# Tris(methylthio)methane Produced by Mortierella hyalina Affects Sulfur Homeostasis in Arabidopsis

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### Abstract

Microbial volatiles are important factors in symbiotic interactions with plants. Mortierella hyalina is a beneficial root-colonizing fungus with a garlic-like smell, and promotes growth of Arabidopsis seedlings. GC-MS analysis of the M. hyalina headspace and NMR analysis of the extracted essential oil identified the sulfur-containing volatile tris(methylthio)methane (TMTM) as the major compound. Its incorporation in seedlings was shown by 34S labeling experiment. Under sulfur deficiency, TMTM downregulated sulfur deficiency-responsive genes, prevented glucosinolate (GSL) and glutathione (GSH) diminishment, and sustained plant growth. However, excess TMTM led to accumulation of GSH and GSL and reduced plant growth. Since TMTM is not directly incorporated into cysteine, we propose that the volatile from M. hyalina influences the plant sulfur metabolism by interfering with the GSH metabolism, and alleviates sulfur imbalances under sulfur stress.

### Introduction

Sulfur is an indispensable macronutrient required for proper plant growth, development and physiology. It is first incorporated into cysteine, and further into methionine, or glutathione (GSH), vitamins and cofactors, such as thiamine and biotin, to carry out important biochemical processes. Notable examples are the iron-sulfur (Fe-S) clusters which are required for electron transport in photosynthesis, reduction and assimilation of sulfur and nitrogen (Raven*et al.* , 1999; Lancaster *et al.* , 1979; Krueger and Siegel, 1982). In *Brassicales* , assimilation of sulfur contributes to the biosynthesis of glucosinolates (GSL), which are essential defense molecules against herbivores and pathogens (Bakhtiari and Rasmann, 2020; Halkier and Gershenzon, 2006; Ting *et al.* , 2020; Wittstock *et al.* , 2016). Although being classified as secondary metabolites, GSLs can hold up to 30% of total sulfur content in the plant body and serves as sulfur reservoir (Falk*et al.* , 2007; Aghajanzadeh *et al.* , 2014).

In natural environments, microorganisms play an important role in providing sulfate ($SO_4^{2-}$), the primary sulfur source accessible, to roots for the biosynthesis of sulfur-containing compounds in plants. As early as in 1877, scientists already knew that elemental sulfur ($S^0$) can be oxidized to sulfate, and microbes were thought to be an essential part of it (Lipman*et al.* , 1916). It was few decades later that scientists isolated the S-oxidizing bacteria *Thiobacillus denitrificans* and *T. thioparus* , and showed that they produce sulfate from $S^0$(Beijerinck, 1904; Lipman *et al.* , 1916; Waksman and Joffe, 1922). It is now known that microorganisms possess sulfatases to mineralize organic sulfur, thereby releasing sulfate into the rhizosphere

(Deng and Tabatabai, 1997; Kertesz, 2000). Furthermore, fungi were shown to mobilize sulfate-esters and activate arylsulfatase activity under sulfur-limiting conditions (Fitzgerald, 1976; Marzluf, 1997; Omar and Abd-Alla, 2000; Baum and Hrynkiewicz, 2006). Fungal symbionts are also crucial in supporting plants with sulfur. Mycorrhizal fungi are notable example for the promotion of sulfur uptake, as shown in maize, clover and tomato (Gray and Gerdemann, 1973; Cavagnaro *et al.* , 2006). The expression of sulfate transporters in plants can also be influenced by mycorrhizal fungi, resulting in improved sulfur status in host plants under sulfur deficient condition (Giovannetti*et al.* , 2014).

Volatile organic compounds (VOCs) from microorganisms present another possible route to provide sulfur to plants. Dimethyl disulfide (DMDS) is produced by the bacteria *Serratia odorifera* and *Bacillus spp.* B55. Under sulfur deficiency, DMDS can sustain plant growth and increase root branching (Meldau*et al.* , 2013). Labeling experiment demonstrated that the S-containing volatile is taken up by the plants (Kai*et al.* , 2010; Meldau *et al.* , 2013). Compared to bacteria, much less is known about sulfur-containing volatiles produced by fungi (Dickschat, 2017). Besides DMDS, mercaptoacetone, 3-methylsulfanylpropan-1-ol, benzothiazole, 2-acetylthiazole, 3,5-dimethyl-1,2,4-trithiolane, 5-(1-propynyl)-thiophen-2-carbaldehyde and sulfur dioxide ($SO_2$) were identified from various fungi (Splivallo*et al.* , 2007; Seifert and King, 1982; Nemcovic *et al.* , 2008; Schalchli *et al.* , 2011; Larsen, 1998; Dickschat, 2017; Citron *et al.* , 2012; Birkinshaw and Chaplen, 1955; Brock *et al.* , 2011). Not much is known about the mechanisms of their incorporation into the plant metabolism, but $SO_2$ can cross cell membranes directly from the surrounding air and influence sulfur distribution within leaf tissue (Randewig*et al.* , 2012; Pfanz *et al.* , 1987; Rennenberg and Polle, 1994).

Incorporation of sulfur is a multi-step process. In soil, it starts primarily with the assimilation of sulfate by sulfate transporters (SULTRs) in the root cells. SULTR1;1 and SULTR1;2 act as the primary sulfate transporters. SULTR2;1 is located in the xylem and pericycle and responsible for root-shoot sulfur transport (Takahashi*et al.* , 2011; Shibagaki *et al.* , 2002; Yoshimoto *et al.* , 2002; Kataoka *et al.* , 2004; Takahashi *et al.* , 1997). Once the sulfate is in root tissue, it is incorporated alongside with ATP into adenosine-5'-phosphosulfate (APS) via the enzyme ATP sulfurylase (ATPS). APS serves as the branching point between primary and secondary metabolism. Through APS reductase, APS is transformed into sulfite ($SO_3^{2-}$), and subsequently reduced to sulfide ($S^{2-}$) by sulfite reductase. With O-acetyl-serine(thiol)lyase (OASTL), sulfide is further incorporated into O-acetylserine (OAS) to form the amino acid cysteine for primary metabolism (Mugford*et al.* , 2011). On the other hand, APS goes into secondary metabolism through APS kinase, which catalyzes the formation of 3'-phosphoadenosine-5'-phosphosulfate (PAPS). PAPS serves as the molecule required for the last step of glucosinolate biosynthesis (Mugford*et al.* , 2009).

Sulfur assimilation and dynamics are highly regulated under sulfur deficiency. In *Arabidopsis* , *SULFUR LIMITATION1* (*SLIM1* ) is a central regulator of sulfur deficiency. The transcription factor of the EIL family induces the expression of genes for sulfur uptake transporters. Furthermore, genes for GSL catabolism are stimulated while those for GSL biosynthesis are repressed, thereby releasing sulfur from the GSL storage for proper plant growth (Maruyama-Nakashita*et al.* , 2006). Correspondingly, mutants defect in *SLIM1* cannot respond to sulfur deficiency, and show reduced root growth (Maruyama-Nakashita*et al.* , 2006). Finally, *SULFUR DEFICIENT INDUCED* (*SDI* )*1* and *SDI2* are often used as marker genes to monitor sulfur deficiency (Aarabi*et al.* , 2016). SDI1 is localized in the nucleus, and can repress GSL biosynthesis by interacting with MYB28, a major transcription factor for aliphatic GSL biosynthesis (Aarabi*et al.* , 2016; Hirai *et al.* , 2007; Gigolashvili *et al.* , 2007). All these components fine tune the sulfur status in the plant body to optimize plant competence in response to sulfur limitation.

*Mortierella hyalina* belongs to the phylum *Mucoromycota* . It possesses a distinctive garlic-like smell in synthetic culture. In the co-cultivation experiments with *Arabidopsis thaliana* seedlings, *M. hyalina* promoted plant growth (Johnson*et al.* , 2019). Similar results were obtained for three other *Mortierella* strains with garlic-like smells, while the growth responses were less for two strains which did not smell (Figure S1). In this study, we address the question whether the volatile from *M. hyalina* interferes with the plant metabolism and might be involved in the regulation of plant growth. The headspace of *M. hyalina* was analyzed by GC-MS to identify those VOCs which are potentially involved in plant nutrition. By NMR, a sulfur-containing volatile, tris(methylthio)methane (TMTM; CAS Number 5418-86-0), was identified as the major chemical in the fungal headspace. Incorporation of the sulfur from the fungal volatile into plant metabolism was shown with stable sulfur isotope labeling experiments. Under sulfur deficiency, TMTM promoted plant growth, reduced the consumption of sulfur-containing metabolites, and reduced the response of seedlings to sulfur deficiency. We propose that TMTM maintains sulfur homeostasis in the plant under sulfur limitation condition. Finally, biochemical analyses examining cysteine biosynthesis did not show direct incorporation of TMTM into O-acetylserine (OAS), suggesting that additional biochemical steps are involved before the sulfur from TMTM is incorporated into cysteine, or non-canonical incorporation mechanisms different from sulfate assimilation are involved.

## Materials and Methods

### Growth medium and conditions for seedlings and fungi

Seeds of wild-type *A. thaliana* (ecotype Columbia-0), and*slim1*(Maruyama-Nakashita*et al.* , 2006) mutant were surface-sterilized for 8 mins in sterilium solution containing lauryl sarcosine (1%) and Clorix cleaner (23%). Surface-sterilized seeds were washed with sterilized water 8 times and placed on Petri dishes with MS medium supplemented with 0.3% gelrite (Murashige and Skoog, 1962). The MS medium contains 1.5 mM $MgSO_4$. After cold treatment at 4 °C for 48 - 72 hours, plates were incubated at 22 °C under long day conditions (16 hours light/ 8 hours dark; 80 µmol $m^{-2}$ $s^{-1}$).

Sulfur deficiency assays were performed with MGRL medium (Fujiwara*et al.* , 1992). 1 L of MGRL medium contains 1.75 mM $NaH_2PO_4$, 1.75 mM $Na_2HPO_4$, 2 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4$, 3 mM $KNO_3$, 67 µM $Na_2EDTA$, 30 µM $H_3BO_3$, 10.3 µM $MnSO_4$, 8.6 µM $FeSO_4$, 1 µM $ZnSO_4$, 1.0 µM $CuSO_4$, 130 nM $CoCl_2$, 24 nM $(NH_4)_6Mo_7O_{24}$, 1% sucrose, 0.3% Gelrite, pH 5.6. For MGRL medium with reduced sulfate, $MgSO_4$ was replaced by $MgCl_2$. The total sulfate concentration in high (HS) and low (LS) sulfate MGRL medium is 1520.9 µM and 20.9 µM, respectively.

*Mortierella* strains (*M. hyalina* , FSU-509; *M. alpina* , SF002698; *M. turficola* , SF009851; *M. vinacea* , SF002701; *M. longicollis* , SF009830) were obtained from Jena Microbial Resource Center (Jena, Germany). They were grown on Potato-Dextrose-Agar (PDA), pH 6.5, and at 23 °C in the dark (Bains and Tewari, 1987) for fresh subcultures and desiccator assays.

For the sulfur labeling assays, *M. hyalina* was grown on KM medium modified from Hill and Käfer (2001): 1 L of the medium contains 7.06 mM $NaNO_3$, 6.98 mM KCl, 11.17 mM $KH_2PO_4$, 177.9 µM $H_3BO_3$, 6.4 µM $CuCl_2$, 76.5 µM $ZnCl_2$, 7.28 µM $CoCl_2$, 0.89 µM $(NH_4)_6Mo_7O_{24}$, 29.6 µM $MnCl_2$, 20 µM $Na_2EDTA$, 20 µM $FeCl_2$, 2% glucose, 0.2% peptone/trypton, 0.1% yeast extract, 0.1% casein hydrolysate, 1% agar. Finally, 2.11 mM ammonium sulfate ($(NH_4)_2{}^{32}SO_4$or $(NH_4)_2{}^{34}SO_4$) was added to make the unlabeled ($^{32}S$)/ labeled ($^{34}S$) medium, respectively.

Tris(methylthio)methane and $^{34}S$ ammonium sulfate were purchased from Sigma-Aldrich (Germany).

### Desiccator assay and sulfur labeling experiment

Twenty-one 10-d old *Arabidopsis* seedlings germinated on MS were transferred to a Petri dish with sucrose-free MS medium (14.5 cm in diameter). In a 2.5 L desiccator, a 7-d old fungal culture grown on PDA was placed at the bottom. A plastic inlay with holes was inserted in the middle of the desiccator, and the big Petri-dish with seedlings was placed on top of it. To ensure sterility of the experiment, the seam of the desiccator was wrapped with 3M$^{TM}$ Micropore tape (Figure S2). Fungus and seedlings were incubated at 22 °C under long day conditions (16 hours light/ 8 hours dark; 80 µmol $m^{-2}s^{-1}$) for 14 days. Number of inflorescence (flower stalk) and shoot fresh weight were measured.

The same procedure was followed for sulfur labeling experiments, in which a 7-d old *M. hyalina* culture grown on labeled/unlabeled KM medium and 16 10-d old *Arabidopsis* seedlings were used. Root and shoot tissues were collected after 14 days for analysis.

### Sulfur deficiency assay with MGRL agar medium

After germinating on MS medium for 5 days, seedlings were rinsed gently with sterilize water and transferred to MGRL agar medium with high (1520.9 µM) or low (20.9 µM) sulfate concentrations and grown for 7 days.

To measure the influence of TMTM on seedling´s fresh and dry weights, a 3-compartments Petri dish (92 mm in diameter, Sarstedt, Germany) was used. Two compartments were filled with MGRL agar medium, both containing either high or low sulfate concentrations. A sheet of sterilized paper was put in the third empty compartment, to which 10 µL sterilized water and a 10 µL mixture with 0, 10, 100 or 1000 µg TMTM dissolved in dichloromethane was applied.

For monitoring root growth, 5 days-old seedlings were transferred onto high or low sulfate MGRL agar medium on a square plate (100 x 100 x 20 mm; Sarstedt, Germany). A sheet of sterilized paper was put on to the bottom of the plate, and TMTM was applied onto it as described above. Plates were incubated vertically. Root length was measured directly after transfer and after 7 days of treatment.

**RNA isolation, and primers and qPCR**

RNA was extracted with TRIzol Reagent (Invitrogen, Germany) following the guideline provided by the manufacturer. Traces of DNA in the RNA samples were digested with TURBO Dnase (Thermo Fisher Scientific, Germany), and cDNA synthesis was performed with Omniscript RT Kit (Qiagen, Germany), following manufacturer's instructions.

Each 20 µL qPCR reaction contained 2 µL of 10 x DreamTaq Buffer (Thermo Fisher Scientific, Germany), 0.2 mM dNTP, 0.5 µM forward/reverse primer, 40 ng cDNA, 1 µL 20 x Evagreen® (Biotum, Germany) and 1.5 U of DreamTaq DNA Polymerase (Thermo Fisher Scientific, Germany). Real-time PCR reaction was conducted with CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Germany). The initial denaturation step was set at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 50 s, and extension at 72 °C for 1 min. Melt curve analysis was performed by incubating at 95 °C for 10 s, 65 °C 5s, and increase to 95 °C at 0.5 °C/5 s increment. Melt curve analysis showed a single peak for all genes analyzed. Values were normalized to the housekeeping gene *RPS18B* (AT1G34030) for gene expression analysis. Gene-specific primer pairs used in this study and the gene accession numbers are listed in Table S1.

**GC-MS analysis of *M. hyalina* headspace**

Headspace volatiles of a slant culture of *M. hyalina* grown on potato dextrose agar (PDA) in a glass tube with stopper were collected 14 days after inoculation with a solid phase micro extraction (SPME) fiber (Aldrich, red fiber, 100 µm PDMS) over 2 hours. As a control, the headspace of the medium alone (PDA) was collected.

SPME fibers were desorbed in the injection port of a GC at 220 °C in splitless mode and a helium flow of 1 mL/min through the chromatographic column connected. The volatiles were separated chromatographically on a ZB-5 ms column (30 m x 0.25 mm x 0.25 µm, Phenomenex) with an GC-oven temperature program starting at 45 °C for 2 min, then heating up to 220 °C with a rate of 10°C/min, followed by a heating rate of 30°C/min to 280°C, and was maintained for 1.83 min. The column was connected to a time-of-flight mass spectrometer (GCT, Micromass) via a transfer line (280 °C). Ion source temperature was set to 250 °C and ionization energy was 70 eV. For high resolution MS (HR-MS), heptacosa was continuously streaming into the source and the calibrated HR-MS profile was locked at m/z 218.9856.

A mixture of n-alkanes $C_8$ – $C_{20}$ in*n* -hexane (Aldrich) was measured before and after a sample sequence under the same conditions except for the injector split ratio (1:50). Retention times of the *n* -alkanes were used to calculate the retention index (RI) for each peak in the GC-MS chromatogram according to the method of Vandendool and Kratz (1963).

Compounds were identified based on their mass spectra (MS) in combination with their individual RIs in comparison to MS and RI database (National Institute of Standards and Technology, 2014) using MassFinder software (Hochmuth, 2010) in combination with NIST MS Search. Authentic reference compounds were used additionally for identification. For relative quantification, identified peaks of the GC-MS total ion chromatogram (TIC) were integrated.

### Identification of the S-containing volatile from *M. hyalina*

To identify the main S-containing volatile produced by *M. hyalina* , the compound needed to be enriched for further analysis. For that, *M. hyalina* was cultured in liquid PDA media for two weeks at 23 °C in the dark without shaking. The fungus mats produced on the surface of the media were collected (total FW [?] 180 g), rinsed twice with tap water and cut into pieces. The fungus material was subjected to hydro-destillation to obtain the essential oil which was further analyzed by NMR and GC-MS.

### GSL analysis by HPLC-UV

Fresh seedlings (20 to 100 mg) were harvested, weighted and freeze-dried until constant weight and ground to fine powder. GSLs were extracted with 1 mL of 80% methanol solution containing 0.05 mM of Sinalbin as internal standard. After centrifugation, 700 µL of extract was loaded onto DEAE Sephadex A 25 columns and treated with arylsulfatase for desulfation (Sigma-Aldrich). The eluted desulfo-GSLs were separated using high performance liquid chromatography (Agilent 1100 HPLC system, Agilent Technologies) on a reversed phase column (Nucleodur Sphinx RP, 250 x 4.6 mm, 5 µm, Macherey-Nagel, Düren, Germany) with a water (A) - acetonitrile (B) gradient: 0 - 1.0 min, 1.5% B; 1.0 - 6.0 min, 1.5-5% B; 6.0 - 8.0 min, 5 - 7% B; 8.0 - 18.0 min, 7 - 21% B; 18.0 - 23.0 min, 21 - 29% B; 23.0 - 23.1 min, 29 - 100% B; 23.1 - 24.0 min 100% B and 24.1 - 28.0 min 1.5% B; flow 1.0 mL $min^{-1}$. Detection was performed with a photodiode array detector and peaks were integrated at 229 nm. Desulfated GSLs were identified by comparison of their retention time and UV spectra to those of purified standards previously extracted from *A. thaliana*(Brown*et al.* , 2003). We used the following molar response factors for quantification of individual GSL relative to the internal standard Sinalbin: 2.0 for aliphatic GSLs and 0.5 for indolic GSLs (Burow*et al.* , 2006).

### Relative quantification of GSH by LC-MS/MS

Relative quantification of GSH was achieved on an Agilent 1200 series HPLC system (Agilent Technologies) coupled to a tandem mass spectrometer API 3200 (Applied Biosystems, Darmstadt, Germany) via electrospray ionization (ESI) in positive ionization mode. An aliquot of the raw extract from GSL analysis (see above) was injected. A Zorbax Eclipse XDB-C18 column (Agilent Technologies) was used for separation. 0.05% formic acid and acetonitrile were used as solvent A and B, respectively, at a flow rate of 1.1 mL/min with the following profile: 0 - 0.5 min, 3 - 15% B; 0.5 - 2.5 min, 15% - 85% B; 2.5 - 2.6 min, 85 - 100% B; 2.6 - 3.5 min, 100% B, 3.5 - 3.6 min, 100% B - 3% B and 3.6 - 6.0 min 3% B. The MS parameters were optimized as follows: ion spray voltage, 5500 V; turbo gas temperature, 650°C; collision gas, 3 psi; curtain gas, 35 psi; ion source gas 1, 60 psi; ion source gas 2, 60 psi. MRM for the parent ion - product ion was set as follows: m/z 308.1 - 179.1 (CE, 17 V; DP, 46 V) for GSH. Relative quantification was accomplished and expressed in relative peak area units of the LC-MS/MS signal per mg fresh weight.

### Determination of incorporation of $^{34}$S into plant metabolites by LC-ESI-Q-ToF-MS

For the determination of $^{34}$S incorporation into plant metabolites, ultra-high-performance liquid chromatography–electrospray ionization– high resolution mass spectrometry (UHPLC–ESI–HRMS) was performed with a Dionex Ultimate 3000 series UHPLC (Thermo Scientific) and a Bruker timsToF mass spectrometer (Bruker Daltonics, Bremen, Germany). UHPLC was used applying a Zorbax Eclipse XDB-C18 column (100 mm × 2.1 mm, 1.8 µm, Agilent Technologies, Waldbronn, Germany) with a solvent system of 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.3 mL/min. The elution profile was the following: 0 to 0.5 min, 5% B; 0.5 to 11.0 min, 5% to 60% B in A; 11.0 to 11.1 min, 60% to 100% B, 11.1 to 12.0 min, 100% B and 12.1 to 15.0 min 5% B. Electrospray ionization (ESI) in positive ionization mode (for GSH) and in negative ionization mode (for GSL) was used for the coupling of LC to MS. The mass spectrometer parameters were set as follows: capillary voltage 4.5/3.5 KV, end plate offset of 500 V, nebulizer pressure 2.8 bar, nitrogen at 280 °C at a flow rate of 8 L/min as drying gas. Acquisition was achieved at 12 Hz with a mass range from m/z 50 to 1500. At the beginning of each chromatographic analysis 10 µL of a sodium formate-isopropanol solution (10 mM solution of NaOH in 50/50 (v/v%) isopropanol- water containing 0.2% formic acid) was injected into the dead volume of the sample injection for recalibration of the mass spectrometer using the expected cluster ion m/z values. Peak areas were integrated from extracted ion chromatogram traces of the monoisotopic molecular ion peak ($[M+H]^+$, $[M-H]^-$) and of the isotopologues that could be detected with an isolation width of m/z +/- 0.002. For details of m/z values of isotopologues see Table S2. First we calculated the percentage of single isotopologues (% isotopologue) as a proportion of the sum of all isotopologues for each single compound (i.e. % of the monoisotopic molecular ion peak = peak area of the monoisotopic molecular ion peak * 100% / (peak area of the monoisotopic molecular ion peak + (peak area of "isotopologue+1") + (peak area of "isotopologue+2") + (peak area of "isotopologue+3") + (peak area of "isotopologue+4"). In order to determine the incorporation of $^{34}S$, the$^{34}S/^{32}S$ ratio was calculated ($^{34}S/^{32}S$ ratio = % "isotopologue + 2"/% of the monoisotopic molecular ion peak).

### OASTL activity assay monitoring cysteine biosynthesis

200 mg *Arabidopsis* wild-type *Col-0* leaves were homogenized in liquid nitrogen. 0.5 mL extraction buffer (50 mM HEPES-KOH, pH 7.5; 10 mM KCl; 1 mM EDTA; 1 mM EGTA; 30 mM DTT; 0.5 mM PMSF and 10% (v/v) glycerol) was added and mixed at 4 °C for 10 min with frequent shaking. After centrifugation at 16,000 g for 10 min, supernatant was collected. Protein concentration was measured with ROTI®Quant (Carl-Roth, Germany) following manufacturer's instruction.

The OASTL activity assay was carried out in a volume of 0.1 mL containing 100 mM HEPES-KOH pH 7.5; 5 mM DTT; 10 mM OAS and 10 mM $Na_2S$ or 4 mM TMTM. The reaction was initiated by the addition of OAS, and was incubated for 10 min at 25 °C. Termination of the reaction was done by adding 50 µL of 20% (w/v) trichloroacetic acid followed by centrifugation at 12500 g.

100 µL of the supernatant was transferred to a new tube and incubated in 200 µL of 134 mM Tris-HCl, pH 8.0 and 1 mM DTT at room temperature for 30 min. The reduced sample was mixed with 200 µL acetic acid and 200 µL ninhydrin reagent (250 mg ninhydrin dissolved in 6 mL acetic acid and 4 mL concentrated HCl). The tube was incubated at 90 °C for 10 min, then cooled rapidly on ice for 2 min. The sample was diluted with 95% ethanol and measured at 560 nm to quantify the synthesized cysteine.

### Measurement of root length

Plates were scanned with an Epson scanner (Perfection V600 Photo, Epson, Germany). Files were imported into ImageJ (Schindelin*et al.* , 2012). Root length was measured by SmartRoot plug-in with semi-automated root tracing method (Lobet*et al.* , 2011).

### Statistical tests

Statistical tests were performed using R studio version 1.1.463 with R version 3.4.4. Figures were plotted using Python 3.7.4 and arranged with LibreOffice Draw 5.1.6.2.

## Results

### *M. hyalina* produces the sulfur-containing volatile tris(methylthio)methane (TMTM)

To identify the volatiles from *M. hyalina* which are responsible for the garlic-like smell, the GC-MS chromatograms of SPME volatile collections of the headspace of slant cultures of *M. hyalina* were compared with the collections from the headspace of the growth medium. Three major constituents could be identified (Figure 1) of which the HR-MS of the molecular ions $M^+$ and $[M+2]^+$ (for the $^{34}S$ isotopologue) revealed the molecular formulas $C_3H_8S_2$ (m/z measured 108.0062, 110.0020 calc. 108.0062, 110.0020; RI 894; 2% rel.), $C_3H_8S_3$ (m/z measured 137.9626, 139.9585 calc. 137.9626, 139.9584; RI 1197; 2% rel.), and $C_4H_{10}S_3$ (m/z measured 153.9942, 155.9910 calc. 153.9939, 155.9897, RI 1217; 96% rel.). $C_3H_8S_2$ and $C_3H_8S_3$ could be identified as bis(methylthio)methane ($RI_{lit.}$ 889) and dimethyl trithiocarbonate ($RI_{lit.}$ 1196), respectively by comparison of their mass spectra and RI with the datasets of the NIST library and additionally with mass spectra and RI of authentic samples recorded under the same conditions.

For $C_4H_{10}S_3$, the major compound of the headspace of *M. hyalina* , library searches in NIST and Wiley mass spectra databases revealed no hit in combination with the RI. Therefore *M. hyalina* was extracted by hydro distillation. The obtained essential oil consisted mainly of three compounds (by GC-MS): Octenol-3-ol (22.4%) 3-octenone (21.7%) and $C_4H_{10}S_3$ (27,3%). NMR analysis of the mixture could reduce the structure motive of $C_4H_{10}S_3$ to $(CH_3\text{-}X)_n\text{-}CH$ (X = S, O, *etc.* ) which in combination with the empirical formula $C_4H_{10}S_3$ from HR-MS led to the structure of tris(thiomethyl)methane. Comparison with an authentic sample of tris(methylthio)methane (Aldrich) showed to be identical with respect to NMR and mass spectra, and RI (Figure 1 and Table 1).

**Sulfur atoms from TMTM are incorporated into plant metabolites**

To test whether sulfur from TMTM is incorporated into plant material, we grew *M. hyalina* on modified KM medium with addition of$^{32}$S- or $^{34}$S-ammonium sulfate, and co-cultivated them together with *Arabidopsis* seedlings in the same desiccator, but without direct physical contact. After 14 days, shoot and root tissues were collected, and the $^{34}$S/$^{32}$S ratio of GSLs and GSH was analyzed with LC-MS. For the shoots, a significant increase of the $^{34}$S/$^{32}$S ratios for the GSLs was detected (8.8%-12.8% for 4MOI3M; 8.4% - 12.6% for I3M; 14% - 16.6% for 8MSOO; 13.7% - 16.7% for 4MSOB; Figure 2; Figure S3 and S4). The ratio was also higher for the GSH in the shoots (4.4% - 6.3%). With the exception of 8MSOO, for which we also observed a significant increase in the roots (11.3% - 15.4%), the increases for the other compounds were much less (4.1% - 4.3%; Figure 2; Figure S3 and S4). In conclusion, sulfur from *M. hyalina* headspace are incorporated into plant GSLs and GSH material.

**TMTM influences plant growth under sulfur deficiency**

Since TMTM contains sulfur, we tested the effect of the volatile on*Arabidopsis* plants. Five days-old seedlings were transferred to MGRL agar medium with either high sulfate (HS, control) or low sulfate (LS) before the application of 0, 10, 100, 1000 µg TMTM (Table 2). After 7 days, the fresh and dry weights of the total seedlings, the shoots and the roots were analyzed. Figure 3 shows the effects of TMTM on the weights of seedlings grown under LS in comparison to seedlings grown sufficient sulfur in the medium (HS). In all instances, TMTM had the strongest growth promoting effect for seedlings grown on LS supplemented with 10 or 100 µg TMTM (Figure 3). The high dose of 1000 µg TMTM reduced plant growth. In summary, low doses of TMTM (10 - 100 µg) had positive effects on plant fresh and dry weights under sulfur deficiency, while the higher dose (1000 µg) had a negative effect.

We also tested whether TMTM promoted growth of seedlings which were grown on medium with sufficient sulfur (cf. Methods and Materials). Different doses of pure TMTM were applied to 10-days old seedlings grown on HS medium in desiccator for 1 or 2 weeks. Although the same trend was visible, the growth promoting effect of the volatile was not significant (data not shown).

**TMTM affects root lengths**

To examine whether TMTM affects the root growth under sulfur deficiency, 5-days old seedlings of wild-type (*Col-0* ) and *slim1* , a mutant which fails to respond to sulfur deficiency (Maruyama-Nakashita*et al.* , 2006), were transferred to LS medium and grown vertically for additional 7 days. Figure 4 shows the increase in the root lengths after 7 days. Compared to LS condition without TMTM, the root lengths of both wild-type (WT) and *slim1* seedlings were significantly higher when they were exposed to 100 µg TMTM ([?] 9% and 7% increase for WT and*slim1* , respectively; Figure 4A and 4B) and reached the level of seedlings which were grown on HS medium without the volatile. In accordance with the fresh and dry weight data, addition of 1000 µg TMTM reduced the root growth rate in WT for about 10% (Figure 4A). Interestingly, the reduction in root growth was not affected in*slim1* ([?] 1% reduction compared to LS without TMTM; Figure 4B). The differences might be due to an effect of TMTM on the sulfur homeostasis.

**TMTM reduces sulfur deficiency responses**

To test whether TMTM serves as sulfur source and affects the sulfur homeostasis of *Arabidopsis* seedlings, we tested the effect of the volatile on the expression of sulfur-responsive genes and the sulfur metabolite dynamics. Under sulfur limitation conditions, expression of sulfur transporters *SULTR1;1* , *SULTR1;2* and *SULTR2;1* was upregulated. Two days after exposure to the volatile, we observed a gradual decrease of their transcript levels and the effect increased with increasing TMTM amounts. Furthermore, the expression of the GSL repressor genes *SDI1* and *SDI2* was significantly down-regulated by TMTM, again in a dosage-dependent manner (Figure 5A). We further examined the expression of genes involved in the GSL and GSH metabolisms (i.e., *BRANCHED-CHAIN AMINOTRANSFERASE4, BCAT4; SULFOTRANSFERASE, SOTs; GLUTAMATE-CYSTEINE LIGASE, GSH1; GLUTATHIONE SYNTHETASE, GSH2;* two *CYTOCHROME P450, CYP79B2 and CYP79F2* ) . With 10 and 100 µg TMTM, their expression levels were similar to those in seedlings grown on HS medium, and with 1000 µg TMTM, their expression levels increased slightly.

Seven days after volatile application, *SDI1* was significantly up-regulated with 10 µg TMTM, while with 100 or 1000 µg TMTM, both *SDI1* and *SDI2* remained down-regulated compared to LS without TMTM (Figure 5B). The expression levels of the GSL metabolism genes *CYP79B2* and *SOTs* increased to the levels in seedlings grown on LS without TMTM, and with 1000 µg TMTM, they showed the highest expression.

In conclusion, after the application of 100 µg TMTM to LS-exposed seedlings, expression of the examined genes is similar to that of the seedlings grown on HS medium, and this is observed from the second to the $7^{th}$ day. We propose that low doses of TMTM (10 and 100 µg) diminish sulfur stress by adjusting the expression of the analyzed genes to the expression levels found under HS conditions. Upregulation of *SDI1* and *SDI2* in LS-grown seedlings exposed to 10 µg TMTM for 7 days indicates that this doses is too low to repress the sulfur-deficiency response after longer time periods.

**TMTM maintained high GSH and GSL levels under sulfur deficiency**

Cysteine is the first metabolite synthesized during sulfur assimilation, while GSH and GSLs contain large portions of the total sulfur pool. Under sulfur deficiency, these metabolites are broken down, and the sulfur is recycled for primary growth (Falk *et al.* , 2007; Sugiyama *et al.* , 2021). We measured the GSH and GSLs level to investigate whether TMTM influences the plant sulfur homeostasis at this level.

Two, 4 and 7 days after application of 1000 µg TMTM, the GSH level was significantly increased compared to the untreated control. Even 100 µg TMTM stimulated the GSH level, which was similar to that found in seedlings grown on HS (Figure 6A).

A similar pattern was observed for the total GSL levels. After 2 days, the GSLs slightly increased with increasing TMTM concentrations (Figure 6B). The effect broadened after 4 days. On LS without TMTM, total GSL level was significantly lowered compared to the rest of the treatments (Figure 6B). Similar to the results obtained for GSH, application of 100 µg TMTM maintained the total GSL level at the same level found in seedlings grown on HS medium without the volatile after 7 days (Figure 6B). We conclude that 100 µg TMTM established sulfur homeostasis in LS-grown seedlings which is comparable to the conditions in seedlings grown on HS. Furthermore, incorporation of TMTM can be observed in seedlings treated with 1000 µg TMTM, since they showed significantly higher amounts of GSH and GSLs than the unexposed controls.

**OASTL does not incorporate sulfur from TMTM into cysteine**

Sulfate is normally reduced to sulfide, which is as substrate for OASTLs to form cysteine. Cysteine is further converted to GSH, methionine or other sulfur-containing metabolites. TMTM is an organosulfide, containing 3 sulfide groups. We tested if plants can synthesize cysteine using TMTM as substrate. An OASTL activity assay was conducted by incubating total protein extract from wild-type *A. thaliana* (ecotype *Col-0* ) leaves with OAS and either $Na_2S$ or TMTM as substrate. Cysteine production was only observed when $Na_2S$ was used as substrate (Figure 7). In another experimental setup, total protein extract, OAS, $Na_2S$ and TMTM were incubated in the same reaction tube. Also under this condition, cysteine was produced, which indicates that OASTL activity was not hindered by TMTM. We conclude that TMTM is not a direct substrate for sulfur incorporation into cysteine by OASTL under our experimental conditions (Figure 7).

**Discussion**

In this study, we identified a fungal volatile, TMTM, as the main component in the headspace of the beneficial fungus *M. hyalina* . Application of TMTM participated in maintaining the sulfur homeostasis in *Arabidopsis* seedlings under sulfur deficiency. At low concentrations (10 – 100 µg), TMTM compensated sulfur-limitation responses of the seedlings: the volatile restored growth and root development which were inhibited under sulfur-limiting conditions, restricted the upregulation of sulfur deficiency marker genes (*SULTRs* , *SDI1* and *SDI2* ), or the breakdown of GSL and the accumulation of GSH. On medium with HS, these TMTM effects were not detectable. TMTM shifted the measured parameters in LS plants to those found in seedlings grown on HS medium without TMTM application. Higher concentration induced toxic or inhibitory effects, altered the sulfur homeostasis, and restricted plant growth. However, TMTM was not directly incorporated into cysteine by OASTLs, and is not inhibiting their function. This suggests that cysteine might not be a direct product of TMTM incorporation, or TMTM must be processed before its sulfur atoms can be incorporated into cysteine.

### *Mortierella* volatiles promote plant growth

Plants reduce the $CO_2$ concentration in closed systems which has to be considered in experimental designs with volatiles (Naznin*et al.* , 2013; Piechulla *et al.* , 2017). In preliminary experiments, we co-cultivated *Arabidopsis* seedlings with 5 different *Mortierella* strains with comparable growth rates and metabolite features. Since the three fungi with distinctive garlic-like smells (*M. hyalina* , *M. alpina* , *M. turficolalis* ) induced a stronger growth promotion compared to two non-smelling strains (*M. vinacea* , *M. longicollis* ; Figure S1), we hypothesized that the sulfur-containing volatiles from the fungi might be involved in the growth regulation. The major volatile in the headspace of one of these fungi, *M. hyalina* , was TMTM, and its abundance prompted us to investigate it in this study. The stronger growth of seedlings which are growing in the presence of the fungus compared to those treated with TMTM demonstrates that the investigated volatile is not the only factor involved in the growth promoting effect. However, it is difficult to design experimental set-ups which allow a quantitative comparison of fungal and volatile effects on plant growth and performance. We assume that the stabilizing effect of TMTM on the sulfur homeostasis allows better plant performance under sulfur stress.

### TMTM maintains the sulfur homeostasis under sulfur limitation

Plants respond to sulfur limitation in various ways. The first response is up-regulation of sulfate transporters (*SULTRs* ) to increase sulfate uptake from root (Takahashi*et al.* , 2011; Shibagaki *et al.* , 2002; Yoshimoto *et al.* , 2002; Kataoka *et al.* , 2004; Takahashi *et al.* , 1997). On the other hand, genes for GSL biosynthesis (e.g., *BCAT4* ,*CYP79B2* and *CYP79F2* ) are down-regulated, while those repressing GSL biosynthesis (*SDI1* and *SDI2* ) are up-regulated. These responses help plants to remobilize sulfur to sustain growth (Lewandowska and Sirko, 2008; Frerigmann and Gigolashvili, 2014; Borpatragohain*et al.* , 2016). Among the inspected genes, the sulfur starvation genes *SULTR1;1* , *SULTR1;2* , *SULTR2;1* and both*SDI1* and *SDI2* were down-regulated in a TMTM dose-dependent manner in plants which suffer from sulfur limitation (Figure 5A and 5B). Since the sulfur in TMTM can be incorporated into the plant material, the expression of the above-mentioned genes and those involved in GSL and GSH metabolism is similar to seedlings grown under HS condition without TMTM. Moreover, excessive TMTM results in the upregulation of these genes, indicating that these plants are actively moving excess sulfur to secondary metabolites. This is in accordance with a recent study by Sugiyama*et al.* (2021), showing a retrograde sulfur flow from glucosinolates to cysteine in *Arabidopsis.* Interestingly, the mRNA levels for GSL and GSH metabolism genes was higher in seedlings after 2 days on LS medium without TMTM compared to seedlings grown on HS medium. This might be caused by higher sulfate influx from the medium due to up-regulation of *SULTRs* . The plants actively metabolize the assimilated sulfate into various metabolites and utilize this as a store to sustain growth under sulfur limitations.

The effect is also observed at the metabolic level. Under sulfur limitation, the GSH and GSL levels decreased. However, 100 µg TMTM maintained the levels high under sulfur starvation conditions (Figure 6). Again, besides maintaining sulfur homeostasis, excess sulfur from the high TMTM dose is largely metabolized into secondary metabolites.

### TMTM sustained root growth under sulfur limitation

Imbalances in the sulfur pool have severe consequences for plant growth and yield (Zhao*et al.* , 1999; Lunde *et al.* , 2008; Jobe *et al.* , 2019). Under LS, biomass production and root growth were significantly reduced (Figure 3 and 4). This could be restored by the application of TMTM in low doses. We propose that TMTM maintains the sulfur homeostasis and allows root growth which is comparable to that under HS conditions (Figure 4). Furthermore, it appears that excess TMTM tilts the sulfur homeostasis and shifts the sulfur towards the secondary metabolite pool, resulting in reduced plant biomass production and root growth (Figure 3 and 4).

Growth regulation by TMTM *via* sulfur homeostasis is further supported by the response of WT and *slim1* seedlings to high TMTM dose. 1000 µg TMTM inhibited root growth in WT seedlings, but not in*slim1* (Figure 4). Apparently, lower doses of TMTM stimulated root growth in LS because the volatile influences the sulfur homeostasis. As a result, the root growth was comparable to seedling´s growth on HS without the volatile. However, the high dose (1000 µg) of TMTM could provide too much sulfur to the LS-grown WT seedlings, which may result in the activation of stress responses and ultimately growth retardation. On the other hand, because *slim1* could not mobilize sulfur from its secondary metabolites (Maruyama-Nakashita*et al.* , 2006), these seedlings showed a higher tolerance to excess TMTM. The different response of the two genotypes to excess TMTM is consistent with the idea that TMTM-induced changes in the sulfur homeostasis influence root growth.

### Metabolism of TMTM may not be a single-step process

It is known that plants are able to assimilate gaseous sulfur compounds, such as $SO_2$ and $H_2S$ (Randewig*et al.* , 2012; Lee *et al.* , 2017; Ausma and De Kok, 2019). They are also able to assimilate other sulfur-containing organic volatiles produced by microbes. One example is dimethyl disulfide (Meldau*et al.* , 2013). Nevertheless, how organosulfides are metabolized inside the plant remains unknown.

Diallyl disulfide (DADS), a volatile from garlic, is perhaps the best studied organosulfide, due to its anticancer ability (Yi and Su, 2013; Xiong *et al.* , 2018; Agassi *et al.* , 2020; Li*et al.* , 2018). It increases GSH levels and regulates antioxidant enzyme activity, leading to reduced oxidative stress in animal models (Demeule*et al.* , 2004; Hassanein *et al.* , 2021; Wei *et al.* , 2021). In plants, DADS also affects sulfur metabolism genes (Cheng*et al.* , 2016; Cheng *et al.* , 2020; Yang *et al.* , 2019). Metabolism of DADS and other organosulfides generates $H_2S$ (Kim*et al.* , 2019; Cai and Hu, 2017; Bolton *et al.* , 2019; Liang*et al.* , 2015). Studies on how DADS and other organosulfides are metabolized suggest the involvement of GSH and cysteine (Bolton*et al.* , 2019; Liang *et al.* , 2015; Cai and Hu, 2017). The reaction between DADS and GSH produces S-allyl GSH and a short-lived intermediate allyl perthio through $\alpha$-carbon nucleophilic substitution. The allyl perthio reacts with a second GSH, resulting in the release of $H_2S$ and S-allyl GSH disulfide (Liang*et al.* , 2015).

We found that 1000 µg TMTM increased both the GSH and GSL levels, and the GSH level responded faster to the volatile treatment (Figure 6A and 6B). A possible explanation could be that incorporation of sulfur from TMTM into the plant metabolism is connected to GSH. We tested if TMTM can be a direct substrate for OASTLs and found that this is not the case (Figure 7). Therefore, unlike sulfate assimilation, TMTM might first interfere with the GSH/GSSG system. This might lead to the cleavage of the C-S bonds and sulfur incorporation into plant. A detailed metabolome analysis of early sulfur-containing compounds after TMTM treatment might elucidate early steps in the role of this novel volatile.

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`image1.emf` available at https://authorea.com/users/420886/articles/527130-tris-methylthio-methane-produced-by-mortierella-hyalina-affects-sulfur-homeostasis-in-arabidopsis

**Figure 1.** GC-MS chromatogram of the headspace of *M. hyalina* (black) and the growth medium alone (blue). Identified signals are not present in the headspace of the growth medium. The three strong signals in the chromatogram of the headspace of the growth medium could be identified by MS and RI as benzaldehyde (7.13 min) nonanal (9.44 min), and decanal (11.00 min).

**Table 1.** Mass spectra and retention indices in comparison with authentic samples.

| RT | Compound | Formula by HR-MS | CAS # | Retention index | RI (lit) | Rel % | Authenic refe |
|---|---|---|---|---|---|---|---|
| 5.83 | Bis(methylthio)methane | $C_3H_8S_2$ | [1618-26-4] | 894 | 889 | 2% | y |

| RT | Compound | Formula by HR-MS | CAS # | Retention index | RI (lit) | Rel % | Authenic refe |
|---|---|---|---|---|---|---|---|
| 10.83 | Dimethyl trithiocarbonate | $C_3H_6S_3$ | [2314-48-9] | 1197 | 1196 | 2% | y |
| 11.25 | Tris(methylthio)methane | $C_4H_{10}S_3$ | [5418-86-0] | 1217 | §) | 98% | y |

§) The RI given in the NIST MS database and by other authors as well as the mass spectrum of Tris(methylthio)methane published there and in the Wiley MS database are not correct. GC-MS of an authentic sample purchased from Aldrich revealed an RI of 1217 and a mass spectrum identical to the mass spectrum of the compound at the identical retention time from the headspace of *M. hyalina* .

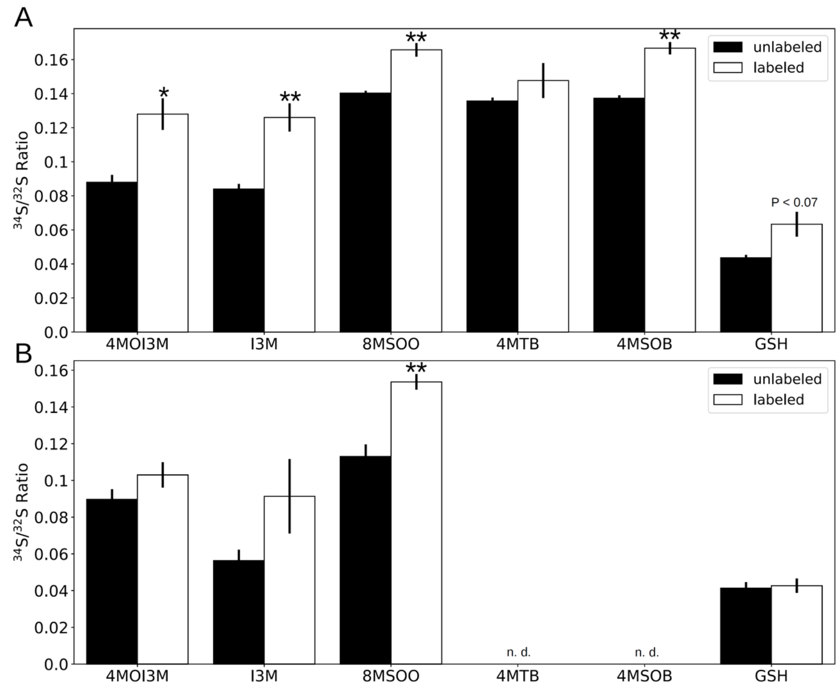



**Figure 2.** Sulfur atoms from fungal volatiles are incorporated into plant tissues. Shown are$^{34}S/^{32}S$ ratios of glucosinolates and glutathione in shoot (A) and root (B) tissues. *M. hyalina* was grown on modified KM plates with addition of $^{32}S$ -ammonium sulfate (unlabeled) or $^{34}S$-ammonium sulfate (labeled), and co-cultivated with *Arabidopsis* seedlings in a desiccator. Ratio of $^{34}S$ over $^{32}S$ of each glucosinolate species and glutathione was computed from M+2 and M peaks in LC-MS chromatogram. Error bars represent SEs from 3 independent biological replicates, each contains 4 technical replicates from 16 seedlings. Asterisks indicate significance level from Student's T-test between unlabeled and labeled samples (*P < 0.05; **P < 0.01). n.d.: not detected.

**Table 2.** Average fresh weight and dry weight per seedling after TMTS application. HS: seedlings grown on high sulfate MGRL medium. 0 µg, 10 µg, 100 µg and 1000 µg: seedlings grown on low sulfate MGRL medium supplied with 0 µg/ 10 µg/ 100 µg/ 1000 µg fungal volatile, respectively. At least 5 independent biological replicates were measured 7 days after treatments, each with 8 seedlings.

| Fresh Weight ± SE (mg) | Fresh Weight ± SE (mg) | Fresh Weight ± SE (mg) | Fresh Weight ± SE (mg) | Fresh Weight |
|---|---|---|---|---|
| Treatments | 0 µg | 10 µg | 100 µg | 1000 µg |
| Shoot | 13.02±1.19 | 12.3±1.21 | 13.46±1.36 | 11.00±1.00 |

| | | | | |
|---|---|---|---|---|
| Root | 6.55±0.62 | 6.40±0.53 | 6.77±0.73 | 5.10±0.64 |
| Total | 19.58±1.78 | 18.70±1.57 | 20.23±2.08 | 16.10±1.62 |
| Dry Weight ± SE (mg) | Dry Weight ± SE (mg) | Dry Weight ± SE (mg) | Dry Weight ± SE (mg) | Dry Weight ± |
| Treatments | 0 µg | 10 µg | 100 µg | 1000 µg |
| Shoot | 0.80±0.08 | 0.72±0.04 | 0.85±0.01 | 0.71±0.07 |
| Root | 0.31±0.03 | 0.33±0.02 | 0.33±0.04 | 0.23±0.04 |
| Total | 1.11±0.09 | 1.04±0.06 | 1.25±0.12 | 0.91±0.09 |

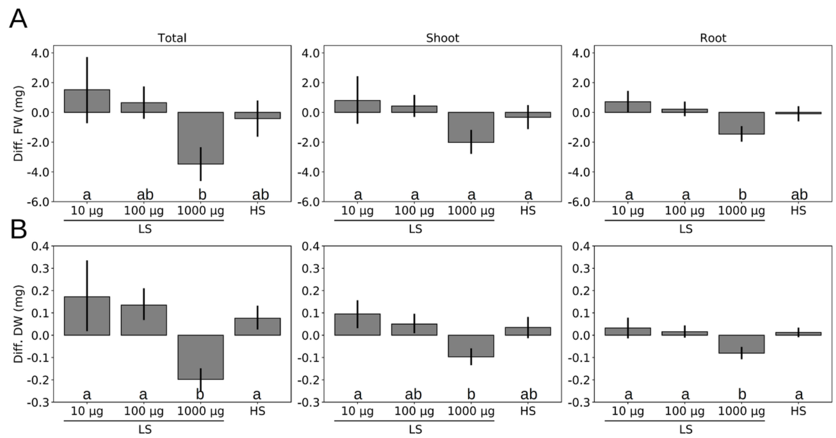



**Figure 3.** TMTS influences *A. thaliana* growth under sulfur deficiency. (A) Difference in fresh weight and (B) difference in dry weight of seedlings grown on low sulfate MGRL medium with addition of TMTS (0, 10, 100 and 1000 µg) compared seedlings grown on high sulfate MGRL medium. Error bars represent SEs from at least 5 independent biological replicates, each with 8 seedlings. Statistical significance was determined by Duncan's multiple range test with p-value $< 0.05$, and indicated with lower-case alphabets.

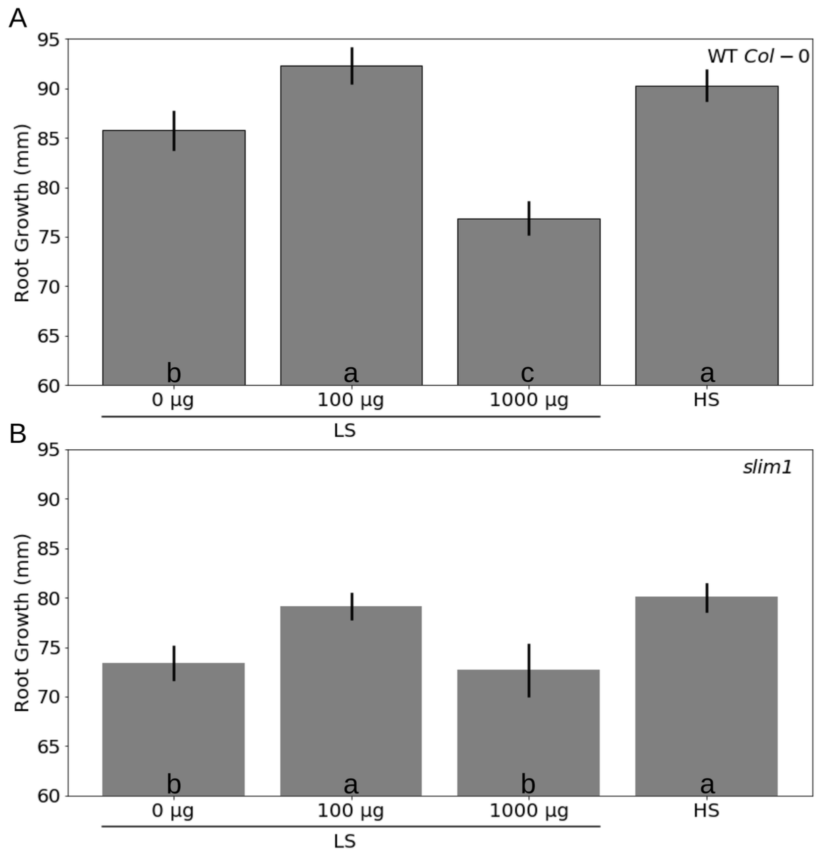
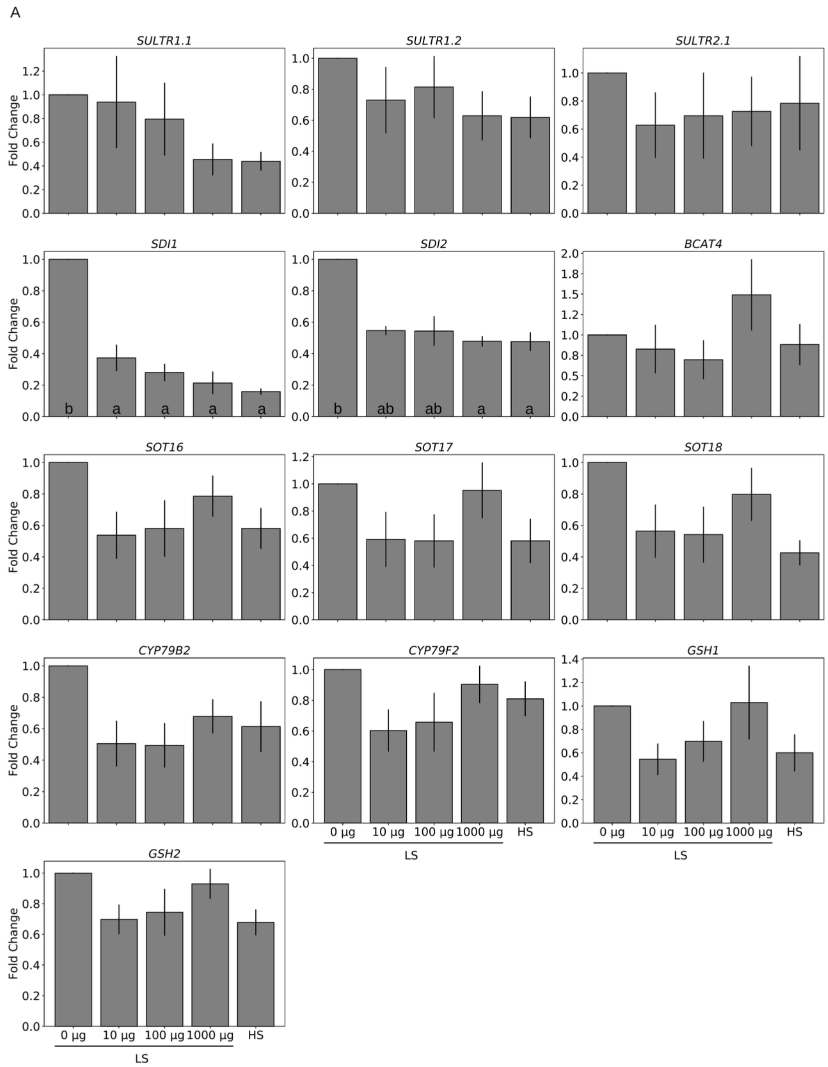



**Figure 4.** TMTS contributes positively to root growth. Wild-type (A) or *slim1* (B) seedling root growth on high sulfate medium (HS) or on low sulfate medium (LS) with addition of 0, 100 or 1000 µg TMTS was measured 7 days after application. Error bars represent SEs from at least 6 independent biological replicates for wildtype and 8 biological replicates for *slim1* . Statistical significance was determined by Duncan's multiple range test with p-vale $< 0.05$, and indicated with lower-case letters.


A
SULTR1.1
SULTR1.2
SULTR2.1
SDI1
SDI2
BCAT4
SOT16
SOT17
SOT18
CYP79B2
CYP79F2
GSH1
GSH2
Fold Change
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0 µg
10 µg
100 µg
1000 µg
HS
LS

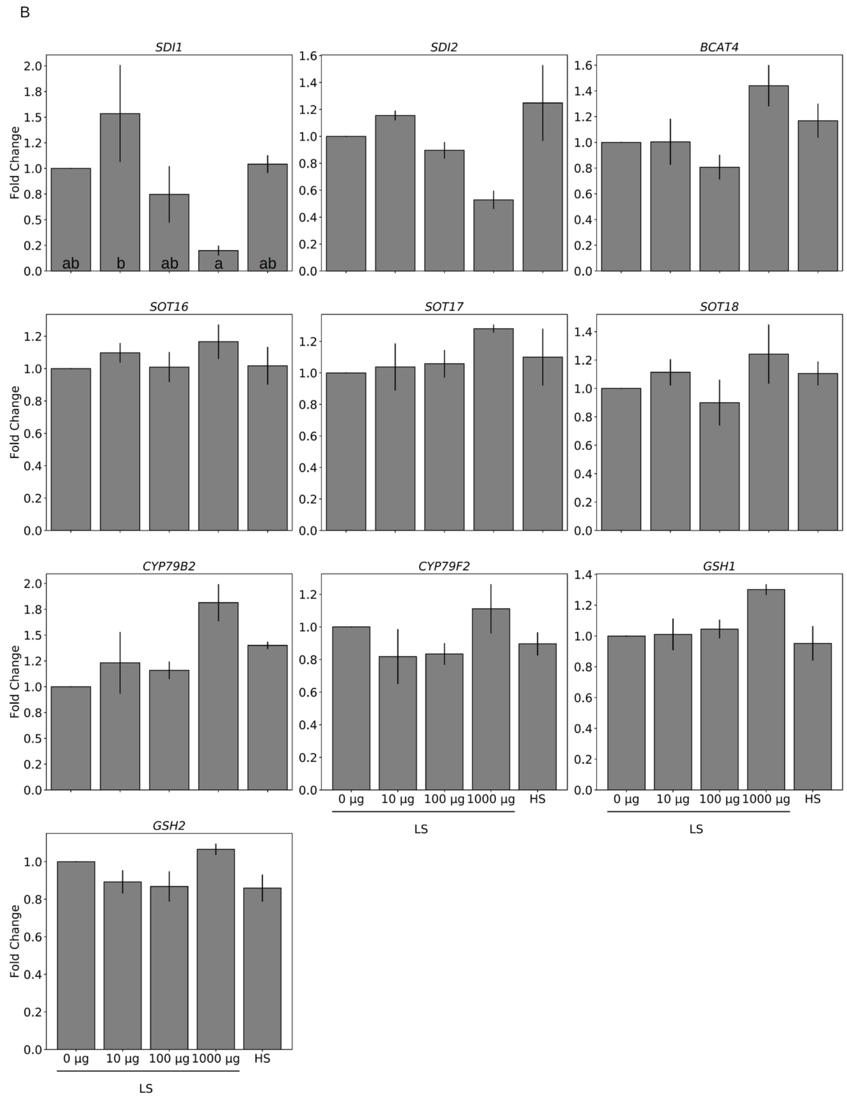


**Figure 5.** TMTS reduces plant response towards sulfur deficiency. Gene expression was analyzed after 2 days (A) and 7 days (B). Values were normalized to seedlings grown on low sulfate (LS) MGRL medium without TMTS (0 µg), and expressed as fold change. RNA for each treatment was extracted from total seedlings (combining root and shoot). Error bars represent SEs from 3 independent biological replicates, each with 8 seedlings. Statistical significance was calculated from dCq values, determined by Duncan's multiple range test with p-value $< 0.05$, and indicated with lower-case alphabets.

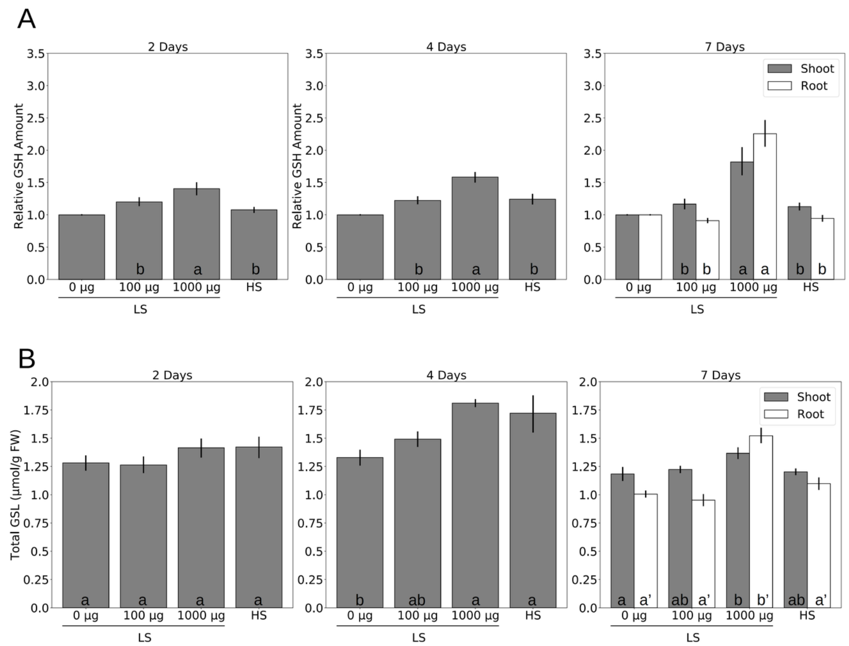


**Figure 6.** TMTS maintains sulfur-containing metabolites under sulfur deficiency. (A) Relative glutathione (GSH) level and (B) total glucosinolate (GSL) level in seedlings grown on low sulfate (LS) MGRL medium with addition of TMTS (0, 100 and 1000 µg) and seedlings grown on high sulfate (HS) MGRL medium 2, 4 and 7 days after treatment. Error bars represent SEs from at least 5 independent biological replicates, each with 8 seedlings. Statistical significance was determined by Duncan's multiple range test with p-value $< 0.05$, and indicated with lower-case alphabets.

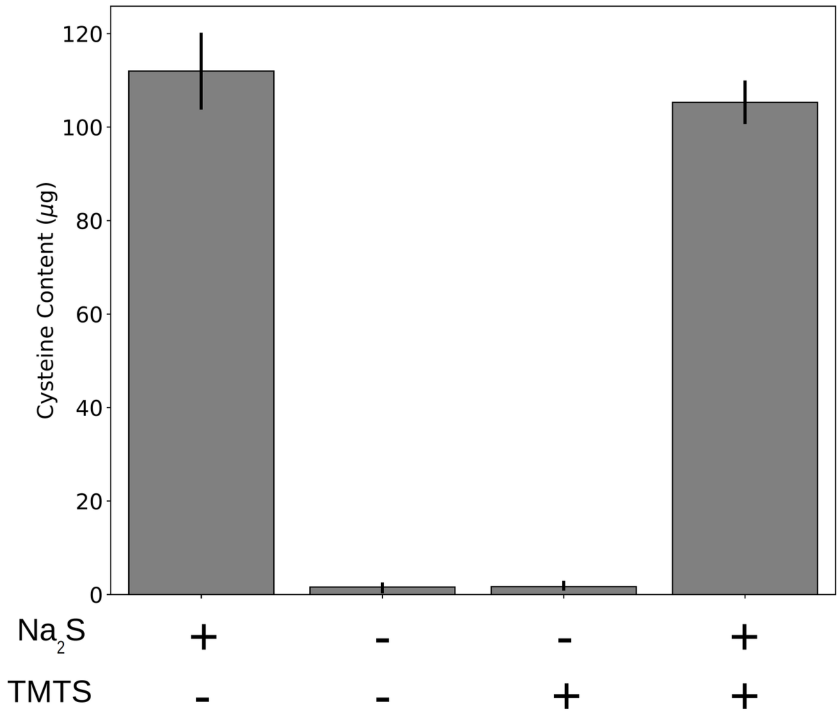


**Figure 7.** Incorporation of TMTS requires more than OASTLs. Cysteine biosynthesis was monitored in 4 parallel samples. In each sample, either $Na_2S$, water, TMTS or both $Na_2S$ and TMTS was added as substrate for OASTLs. Error bars represent SEs from 3 independent measurement using total protein extract from 3 independent biological replicates.