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Research paper

Regulation of sulfur metabolism in seedlings of the C_4 plant maize upon sulfate deprivation and atmospheric H_2S exposure

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ABSTRACT

The increased cultivation of highly productive C_4 crop plants may contribute to a second green revolution in agriculture. However, the regulation of mineral nutrition is rather poorly understood in C_4 plants. To understand the impact of C_4 photosynthesis on the regulation of sulfate uptake by the root and sulfate assimilation into cysteine at the whole plant level, seedlings of the monocot C_4 plant maize (*Zea mays*) were exposed to a non-toxic level of 1.0 μl l^{-1} atmospheric H_2S at sulfate-sufficient and sulfate-deprived conditions. Sulfate deprivation not only affected growth and the levels of sulfur- and nitrogen-containing compounds, but it also enhanced the expression and activity of the sulfate transporters in the root and the expression and activity of APS reductase (APR) in the root and shoot. H_2S exposure alleviated the establishment of sulfur deprivation symptoms and seedlings switched, at least partly, from sulfate to H_2S as sulfur source. Moreover, H_2S exposure resulted in a downregulation of the expression and activity of APR in both shoot and root, though it hardly affected that of the sulfate transporters in the root. These results indicate that maize seedlings respond similarly to sulfate deprivation and atmospheric H_2S exposure as C_3 monocots, implying that C_4 photosynthesis in maize is not associated with a distinct whole plant regulation of sulfate uptake and assimilation into cysteine.

1. Introduction

In the upcoming century, agricultural practices will be aimed at sustainably enhancing crop yields. C_4 crop plants are particularly suited for achieving this aim, because C_4 plants can achieve high growth rates, while using water and nitrogen efficiently (Brown, 1978; Jobe et al., 2019, 2020). C_4 plants are characterized by a spatial separation of photosynthetic processes between the leaf's mesophyll and bundle sheath cells (Hatch and Slack, 1966). Whereas the initial assimilation of CO_2 in C_4 acids is located in the mesophyll, CO_2 assimilation in the Calvin cycle is restricted to the bundle sheath (Hatch and Slack, 1966). This spatial separation is supported by a Kranz anatomy, with an enlargement of the bundle sheath cells (Bräutigam et al., 2011; Gowik and Westhoff, 2011). The cellular differentiation of photosynthesis and the Kranz anatomy are absent in C_3 plants (Bräutigam et al., 2011; Gowik and Westhoff, 2011).

The regulation of mineral nutrient metabolism has hardly been studied in C_4 plants. Although it is known that C_4 plants feature a higher nitrogen (N) use efficiency than C_3 plants, mineral nutrient research focused on C_3 plants. Nevertheless, it is particularly relevant to understand how C_4 photosynthesis affects the regulation of the metabolism of the macronutrient sulfur (S), for S deficiency frequently limits the growth and reproduction of crops and other plants (Johnson, 1984; Schnug and Haneklaus, 2005; Ausma et al., 2021). For instance, S

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limitation undermines the development of flowers and particularly the color of yellow, carotenoid-based flowers, which will decrease plant reproductive success and crop yield (Ausma et al., 2021). This effect may cascade to pollinators, thus causing S deficiency to potentially hamper (agro-)ecosystem functioning (Ausma et al., 2021). Understanding the regulation of S metabolism in C_4 plants helps to optimally grow C_4 crops, e.g., by identifying breeding targets for the efficient use of S fertilizers (Takahashi et al., 2011).

Sulfur is generally acquired by plants as sulfate taken up by the root and distinct transporters are involved in its uptake and distribution across the plant (Takahashi et al., 2011). Whereas Sultr1;1 and Sultr1;2 are involved in the primary uptake of sulfate by the root, Sultr2;1 mediates sulfate distribution across the plant via the xylem (Buchner et al., 2004; Takahashi, 2019). After its activation to APS by ATP sulfurylase (ATPS), sulfate is reduced to sulfite by APS reductase (APR). Sulfite is subsequently reduced by sulfite reductase (SiR) to sulfide, which is finally assimilated into cysteine via a reaction with *O*-acetylserine (OAS), catalyzed by the enzyme OAS-(thiol)lyase (OAS-TL). Cysteine is the precursor and/or reduced S donor for several organic S compounds. For instance, cysteine is used for methionine synthesis and both amino acids are essential for protein synthesis (Bergmann and Rennenberg, 1993; Hawkesford and De Kok, 2006: Ausma and De Kok, 2019).

C_4 photosynthesis might affect the regulation of S metabolism. In maize (*Zea mays*) and other C_4 monocots the foliar metabolism of sulfate into cysteine is exclusively located in bundle sheath cells (Gerwick et al., 1980; Passera and Ghisi,1982; Schmutz and Brunold, 1984; Kopriva et al., 2001). However, in C_4 species from the dicot genus *Flaveria* this metabolism is located in all leaf cells (Koprivova et al., 2001). Thus, C_4 photosynthesis is not universally associated with a cellular compartmentalization of foliar sulfate metabolism. Nevertheless, C_4 photosynthesis might still affect the regulation of sulfate uptake and assimilation. C_3 and C_4 plants have a highly different leaf biochemistry and anatomy and, moreover, the evolution of C_4 photosynthesis occurred under specific abiotic conditions, such as heat and drought (Jobe et al., 2019, 2020). These environmental conditions are known to impact the regulation of sulfate metabolism (De Kok et al., 1991; Ahmad et al., 2016; Batool et al., 2018).

The fumigation of plants with non-toxic atmospheric H_2S levels represents a powerful tool to obtain insights into the regulatory aspects of sulfate uptake and assimilation at the whole plant level (Ausma and De Kok, 2019). Plants may use atmospheric H_2S as S source for growth, since upon its foliar absorbance, H_2S may directly be used for cysteine synthesis (Ausma and De Kok, 2019). Plants may also switch from sulfate to H_2S as S source and grow with H_2S as sole S source (Ausma and De Kok, 2019). A previous study, on the impact of H_2S on stomatal aperture in maize seedlings, suggested that atmospheric H_2S levels ≤ 1 ppm were non-toxic to maize (Ausma et al., 2020). Maize seedlings were able to utilize atmospheric H_2S as S source for growth, and exposure to 1.0 μl l^{-1} fully prevented the development of S deficiency symptoms upon sulfate deprivation (Ausma et al., 2020).

In the current paper, the hypothesis was tested that C_4 photosynthesis is associated, at the whole plant level, with a distinct regulation of sulfate uptake and assimilation into cysteine (primary sulfate metabolism). The impact of 1.0 μl l^{-1} H_2S, in the presence and absence of a sulfate supply to the root, on this regulation was studied in maize seedlings. This impact was compared with that previously observed in C_3 monocots. From this comparison, it was concluded that C_4 metabolism in maize, at the whole plant level, was not associated with a distinct regulation of sulfate uptake and assimilation into cysteine.

2. Materials and methods

2.1. Plant material and growth conditions

Maize (*Zea mays*, cv. 669, van der Wal, Hoogeveen, The Netherlands) was germinated between moistened filter paper in closed germination trays for 3 days. Subsequently, the germinated seedlings were grown on containers, holding 15 l aerated tap water. After 7 days, the seedlings were transferred to stainless-steel containers (60 plants per container) holding a 13 l aerated 50 % Hoagland nutrient solution at either 0 or 1 mM sulfate (for details on solution composition, see Ausma et al., 2020). Containers with plants were placed in 150 l cylindrical stainless-steel cabinets (0.6 m diameter) with a polymethyl methacrylate top (for an impression of the experimental design, see Fig. 1). Day and night air temperatures were 21 and 18 °C (± 1 °C), respectively, relative humidity was 30–40 %, and the photoperiod was 16 h at a photon fluency rate of 300 ± 20 μmol m^{-2} s^{-1} (within the 400–700 nm range) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules. Air exchange inside the cabinets was 40 l min^{-1} and the air inside the cabinets was stirred continuously by a ventilator. Plants were exposed to 0 or 1.0 μl l^{-1} atmospheric H_2S (for details see Ausma et al., 2020). Sealing of the lid of the boxes and plant sets prevented absorption of H_2S by the nutrient solutions. In $\delta^{34}S$ signature experiments plants were grown on 25 % Hoagland nutrient solutions that were enriched with ^{34}S-sulfate at 2.5 atom% excess (Abdallah et al., 2010) and plants were exposed to 0 or 1.0 μl l^{-1} atmospheric H_2S. The ^{34}S-sulfate was a kind gift from Jean-Christophe Avice (Université de Caen Normandie). The plants were harvested after 10 days and the shoot and root were separated and weighted. Biomass production was calculated by subtracting the weight at the start of the experiment from the weight at harvest. The plants that were exposed to ^{34}S-sulfate were lyophilized at −60 °C for 96 h for the determination of the S isotope signature. The plants that were not exposed to ^{34}S-sulfate were either dried at 80 °C for 24 h (to determine dry matter content) or frozen at −80 °C for the determination of other physiological parameters.

2.2. S and N metabolite content and $\delta^{34}S$ signature

S isotope signatures ($\delta^{34}S$ values) were determined in pulverized lyophilized plant material with a continuous flow isotope-ratio mass spectrometer (IRMS, Isoprime, GV Instruments, Manchester, UK) coupled to an elemental analyzer (EA3000, EuroVector, Milan, Italy, Abdallah et al., 2010). The $\delta^{34}S$ signatures of the shoot and root were expressed as permille (‰) difference compared to the ratio in the Vienna-CDT isotope standard (Dubousset et al., 2010). Additionally, tissue ^{34}S concentrations were calculated following the formulas in Dubousset et al. (2010). Total S and N levels were determined from pulverized dried material. Total S was measured with inductively coupled plasma mass spectrometry (ICP-MS) with an Agilent 7700 ICP-MS (Agilent Technologies, Santa Clara, CA, USA) (Almario et al., 2017). Total N was determined via the Dumas procedure, using an automated elemental analyzer (model EA 1110; Interscience, New York, NY, USA) with Eager 200 for Windows (van Klink et al., 2020). Sulfate and nitrate were extracted from frozen plant material, according to Aghajanzadeh et al. (2016), and their contents were determined via ion chromatography (IC), following Huang et al. (2016). Water-soluble non-protein thiols were isolated from fresh plant material, according to Aghajanzadeh et al. (2016), and their total level was determined via a reaction with the Ellman's reagents, following De Kok et al. (1988). Moreover, cysteine levels were measured by quantifying the difference in thiol content before and after the reaction of cysteine's sulfhydryl group with methylglyoxal (De Kok et al., 1988). Free amino acids were extracted similarly as sulfate and nitrate and their total level was determined with the ninhydrin reagent (Rosen, 1957). Soluble proteins were extracted in a 0.1 M K_2PO_4 buffer (pH 7.5) and subsequently quantified with the Bradford reagents (Bradford,1976).

2.3. Expression and activity of APR and sulfate transporters

RNA was extracted from frozen plant material using the procedure of Verwoerd et al. (1989) with an additional phenol-chloroform

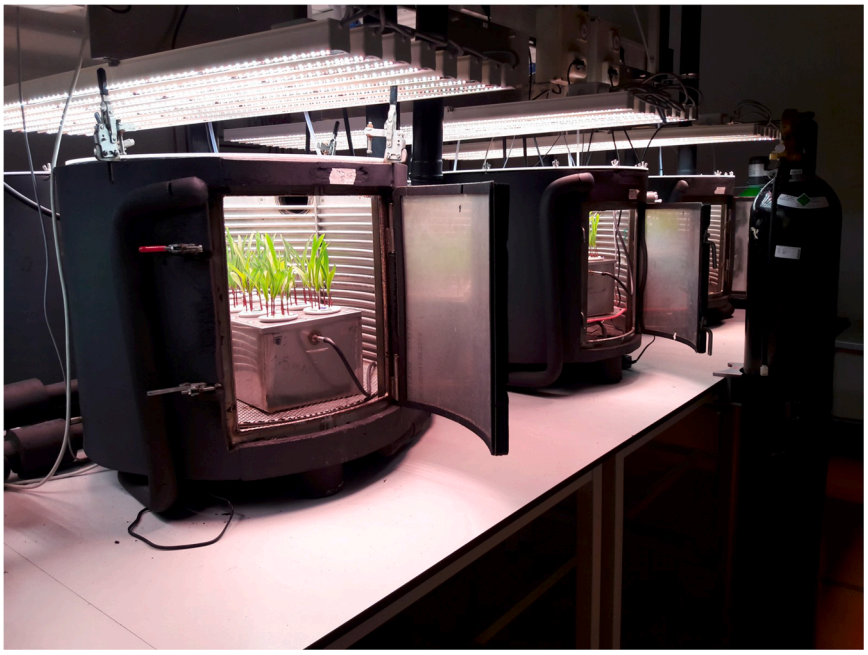

Fig. 1. The experimental set-up used in the outlined experiments.

isoamylalcohol extraction of the aqueous phase after the first centrifugation step. After removing possible gDNA contaminations using DNAse I (Thermo-Fisher, Waltham, MA, USA), RNA was converted to cDNA with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo-Fisher). Gene expression levels were quantified via quantitative PCR (qPCR), using elongation factor 1α (*EF1α*) as reference gene, because its expression level is comparable in the shoot and root, and unaffected by several environmental factors (see the maize eFP browser, Winter et al., 2007; Sekhon et al., 2011; Lin et al., 2014). Primers for *EF1α* were retrieved from Lin et al. (2014) and primers for *Sultr1;1* and *Sultr1;2* from Huang et al. (2018). Primers for *Sultr2;1* and *APR2* were designed on the coding sequence of the gene with NCBI's Primer BLAST. For primer sequences, see Table 1. The qPCR reaction mixtures contained 2 μl 1:50 diluted cDNA, 12.5 μl 2x Bio-Star qPCR-Mastermix SYBR Blue (GeneON, Ludwigshafen, Germany), 0.75 μl ROX (GeneON), 0.75 μl of the forward and reverse primer (10 μM stock) and 8.25 μl deionized water. Reactions were run in triplicate on an Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). After an initial denaturation of 5 min at 95 °C, 50 cycles followed of 15 s denaturation at 95 °C, 15 s annealing at 60 °C or 64 °C (in the case of *Sultr2;1*), and 30 s elongation at 72 °C. The program was finished by denaturation from 65 to 95 °C to generate melting curves for the verification of the primers' gene specificity. Obtained qPCR data were baseline-corrected using the LinRegPCR software (version 2014.2, Heart Failure Research Centre, Amsterdam, The Netherlands) and, subsequently, the initial number of gene transcripts (N_0) in each sample was determined with the mean PCR efficiency per primer set (Ramakers et al., 2003; Ruijter et al., 2009; Dalla Benetta et al., 2019). For the calculation of the relative expression level of a gene, the N_0 value of the gene-of-interest was divided by the N_0 value of *EF1α*. APR activity was quantified from pulverized frozen plant material that was homogenized in a 50 mM K_2HPO_4 buffer (pH 8.0) containing 30 mM Na_2SO_4, 500 μM AMP and 10 mM DTE. After adding ^{35}S-APS to the homogenates, APR activity was quantified as the production of ^{35}S-sulfite (according to Brunold and Suter, 1990). The sulfate uptake capacity was assessed by incubated plants for 1 h on a 25 % Hoagland nutrient solution, containing 0.5 mM sulfate that was labeled with ^{35}S-sulfate (2 MBq l^{-1}), as detailed by Zuidersma et al. (2020).

2.4. Statistical analyses

Statistical analyses were performed with GraphPad Prism (version 8.4.2, GraphPad Software, San Diego, CA, USA). Treatment means were analyzed for statistically significant differences with either an unpaired Student's *t*-test (in case of comparing two groups) or a two-way analysis of variance (ANOVA) with a Tukey's HSD post-hoc test (in case of comparing more than two groups) at the $P \leq 0.05$ level.

Table 1
Oligonucleotide primers used in the quantitative PCR (qPCR) assays. Locus IDs have been retrieved from the Maize Genome Database (version 5.0).

Gene name	Locus ID	Primer sequences (forward+reverse)	PCR efficiency (%)
EF1α	Zm00001eb385900	F: TGGGCCTACTGGTCTTACTACTGA R: ACATACCCACGCTTCAGATCCT	96
Sultr1;1	Zm00001eb008160	F: ATCAACCCACCTTCAGCTAGTCT R: TCTTTGTTCCCATCTATCTGGTAATC	94
Sultr1;2	Zm00001eb178810	F: TGGTAGCACTTGGGACGATGAA R: AACAGCGGCGTGATGAGCAG	88
Sultr2;1	Zm00001eb008170	F: GCGAGGGAGAGGATACAAGC R: TCCAGTCCTGTCCTACCCTG	94
APR2	Zm00001eb105800	F: CAGGTGCCCAAAACACGT R: CCAATCCTCGCAGCTTTGAC	93

3. Results

3.1. Atmospheric H_2S as sulfur source for growth

In this study maize seedlings were exposed to sulfate deprivation and H_2S fumigation. When maize seedlings were sulfate-deprived for 10 days, biomass production was reduced by approximately 20 % (Fig. 2). Shoot and root biomass production were equally reduced, implying that upon sulfate deprivation the shoot-to-root ratio was unaffected. Sulfate deprivation additionally enhanced shoot and root dry matter content (DMC) by 1.1- and 1.3-fold, respectively (Fig. 2). At sulfate-sufficient conditions, the fumigation with 1.0 μl l^{-1} H_2S did not affect biomass production and DMC (Fig. 2), but at sulfate-deprived conditions it alleviated the negative impacts of sulfate deprivation on biomass production and DMC. This means that, upon sulfate deprivation, sulfide absorbed in the foliage could replace sulfate taken up by the root as S source for growth (Fig. 2).

When maize seedlings were grown on a Hoagland nutrient solution that was enriched with ^{34}S-sulfate at 2.5 atom% excess, H_2S fumigation of sulfate-sufficient maize seedlings resulted in a 60 % and 10 % lower shoot and root $\delta^{34}S$ value, respectively (Fig. 3). This demonstrated that also at sulfate-sufficient conditions seedlings switched from sulfate to H_2S as sulfur source.

3.2. Impact of sulfate deprivation and H_2S fumigation on S and N metabolite content

Both sulfate deprivation and H_2S fumigation had a significant impact on the sulfate content of maize. Sulfate deprivation resulted in an 80 % and 85 % reduction in shoot and root sulfate content, respectively (Table 2). Consequently, the shoot and root total S contents decreased by 60 % and 55 %, respectively (Table 2). H_2S fumigation at sulfate-sufficient conditions resulted in a 1.2-fold to 1.6-fold increase in shoot total S content, because sulfate contents increased 1.7-fold (Table 2). Whereas H_2S fumigation at sulfate-deprived conditions alleviated the sulfate-deprived decreases in shoot sulfate and total S content, it did not alleviate these decreases in the root (Table 2).

S supply also profoundly impacted the (water-soluble non-protein) thiol content of maize. Sulfate deprivation resulted in a 70 % and 65 % decreased thiol content in the shoot and root, respectively (Table 2). Generally, glutathione is the major water-soluble non-protein thiol in plant tissues, though cysteine constitutes a minor thiol (Bergmann and Rennenberg, 1993). In maize, cysteine accounted for 8 % and 15 % of the thiol content of sulfate-sufficient shoots and roots, respectively (Table 2). The sulfate-deprived decrease in thiol levels could only partly be attributed to decreased cysteine contents, which indicated that sulfate deprivation also resulted in a decreased glutathione content (Table 2). H_2S fumigation resulted in a 1.8- and 1.9-fold increased thiol content in sulfate-sufficient and sulfate-deprived shoots, respectively (Table 2). The enhancements could be ascribed to both increased cysteine and glutathione contents, though cysteine contents were relatively more enhanced than glutathione contents (Table 2). Potentially, H_2S is metabolized into cysteine and glutathione in another subcellular compartment than sulfate. Consequently, the H_2S metabolism could be beyond control of existing regulatory feedback mechanisms, resulting in increased thiol levels (Hesse et al., 1997).

S and N metabolism are metabolically linked to each other, and S supply thus affected maize's N metabolism (Stulen and De Kok, 2012). Sulfate deprivation resulted in a 15 % and 40 % decreased shoot and root nitrate content and a 50 % decreased shoot (water-soluble) protein content (Table 2). Sulfate deprivation further enhanced shoot and root free amino acid contents with 4.9- and 3.9-fold, respectively (Table 2). Additionally, it slightly enhanced shoot and root N contents with 1.1-fold (Table 2). These phenomena are characteristic for sulfate-deprived plants. Sulfate deprivation results in a decreased availability of cysteine, which depresses protein synthesis. This causes the accumulation of non-S-containing amino acids as well as carbohydrates (Stuiver et al., 1997). The accumulation of these compounds contributes to the increased dry matter content upon sulfate deprivation. Whereas H_2S fumigation at sulfate sufficient conditions did not affect

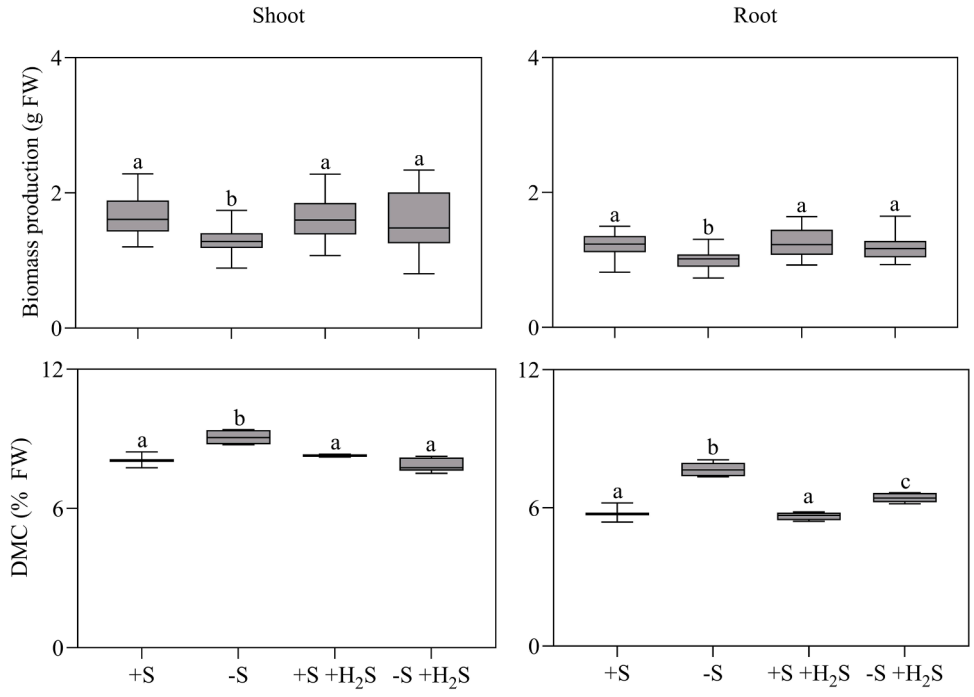

Fig. 2. Impact of sulfate deprivation and H_2S fumigation on the biomass production and dry matter content (DMC) of maize seedlings. The initial shoot and root weight were 0.21 ± 0.03 and 0.17 ± 0.03 g FW, respectively. Data on biomass production represent 2 experiments with 12–14 measurements with 2–3 plants in each. Data on DMC represent 3–5 measurements with 3 plants in each. Data are presented as boxes with a 5–95 percentile and whiskers. Different letters indicate significant differences between treatments ($P \leq 0.05$, Two-way ANOVA, Tukey's HSD test as a post-hoc test).

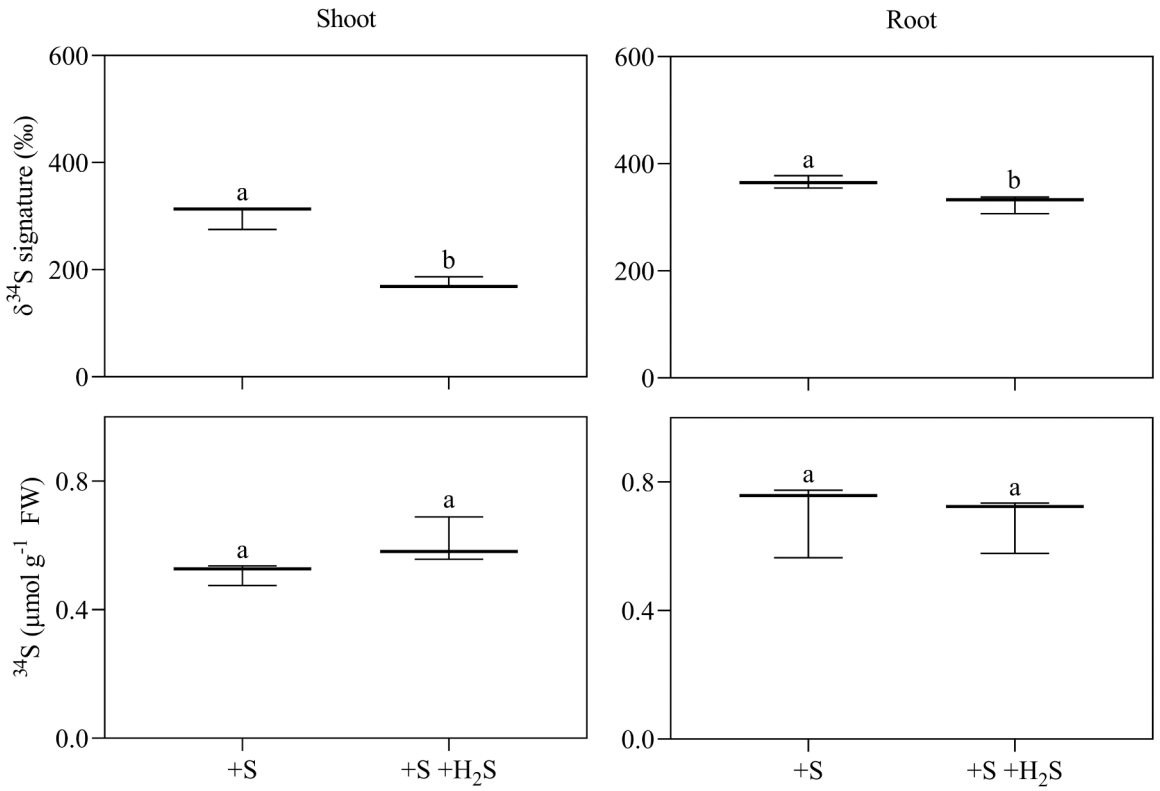

Fig. 3. Impact of H_2S fumigation on the S isotope signature of maize seedlings. Data, representing 3 measurements with 11–16 plants in each, are presented as boxes with a 5–95 percentile and whiskers. Different letters indicate significant differences between treatments ($P \leq 0.05$, Student's *t*-test).

total N, nitrate, amino acid, and soluble protein levels, at sulfate-deprived conditions it largely alleviated the impacts of sulfate deprivation on the levels of these N metabolites (Table 2).

3.3. Impact of sulfate deprivation and H_2S fumigation on the activity and expression of APR and sulfate transporters

APR is the key regulating enzyme in the sulfate reduction pathway (Kopriva and Koprivova, 2004; Hawkesford and De Kok, 2006; Ausma and De Kok, 2019) and hence its regulation was assessed. Sulfate deprivation resulted in a 1.8- and 3.4-fold enhanced APR activity in the shoot and root, respectively (Fig. 4). H_2S fumigation of sulfate-sufficient plants, however, resulted in a downregulation of the APR activity in the shoot and root by 50 % and 85 %, respectively (Fig. 4). In H_2S-fumigated sulfate-deprived plants, the enhanced APR activity was partially alleviated, though APR activity was still higher than in sulfate-sufficient plants (Fig. 4). Sulfate deprivation and H_2S fumigation had similar impacts on the expression of *APR2* (major APR isoform in maize; Chorianopoulou et al., 2020), with the exception that H_2S fumigation did not alleviate the sulfate-deprived increased expression of *APR2* in the root (Fig. 4). These data indicate that upon H_2S fumigation the reduction and hence assimilation of sulfate was, at least partly, downregulated and that maize seedlings transferred to sulfide, absorbed by the leaf, as S source for growth. The latter was supported by the experiments where the Hoagland nutrient solution was enriched with ^{34}S-sulfate (at 2.5 atom% excess). H_2S fumigation resulted in a 60 % and 10 % lower shoot and root δ^{34}S value, respectively (Fig. 3).

Despite the decreased δ^{34}S value in H_2S-fumigated plants that were grown on a ^{34}S-sulfate-enriched Hoagland nutrient solution, the total content of the ^{34}S isotope in the shoot and root remained unaffected by H_2S fumigation (Fig. 3). This indicated that the sulfate uptake rate was unaffected upon H_2S fumigation. Analogously, the sulfate uptake capacity of the root of sulfate-sufficient plants, which represents the activity of the sulfate transporters at an optimal sulfate supply, was also not affected by H_2S fumigation (Fig. 5). Nevertheless, sulfate deprivation resulted in an enhanced sulfate uptake capacity of the roots by 8.4-fold and this increase was partly alleviated by H_2S fumigation (Fig. 5).

The transcript levels of *Sultr1;1*, *Sultr1;2* and *Sultr2;1* were also determined. The first two transporters together facilitate sulfate uptake by the root (Buchner et al., 2004; Takahashi, 2019). In sulfate-sufficient roots the expression of *Sultr1;1* was 4.0-fold higher than that of *Sultr1;2* (Fig. 6). Sulfate deprivation enhanced the expression of *Sultr1;1* and *Sultr1;2* in the root by 5.8- and 3.0-fold, respectively (Fig. 6). H_2S fumigation did not affect the expression of these transporters (Fig. 6). The expression of *Sultr2;1*, which facilitates the xylem loading of sulfate (Takahashi, 2019), was enhanced by sulfate deprivation (Fig. 6). H_2S fumigation partly alleviated the increased *Sultr2;1* expression in sulfate-deprived roots (Fig. 6). From the above data it can be deduced that there generally is a poor shoot-to-root interaction between the metabolism of foliar absorbed atmospheric H_2S in the shoot and the uptake of sulfate by the root in maize seedlings.

4. Discussion

Cultivating more C_4 crops is part of the strategy to sustainably enhance agricultural yields (Jobe et al., 2019, 2020). However, crop yield is frequently limited by S deficiency (Johnson, 1984; Schnug and Haneklaus,2005; Ausma et al., 2021). Therefore, to optimally grow C_4 plants and to sustainably enhance agricultural yields, the regulation of S metabolism in C_4 plants should be understood. This understanding is fundamental to identifying breeding targets for the efficient use of S fertilizers (Takahashi et al., 2011). Our research shows that at the whole plant level the regulation of sulfate metabolism does not differ between C_3 and C_4 plants.

The impact of sulfate deprivation and atmospheric H_2S on sulfate uptake and its assimilation into cysteine was compared between maize

Table 2
Impact of sulfate deprivation and H_2S fumigation on the sulfur and nitrogen metabolite content of maize seedlings. Data represent the mean of 3 measurements with 3 plants in each (± SD). Different letters indicate significant differences between treatments ($P \leq 0.05$, Two-way ANOVA, Tukey's HSD test as a post-hoc test).

	0 µl l^{-1} H_2S		1.0 µl l^{-1} H_2S	
	+S	-S	+S	-S
Shoot				
Total S (µmol g^{-1} DW)	72 ± 3a	29 ± 3b	118 ± 11c	69 ± 8a
Sulfate (µmol g^{-1} FW)	4.3 ± 0.3a	0.9 ± 0.3b	7.6 ± 1.4c	3.3 ± 0.6a
Cysteine (µmol g^{-1} FW)	0.03 ± 0.01a	0.00 ± 0.02b	0.13 ± 0.02c	0.15 ± 0.02c
Thiols (µmol g^{-1} FW)	0.33 ± 0.04a	0.11 ± 0.02b	0.59 ± 0.02c	0.64 ± 0.03c
Total N (µmol g^{-1} DW)	3506 ± 68a	3771 ± 56b	3331 ± 92c	3333 ± 22c
Nitrate (µmol g^{-1} FW)	33.3 ± 0.3a	28.8 ± 0.4ab	33.3 ± 0.1a	32.9 ± 0.7a
Amino acids (µmol g^{-1} FW)	4.6 ± 0.3a	33.2 ± 1.8b	5.4 ± 0.3a	7.8 ± 0.8c
Soluble proteins (mg g^{-1} FW)	3.83 ± 0.23a	1.94 ± 0.22b	3.63 ± 0.19a	3.75 ± 0.12a
Root				
Total S (µmol g^{-1} DW)	92 ± 7a	36 ± 1b	80 ± 9a	41 ± 3b
Sulfate (µmol g^{-1} FW)	6.7 ± 0.3a	1.0 ± 0.2b	5.0 ± 0.6c	1.0 ± 0.1b
Cysteine (µmol g^{-1} FW)	0.07 ± 0.01a	0.00 ± 0.02b	0.05 ± 0.00a	0.05 ± 0.02a
Thiols (µmol g^{-1} FW)	0.36 ± 0.05a	0.12 ± 0.01b	0.44 ± 0.01c	0.36 ± 0.03a
Total N (µmol g^{-1} DW)	3140 ± 161ab	3314 ± 68b	2962 ± 58a	3017 ± 65a
Nitrate (µmol g^{-1} FW)	34.3 ± 0.7ab	21.0 ± 2.4b	35.4 ± 0.7a	35.6 ± 2.0c
Amino acids (µmol g^{-1} FW)	5.5 ± 0.2a	29.1 ± 4.1b	6.4 ± 0.6a	10.1 ± 0.7a
Soluble proteins (mg g^{-1} FW)	2.10 ± 0.13a	1.57 ± 0.07b	2.13 ± 0.35a	2.31 ± 0.12a

and C_3 plants. Similar to C_3 plants, maize seedlings could metabolize atmospheric H_2S. In agreement with previous observations (Ausma et al., 2020), exposure of maize to 1.0 µl l^{-1} H_2S alleviated the impacts of sulfate deficiency on biomass production, dry matter content, and the levels of S and N metabolites (Fig. 2; Table 2). Additionally, H_2S exposure resulted in enhanced cysteine, glutathione, and sulfate levels (Ausma et al., 2020; Table 2). Finally, in plants where the S supply to the root was enriched with ^{34}S-sulfate, the δ^{34}S values of both shoot and root were lowered upon H_2S fumigation (Fig. 3). Similar H_2S-impacts have been observed in C_3 plants, such as *Brassica* species, onion (*Allium cepa*), and barley (*Hordeum vulgare*; Durenkamp et al., 2007; Durenkamp and De Kok, 2004; Ausma and De Kok, 2019, 2020; Ausma et al., 2020).

The observation that maize can use H_2S as S source provides insights into the transport of S metabolites within a C_4 leaf. In C_4 monocots, including maize, the foliar metabolism of sulfate in cysteine is restricted to the bundle sheath (Gerwick et al., 1980; Passera and Ghisi, 1982; Schmutz and Brunold, 1984; Kopriva et al., 2001). The first enzyme of sulfate metabolism, ATPS, is only located in these cells (Gerwick et al., 1980). Consequently, the highly reactive intermediates of sulfate metabolism, and thus the complete metabolism, are restricted to the bundle sheath. In maize APR is also restricted to the bundle sheath

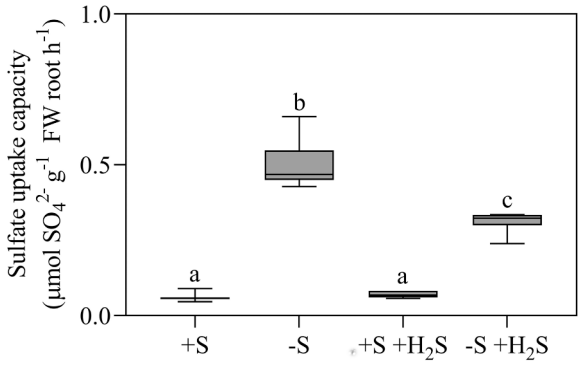

Fig. 5. Impact of sulfate deprivation and H_2S fumigation on the sulfate uptake capacity of the root of maize seedlings. Data, representing 7 measurements with 3 plants in each, are presented as boxes with a 5–95 percentile and whiskers. Different letters indicate significant differences between treatments ($P \leq 0.05$, Two-way ANOVA, Tukey's HSD test as a post-hoc test).

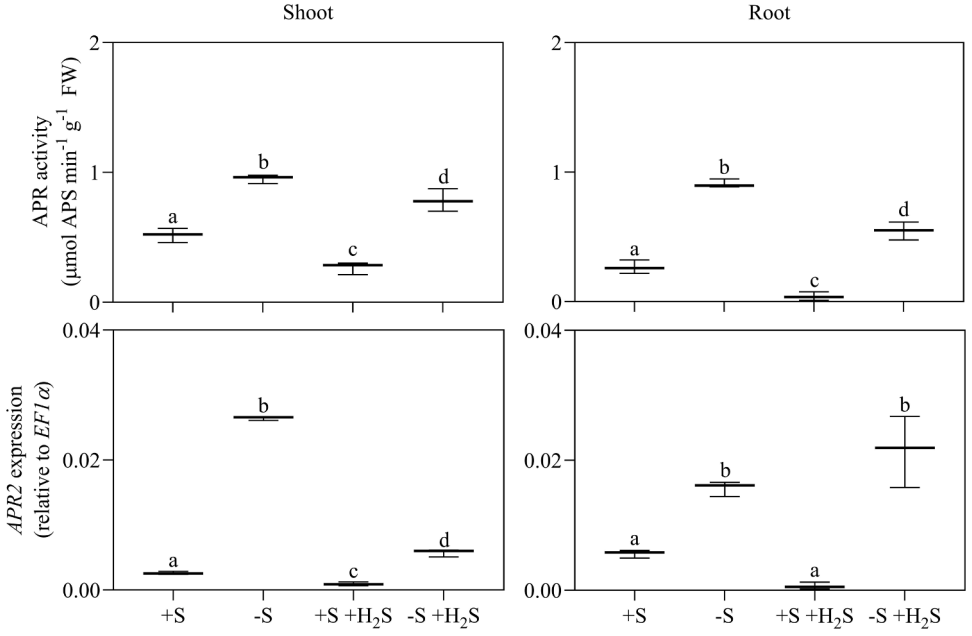

Fig. 4. Impact of sulfate deprivation and H_2S fumigation on *APR2* expression and activity in maize seedlings. Data, representing 3 measurements with 3 plants in each, are presented as boxes with a 5–95 percentile and whiskers. Different letters indicate significant differences between treatments ($P \leq 0.05$, Two-way ANOVA, Tukey's HSD test as a post-hoc test).

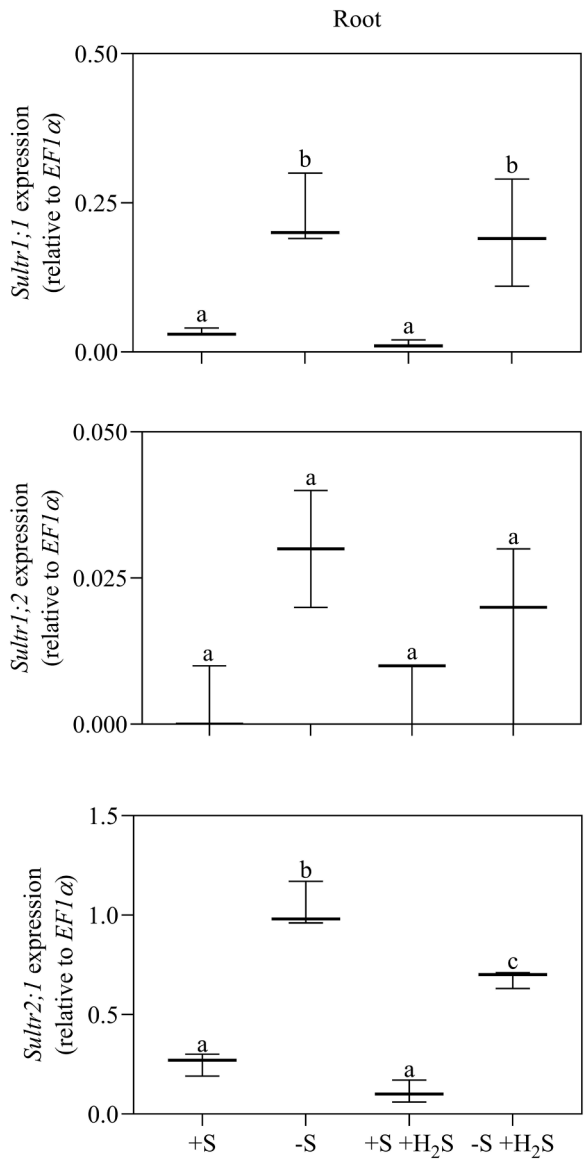

Fig. 6. Impact of sulfate deprivation and H_2S fumigation on the expression of sulfate transporters in the root of maize seedlings. Data, representing 3 measurements with 3 plants in each, are presented as boxes with a 5–95 percentile and whiskers. Different letters indicate significant differences between treatments ($P \leq 0.05$, Two-way ANOVA, Tukey's HSD test as a post-hoc test).

(Kopriva et al., 2001) though SiR and OAS-TL are located in both the bundle sheath and mesophyll (Passera and Ghisi, 1982; Schmutz and Brunold, 1984; Kopriva et al., 2001). Most likely, atmospheric H_2S, absorbed in the foliage of maize, was metabolized into cysteine by mesophilic OAS-TL, because (1) at the pH of the mesophyll the absorbed H_2S remains mostly undissociated and the bundle sheath is highly impermeable to gases and (2) H_2S cannot diffuse long distances, such as from the mesophyll to stem or root tissues, independently from the bundle sheath, as it is highly reactive (De Kok et al., 1991; Ausma and De Kok, 2019). It is thus likely that S metabolites have been transported to the leaf's bundle sheath and next to the root. Whereas in maize cysteine is transported from the bundle sheath to the mesophyll (Burgener et al., 1998), future research should elucidate the nature of the transport processes in the opposite direction.

Sulfate assimilation into cysteine was not distinctively regulated at the whole plant level in C_3 and C_4 plants. Maize switched, at least partly, from sulfate to H_2S as S source for growth, because H_2S fumigation of sulfate-sufficient maize downregulated APR activity – the key regulator of the rate of sulfate metabolism into cysteine (Fig. 4; Kopriva and Koprivova, 2004). Moreover, H_2S fumigation partly alleviated the sulfate-deprived increase in APR activity (Fig. 4). Similar findings have been observed in C_3 plants from the genus *Brassica* as well as in the C_3 plants spinach (*Spinacia oleracea*), barley, and onion (Herschbach et al., 1995a,b; Durenkamp et al., 2007; Durenkamp and De Kok, 2004; Koralewska et al., 2008; Ausma and De Kok, 2020).

The uptake of sulfate was also not distinctively regulated at the whole plant level in C_3 and C_4 plants. Firstly, the impact of H_2S fumigation on sulfate-sufficient plants was not different between C_3 and C_4 plants. H_2S fumigation did not affect the sulfate uptake *capacity* and *rate* of sulfate-sufficient maize, which were measured by incubating plants on labeled nutrient solutions (Figs. 3, 5). It remains ambiguous why H_2S exposure did not affect the sulfate uptake capacity and rate of sulfate-sufficient maize. This impact is not related to C_4 metabolism, for similar impacts have been observed in C_3 plants. Although H_2S fumigation downregulated the sulfate uptake capacity in *Brassica* and barley, it did not affect onion's sulfate uptake capacity (Westerman et al., 2000a,b; Buchner et al., 2004; Koralewska et al., 2008; Durenkamp et al., 2007; Durenkamp and De Kok, 2004; Ausma and De Kok, 2020).

The impact of H_2S fumigation on sulfate-deprived plants was also not different for C_3 and C_4 plants. H_2S fumigation partly alleviated the sulfate-deprived increase in sulfate uptake capacity in maize (Fig. 5). Sulfate-deprived C_3 plants feature a range of different responses to H_2S fumigation. For instance, H_2S fumigation fully alleviated the sulfate-deprived increase in sulfate uptake capacity in barley, whereas it hardly alleviated this increase in *Brassica* (Westerman et al., 2000a; Buchner et al., 2004; Koralewska et al., 2008; Ausma and De Kok, 2020).

Although the regulation of sulfate uptake and its assimilation into cysteine are not different at the whole plant level in C_4 and C_3 plants, it remains unclear if regulatory patterns differ at the cellular level. Root sulfate uptake may in maize principally be mediated by Sultr1;1, because sulfate-sufficient maize was characterized by a significantly higher root *Sultr1;1* than *Sultr1;2* expression (Fig. 6; Ahmad et al., 2016; Huang et al., 2018). However, this expression pattern may be a monocot-specific trait (instead of a C_4-specific trait), because a similar expression pattern was obtained for the C_3 monocots wheat (*Triticum aestivum*) and rice (*Oryza sativa*; Buchner et al., 2010; Takahashi, 2019). By contrast, in species from the dicot C_3 genus *Brassica Sultr1;1* was not expressed at sulfate-sufficient conditions (Buchner et al., 2004; Koralewska et al., 2007, 2008; Takahashi, 2019). In maize, sulfate uptake and assimilation were regulated via transcriptional and post-transcriptional mechanisms, which agrees with observations in barley and species from the genus *Brassica* (Ausma and De Kok, 2019, 2020). For instance, *APR2* expression and APR activity in the shoot responded identically to variation in sulfate and H_2S supply, indicating regulation of shoot APR activity at the transcriptional level (Fig. 4). However, whereas H_2S fumigation partly alleviated the sulfate-deprived increase in root APR activity, it did not alleviate the sulfate-deprived increase in root *APR* expression (Fig. 4). Hence, root APR activity was controlled (at least partly) via a post-transcriptional mechanism.

The exact nature of the regulatory signals remains unclear. Apart from being a nutrient (*viz.* S source for growth), endogenous H_2S levels may coordinate various physiological processes (Huang and Xie, 2023, Yu et al., 2024). H_2S might function as an endogenous gaseous transmitter co-regulating cellular sulfate uptake and its reduction in the plastids (De Kok et al., 2012; Shahbaz et al., 2013; Ausma and De Kok, 2019). Cysteine has also been proposed to regulate sulfate uptake in maize (Bolchi et al., 1999). However, there was no correlation between cysteine content and sulfate uptake transporter expression and activity (Figs. 5 and 6; Table 2). Cysteine contents did also not correlate with *APR* expression and activity nor with *Sultr2;1* expression (Figs. 4 and 6; Table 2). Although sulfate, glutathione, and metabolites from N metabolism may regulate sulfate uptake and assimilation, there were also no correlations between sulfate, nitrate, glutathione, and free amino acid contents on the one hand and the activity and expression of APR and the

sulfate transporters on the other hand (Figs. 4, 5 and 6; Table 2; Takahashi et al., 2011; Ausma and De Kok, 2019; De Jager et al., 2023). Future studies should assess the signal transduction pathways via which H_2S affects maize's sulfate utilization. Whereas these pathways are also poorly understood in C_3 plants, these remain almost entirely elusive for C_4 plants (Takahashi et al., 2011; Jobe et al., 2019; Takahashi, 2019; De Jager et al., 2023; Zenzen et al., 2024).

5. Conclusions

Our results show that C_4 metabolism is not associated with a distinct whole plant regulation of sulfate uptake and assimilation into cysteine, because sulfate metabolism responds similarly to H_2S supply and sulfate deprivation in maize as in C_3 monocots. Further studies should analyze the (molecular) signal pathways that govern sulfate metabolism at the whole plant level in C_4 plants. Insights from such studies will help to combat S deficiency problems, which is fundamental to optimally grow C_4 plants and to sustainably enhance agricultural yields.

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CRediT authorship contribution statement

Rahimzadeh Karvansara Parisa: Investigation, Formal analysis, Data curation. **van der Kooi Casper J.:** Writing – review & editing, Supervision. **Ausma Ties:** Writing – original draft, Investigation, Formal analysis, Data curation. **Riezebos Chiel-Jan:** Investigation, Formal analysis, Data curation. **De Kok Luit J.:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no affiliation with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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Data availability

Data will be made available on request.

References

Abdallah, M., Dubousset, L., Meuriot, F., Etienne, P., Avice, J.C., Ourry, A., 2010. Effect of mineral sulphur availability on nitrogen and sulphur uptake and remobilization during the vegetative growth of *Brassica napus* L. J. Exp. Bot. 61, 2635–2646. https://doi.org/10.1093/jxb/erq096.

Aghajanzadeh, T., Hawkesford, M.J., De Kok, L.J., 2016. Atmospheric H_2S and SO_2 as sulfur sources for *Brassica juncea* and *Brassica rapa*: Regulation of sulfur uptake and assimilation. Environ. Exp. Bot. 124, 1–10. https://doi.org/10.1016/j.envexpbot.2015.12.001.

Ahmad, N., Malagoli, M., Wirtz, M., Hell, R., 2016. Drought stress in maize causes differential acclimation responses of glutathione and sulfur metabolism in leaves and roots. BMC Plant. Biol. 16, 247. https://doi.org/10.1186/s12870-016-0940-z.

Almario, J., Jeena, G., Wunder, J., Langen, G., Zuccaro, A., Coupland, G., Bucher, M., 2017. Root-associated fungal microbiota of nonmycorrhizal Arabis alpina and its contribution to plant phosphorus nutrition. Proc. Natl. Acad. Sci. USA 114, 9403–9412. https://doi.org/10.1073/pnas.1710455114.

Ausma, T., Bansal, V., Kraaij, M., Verloop, A.C.M., Gasperl, A., Müller, M., Kopriva, S., De Kok, L.J., van der Kooi, C.J., 2021. Floral displays suffer from sulphur deprivation. Environ. Exp. Bot. 192, 104656. https://doi.org/10.1016/j.envexpbot.2021.104656.

Ausma, T., De Kok, L.J., 2019. Atmospheric H_2S: impact on plant functioning. Front. Plant Sci. 10, 743. https://doi.org/10.3389/fpls.2019.00743.

Ausma, T., De Kok, L.J., 2020. Regulation of sulfate uptake and assimilation in barley (*Hordeum vulgare*) as affected by rhizospheric and atmospheric sulfur nutrition. Plants 9, 1283. https://doi.org/10.3390/plants9101283.

Ausma, T., Mulder, J., Polman, T.R., van der Kooi, C.J., De Kok, L.J., 2020. Atmospheric H_2S exposure does not affect stomatal aperture in maize. Planta 252, 1–9. https://doi.org/10.1007/s00425-020-03463-6.

Batool, S., Uslu, V.V., Rajab, H., Ahmad, N., Waadt, R., Geiger, D., Malagoli, M., Xiang, C. B., Hedrich, R., Rennenberg, H., Herschbach, C., Hell, R., Wirtz, M., 2018. Sulfate is incorporated into cysteine to trigger ABA production and stomatal closure. Plant Cell 30, 2973–2987. https://doi.org/10.1105/tpc.18.00612.

Bergmann, L., Rennenberg, H., 1993. Glutathione metabolism in plants. In: De Kok, L.J., Rennenberg, H., Brunold, C., Rauser, W.E. (Eds.), Sulfur Nutrition and Sulfur Assimilation in Higher Plants: Regulatory, Agricultural and Environmental Aspects. SPB Publishers, The Hague, pp. 109–123.

Bolchi, A., Petrucco, S., Tenca, P.R., 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 Mol. Biol. 39, 527–537. https://doi.org/10.1023/a:1006148815106.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1006/abio.1976.9999.

Bräutigam, A., Kajala, K., Wullenweber, J., Sommer, M., Gagneul, D., Weber, K.L., Carr, K.M., Gowik, U., Maß, J., Lercher, M.J., Westhoff, P., Hibberd, J.M., Weber, A. P.M., 2011. An mRNA blueprint for C_4 photosynthesis derived from comparative transcriptomics of closely related C_3 and C_4 species. Plant Phys. 155, 142–156. https://doi.org/10.1104/pp.110.159442.

Brown, R.H., 1978. A difference in the nitrogen use efficiency of C_3 and C_4 plants and its implications in adaptation and evolution. Crop Sci. 18, 93–98. https://doi.org/10.2135/cropsci1978.0011183X001800010025x.

Brunold, C., Suter, M., 1990. Sulphur metabolism: adenosine 5-phosphosulfate sulfotransferase. Methods Plant Biochem 3, 339–343. https://doi.org/10.1016/B978-0-12-461013-2.50029-0.

Buchner, P., Parmar, S., Kriegel, A., Carpentier, M., Hawkesford, M.J., 2010. The sulfate transporter family in wheat: tissue-specific gene expression in relation to nutrition. Mol. Plant 3, 374–389. https://doi.org/10.1093/mp/ssp119.

Buchner, P., Stuiver, C.E.E., Westerman, S., Wirtz, M., Hell, R., Hawkesford, M.J., De Kok, L.J., 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 Physiol. 136, 3396–3408. https://doi.org/10.1104/pp.104.046441.

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 Physiol. 116, 1315–1322. https://doi.org/10.1104/pp.116.4.1315.

Chorianopoulou, S.N., Sigalis, P.P., Tsoutsoura, N., Apodiakou, A., Saridis, G., Ventouris, Y.E., Bouranis, D.L., 2020. Regulation of sulfur homeostasis in mycorrhizal maize plants grown in a Fe-limited environment. Int. J. Mol. Sci. 21, 3249. https://doi.org/10.3390/ijms21093249.

Dalla Benetta, E., Beukeboom, L.W., van de Zande, L., 2019. Adaptive differences in circadian clock gene expression patterns and photoperiodic diapause induction in *Nasonia vitripennis*. Am. Nat. 193, 881–896. https://doi.org/10.1086/703159.

De Jager, N., Shukla, V., Koprivova, A., Lycka, M., Bilalli, L., You, Y., Zeier, J., Kopriva, S., Ristova, D., 2023. Traits linked to natural variation of sulfur content in *Arabidopsis thaliana*. J. Exp. Bot. 75, 1036–1050. https://doi.org/10.1093/jxb/erad401.

De Kok, L.J., Buwalda, F., Bosma, W., 1988. Determination of cysteine and its accumulation in spinach leaf tissue upon exposure to excess sulfur. J. Plant Physiol. 133, 502–505. https://doi.org/10.1016/S0176-1617(88)80045-2.

De Kok, L.J., Rennenberg, H., Kuiper, P.J.C., 1991. The internal resistance in spinach shoots to atmospheric H_2S deposition is determined by metabolism processes. Plant Physiol. Biochem. 29, 463–470.

De Kok, L.J., Stuiver, C.E.E., Shahbaz, M., Koralewska, A., 2012. Regulation of expression of sulfate transporters and APS reductase in leaf tissue of Chinese cabbage (*Brassica pekinensis*). In: De Kok, L.J., Tausz, M., Hawkesford, M.J., Hoefgen, R., McManus, M. T., Norton, R.M., Rennenberg, H., Saito, K., Schnug, E., Tabe, L. (Eds.), Sulfur Metabolism in Plants – Mechanisms and Applications to Food Security and Responses to Climate Change. Springer, Berlin, Germany, pp. 47–52.

Dubousset, L., Etienne, P., Avice, J.C., 2010. Is the remobilization of S and N reserves for seed filling of winter oilseed rape modulated by sulphate restrictions occurring at different growth stages? J. Exp. Bot. 61, 4313–4324. https://doi.org/10.1093/jxb/erq233.

Durenkamp, M., De Kok, L.J., 2004. Impact of pedospheric and atmospheric sulphur nutrition on sulphur metabolism of *Allium cepa* L., a species with a potential sink capacity for secondary sulphur compounds. J. Exp. Bot. 55, 1821–1830. https://doi.org/10.1093/jxb/erh187.

Durenkamp, M., De Kok, L.J., Kopriva, S., 2007. Adenosine 5′-phosphosulphate reductase is regulated differently in *Allium cepa* L. and *Brassica oleracea* L. upon exposure to H_2S. J. Exp. Bot. 58, 1571–1579. https://doi.org/10.1093/jxb/erm031.

Gerwick, B.C., Ku, S.B., Black, C.C., 1980. Initiation of sulfate activation: a variation in C_4 photosynthesis plants. Science 209, 513–515. https://doi.org/10.1126/science.209.4455.513.

Gowik, U., Westhoff, P., 2011. The path from C_3 to C_4 photosynthesis. Plant Physiol. 155, 56–63. https://doi.org/10.1104/pp.110.165308.
Hatch, M.D., Slack, C.R., 1966. Photosynthesis by sugarcane leaves. A new carboxylation reaction and the pathway of sugar formation. Biochem. J. 101, 103–111. https://doi.org/10.1042/bj1010103.
Hawkesford, M.J., De Kok, L.J., 2006. Managing sulphur metabolism in plants. Plant Cell Environ. 29, 382–395. https://doi.org/10.1111/j.1365-3040.2005.01470.x.
Herschbach, C., De Kok, L.J., Rennenberg, H., 1995. Net uptake of sulfate and its transport to the shoot in spinach plants fumigated with H_2S or SO_2: does atmospheric sulfur affect the "inter-organ" regulation of sulfur nutrition? Bot. Acta 108, 41–46. https://doi.org/10.1111/j.1438-8677.1995.tb00829.x.
Hesse, H., Lipke, J., Altman, T., Hoefgen, R., 1997. Expression analysis and subcellular localization of cysteine synthase isoforms from *Arabidopis thaliana*. In: Cram, W.J., De Kok, L.J., Stulen, I., Brunold, C., Rennenberg, H. (Eds.), In: Sulphur Metabolism in Higher Plants: Molecular Ecophysiological and Nutritional Aspects. Backhuys Publishers, Leiden, pp. 227–230.
Huang, X.Y., Chao, D.Y., Koprivova, A., Dank, J., Wirtz, M., Müller, S., Sandoval, F.J., Bauwe, H., Roje, S., Dilkes, B., Hell, R., Kopriva, S., Salt, D.E., 2016. Nuclear localized MORE SULPHUR ACCUMULATION1 epigenetically regulates sulphur homeostasis in *Arabidopsis thaliana*. PLoS Genet 12, e1006298. https://doi.org/10.1371/journal.pgen.1006298.
Huang, Q., Wang, M., Xia, Z., 2018. The SULTR gene family in maize (*Zea mays* L.): gene cloning and expression analyses under sulfate starvation and abiotic stress. J. Plant Physiol. 220, 24–33. https://doi.org/10.1016/j.jplph.2017.10.010.
Huang, J., Xie, Y., 2023. Hydrogen sulfide signaling in plants. Antioxid. Redox Signal. 39, 40–58. https://doi.org/10.1089/ars.2023.026.
Jobe, T.O., Rahimzadeh Karvansara, P., Zenzen, I., Kopriva, S., 2020. Ensuring nutritious food under elevated CO_2 conditions: a case for improved C_4 crops. Front. Plant Sci. 11, 1267. https://doi.org/10.3389/fpls.2020.01267.
Jobe, T.O., 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. J. Exp. Bot. 70, 4211–4221. https://doi.org/10.1093/jxb/erz250.
Johnson, D.W., 1984. Sulphur cycling in forests. Biogeochemistry 1, 29–43.
Kopriva, S., Jones, S., Koprivova, A., Suter, M., von Ballmoos, P., Brander, K., Flückiger, J., Brunold, C., 2001. Influence of chilling stress on the intercellular distribution of assimilatory sulfate reduction and thiols in *Zea mays*. Plant Biol. 3, 24–31. https://doi.org/10.1055/s-2001-11745.
Kopriva, S., Koprivova, A., 2004. Plant adenosine 5′-phosphosulphate reductase: the past, the present, and the future. J. Exp. Bot. 55, 1775–1783. https://doi.org/10.1093/jxb/erh185.
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 Physiol. 127, 543–550. https://doi.org/10.1104/pp.010144.
Koralewska, A., Posthumus, F.S., Stuiver, C.E.E., Buchner, P., Hawkesford, M.J., De Kok, L.J., 2007. The characteristic high sulfate content in *Brassica oleracea* is controlled by the expression and activity of sulfate transporters. Plant Biol. 9, 654–661. https://doi.org/10.1055/s-2007-965438.
Koralewska, A., Stuiver, C.E.E., Posthumus, F.S., Kopriva, S., Hawkesford, M.J., De Kok, L.J., 2008. Regulation of sulfate uptake, expression of the sulfate transporters Sultr1;1 and Sultr1;2, and APS reductase in Chinese cabbage (*Brassica pekinensis*) as affected by atmospheric H_2S nutrition and sulfate deprivation. Funct. Plant Biol. 35, 318–327. https://doi.org/10.1071/fp07283.
Lin, Y., Zhang, C., Lan, H., Gao, S., Liu, H., Liu, J., Cao, M., Pan, G., Rong, T., Zhang, S., 2014. Validation of potential reference genes for qPCR in maize across abiotic stresses, hormone treatments, and tissue types. PLoS One 9, e95445. https://doi.org/10.1371/journal.pone.0095445.
Passera, C., Ghisi, R., 1982. ATP sulphurylase and *O*-acetylserine sulphydrylase in isolated mesophyll protoplasts and bundle sheath strands of S-deprived maize leaves. J. Exp. Bot. 33, 432–438. https://doi.org/10.1093/jxb/33.3.432.
Ramakers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62–66. https://doi.org/10.1016/s0304-3940(02)01423-4.
Rosen, H., 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. 67, 10–15. https://doi.org/10.1016/0003-9861(57)90241-2.
Ruijter, J.M., Ramakers, C., Hoogaars, W.M., Karlen, Y., Bakker, O., van den Hoff, M.J.B., Moorman, A.F.M., 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37, e45. https://doi.org/10.1093/nar/gkp045.
Schmutz, D., Brunold, C., 1984. Intercellular organization of assimilatory sulfate reduction in leaves of *Zea mays* and *Triticum aestivum*. Plant Phys. 74, 866–870. https://doi.org/10.1104/pp.74.4.866.
Schnug, E., Haneklaus, S.H., 2005. Sulphur deficiency symptoms in oilseed rape (*Brassica napus* L.) – The aesthetics of starvation. Phyton 45, 79–95.
Sekhon, R.S., Lin, H., Childs, K.L., Hansey, C.N., Buell, C.R., de Leon, N., Kaeppler, S.M., 2011. Genome-wide atlas of transcription during maize development. Plant J. 66, 553–563. https://doi.org/10.1111/j.1365-313x.2011.04527.x.
Shahbaz, M., Stuiver, C.E.E., Posthumus, F.S., Parmar, S., Hawkesford, M.J., De Kok, L.J., 2013. Copper toxicity in Chinese cabbage is not influenced by plant sulphur status, but affects sulphur metabolism-related gene expression and the suggested regulatory metabolites. Plant Biol. 16, 68–78. https://doi.org/10.1111/plb.12019.
Stuiver, C.E.E., De Kok, L.J., Westerman, S., 1997. Sulfur deficiency in *Brassica oleracea* L.: Development, biochemical characterization, and sulfur/nitrogen interactions. Russ. J. Plant Phys. 44, 505–513.
Stulen, I., De Kok, L.J., 2012. Foreword: Exploring interactions between sulfate and nitrate uptake at a whole plant level. In: De Kok, L.J., Tausz, M., Hawkesford, M.J., Hoefgen, R., McManus, M.T., Norton, R.M., Rennenberg, H., Saito, K., Schnug, E., Tabe, L. (Eds.), Sulfur Metabolism in Plants: Mechanisms and Applications to Food Security and Responses to Climate Change. Springer, Dordrecht, pp. 1–8.
Takahashi, H., 2019. Sulfate transport systems in plants: functional diversity and molecular mechanisms underlying regulatory coordination. J. Exp. Bot. 70, 4075–4087. https://doi.org/10.1093/jxb/erz132.
Takahashi, H., Kopriva, S., Giordana, M., Saito, K., Hell, R., 2011. Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu. Rev. Plant Biol. 62, 157–184. https://doi.org/10.1146/annurev-arplant-042110-103921.
van Klink, R., van Laar-Wiersma, J., Vorst, O., Smit, C., 2020. Rewilding with large herbivores: positive direct and delayed effects of carrion on plant and arthropod communities. PLoS One 15, e0226946. https://doi.org/10.1371/journal.pone.0226946.
Verwoerd, T.C., Dekker, B.M., Hoekema, A., 1989. A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. 17, 2362. https://doi.org/10.1093/nar/17.6.2362.
Westerman, S., De Kok, L.J., Stuiver, C.E.E., Stulen, I., 2000. Interaction between metabolism of atmospheric H_2S in the shoot and sulfate uptake by the roots of curly kale (*Brassica oleracea*). Physiol. Plant. 109, 443–449. https://doi.org/10.1034/j.1399-3054.2000.100411.x.
Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., Provart, N.J., 2007. An "electronic fluorescent pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS One 2, e718. https://doi.org/10.1371/journal.pone.0000718.
Yu, Y., Fotopoulos, V., Zhou, K., Fernie, A.R., 2024. The role of gasotransmitter hydrogen sulfide in plant cadmium stress responses. Trends Plant Sci. https://doi.org/10.1016/j.tplants.2024.08.003.
Zenzen, I., Cassol, D., Westhoff, P., Kopriva, S., Ristova, D., 2024. Transcriptional and metabolic profiling of sulfur starvation response in two monocots. BMC Plant Biol. 24, 257. https://doi.org/10.1186/s12870-024-04948-2.
Zuidersma, E.I., Ausma, T., Stuiver, C.E.E., Prajapati, D.H., Hawkesford, M.J., De Kok, L. J., 2020. Molybdate toxicity in Chinese cabbage is not the direct consequence of changes in sulphur metabolism. Plant Biol. 22, 331–336. https://doi.org/10.1111/plb.13065.