathway of plant primary metabolism, regulated by the demand for reduced sulfur (S). The S-containing tripeptide glutathione (GSH) is the key signal for such regulation in Arabidopsis, but little is known about the conservation of these regulatory mechanisms beyond this model species. Using two model monocot species, C_3 rice (*Oryza sativa*) and C_4 *Setaria viridis*, and feeding of cysteine or GSH, we aimed to find out how conserved are the regulatory mechanisms described for Arabidopsis in these species. We showed that while in principle the regulation is similar, there are many species-specific differences. For example, thiols supplied by the roots are translocated to the shoots in rice but remain in the roots of *Setaria*. Cysteine and GSH concentrations are highly correlated in *Setaria*, but not in rice. In both rice and *Setaria*, GSH seems to be the signal for demand-driven regulation of sulfate assimilation. Unexpectedly, we observed cysteine oxidation to sulfate in both species, a reaction that does not occur in Arabidopsis. This reaction is dependent on sulfite oxidase, but the enzyme(s) releasing sulfite from cysteine still need to be identified. Altogether our data reveal a number of unique features in the regulation of S metabolism in the monocot species and indicate the need for using multiple taxonomically distinct models to better understand the control of nutrient homeostasis, which is important for generating low-input crop varieties.

Keywords: C_4 photosynthesis, cysteine, glutathione, monocots, plant nutrition, regulation, rice, *Setaria viridis*, sulfate assimilation.

Introduction

Sulfur (S) is essential for life, and involved in the primary and secondary metabolism of plants. Cys, the first product of the sulfate assimilation pathway, serves as a S donor for synthesis of methionine, glutathione (GSH), vitamins, and many cofactors (Takahashi *et al.*, 2011). Plants take up inorganic sulfate from the soil by sulfate transporters. The initial step of sulfate

assimilation is the activation of inorganic sulfate by ATP sulfurylase (ATPS) to adenosine 5′-phosphosulfate (APS) which is reduced to sulfite by APS reductase (APR). Sulfite is further reduced by sulfite reductase (SIR) to sulfide, which can further be integrated into the amino acid backbone of *O*-acetylserine (OAS), formed by Ser acetyltransferase (SAT) from Ser and acetyl-CoA, by OAS (thiol) lyase (OAS-TL) forming Cys (Takahashi *et al.*, 2011). The main storage and transport form of reduced S is the tripeptide GSH (Noctor *et al.*, 2012). GSH biosynthesis is mediated by the subsequent action of γ-glutamylcysteine synthetase (γECS) and GSH synthetase, which join its components, Glu, Cys, and Gly, in an ATP-dependent manner (Noctor *et al.*, 2012).

S metabolism is tightly connected with the assimilation of carbon and nitrogen. Both sulfate and nitrate assimilatory pathways were shown to undergo spatial separation between mesophyll cells (MCs) and bundle sheath cells (BSCs) in C_4 species (Jobe *et al.*, 2019). C_4 photosynthesis diminishes the oxygenation reaction of Rubisco and advances the photosynthesis performance. This is achieved by avoiding the loss of assimilated CO_2 by separating the CO_2-fixing reactions between two differentiated cell types, the MCs and the BSCs (Hatch and Slack, 1966; Slack *et al.*, 1969; Sage *et al.*, 2012). MCs and BSCs differ from each other by their morphological characteristics, dispensation within the plant tissue, and cell-specific localization of many enzymes. The initial CO_2 fixation in MCs is catalyzed by phosphoenolpyruvate carboxylase, resulting in oxaloacetate, a C_4 compound (Laetsch, 1974). Oxaloacetate is promptly turned to malate or aspartate, C_4 acids, which enter BSCs and are decarboxylated, resulting in enriched CO_2 concentrations at the Rubisco site in the BSCs (Edwards *et al.*, 2001; Sage *et al.*, 2012). Thus, the oxygenase reaction of Rubisco and photorespiration are strongly diminished, leading to higher photosynthetic efficiency and consequently enhanced biomass production, as seen, for example, in the productivity of C_4 crops maize or sorghum (Laetsch, 1974; Wingler *et al.*, 2000; Jobe *et al.*, 2020). However, enhanced biomass production also requires a steady acquisition of soil-derived mineral nutrients and, correspondingly, adaptations in nitrogen and S metabolism of C_4 plants have been observed (Brown, 1978; Gerwick *et al.*, 1980; Koprivova *et al.*, 2001; Kopriva, 2011; Gerlich *et al.*, 2018; Jobe *et al.*, 2019). Apart from differential localization of sulfate assimilation between MCs and BSCs (Gerwick *et al.*, 1980; Passera and Ghisi, 1982; Schmutz and Brunold, 1984; Burgener *et al.*, 1998), a gradient in concentration of Cys and GSH, and enzyme activity of APR increasing from C_3 to C_4 *Flaveria* species was described (Gerlich *et al.*, 2018; Koprivova *et al.*, 2001). Thus it appears that the sulfate assimilation pathway might be under different regulatory circuits in C_3 and C_4 plants. Sulfate assimilation is mainly regulated in a demand-driven manner (Lappartient and Touraine, 1996). The pathway is induced under a strong demand for growth and development, as well as when sulfate supply is limited; however, the excess amounts of reduced S compounds suppress the pathway (Vauclare *et al.*, 2002; Takahashi *et al.*, 2011). In addition, many environmental factors, for example biotic and abiotic stresses, increase the demand for reduced S in plants (Takahashi *et al.*, 2011; Koprivova and Kopriva, 2014). Sulfate transport and APS reduction by APR are the main control points of the pathway (Vauclare *et al.*, 2002). GSH was shown to be a systemic signal in the demand-driven regulation of sulfate assimilation in *Brassica napus* and Arabidopsis, reducing transcript levels and activity of ATPS and/or APR (Lappartient and Touraine, 1996; Lappartient *et al.*, 1999; Vauclare *et al.*, 2002). These experiments exploited the inhibitor of GSH synthesis buthionine sulfoximine (BSO) to assign the signaling role to GSH and not Cys, since in the presence of BSO Cys was not able to trigger the regulation. However, in *Zea mays*, Cys and not GSH was able to decrease the ATPS transcript level (Bolchi *et al.*, 1999). Possibly Cys acquired the signaling role, since maize is a C_4 plant with sulfate assimilation localized in BSCs, and Cys was shown to be exported from BSs (Burgener *et al.*, 1998). Thus, to test this hypothesis and to obtain more robust and general understanding of the mechanisms of demand-driven control of sulfate assimilation, it is necessary to analyze additional species.

Here we addressed the demand-driven regulation of sulfate assimilation using two model monocot species, C_3 rice (*Oryza sativa*) and C_4 *Setaria* (*Setaria viridis*). We show that in the monocot species both Cys and GSH are equally capable of triggering the demand-driven regulation of sulfate assimilation. We also reveal an unexpected oxidation of Cys and GSH to sulfate in two monocot species, which has not been observed in Arabidopsis. Altogether, the results broaden our understanding of the molecular mechanisms of regulation of sulfate assimilation.

Materials and methods

Plant material and growth conditions

The seeds of rice (*Oryza sativa* ssp. *japonica* variety KitaakeX) and *Setaria viridis* variety A10,1 were used for all experiments. Seeds were germinated on distilled water-soaked filter papers, the *S. viridis* seeds having been treated by liquid smoke to improve germination (Sebastian *et al.*, 2014). One-week-old seedlings were transferred to Falcon tubes with 40 ml of hydroponic half-strength modified Hoagland medium (for composition, see Supplementary Table S1) with sulfate concentration adjusted to 1 mM for control and 12.5 μM for low S medium. The plants were grown in a growth cabinet with a 16 h light/8 h dark photoperiod, at a temperature of 30/22 °C day/night, for 10 d under a light intensity of 150 μmol photons m^{-2} s^{-1}. After 10 d, the seedlings of both species reached stage 13 or 14 of the BBSH scale (defined by Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie), used to identify the phenological development stages of plants. The low S-grown plants were then divided into six groups, supplemented with fresh low S medium and 2 mM BSO, 0.3 mM GSH, 0.3 mM L-Cys, 2 mM BSO+0.3 mM GSH, 2 mM BSO+0.3 mM L-Cys, and no supplement for control, while the control plants were given a fresh control medium, and all plants were further grown for 48 h (Fig. 1A). The concentration of BSO was chosen to rapidly and fully inhibit GSH synthesis, while the concentrations of Cys and GSH were intended to increase the concentration of thiols in the tissue with only mild inhibition of the sulfate assimilation pathways (Vauclare *et al.*, 2002). To test

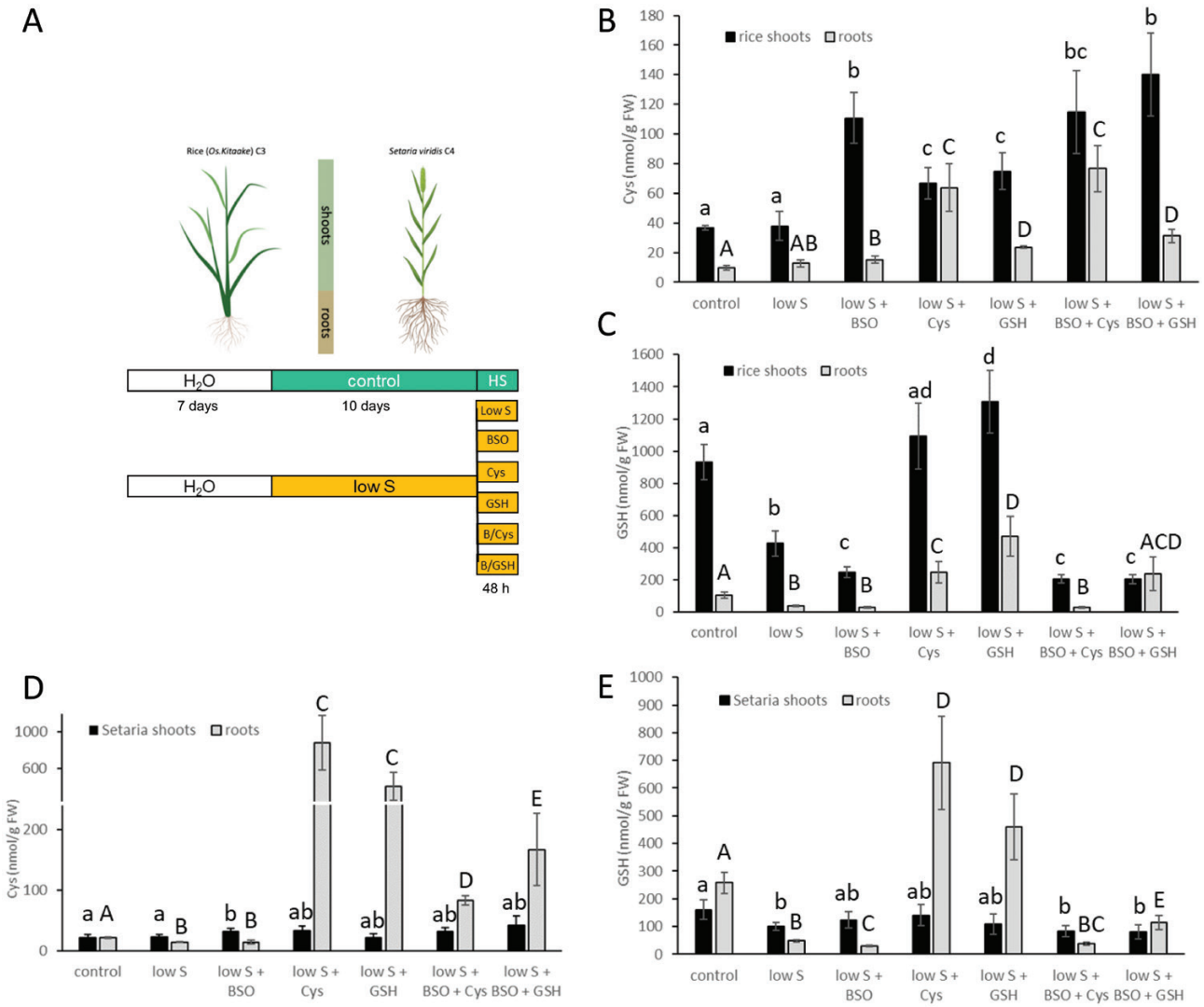

Fig. 1. Experimental design and thiol concentrations in rice and *S. viridis*. (A) Experimental design: 7-day-old plants were transferred to control (1 mM sulfate) or low S (12.5 µM) hydroponic medium and grown for 10 d. Afterwards the low S plants were divided into six groups and supplemented with fresh medium with additions of 2 mM BSO, 0.3 mM L-Cys, 0.3 mM GSH, 2 mM BSO+0.3 mM L-Cys, or 2 mM BSO+0.3 mM GSH, and grown for 48 h. (B, D) Cys and (C, E) total GSH were determined in shoots and roots of rice (B, C) and *S. viridis* (D, E). Data are shown as means and SDs from five biological replicates. A typical experiment of at least two independent experiments is presented. Statistical significance of differences between values is indicated with different letters ($P<0.05$, ANOVA).

the contribution of bacteria to Cys oxidation, the plants were grown on low S medium for 10 d and treated with low S medium including 0.3 mM L-Cys with or without 50 mg ml^{-1} ampicillin. To inhibit sulfite oxidase, the plants were grown on control or low S medium for 10 d and were then treated for 48 h with control, low S, or low S with 0.3 mM L-Cys including 1 mM sodium tungstate (Brychkova *et al.*, 2012).

Measurement of sulfate and sulfur-containing metabolites

Sulfate was determined from ~30 mg of plant material by ion chromatography as described in Huang *et al.* (2016). The low molecular weight thiols, Cys and GSH, were extracted by HCl and quantified by HPLC-based analysis as their monobromobimane-derivatized products from ~30 mg of plant material as described by Gerlich *et al.* (2018).

Isolation of total RNA and expression analysis

Total RNA was isolated by standard phenol/chlorophorm extraction and LiCl precipitation. First-strand cDNA was synthesized from 800 ng of total RNA using the QuantiTect ReverseTranscription Kit (Qiagen) that contains a DNase step to eliminate DNA, according to the manufacturer's instruction, and diluted with water to a final volume of 200 µl. The concentration of nucleic acids was determined by using Nanodrop (Nanodrop ND1000, Peqlab). Quantitative real-time reverse transcription–PCR (qPCR) was performed using gene-specific primers (Supplementary Table S2) and the GoTaq® qPCR Master Mix (Promega, Germany), employing the fluorescent intercalating dye SYBR Green, as described in the technical manual. Each reaction in the 96-well plate included 4 µl of 1 µM Primer mix, 1 µl of cDNA (corresponding to 4 ng of RNA), and 5 µl of GoTaq® qPCR Master Mix. The PCR was performed using a CFX96 Touch Real-Time PCR Detection System (Bio

Rad, Germany) and 40 cycles of 15 s 95 °C, 30 s 58 °C, and 30 s 60 °C, after denaturation at 95 °C for 30 s. To quantify the transcription levels, CFX Manager Software (Bio Rad, Germany) was used and the relative gene expression was calculated as 2-ΔΔCt relative to the *TIP41* and *UBQ* genes for *O. sativa*, as well as *DUSP* and *PP2A* for *S. viridis*.

Determination of sulfate uptake

Sulfate uptake and root to shoot translocation were measured in plants grown at low S and treated for 48 h with 2 mM BSO, 0.3 mM GSH, 0.3 mM L-Cys, 2 mM BSO+0.3 mM GSH, and 2 mM BSO+0.3 mM L-Cys. The growth medium was exchanged by 20 ml of nutrient solution containing 0.2 mM sulfate supplemented with 9 µCi of [^{35}S]sulfuric acid and incubated for 30 min in the light. The seedlings were washed thoroughly, blotted dry, and shoot and root samples were stored separately in liquid nitrogen until further processing on the same day. Samples were extracted in a 10-fold volume of 0.1 M HCl. A 100 µl aliquot of extract was used to determine sulfate uptake and translocation to shoots as described in Dietzen *et al.* (2020).

APR activity

APR activity was determined as in Koprivova *et al.* (2008). Approximately 30 mg of plant material were extracted in 300 µl of 50 mM Na/K phosphate buffer, pH 8, supplemented with 30 mM Na_2SO_3, 0.5 mM AMP, and 10 mM DTT, and the extract was centrifuged for 30 s at 2000 rpm to remove cell debris. A 25 µl aliquot of 1 M Tris–HCl adjusted to pH 9, 100 µl of 2 M $MgSO_4$, 10 µl of 0.2 M DTT, 5 µl 3.75 mM [^{35}S]APS (specific activity ~ 1.6 kBq/5 µl), 100 µl of H_2O, and 10 µl of extract were added to tubes without a lid. The samples were vortexed and then incubated at 37 °C for 30 min. Afterwards 100 µl of 1 M Na_2SO_3 was added and the tubes were transferred into 20 ml scintillation vials containing 1 ml of 1 M triethanolamine. Then 200 µl of 1 M H_2SO_4 was added to the reactions in the tubes before quickly closing the scintillation vials. The vials were incubated overnight at room temperature. The tubes were removed from the scintillation vials and the bottoms were washed with 200 µl of H_2O. Finally, 2.5 ml of scintillation cocktail was added, mixed, and the radioactivity was measured using the LS 6500 Multi-Purpose Scintillation Counter by Beckman Coulter. Protein concentration in the extracts was determined by Bio-Rad protein assay with BSA as a standard (Koprivova *et al.*, 2008).

Statistical analyses

The metabolite measurements were conducted with 4–5 independent biological replicates constituted of individual roots and shoots, while for the expression analysis three biological replicates were used. The experiments were independently replicated; data from the second experiment are presented in Supplementary Figs S1–S3. The data were analyzed by ANOVA or Student's *t*-test (Excel software). The Pearson correlation analysis was performed using the 'Hmisc' and 'corrplot' packages in R (https://www.R-project.org).

Results

The primary aim of the study was to distinguish whether Cys or GSH is the signal of reduced S in the demand-driven regulation of sulfate assimilation in the two model monocot species, rice and *S. viridis*. To this end, we adapted the experimental design of Lappartient *et al.* (1999) and tested the ability of Cys and GSH, alone or in combination with BSO, to repress the transcript levels of sulfate starvation markers in low sulfate supply (Fig. 1A; Supplementary Fig. S4).

Sulfur-containing metabolites

First we determined the accumulation of thiols, to confirm that the treatments modulated the thiol levels as expected (Supplementary Fig. S4). Interestingly, the two species varied in the response to the treatments, showing different capacity for uptake and root to shoot translocation of Cys and GSH. Shoot Cys concentrations were unchanged in both species under S deprivation compared with control media. On the other hand, although S deficiency did not modify rice root cysteine levels, it resulted in decreased cysteine concentrations in *S. viridis* roots (Fig. 1). In shoots of both rice and *Setaria*, Cys concentration was significantly increased by treatment with BSO; however, a concomitant decrease in GSH was observed only in rice shoots (Fig. 1B, D). Root GSH content was diminished in both species upon BSO treatment, but Cys increased only in rice (Fig. 1). In rice, treatment with GSH or Cys alone resulted in an increase in both thiols in roots and shoots. On the other hand, in *S. viridis*, the same treatments led to an increase in both thiols only in the roots and not in the shoots, indicating a lack of translocation from roots to shoots. When the thiols were supplied together with BSO, the increases in thiol concentrations detected in plants fed with the thiols alone were very strongly diminished, particularly for GSH. It seems that BSO reduced the ability of both rice and *Setaria* to take up the thiols (Fig. 1). Generally, the expected pattern of accumulation of thiols, derived from experiments with Arabidopsis (Vauclare *et al.*, 2002), has been observed in rice for Cys and for GSH in the roots but in *Setaria* only for GSH in the roots (Fig. 1; Supplementary Fig. S4).

To explore further differences in regulation of S metabolism in the C_3 and C_4 models, *O. sativa* and *S. viridis*, sulfate concentrations in roots and shoots of both species were determined. In both species, growth with a low S concentration resulted in a substantial decrease of sulfate pools in both organs. Unexpectedly, however, sulfate content in shoots and roots of S-deficient rice plants increased in response to all exogenous supplementations, with the highest levels of sulfate in shoots of plants exposed to Cys (Fig. 2A). In *S. viridis*, this was true for all treatments containing thiols and sulfate in the roots, but this sulfate was not translocated to the shoots, where sulfate remained low and not altered compared with low S-grown plants (Fig. 2B). Thus, rice and *Setaria* seem to be able to oxidize Cys and/or GSH to sulfate.

Sulfate uptake and APR activity

Since we observed unexpected alterations in the accumulation of S-containing metabolites in the different treatments and also significant differences between the species, we compared the

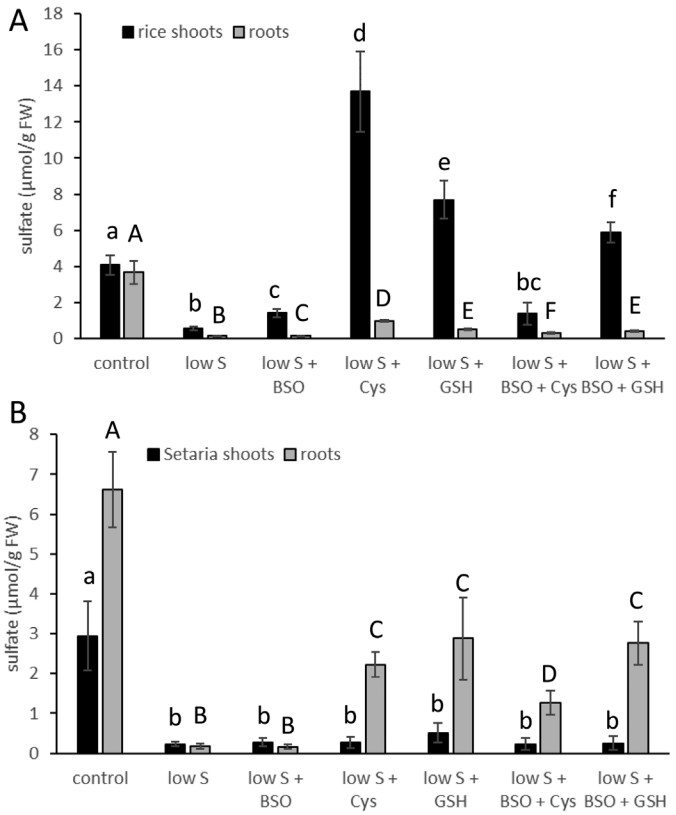

Fig. 2. Sulfate concentrations in rice and *S. viridis*. Seven-day-old plants were transferred to control (1 mM sulfate) or low S (12.5 µM) hydroponic medium and grown for 10 d. Afterwards the low S plants were divided into six groups and supplemented with fresh medium with additions of 2 mM BSO, 0.3 mM L-Cys, 0.3 mM GSH, 2 mM BSO+0.3 mM L-Cys, and 2 mM BSO+0.3 mM GSH, and grown for 48 h. Sulfate concentration were determined in shoots and roots of rice (A) and *S. viridis* (B). Data are shown as means and SDs from five biological replicates. A typical experiment of at least two independent experiments is presented. Statistical significance of differences between values is indicated with different letters (P<0.05, ANOVA).

effects of the treatments on the two key regulatory points of sulfate assimilation, sulfate uptake and APR activity (Vauclare *et al.*, 2002), in rice and *Setaria*. As expected, when exposed to low S conditions, the total uptake and translocation rates were higher compared with control S supply in both species. Exposure to BSO led to further increased sulfate uptake rates, with a markedly higher extent in rice than in *Setaria* (8.6-fold versus 2.2-fold) (Fig. 3A, B). The effect of BSO on the sulfate translocation rate was even stronger in rice, 19-fold higher than in low S plants, whereas in *Setaria* it was not significant (Fig. 3C, D). Surprisingly, incubation of the low S-grown plants with thiols did not reduce sulfate uptake or translocation in rice. In *S. viridis*, Cys diminished the uptake and translocation, although not to the control levels, but GSH did not (Fig. 3). Simultaneous treatment with the thiols and BSO showed further differences between the species. In rice, the sulfate uptake and translocation rate were reduced compared with BSO alone but higher compared with low S, with GSH having a stronger effect (Fig. 3A, C). In *Setaria*, addition of GSH did not influence the stimulating effect of BSO on sulfate uptake, but Cys again reduced it to the level of low S plants, while the translocation remained at the level of low S- and BSO-treated plants. Thus, sulfate uptake and translocation seem to be regulated differently in the two species, and the negative feedback regulation of sulfate uptake by thiols as observed in Arabidopsis might not be universal. It also needs to be noted that sulfate uptake and translocation in *Setaria* were much higher than in rice in all conditions (Fig. 3).

The partitioning of APR activity between roots and shoots markedly differed between the two species, with a much higher portion of the activity in shoots in the C_4 *Setaria* than in C_3 rice (Fig. 4), which corresponds to the earlier observations with C_3 and C_4 *Flaveria* species (Gerlich *et al.*, 2018). Against expectations, S deficiency did not affect the activity in the shoots but only led to a 5-fold and 3.4-fold increase in APR in the roots of rice and *Setaria*, respectively. Surprisingly, BSO did not affect the activity compared with low S-grown plants (Fig. 4). GSH and Cys attenuated the increase in APR by low S in the roots of rice, but in the shoots only GSH was effective (Fig. 4A). In *Setaria*, compared with low S-grown plants, the thiols did not affect the activity in roots and only Cys reduced the activity in the shoots (Fig. 4B). Interestingly, in both species in combination with BSO, GSH but not Cys affected the activity in roots, pointing to a role for GSH in regulation of APR activity.

Expression analysis

A number of genes change their expression according to the sulfur status of the plants (Bielecka *et al.*, 2014) and are thus great tools to dissect the signaling pathways of demand-driven regulation of sulfate assimilation. We chose three genes for the analysis: *APR*, *SULTR4;1* encoding a vacuolar sulfate transporter, and *SDI1* (sulfur deficiency induced 1), that in Arabidopsis are induced by S deficiency and the induction is repressed by resupply of sulfate (Bielecka *et al.*, 2014). Indeed, in roots of both species, the three genes were induced by low S treatment and the induction was reverted by the treatment with thiols, except for *APR* and GSH, where the reduction was not significant at the chosen significance level of 0.05 (Fig. 5). Generally, the regulation of *APR* was slightly different from that of *SULTR4;1* and *SDI1*, most probably because APR activity is regulated on multiple levels beyond the gene expression (Koprivova *et al.*, 2008). Thus, treatment with the thiols together with BSO resulted in an increase in APR transcript levels in rice roots compared with low S- and BSO-treated plants, whereas the transcript levels of the other two genes were intermediate between low S and control plants. In *Setaria*, treatment with BSO and GSH was significantly more effective in reducing the transcript levels of the three genes than BSO and Cys (Fig. 5). The expression analysis thus again points to differences in regulation of sulfate assimilation between rice

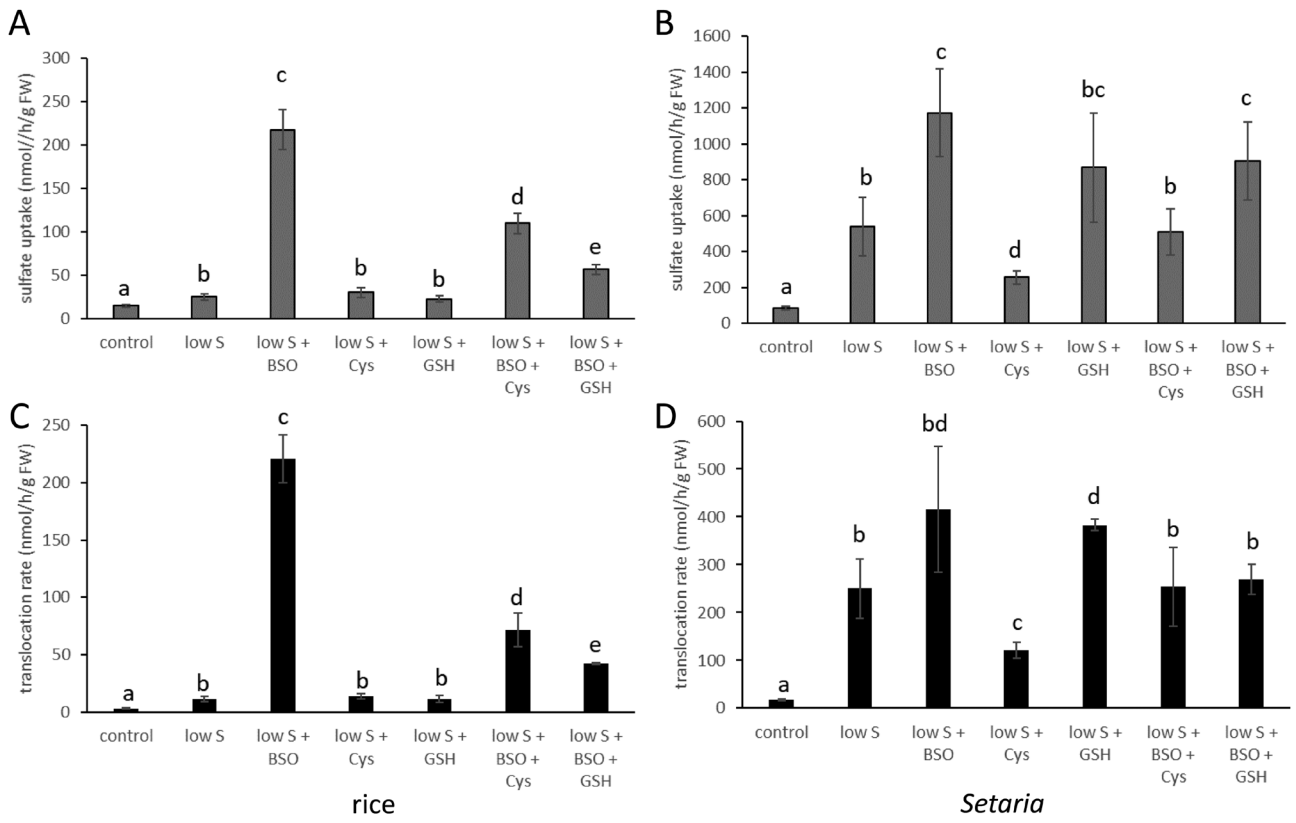

Fig. 3. Sulfate uptake and root to shoot translocation. Seven-day-old plants were transferred to control (1 mM sulfate) or low S (12.5 µM) hydroponic medium and grown for 10 d. Afterwards the low S plants were divided into six groups and supplemented with fresh medium with additions of 2 mM BSO, 0.3 mM L-Cys, 0.3 mM GSH, 2 mM BSO+0.3 mM L-Cys, and 2 mM BSO+0.3 mM GSH, and grown for 48 h. The plants were then further incubated with [^{35}S]sulfate for 30 min. Sulfate uptake (A, B) and root to shoot translocation (C, D) in rice (A, C) and *S. viridis* (B, D) were determined via a scintillation counter. Data are shown as means and SDs from four biological replicates. Statistical significance of differences between values is indicated with different letters (P<0.05, ANOVA).

and *Setaria*. While the expression pattern in *Setaria* is consistent with GSH being the signal of S status (Fig. 5; Supplementary Fig. S4) in rice, it seems that both thiols are involved in the regulation.

Correlation analysis

To obtain a better overview of the regulation of S homeostasis in rice and *Setaria*, the data from all experiments were combined and subjected to correlation analysis (Fig. 6). The analysis clearly confirmed the differences between the two species in responses to the treatments. Generally, the S-related traits seemed to be better coordinated in *Setaria* than in rice. Both species show a good reciprocal correlation of the typical responses to sulfur deficiency—the expression of the marker genes, sulfate uptake, and APR activity—although the transcript of APR in the root was much better correlated in *Setaria* than in rice (Fig. 6). These traits are also strongly negatively correlated to sulfate content in both roots and shoots and in rice also to GSH. Among the most striking differences is a high correlation between Cys and GSH content in roots of *Setaria* versus no correlation in rice. Comparing root and shoot traits, in *Setaria* the sulfate content in the two organs is well correlated, whereas in rice it is the thiols, particularly GSH (Fig. 6). Thus, the root–shoot allocation of S metabolites might have different drivers in the C_3 and C_4 species as suggested for *Flaveria* previously (Gerlich *et al.*, 2018). Interestingly, the conclusions on signaling of S status derived from the expression pattern of the marker genes in the individual treatments described above were not strongly supported by the correlations over the whole dataset.

Cys oxidation to sulfate

The oxidative retrograde flow of S from thiols to sulfate (Fig. 2) was not described in plants previously; however, it is a common part of microbial S metabolism. We therefore asked whether the sulfate production might be due to bacterial contamination in the hydroponics solution, and performed the Cys feeding with or without addition of antibiotics. Incubation of S-deprived rice with Cys for 48 h resulted in an increase in sulfate content as seen previously, and the increase was attenuated

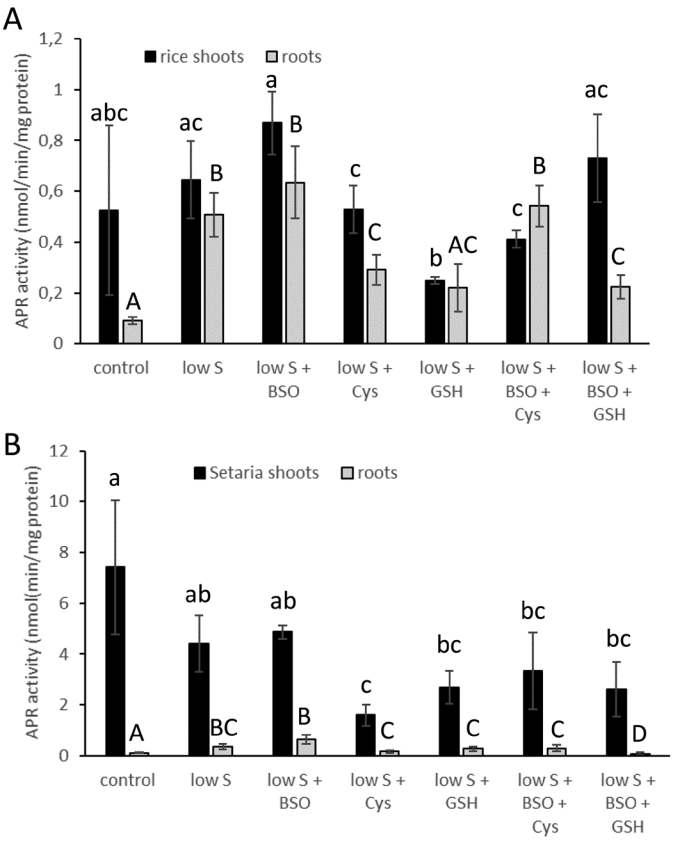

Fig. 4. APR activity in rice and *S. viridis*. Seven-day-old plants were transferred to control (1 mM sulfate) or low S (12.5 μM) hydroponic medium and grown for 10 d. Afterwards the low S plants were divided into six groups and supplemented with fresh medium with additions of 2 mM BSO, 0.3 mM L-Cys, 0.3 mM GSH, 2 mM BSO+0.3 mM L-Cys, or 2 mM BSO+0.3 mM GSH, and grown for 48 h. APR activity was determined in shoots and roots of rice (A) and *S. viridis* (B). Data are shown as means and SDs from four biological replicates. A typical experiment of at least two independent experiments is presented. Statistical significance of differences between values is indicated with different letters (P<0.05, ANOVA).

but not lost by treatment with ampicillin (Fig. 7A). Thus, rice is indeed capable of oxidation of Cys to sulfate, but the reaction might be additionally supported by bacterial Cys oxidation. The increased sulfate in the shoots could be caused by oxidation of Cys or, alternatively, by stimulation of sulfate uptake. To distinguish between these possibilities, we transferred the low S-grown plants to nutrient solution with low S (12.5 μM) or without sulfate addition and treated them with Cys. The sulfate content in Cys-treated roots grown in low S or no S remained identical and higher than without Cys addition (Fig. 7B). In the shoots, the accumulation of sulfate caused by Cys was lower in plants grown without sulfate than with low S, but still significantly increased (Fig. 7B), showing that the sulfate in shoots of Cys-treated plants originates at least partly from oxidation of Cys. To find out if this reaction is ubiquitous in plants, we tested whether we can observe a similar increase in sulfate after Cys feeding of S-deprived Arabidopsis. This, however, was not the case; only a minimal increase in sulfate was seen in the shoots (Fig. 7C), that is not comparable with the massive accumulation of sulfate in Cys-fed rice and *Setaria*. Thus, it seems that the ability to oxidize reduced S compounds to sulfate is not universal and might be specific to monocots.

In animal systems, Cys oxidation is initiated by cysteine dioxygenase (CDO) (Stipanuk *et al.*, 2009), which, however, does not occur in plants. Plants possess a different Cys degradation pathway in the mitochondria, in which Cys is initially transaminated to 3-mercaptopyruvate. The sulfhydryl group is then transferred to GSH by sulfurtransferase 1 (STR1) and oxidized to sulfite by the S dioxygenase ETHE1 (Hofler *et al.*, 2016). In the canonical pathway, the sulfite is converted to thiosulfate by STR1; however, the sulfite might possibly also be oxidized to sulfate by sulfite oxidase (Eilers *et al.*, 2001). We therefore tested whether inhibition of sulfite oxidase by tungstate affects the increase in sulfate after Cys treatment of rice. Interestingly, growth in the presence of tungstate in control medium resulted in lower sulfate accumulation in both roots and shoots (Fig. 7D, E). Importantly, however, while the small increase in sulfate levels in roots of Cys-grown plants was not affected by tungstate, the considerable accumulation of sulfate in shoots was abolished by the sulfite oxidase inhibitor (Fig. 7D). Thus, the retrograde pathway of S from Cys to sulfate most probably includes sulfite oxidase. To assess whether ETHE1 or the two main sulfurtransferases STR1 and STR2 may play a role in the sulfate production, we compared transcript levels of the corresponding genes in Cys-treated rice and *Setaria* plants. However, somewhat surprisingly, none of the three genes was significantly affected in roots of Cys-treated rice or *Setaria* (Supplementary Fig. S5). Whether these enzymes are not involved in the retrograde S flow from Cys to sulfate or whether they are regulated by mechanisms other than transcriptionally needs to be determined.

Discussion

Plant sulfate assimilation is an essential pathway of primary metabolism and, since several of the pathway intermediates are toxic in higher concentrations, it is highly and coordinately regulated (Takahashi *et al.*, 2011). Generally, the pathway is controlled in a demand-driven manner; it is up-regulated when sulfate supply is low or during various stresses, and repressed in the presence of reduced S compounds (Vauclare *et al.*, 2002; Jost *et al.*, 2005; Nikiforova *et al.*, 2005). Despite the general concepts of the regulation of sulfate assimilation being known for decades, surprisingly little is known about the molecular mechanisms of sensing and signaling so that even the highly studied response to sulfate deficiency has not been fully resolved (Aarabi *et al.*, 2020). This is even more apparent for the repression of the pathway by reduced S compounds. Sulfate uptake and APR activity are inhibited by sulfite, sulfide, Cys,

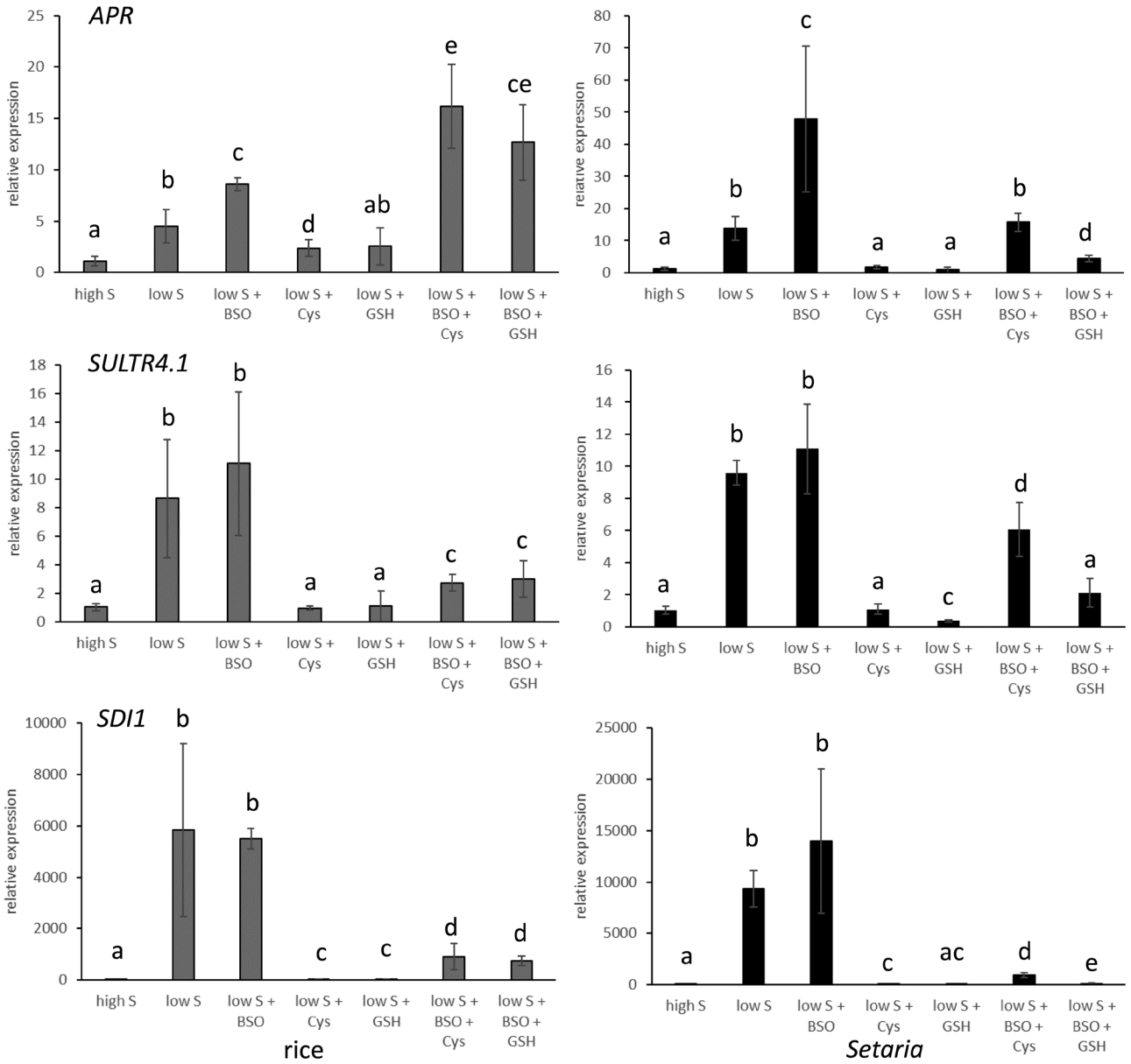

Fig. 5. Expression analysis. Seven-day-old plants were transferred to control (1 mM sulfate) or low S (12.5 µM) hydroponic medium and grown for 10 d. Afterwards the low S plants were divided into six groups and supplemented with fresh medium with additions of 2 mM BSO, 0.3 mM L-Cys, 0.3 mM GSH, 2 mM BSO+0.3 mM L-Cys, or 2 mM BSO+0.3 mM GSH, and grown for 48 h. Total RNA was isolated and the relative gene expression (2-ΔΔCt) of *APR*, *SULTR4;1*, and *SDI1* in the roots was determined via qRT–PCR. Data are shown as means and SDs from four biological replicates. The values in Col-0 under control conditions were set to 1. A typical experiment of at least two independent experiments is presented. Statistical significance of differences between values is indicated with different letters (P<0.05, ANOVA).

and GSH (Brunold, 1978; Tschanz *et al.*, 1986; Lappartient and Touraine, 1996; Lappartient *et al.*, 1999; Vauclare *et al.*, 2002; Buchner *et al.*, 2004; Durenkamp *et al.*, 2007). It is, however, not clear which of these compounds is sensed and which may act as a signal to affect the gene expression. This is not a trivial issue to dissect, because these compounds are rapidly interconverted and metabolized; for example, treatment with any of these four compounds results in an increase in Cys accumulation (Tschanz *et al.*, 1986; Lappartient and Touraine, 1996; Ausma and De Kok, 2019), a situation similar to the long discussions of the role of OAS as a signal (Hubberten *et al.*, 2012). Hydrogen sulfide (H_2S) has long been considered a signal in stress response with the potential of mitigating damage by oxidative stress, in autophagy, and in stomatal closure, but this has often been controversially discussed because its role as an intermediate in Cys synthesis, leading to increased GSH, has often been neglected (Calderwood and Kopriva, 2014). Regulation of sulfate assimilation by H_2S is highly dependent on plant species,

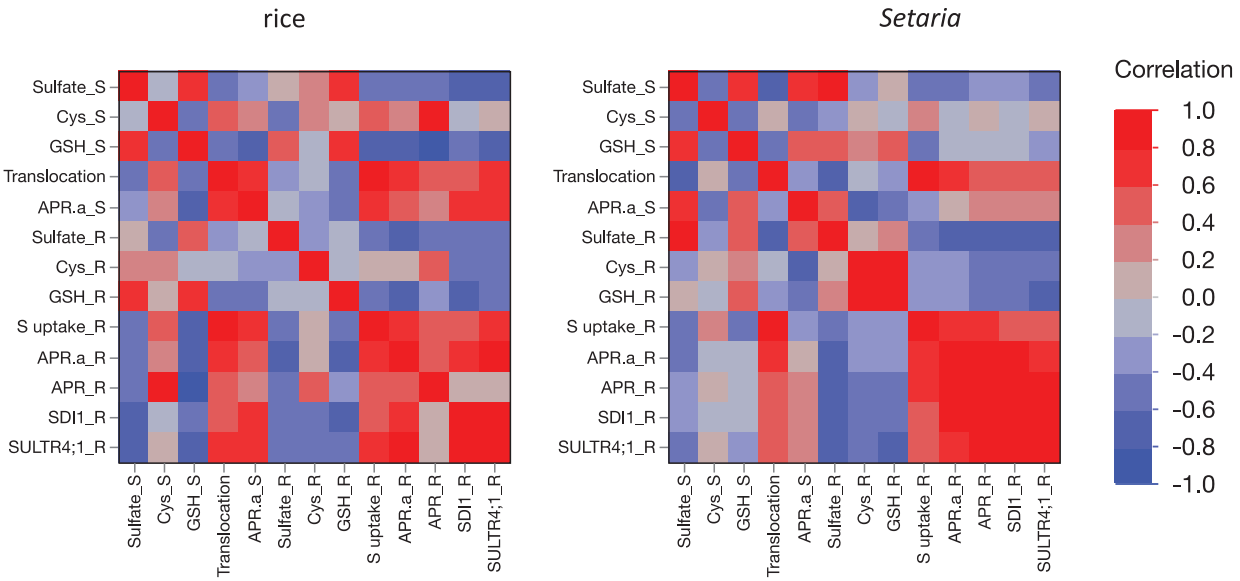

Fig. 6. Correlation analysis of multiple variables. Correlation matrix of sulfur-related trait data from rice and *S. viridis* plants grown in control (1 mM sulfate) or low S (12.5 µM sulfate), and low S plants treated with 2 mM BSO, 0.3 mM L-Cys, 0.3 mM GSH, 2 mM BSO+0.3 mM L-Cys, and 2 mM BSO+0.3 mM GSH. Shoots and roots were harvested separately as indicated by the letters 'S' and 'R' for the corresponding variable. The results are presented as a heat map with the Pearson correlation score from −1 to 1. The correlation coefficients are given in Supplementary Tables S3 and S4.

developmental stage, tissue, and on the supply of sulfate, as plants can use it as a sole S source (Ausma and De Kok, 2019). The most likely signals are thus Cys and GSH, and both have been described as such previously (Lappartient and Touraine, 1996; Bolchi *et al.*, 1999).

Both Cys and GSH were shown to inhibit sulfate uptake, APR activity, and flux through sulfate assimilation (Tschanz *et al.*, 1986; Herschbach and Rennenberg, 1994; Lappartient and Touraine, 1996; Vauclare *et al.*, 2002). Addition of Cys leads to increased accumulation of GSH, as Cys is a precursor for GSH and treatment with GSH results in an increase in Cys content as excess GSH is degraded into its constituent amino acids. However, using BSO, the inhibitor of GSH synthesis (Griffith and Meister, 1979), it is possible to distinguish which thiol is responsible for the regulation, since, in the presence of BSO, addition of Cys does not increase GSH content (Herschbach and Rennenberg, 1994; Bolchi *et al.*, 1999; Lappartient *et al.*, 1999). In the initial experiments with tobacco and Arabidopsis, active synthesis of GSH was necessary for Cys to cause inhibition of sulfate uptake and ATPS activity (Herschbach and Rennenberg, 1994; Lappartient *et al.*, 1999). The same was shown for APR activity and transcript levels in Arabidopsis and poplar (Vauclare *et al.*, 2002; Hartmann *et al.*, 2004), pointing clearly to GSH being the signal for feedback inhibition of sulfate assimilation. However, Bolchi *et al.* (1999) showed that this conclusion is not general and that in maize Cys acts as a signal independent of GSH. This finding was attributed to the different spatial organization of sulfate assimilation in C_4 plants and differential connectivity of Cys and GSH pools in the different cell types (Kopriva and Koprivova, 2005). In this work, however, this hypothesis was not confirmed, as in the C_4 species *S. viridis* the regulation of APR, sulfate uptake, and expression of S starvation marker genes were consistent with GSH being the signal (Fig. 5). Also in rice, the results of BSO treatment point to GSH being the signal, in particular when the correlation between thiol levels and the S starvation-related traits is taken into account (Fig. 6); however, direct signaling by Cys cannot be excluded, since the treatment with BSO and Cys led to a significant albeit not full repression of sulfate uptake and transcript levels of the marker genes (Figs 3, 5). While in *Setaria* simultaneous treatment with Cys and BSO has a lower impact on the traits than Cys alone, the correlation analysis shows a good correlation not only with GSH but also with Cys, making a direct involvement also possible. Collectively, the results indicate a certain plasticity of the regulation of sulfate assimilation in the two monocot species and a mechanism that is not strictly dependent on a single signaling compound. To resolve the molecular mechanisms unequivocally, therefore, it is necessary to identify the downstream components of the signaling and test their affinities for the two thiols.

The differences in response of these two species to the treatments confirm previous observations on variation in organization and regulation of sulfate assimilation in plants. While some responses are conserved across large phylogenetic distances between higher plants and diatoms, such as the up-regulation of genes for sulfate transporters and SAT (Bochenek *et al.*, 2013), some seem to be rather specific, such as the Cys-based signaling in maize (Bolchi *et al.*, 1999). The species selected for this study were chosen based on their different photosynthesis type, C_3 for rice and C_4 for *S. viridis*. Differences in sulfate assimilation

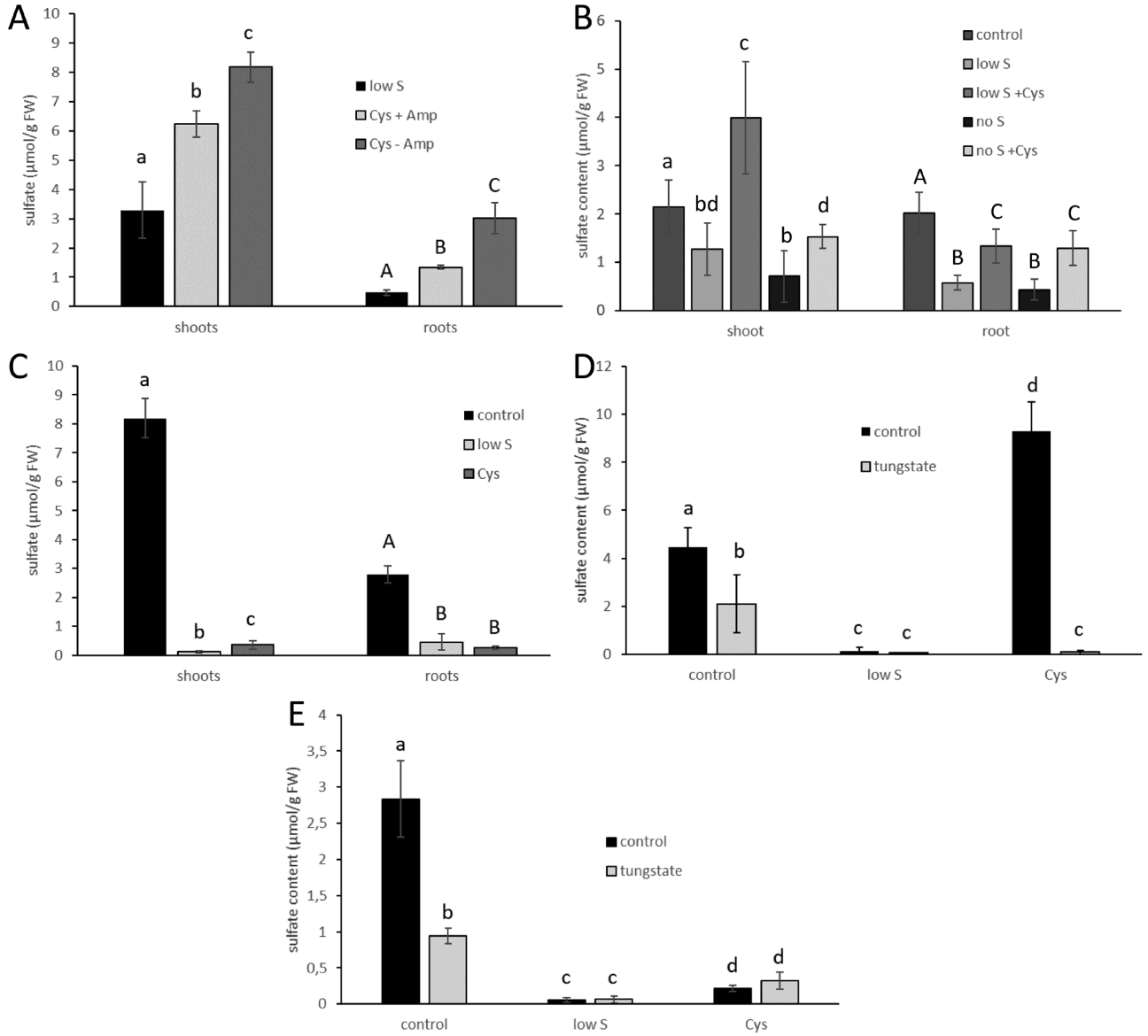

Fig. 7. Cys oxidation to sulfate. (A) Rice plants were grown in low S (12.5 µM) hydroponic medium supplemented with 50 µg ml^{-1} ampicillin for 10 d and treated with 0.3 mM L-Cys for 48 h, also in the presence of the antibiotics. In parallel, the rice plants were grown and treated without antibiotics, 10 d in low S and 48 h with L-Cys. Sulfate concentration was determined in shoots and roots. (B) Rice plants were grown in control (1 mM sulfate) or low S (12.5 µM) hydroponic medium for 10 d. Low S plants were transferred into low S or no S nutrient solution and treated or not with 0.3 mM L-Cys for 48 h. Sulfate concentration was determined in shoots and roots. (C) Arabidopsis seedlings were grown for 2 weeks in control (1 mM sulfate) or low S (12.5 µM) hydroponic medium. Afterwards the low S plants were supplied either with 0.3 mM L-Cys or with fresh low S medium and incubated for 48 h. Sulfate concentration was determined in pools of shoots and roots combined from at least 20 seedlings. (D, E) Rice plants were grown in control (1 mM sulfate) or low S (12.5 µM) hydroponic medium, supplemented or not with 1 mM sodium tungstate for 10 d and treated with 0.3 mM L-Cys for 48 h, again in the presence of the tungstate or not. Sulfate concentration was determined in shoots (D) and roots (E). Data are shown as means and SDs from one typical experiment from at least two full experimental replications with three (C) or five (A, B, D, E) biological replicates. Statistical significance of differences between values is indicated with different letters (P<0.05, ANOVA).

between C_3 and C_4 species of the genus *Flaveria* were observed before, with a higher accumulation of thiols and APR activity in C_4 species (Koprivova *et al.*, 2001; Gerlich *et al.*, 2018). Rice and *Setaria* did not show such a trend consistently: while APR activity and sulfate uptake were indeed higher in *Setaria* than in rice, foliar Cys and GSH in shoots was higher in rice than in *Setaria*. However, this does not automatically disqualify the previous conclusions on C_3/C_4-specific differences in sulfate assimilation since the species analyzed here were monocots and the previous conclusions are based on the dicot genus *Flaveria*. C_4 dicots and monocots differ in the spatial organization of sulfate reduction. While numerous studies showed a

bundle sheath-specific sulfate reduction in C_4 monocots (Gerwick *et al.*, 1980), *in situ* hybridizations and cell type-specific sequencing revealed that in C_4 dicots the pathway is present in both cell types (Koprivova *et al.*, 2001; Aubry *et al.*, 2014a). Interestingly, however, in C_3 plants the spatial organization is also not conserved, in wheat the enzymes for sulfate reduction seem to occur in both cell types, and in Arabidopsis and rice they seem to be at least preferentially, if not exclusively, localized to the bundle sheath (Schmutz and Brunold, 1984; Aubry *et al.*, 2014b; Hua *et al.*, 2021). Thus, it seems that there is a substantial variation in different aspects of sulfate assimilation and that such variation does not run along the major phylogenetic lineages, but is rather specific to smaller taxonomic units.

The most striking difference among the two species was the very low translocation of the added thiols to the shoot in *Setaria* compared with rice (Fig. 1). In most conditions, the concentration of thiols was higher in shoots than in roots in rice, and vice versa in *S. viridis*. This is particularly interesting for GSH in the control condition, where this pattern is opposite to *Flaveria*, where C_4 species contained more GSH in leaves than in roots (Gerlich *et al.*, 2018). The accumulation of Cys and GSH in roots of *S. viridis* treated with the thiols indicates a low capacity of this species to transport Cys and GSH from roots to the shoots. Both thiols have been detected in xylem sap of various tree species and tobacco at a very low level compared with sulfate (Rennenberg *et al.*, 1994; Kruse *et al.*, 2007), and feeding of beans with GSH resulted in its accumulation in the shoots (Tausz *et al.*, 2004). Therefore, it seems that rice behaves in the default manner and transports the thiols to the shoot, whereas in *S. viridis* the low capacity of xylem loading of thiols is the exception. The transporters needed for the xylem loading of thiols are not known in any plant species; therefore, the mechanistic understanding of these differences remains to be clarified.

An unexpected finding from the feeding experiments was the sulfate production from cysteine and GSH in both rice and *Setaria*. It is generally accepted that in plants sulfate assimilation to Cys is irreversible and oxidative S metabolism from Cys to sulfate has not been observed before. This is in contrast to bacteria that are able to mineralize different organic S sources, including reduced ones, and generate sulfate that in turn can be utilized by plants (Kertesz and Mirleau, 2004; Santana *et al.*, 2016). Therefore, it was important to exclude that the sulfate is a result of activity by bacterial contamination (Fig. 7A) or enhanced translocation to the shoot (Fig. 7B). Indeed, plants also have pathways of Cys degradation that may theoretically lead to sulfate production. Cys desulfurylation to sulfide, ammonia, and pyruvate is an important source of H_2S as a signal regulating a number of processes from autophagy to stomatal aperture (Alvarez *et al.*, 2012; Scuffi *et al.*, 2014). Oxidation of sulfide to sulfite is possible through subsequent action of sulfide:quinone oxidoreductase (SQR) and ETHE1 in many organisms (e.g. mammalian mitochondria; Zhang *et al.*, 2021), but SQR is not present in plants. Instead, in plants, the persulfide as substrate for ETHE1 is synthesized through sulfurtransferases, for example the STR1 which uses mercaptopyruvate as S donor (Hofler *et al.*, 2016). However, the usual fate of the sulfite generated by ETHE1 is its conversion to thiosulfate through another reaction with persulfide and STR1. Nevertheless, plants (and other organisms) have an alternative way of detoxifying sulfite, through oxidation to sulfate by sulfite oxidase (Eilers *et al.*, 2001; Brychkova *et al.*, 2013). This enzyme is present in peroxisomes, and its involvement in sulfate production would thus require diffusion of sulfite across the membranes, which is possible at least for plasma membranes (Furihata *et al.*, 1997). The inhibition of sulfate accumulation upon Cys treatment by tungstate (Fig. 7D) confirmed the involvement of sulfite oxidase in this process. Sulfite oxidase contains a molybdenum cofactor, the function of which is strongly inhibited by tungstate (Kuper *et al.*, 2004). However, how the S from Cys or GSH was oxidized to sulfite is not clear, as none of the candidates ETHE1 and STR1 was up-regulated by Cys, at least on the transcript level. Interestingly, the sulfate produced from the thiols remained in the roots of *Setaria*, whereas it was transported to shoots of rice. This is consistent with the allocation of the thiols as discussed before, but in contrast to results of feeding with [^{35}S]sulfate, which showed a higher sulfate root to shoot translocation rate in *Setaria* compared with rice (Fig. 3C, D). The Cys oxidation and the fate of the generated sulfate thus require further detailed investigation to identify the responsible genes, to find how conserved the pathway is among plants and if it is indeed specific to monocots as suggested by the results obtained with a limited number of plant species, and to determine the contribution of these reactions to general plant S homeostasis.

In conclusion, this study aimed to test the hypothesis that C_4 plants use Cys as a signal for demand-driven regulation of sulfate assimilation while C_3 plants use GSH. Our data refuted the hypothesis pointing to GSH being the signal in both C_3 rice and C_4 *Setaria*. The two species showed a number of differences in the regulation of the sulfate assimilation pathway and in the allocation of S-containing compounds between roots and shoots. Interestingly, we unraveled a new reaction in plant S metabolism, the oxidation of Cys to sulfate, which seems to occur in monocots but not dicots. Thus, the sulfate assimilation pathway in plants might be more varied than anticipated and new pathways and metabolic fluxes might still be discovered.

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. Independent replication of data from Figs 1, 2, and 4.
Fig. S2. Independent replication of data from Fig. 5.
Fig. S3. Independent replication of data from Fig. 7.
Fig. S4. Expected pattern of S-related traits.
Fig. S5. Expression analysis of genes potentially oxidizing Cys.
Table S1. Composition of nutrient solutions.

Table S2. Primers used for qRT–PCR.
Table S3. Correlation matrix of S-related traits in rice.
Table S4. Correlation matrix of S-related traits in *S. viridis.*

Author contributions

SK: conceptualization; PRK, CK, RK, IZ, and ES: investigation; PRK, DR, and SK: formal analysis; PRK and SK: writing—original draft preparation; all authors: writing—review and editing; SK: funding acquisition; SK, IZ, and TOJ: supervision.

Conflict of interest

No conflict of interest declared.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) under Germany´s Excellence Strategy—EXC 2048/1—project 390686111 and by a DFG grant no. 436380415 to SK. IZ is recipient of a DAAD fellowship.

Data availability

All data supporting the findings of this study are available within the paper and within its supplementary data published online.

References

Aarabi F, Naake T, Fernie AR, Hoefgen R. 2020. Coordinating sulfur pools under sulfate deprivation. Trends in Plant Science **25**, 1227–1239.

Alvarez C, Garcia I, Moreno I, Perez-Perez ME, Crespo JL, Romero LC, Gotor C. 2012. Cysteine-generated sulfide in the cytosol negatively regulates autophagy and modulates the transcriptional profile in Arabidopsis. The Plant Cell **24**, 4621–4634.

Aubry S, Kelly S, Kumpers BM, Smith-Unna RD, Hibberd JM. 2014a. Deep evolutionary comparison of gene expression identifies parallel recruitment of trans-factors in two independent origins of C4 photosynthesis. PLoS Genetics **10**, e1004365.

Aubry S, Smith-Unna RD, Boursnell CM, Kopriva S, Hibberd JM. 2014b. Transcript residency on ribosomes reveals a key role for the *Arabidopsis thaliana* bundle sheath in sulfur and glucosinolate metabolism. The Plant Journal **78**, 659–673.

Ausma T, De Kok LJ. 2019. Atmospheric H_2S: impact on plant functioning. Frontiers in Plant Science **10**, 743.

Bielecka M, Watanabe M, Morcuende R, Scheible WR, Hawkesford MJ, Hesse H, Hoefgen R. 2014. Transcriptome and metabolome analysis of plant sulfate starvation and resupply provides novel information on transcriptional regulation of metabolism associated with sulfur, nitrogen and phosphorus nutritional responses in Arabidopsis. Frontiers in Plant Science **5**, 805.

Bochenek M, Etherington GJ, Koprivova A, Mugford ST, Bell TG, Malin G, Kopriva S. 2013. Transcriptome analysis of the sulfate deficiency response in the marine microalga *Emiliania huxleyi*. New Phytologist **199**, 650–662.

Bolchi A, Petrucco S, Tenca PL, Foroni C, Ottonello S. 1999. Coordinate modulation of maize sulfate permease and ATP sulfurylase mRNAs in response to variations in sulfur nutritional status: stereospecific down-regulation by L-cysteine. Plant Molecular Biology **39**, 527–537.

Brown RH. 1978. A difference in the nitrogen use efficiency of C_3 and C_4 plants and its implications in adaptation and evolution. Crop Science **18**, 93–98.

Brunold C. 1978. Regulation of sulfate assimilation in plants: 7. Cysteine inactivation of adenosine 5′-phosphosulfate sulfotransferase in *Lemna minor* L. Plant Physiology **61**, 342–347.

Brychkova G, Grishkevich V, Fluhr R, Sagi M. 2013. An essential role for tomato sulfite oxidase and enzymes of the sulfite network in maintaining leaf sulfite homeostasis. Plant Physiology **161**, 148–164.

Brychkova G, Yarmolinsky D, Ventura Y, Sagi M. 2012. A novel in-gel assay and an improved kinetic assay for determining in vitro sulfite reductase activity in plants. Plant & Cell Physiology **53**, 1507–1516.

Buchner P, Stuiver CE, Westerman S, Wirtz M, Hell R, Hawkesford MJ, De Kok LJ. 2004. Regulation of sulfate uptake and expression of sulfate transporter genes in *Brassica oleracea* as affected by atmospheric H_2S and pedospheric sulfate nutrition. Plant Physiology **136**, 3396–3408.

Burgener M, Suter M, Jones S, Brunold C. 1998. Cyst(e)ine is the transport metabolite of assimilated sulfur from bundle-sheath to mesophyll cells in maize leaves. Plant Physiology **116**, 1315–1322.

Calderwood A, Kopriva S. 2014. Hydrogen sulfide in plants: from dissipation of excess sulfur to signaling molecule. Nitric Oxide **41**, 72–78.

Dietzen C, Koprivova A, Whitcomb SJ, Langen G, Jobe TO, Hoefgen R, Kopriva S. 2020. The transcription factor EIL1 participates in the regulation of sulfur-deficiency response. Plant Physiology **184**, 2120–2136.

Durenkamp M, De Kok LJ, Kopriva S. 2007. Adenosine 5′-phosphosulphate reductase is regulated differently in *Allium cepa* L. and *Brassica oleracea* L. upon exposure to H_2S. Journal of Experimental Botany **58**, 1571–1579.

Edwards GE, Furbank RT, Hatch MD, Osmond CB. 2001. What does it take to be C_4? Lessons from the evolution of C_4 photosynthesis. Plant Physiology **125**, 46–49.

Eilers T, Schwarz G, Brinkmann H, Witt C, Richter T, Nieder J, Koch B, Hille R, Hansch R, Mendel RR. 2001. Identification and biochemical characterization of *Arabidopsis thaliana* sulfite oxidase. A new player in plant sulfur metabolism. Journal of Biological Chemistry **276**, 46989–46994.

Furihata T, Pomprasirt S, Sakurai H. 1997. Characteristics of sulfite transport by *Chlorella vulgaris*. Plant & Cell Physiology **38**, 398–403.

Gerlich SC, Walker BJ, Krueger S, Kopriva S. 2018. Sulfate metabolism in C_4 *Flaveria* species is controlled by the root and connected to serine biosynthesis. Plant Physiology **178**, 565–582.

Gerwick BC, Ku SB, Black CC. 1980. Initiation of sulfate activation: a variation in C4 photosynthesis plants. Science **209**, 513–515.

Griffith OW, Meister A. 1979. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). Journal of Biological Chemistry **254**, 7558–7560.

Hartmann T, Honicke P, Wirtz M, Hell R, Rennenberg H, Kopriva S. 2004. Regulation of sulphate assimilation by glutathione in poplars (*Populus tremula×P.alba*) of wild type and overexpressing gamma-glutamylcysteine synthetase in the cytosol. Journal of Experimental Botany **55**, 837–845.

Hatch MD, Slack CR. 1966. Photosynthesis by sugar-cane leaves. A new carboxylation reaction and the pathway of sugar formation. The Biochemical Journal **101**, 103–111.

Herschbach C, Rennenberg H. 1994. Influence of glutathione (GSH) on net uptake of sulfate and sulfate transport in tobacco plants. Journal of Experimental Botany **45**, 1069–1076.

Hofler S, Lorenz C, Busch T, Brinkkotter M, Tohge T, Fernie AR, Braun HP, Hildebrandt TM. 2016. Dealing with the sulfur part of cysteine: four enzymatic steps degrade L-cysteine to pyruvate and thiosulfate in Arabidopsis mitochondria. Physiologia Plantarum **157**, 352–366.

Hua L, Stevenson SR, Reyna-Llorens I, Xiong H, Kopriva S, Hibberd JM. 2021. The bundle sheath of rice is conditioned to play an active role in water transport as well as sulfur assimilation and jasmonic acid synthesis. The Plant Journal **107**, 268–286.

Huang XY, Chao DY, Koprivova A, *et al*. 2016. Nuclear localised MORE SULPHUR ACCUMULATION1 epigenetically regulates sulphur homeostasis in *Arabidopsis thaliana*. PLoS Genetics **12**, e1006298.

Hubberten HM, Klie S, Caldana C, Degenkolbe T, Willmitzer L, Hoefgen R. 2012. Additional role of O-acetylserine as a sulfur status-independent regulator during plant growth. The Plant Journal **70**, 666–677.

Jobe TO, Rahimzadeh Karvansara P, Zenzen I, Kopriva S. 2020. Ensuring nutritious food under elevated CO_2 conditions: a case for improved C_4 crops. Frontiers in Plant Science **11**, 1267.

Jobe TO, Zenzen I, Rahimzadeh Karvansara P, Kopriva S. 2019. Integration of sulfate assimilation with carbon and nitrogen metabolism in transition from C_3 to C_4 photosynthesis. Journal of Experimental Botany **70**, 4211–4221.

Jost R, Altschmied L, Bloem E, *et al*. 2005. Expression profiling of metabolic genes in response to methyl jasmonate reveals regulation of genes of primary and secondary sulfur-related pathways in *Arabidopsis thaliana*. Photosynthesis Research **86**, 491–508.

Kertesz MA, Mirleau P. 2004. The role of soil microbes in plant sulphur nutrition. Journal of Experimental Botany **55**, 1939–1945.

Kopriva S. 2011. Nitrogen and sulfur metabolism in C_4 plants. In: Raghavendra AS, Sage RF, eds. C_4 photosynthesis and related CO2 concentrating mechanisms. Dordrecht: Springer, 109–128.

Kopriva S, Koprivova A. 2005. Sulfate assimilation and glutathione synthesis in C-4 plants. Photosynthesis Research **86**, 363–372.

Koprivova A, Kopriva S. 2014. Molecular mechanisms of regulation of sulfate assimilation: first steps on a long road. Frontiers in Plant Science **5**, 589.

Koprivova A, Melzer M, von Ballmoos P, Mandel T, Brunold C, Kopriva S. 2001. Assimilatory sulfate reduction in C_3, C_3–C_4, and C_4 species of *Flaveria*. Plant Physiology **127**, 543–550.

Koprivova A, North KA, Kopriva S. 2008. Complex signaling network in regulation of adenosine 5′-phosphosulfate reductase by salt stress in Arabidopsis roots. Plant Physiology **146**, 1408–1420.

Kruse J, Kopriva S, Hansch R, Krauss GJ, Mendel RR, Rennenberg H. 2007. Interaction of sulfur and nitrogen nutrition in tobacco (*Nicotiana tabacum*) plants: significance of nitrogen source and root nitrate reductase. Plant Biology **9**, 638–646.

Kuper J, Llamas A, Hecht HJ, Mendel RR, Schwarz G. 2004. Structure of the molybdopterin-bound Cnx1G domain links molybdenum and copper metabolism. Nature **430**, 803–806.

Laetsch WM. 1974. The C_4 syndrome: a structural analysis. Annual Review of Plant Physiology **25**, 27–52.

Lappartient AG, Touraine B. 1996. Demand-driven control of root ATP sulfurylase activity and SO_4^{2-} uptake in intact canola (the role of phloem-translocated glutathione). Plant Physiology **111**, 147–157.

Lappartient AG, Vidmar JJ, Leustek T, Glass AD, Touraine B. 1999. Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. The Plant Journal **18**, 89–95.

Nikiforova VJ, Kopka J, Tolstikov V, Fiehn O, Hopkins L, Hawkesford MJ, Hesse H, Hoefgen R. 2005. Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidopsis plants. Plant Physiology **138**, 304–318.

Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Garcia B, Queval G, Foyer CH. 2012. Glutathione in plants: an integrated overview. Plant, Cell & Environment **35**, 454–484.

Passera C, Ghisi R. 1982. ATP sulphurylase and O-acetylserine sulphydrylase in isolated mesophyll protoplasts and bundle sheath strands of S-deprived maize leaves. Journal of Experimental Botany **33**, 432–438.

Rennenberg H, Schupp R, Glavac V, Jochheim H. 1994. Xylem sap composition of beech (*Fagus sylvatica* L.) trees: seasonal changes in the axial distribution of sulfur compounds. Tree Physiology **14**, 541–548.

Sage RF, Sage TL, Kocacinar F. 2012. Photorespiration and the evolution of C_4 photosynthesis. Annual Review of Plant Biology **63**, 19–47.

Santana MM, Gonzalez JM, Clara MI. 2016. Inferring pathways leading to organic-sulfur mineralization in the Bacillales. Critical Reviews in Microbiology **42**, 31–45.

Schmutz D, Brunold C. 1984. Intercellular localization of assimilatory sulfate reduction in leaves of *Zea mays* and *Triticum aestivum*. Plant Physiology **74**, 866–870.

Scuffi D, Alvarez C, Laspina N, Gotor C, Lamattina L, Garcia-Mata C. 2014. Hydrogen sulfide generated by L-cysteine desulfhydrase acts upstream of nitric oxide to modulate abscisic acid-dependent stomatal closure. Plant Physiology **166**, 2065–2076.

Sebastian J, Wong MK, Tang E, Dinneny JR. 2014. Methods to promote germination of dormant *Setaria viridis* seeds. PLoS One **9**, e95109.

Slack CR, Hatch MD, Goodchild DJ. 1969. Distribution of enzymes in mesophyll and parenchyma-sheath chloroplasts of maize leaves in relation to the C_4-dicarboxylic acid pathway of photosynthesis. The Biochemical Journal **114**, 489–498.

Stipanuk MH, Ueki I, Dominy JE Jr, Simmons CR, Hirschberger LL. 2009. Cysteine dioxygenase: a robust system for regulation of cellular cysteine levels. Amino Acids **37**, 55–63.

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R. 2011. Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annual Review of Plant Biology **62**, 157–184.

Tausz M, Pilch B, Rennenberg H, Grill D, Herschbach C. 2004. Root uptake, transport, and metabolism of externally applied glutathione in *Phaseolus vulgaris* seedlings. Journal of Plant Physiology **161**, 347–349.

Tschanz A, Landoit W, Bleuler P, Brunold C. 1986. Effect of SO_2, on the activity of adenosine 5′-phosphosulfate sulfotransferase from spruce trees (*Picea abies*) in fumigation chambers and under field conditions. Physiologia Plantarum **67**, 235–241.

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos P, Krahenbuhl U, den Camp RO, Brunold C. 2002. Flux control of sulphate assimilation in *Arabidopsis thaliana*: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. The Plant Journal **31**, 729–740.

Wingler A, Lea PJ, Quick WP, Leegood RC. 2000. Photorespiration: metabolic pathways and their role in stress protection. Philosophical Transactions of the Royal Society B: Biological Sciences **355**, 1517–1529.

Zhang X, Xin Y, Chen Z, Xia Y, Xun L, Liu H. 2021. Sulfide-quinone oxidoreductase is required for cysteine synthesis and indispensable to mitochondrial health. Redox Biology **47**, 102169.