Roles of SULTR3s in sulfur and ABA metabolism

SULTR3s function in chloroplast sulfate uptake and affect ABA biosynthesis and the stress response

One sentence summary: The sultr3 quintuple mutant unequivocally demonstrates that sulfate transporter subfamily 3 is responsible for more than half of the chloroplast sulfate uptake and influences downstream sulfate assimilation and ABA biosynthesis as well as sulfate-induced stomatal closure.

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Abstract

Plants are major sulfur reducers in the global sulfur cycle. Sulfate, the major natural sulfur source in soil, is absorbed by plant roots and transported into plastids, where it is reduced and assimilated into cysteine for further metabolic processes. In spite of its importance, how sulfate is transported into plastids is poorly understood. We previously demonstrated using single Arabidopsis (Arabidopsis thaliana) genetic mutants that each member of the sulfate transporter (SULTR) subfamily 3 was able to transport sulfate across the chloroplast envelope membrane. To resolve the function of SULTR3s, we constructed a sultr3 quintuple mutant completely knocking out all five members of the subfamily. Here we report that all members of the SULTR3 subfamily show chloroplast membrane localization. Sulfate uptake by chloroplasts of the quintuple mutant is reduced by more than 50% compared with the wild type. Consequently, cysteine and ABA content are reduced to ~67 and ~20% of the wild-type level, respectively, and strong positive correlations are found among sulfate, cysteine, and ABA content. The sultr3 quintuple mutant shows obvious growth retardation with smaller rosettes and shorter roots. Seed germination of the sultr3 quintuple mutant is hypersensitive to exogenous ABA and salt stress, but is rescued by sulfide supplementation. Furthermore, sulfate-induced stomatal closure is abolished in the quintuple mutant, strongly suggesting that chloroplast sulfate is required for stomatal closure. Our genetic analyses unequivocally demonstrate that sulfate transporter subfamily 3 is responsible for more than half of the chloroplast sulfate uptake and influences downstream sulfate assimilation and ABA biosynthesis.

Key words: sulfate transporter, SULTR3, chloroplast, cysteine, ABA, Arabidopsis
**Introduction**

Sulfur is an essential macronutrient for plants as it participates in many biological processes, including the biosynthesis of cysteine and methionine, the resistance against diseases and pests, and the detoxification of reactive oxygen species (ROS), xenobiotics, and heavy metals (Leustek et al., 2000; Saito, 2000; Xiang et al., 2001; Takahashi et al., 2011; Alvarez et al., 2012). Sulfate is the main form of inorganic sulfur in the natural environment, and the oxidized sulfur in sulfate must be reduced and assimilated to cysteine before entering other metabolic processes (Leustek, 2002). Incorporation of sulfur into plant metabolism requires uptake from the soil and coordinated transport of sulfate through dedicated sulfate transporters (Takahashi et al., 2000; Yoshimoto et al., 2002). Then, sulfate is reduced to sulfide in plastids by APS reductase and sulfite reductase and finally fixed by O-acetylserine (thiol) lyase into cysteine (Khan et al., 2010; Hell and Wirtz, 2011).

Sulfate transporters are anion transporters dedicated to sulfate uptake and transport in plants. The typical sulfate transporters are pH-dependent proton/sulfate co-transporters with 10 – 12 membrane-spanning helices and a STAS domain (Smith et al., 1995; Takahashi et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002). The STAS domain is thought to be critical for the function of sulfate transport since MOT1 and MOT2, molybdate transporters previously named SULTR5;2 and SULTR5;1, lack the STAS domain (Shibagaki and Grossman, 2004; Tomatsu et al., 2007; Gasber et al., 2011). In *Arabidopsis thaliana* (hereafter Arabidopsis), 12 sulfate transporters were initially identified and subdivided into four groups based on their phylogenetic relationships and kinetic properties (Takahashi et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002). Two root-specific, high-affinity transporters of group 1, SULTR1;1 and SULTR1;2, are involved in sulfate uptake from soil (Yoshimoto et al., 2002). SULTR2;1 and SULTR2;2, two low-affinity transporters of group 2, transport sulfate to xylem for root-to-shoot transport (Takahashi et al., 2000), while SULTR1;3 mediates shoot-to-root sulfate translocation through phloem (Yoshimoto et al., 2003). SULTR4;1 and SULTR4;2 of group 4 are both tonoplast-localized transporters and...
coordinately facilitate the efflux of sulfate from the vacuole, the main site for cellular sulfate storage (Kataoka et al., 2004). However, the function of group 3 transporters remains fragmentary. SULTR3;5 was reported to reinforce SULTR2;1’s function in root-to-shoot sulfate translocation, with more sulfate accumulating in roots of the sultr3;5 mutant under low sulfur conditions (Kataoka et al., 2004). Other work also reported increased sulfate and decreased free cysteine content in Arabidopsis seeds of the single defective mutant of group 3 sulfate transporters, with total sulfur supply unaffected, indicating a decline in sulfur reduction and assimilation in these defective mutants (Zuber et al., 2010). Our previous work demonstrated that SULTR3;1 is chloroplast-localized and involved in sulfate uptake across the chloroplast envelope membrane (Cao et al., 2013). Single knockout mutants of group 3 sulfate transporters show decreased chloroplast sulfate uptake, indicating that these sulfate transporters may also be involved in chloroplast sulfate transport (Cao et al., 2013). Since chloroplasts are the main site for sulfate reduction in plants (Hell and Wirtz, 2011; Takahashi et al., 2011), cysteine levels also decreased in the sultr3;1 mutant due to a decline in sulfur assimilation (Cao et al., 2013).

Sulfate was reported to be a signal under drought stress that reinforces the effect of ABA in stomatal closure (Goodger et al., 2005; Ernst et al., 2010). Sulfate can keep the R-Type anion channel QUAC1 open, which regulates stomata movement (Meyer et al., 2010) and induces the expression of NCED3, a rate-limiting enzyme for ABA synthesis, in guard cell through unknown pathways (Malcheska et al., 2017). Besides sulfate, sulfide was also reported to act as a signaling molecule to induce stomatal closure (Lisjak et al., 2010; Jin et al., 2013; Honda et al., 2015). ABA biosynthesis is connected with the availability of cysteine because the activity of AAO3, a key enzyme in ABA biosynthesis, relies on cysteine as the sulfur donor for its Moco-factor sulfuration catalyzed by sulfurase ABA3 (Bittner et al., 2001; Xiong et al., 2001; Mendel and Hansch, 2002; Llamas et al., 2006). AAO3 activity is reduced in sultr3;1 and can be restored by exogenous application of cysteine (Cao et al., 2014). In addition, SULTR3;5 single mutants were reported to show reduced ABA levels under
normal and salt stress conditions and were hypersensitive to exogenous ABA and salt during the germination stage (Cao et al., 2014). Two other mutants in sulfur assimilation, *sir1-1* and *apr2*, also showed delayed germination under ABA and salt stress conditions (Cao et al., 2014).

In a companion study, we identified cysteine as a trigger of ABA biosynthesis in Arabidopsis, which explains the drought-sensitive phenotype of cysteine-synthesis depleted mutants and provides a mechanism for sulfate-induced stomatal closure (Batool et al., 2018). Here, we address the biological relevance of the remaining SULTR3 group members for sulfate allocation in plant cells and its potential impact for production of the phytohormone ABA. We demonstrate that the four remaining sulfate transporters of SULTR3 subfamily, SULTR3;2 (AT4G02700), SULTR3;3 (AT1G23090), SULTR3;4 (AT3G15990), and SULTR3;5 (AT5G19600), are localized in plastids. Thus, we generated double, triple, quadruple, and quintuple mutants of the SULTR3 subfamily and provided direct evidence for the redundant function of all SULTR3s in sulfate uptake into the chloroplast. All SULTR3s contributed almost equally to the sulfate uptake rate in isolated chloroplasts. The decreased sulfate allocation into chloroplasts significantly impacted steady state levels of cysteine and ABA in double, triple, quadruple, and quintuple mutant of SULTR3. The quintuple mutant displayed the lowest chloroplast sulfate uptake rate, and significantly reduced cysteine and ABA content, but was viable. These findings highlight the biological relevance of SULTR3s for subcellular partitioning of sulfate in plant cells and uncover the existence of a yet unidentified back-up system for sulfate uptake into plastids. The remarkably high correlation between the sulfate uptake rate into plastids and the syntheses of cysteine and ABA prompted us to test the germination rates of mutant seeds on high ABA and salt stress. As expected, the higher-order *sultr3* mutants displayed progressively increasing sensitivity to both stresses compared to the wild type, and the *sultr3* quintuple mutant was most strongly affected. Furthermore, the sensitive germination phenotype of the mutants can be rescued by feeding sulfide. As a result of its 50% decreased sulfate uptake rate, the *sultr3* quintuple mutants failed to close stomata upon sulfate administration. Our
findings demonstrate a crucial role of SULTR3s for dynamic transport of sulfate into the chloroplasts to promote stress-induced synthesis of cysteine, which in turn triggers biosynthesis of the phytohormone ABA to coordinate rapid adaptive responses such as stomatal closure.

Results

Subcellular localization of SULTR3 in plants

The initial characterization of group 3 SULTRs revealed that SULTR3;1 is localized in the chloroplast envelope and that loss-of-function mutants for most SULTR3 family-members suffer from decreased sulfate uptake into isolated chloroplast (Cao et al., 2013). To provide direct evidence for the action of the five SULTR3s in the chloroplast envelope, we fused the remaining SULTR3;2, SULTR3;3, SULTR3;4, and SULTR3;5 to the N-terminus of GFP (Supplemental Figure S1), and determined the subcellular localization of the fusion proteins in hypocotyls of T2 transgenic lines (Figure 1). The GFP-specific signal of all tested SULTR3-GFP fusion proteins co-localized with the autofluorescence of chlorophyll. We did not observe any significant signal at the plasma membrane or the tonoplast. These results suggested that SULTR3;2 to SULTR3;5 were also integrated in the chloroplast envelope as shown previously for SULTR3;1.

sultr3 quintuple mutant of subfamily 3 sulfate transporters

To address the biological-function of the SULTR3 subfamily, we aimed to construct double, triple, quadruple, and quintuple mutants of group 3 SULTRs by genetic crossing of established T-DNA insertion lines of SULTR3;1, SULTR3;2, SULTR3;3, SULTR3;4, and SULTR3;5 ordered from Arabidopsis Biological Resource Center (ABRC). We obtained four double, three triple, and two quadruple mutant lines as described in Supplemental Table S1. The T-DNA insertion in the 5′UTR region of SULTR3;4 in the sultr3;4 line did not entirely abolish expression of SULTR3;4. Thus, the steady state SULTR3;4 transcript level was decreased to 33% of wild type level in...
the first quadruple mutant (sultr3;2 sultr3;3 sultr3;4 sultr3;5). Since transcription of 
SULTR3;1, SULTR3;2, SULTR3;3, and SULTR3;5 in the second quadruple mutant 
(sultr3;1 sultr3;2 sultr3;3 sultr3;5) was absent (Figure 2a), we decided to eliminate 
SULTR3;4 activity by the CRISPR/Cas9 technology in this quadruple mutant. This 
strategy resulted in creation of two independent SULTR3 quintuple mutant lines 
lacking activities of all SULTR3 members. The identities of the SULTR3;1, SULTR3;2, 
SULTR3;3, and SULTR3;5 loci in the quintuple mutants were confirmed by PCR 
amplification of the T-DNA insertion sites in the respective loci from genomic DNA 
(Figure 2b). The CRISPR-Cas9-mediated genomic editing of the SULTR3;4 locus in 
both sultr3 quintuple lines, 1-2 and 16-29, was verified by sequencing of the genomic 
region (Figure 2c) and the transcribed mRNA (Figure 2d). As expected, a frameshift 
mutation was created by a thymidine (T) insertion in the sgRNA-targeted region, 
resulting in early termination at the 59th codon of SULTR3;4 mRNA translation 
(Figure 2e).

Knockout of sulfate transporter subfamily 3 results in growth retardation

While single knockout mutants of the SULTR3s grew like the wild type, as 
reported previously (Cao et al., 2013), the higher-order mutants of SULTR3 showed 
various degrees of growth retardation resulting in smaller rosettes and shorter roots 
when compared to wild type plants (Figure 3a and 3b). A progressive reduction in 
fresh weight, rosette diameter, and primary root length from double mutants to the 
SULTR3 quintuple mutant was observed. Comparison of the various higher-order 
SULTR3 mutants did not reveal a dominant isoform of SULTR3 responsible for the 
observed growth retardation. As expected, the SULTR3 quintuple mutant suffered the 
most severe growth retardation (Figure 3c to 3e). However, organ development in 
the vegetative and generative phases of growth was normal in all SULTR3 mutants 
when compared to wild type, explaining the fertility of the SULTR3 quintuple mutant. 
These results showed that the depletion of SULTR3 activity located at the chloroplast 
envelope strongly affects plant growth. Surprisingly, loss of all SULTR3 isoforms did
not cause lethality, suggesting that sulfate import into the chloroplast was not entirely abolished in the SULTR3 quintuple mutant or that sulfate is reduced and incorporated into cysteine in another subcellular compartment.

**Group 3 sulfate transporters show redundancy in chloroplast sulfate uptake, cysteine level, and ABA content**

To address the contribution of group 3 sulfate transporters in transporting sulfate into chloroplast, we determined incorporation of radioactive $^{35}$S-labeled sulfate into freshly isolated chloroplasts from the five higher-order SULTR3 loss-of-function mutants and the wild type. The Hill reaction was applied to ensure that the isolated chloroplasts were intact and photosynthetically active (Supplemental Figure S2). The number of chloroplasts was adjusted to the same concentration for each line before the $^{35}$S-uptake assay. The *in organello* sulfate uptake was assayed at three exogenous sulfate concentrations: 21.25, 42.5, and 170 µM. Under all tested external sulfate regimes, the sulfate uptake rates of the isolated chloroplasts from single, double, triple, quadruple, and quintuple mutants were decreased when compared to the wild type control (Col-0). $^{35}$S-sulfate uptake rates were negatively correlated with the number of disrupted SULTR3 genes, with a progressive reduction from the single mutant to the sultr3 quintuple mutant, the latter displaying the lowest sulfate uptake rate (Figure 4a). We also measured the sulfate levels in isolated chloroplasts (Supplemental Figure S3a). Despite the technical difficulty of accurately measuring chloroplast sulfate levels, a negative correlation of sulfate level in chloroplasts was observed with the number of disrupted SULTR3 genes. These results demonstrated the functional redundancy of group 3 sulfate transporters in chloroplast sulfate uptake, indicating that each of the SULTR3 members contributes to sulfate transport across the chloroplast envelop membrane. This finding directly linked the decreased sulfate uptake rate into chloroplasts with the observed growth retardation of the allelic series of SULTR3 mutants. Under 170 µM, sulfate uptake of chloroplasts from the sultr3 quintuple mutant decreased to less than half (45%) of the wild type level, which indicates the...
existence of another yet unidentified sulfate transporter system in chloroplasts that 
safeguards sulfate import in the absence of \textit{SULTR3}.

Since plastidic sulfate reduction provides the precursor for cysteine biosynthesis in other subcellular compartments, we determined the foliar cysteine concentration in the \textit{SULTR3} double, triple, quadruple, and quintuple mutants and found it decreased to 90%, 84%, 70%, and 65% of that of wild type plant, respectively (Figure 4b). These results strongly support the hypothesis that growth retardation in the higher-order \textit{SULTR3} mutants was a direct consequence of depleted sulfur fixation into cysteine, due to decreased sulfate reduction capability in plastids. In agreement, we found a very stringent correlation of sulfate import rate into chloroplasts with the growth retardation of plants ($R^2 = 0.9049$, Figure 4d) and the foliar cysteine steady state level ($R^2 = 0.9149$, Figure 4e). To validate sulfate assimilation into cysteine, we measured the more robust metabolite glutathione (Supplemental Figure S3b). Similar to cysteine levels, GSH levels were negatively correlated with the number of disrupted \textit{SULTR3} genes. The quintuple mutant had the lowest GSH level, as expected.

Our previous work showed that steady state ABA levels in \textit{SULTR3} single mutants decreased (Cao et al., 2014), which could be explained by the stimulating effect of cysteine on ABA biosynthesis. In support of this hypothesis, the ABA content of the sultr3 double, triple, quadruple, and quintuple mutants decreased to 68%, 50%, 36%, and 20% of that of wild type plants, respectively (Figure 4c). Remarkably, the foliar steady state ABA level correlated well with the sulfate uptake rate into chloroplast ($R^2 = 0.9862$, Figure 4f) and the cysteine supply of the cells ($R^2 = 0.9432$, Figure 4g).

**Loss of SULTR3 transporters alters responses to ABA and salt stress during seed germination**

It is known that loss of single SULTR3 transporters can alter seed germination response to exogenous ABA and salt conditions. We assayed seed germination of the multi-gene defective mutants under ABA and salt conditions (Figure 5a). Again, we observed a progressive reduction in germination rate from single to SULTR3.
quintuple mutants, with the latter again having the lowest rate under both ABA and NaCl treatment (Figure 5b-d and 5e-g). Five days after sowing, seed germination rate of the SULTR3 quintuple mutant was only 10% under 0.3 µM ABA, whereas more than 60% of wild type seeds germinated. Eight days after sowing, only 20% of the SULTR3 quintuple mutant seeds germinated, while more than 90% of wild type seeds germinated (Figure 5c). Seed germination under salt stress showed similar patterns. Compared with 0.3 µM ABA, seed germination of all sultr3 mutants was more sensitive to 180 mM NaCl than that of the wild type. The germination rate of sultr3;1 knockout mutant seed was only 33% of that of the wild type, and no radical emergence of SULTR3 quintuple mutant seeds was found even five days after sowing (Figure 5d). Eight days after sowing, only 5% of SULTR3 quintuple mutant seeds germinated, whereas more than 75% of the wild type seeds germinated. Consistent with alterations in cysteine and ABA content, seed germination inhibition upon treatment with exogenous ABA and salt was directly proportional to the number of mutated SULTR3 genes, demonstrating the correlation of cysteine and ABA content with the plant abiotic stress response during germination stages.

Seed germination of SULTR3 quadruple and quintuple mutants was also delayed under normal conditions compared with that of the wild type. For triple mutants, sultr3;1 sultr3;2 sultr3;3, but not sultr3;2 sultr3;3 sultr3;4 or sultr3;3 sultr3;4 sultr3;5, showed a germination delay on day three after sowing (Figure 5b), which indicates that SULTR3;1 may have a more critical role than other SULTR3 genes in the early germination stage.

If disruption of SULTR3 genes caused the ABA- and salt-sensitive germination phenotypes of the mutants, one would predict that the observed defects could be partially rescued by feeding sulfide. Indeed, 1 mM NaHS could rescue the ABA- and salt-sensitive germination phenotypes of the mutants (Supplemental Figure S4), which further confirms the role of SULTR3 transporters in the seed germination response to ABA and salt stress.

**SULTR3 transporters are essential for sulfate-induced stomatal closure**

In our companion manuscript, we demonstrated that sulfate triggers ABA...
production after incorporation into cysteine (Batool et al., 2018). These findings prompted us to test if the sultr3 quintuple mutant is defective in dynamic induction of stomatal closure upon short-term sulfate application. The wild type rapidly closed stomata upon addition of sulfate (15 mM MgSO₄, P<0.001), cysteine (0.5 mM, P<0.001), or ABA (50 µM, P<0.001). As expected, addition of glycine (0.5 mM) as a control had only marginal influence on stomatal aperture of the wild type or the sultr3 quintuple mutant. In contrast to the wild type, application of sulfate to the sultr3 quintuple mutant failed to induce stomatal closure, strongly suggesting that the sultr3 mutant was unable to incorporate the sulfate into cysteine for promotion of ABA biosynthesis (Figure 6). In order to rule out a potential impact of sultr3 quintuple mutations on ABA sensing or promotion of ABA biosynthesis by cysteine, we also applied cysteine and ABA to the sultr3 quintuple mutant. ABA and cysteine were able to induce stomatal closure proving that ABA sensing and promotion of ABA biosynthesis by cysteine was functional in the sultr3 quintuple mutant. Taken together these findings demonstrate that loss of all SULTR3 isoforms strongly impairs dynamic and efficient transport of sulfate into the chloroplast for stress-induced cysteine biosynthesis.

Discussion

Sulfate transporters of group 3 control sulfate import into chloroplasts

Plants rely on a series of pH-dependent proton/sulfate co-transporters, simply referred to as sulfate transporters, for transport of the charged oxyanion sulfate across plasma membranes. In Arabidopsis, 12 known sulfate transporters are sub-divided into four groups based on their phylogenetic relationships. The biological function of the five plasma membrane-localized transporters of group 1 and 2 in the uptake and partitioning of sulfate at the whole plant level is well established, while tonoplast-embedded SULTR4;1 and SULTR4;2 facilitate intracellular remobilization of vacuolar stored sulfate (reviewed in Takahashi et al., 2011).

In contrast, the role of group 3 SULTRs is poorly understood. Our previous work...
demonstrated that SULTR3;1 localized to the chloroplast envelop membrane and was involved in sulfate influx into chloroplasts. Here, we demonstrate that the remaining four SULTR3 isoforms also localize to plastids (Figure 1). This localization provides a molecular explanation for the decreased sulfate uptake rate of chloroplasts isolated from single loss-of-function mutants for the other four SULTR3 subfamily members (Cao et al., 2013). We applied CRISPR-Cas9-based gene editing and genetic crossing of established SULTR3 loss-of-function mutants to successively eliminate all group 3 SULTRs to explore the relevance of each isoform for sulfate import into chloroplasts during vegetative growth under non-stressed conditions. The sulfate influx rate continually decreased upon elimination of additional SULTR3 isoforms, revealing the functional redundancy of SULTR3 proteins (Figure 3). These results strengthen the conclusion that not only SULTR3;1, but all group 3 SULTRs, contribute to sulfate transport across the chloroplast membrane. Remarkably, the comparatively large number of group 3 SULTRs found in Arabidopsis is conserved in the reference grass, Brachypodium distachyon (SULTR3;1 to SULTR3;5), while redundant SULTRs in group 2 and group 4 are absent in this species (Tombuloglu et al., 2017). In Medicago truncalata, the number of group 3 sulfate transporter is even higher (Gallardo et al., 2014). Gene duplication often allows specification of function, e.g., via modulation of transcriptional responses. Indeed, SULTR3 transporters respond differentially to diverse stresses (Gallardo et al., 2014). Notably, the transcriptional regulation of SULTR1 subfamily is the hallmark of the sulfate-deficiency response and the key to surviving amidst a limited sulfur supply, strongly suggesting that stress-induced transcriptional regulation of SULTRs is biologically relevant and controlled by highly specific signal transduction pathways (Maruyama-Nakashita et al., 2004; Rouached et al., 2008). Spatial separation of SULTR3 transcription has been reported in vegetative and generative organs and will add to the specification of SULTR3 transporter activity in vascular plants (Kataoka et al., 2004; Zuber et al., 2010).

Plastids, especially chloroplasts, are the exclusive site for de novo reduction of APS-bound sulfur to sulfide in plants. Thus, transport of sulfate into chloroplasts is essential for plants. Chloroplasts isolated from the SULTR3 quintuple mutant
displayed only 45% of wild-type sulfate uptake, revealing that SULTR3 contributed significantly to chloroplast sulfate influx. Impaired plastid sulfate uptake capacity caused a significant decrease in plant growth (Figure 4). However, the viability of the sultr3 quintuple mutant provides evidence for the existence of a yet unidentified backup system for chloroplast sulfate influx, which safeguards sulfate incorporation into plastids in the absence of SULTR3s. The contribution of this system to sulfate import into plastids in the presence of the five SULTR3s remains elusive since it fails to facilitate fast transport of sulfate required for sulfate-induced stomatal closure in the sultr3 quintuple mutant (Figure 6). Candidates for this backup system are plastid localized ABC-type transporters or the triose-phosphate/phosphate translocator. Evidence for the involvement of chloroplast-localized ABC-type transporter complexes evolved from the analysis of sulfate uptake into chloroplasts from green algae (Chen et al., 2003; Chen and Melis, 2004; Chen et al., 2005). However, no homologs of these ABC type transporters were identified in vascular plants (Buchner et al., 2004).

Exchange of sulfate specifically with triose-phosphate and not glucose-6-phosphate was shown previously in isolated plastids from wild-type Spinacia oleracea (Hampp and Ziegler, 1977). Furthermore, isolated spinach chloroplasts are capable of exchanging sulfate with phosphate. This strict counter-exchange followed a saturation kinetic with a $K_m$ of 2.5 mM and a rate of 25 $\mu$mol sulfate mg$^{-1}$ chlorophyll h$^{-1}$ and external phosphate acted as a competitive inhibitor ($K_I$: 0.7 mM) of sulfate import by chloroplasts (Mourioux and Douce, 1979). Both results suggest that the triose-phosphate/phosphate translocator (TPT) is involved in the loading of sulfate into plastids. In Arabidopsis, the tpt-1 mutant, which is defective in the chloroplast triose phosphate/phosphate translocator (AT5g46110), displays less than 5% of wild-type triose phosphate-specific transport but shows no apparent growth retardation under non-stressed conditions (Schneider et al., 2002). Together with our findings, these independent studies point towards a significant contribution of plastid membrane-localized TPT1 for sulfate import into plastids. The established sultr3 quintuple mutant will open new avenues to address...
the function of the potential plastidic sulfate-import systems in planta mentioned above.

Uptake of sulfate into chloroplasts impacts cysteine and ABA biosynthesis

Restriction of sulfate reduction in plastids strongly limits cysteine synthesis and downstream processes like glutathione biosynthesis and translation (Khan et al., 2010; Dong et al., 2017; Speiser et al., 2018). On the one hand, sulfate reduction is controlled by regulation of APR and APS kinase to effectively control the flux of sulfur into primary sulfur metabolism or secondary sulfur metabolism (Loudet et al., 2007; Mugford et al., 2009; Ravilious et al., 2012). On the other hand, it might be restricted by sulfate transport from the cytosol into chloroplasts. The strict correlation between the steady-state cysteine level and the sulfate-uptake capacity into chloroplasts (Figure 3) strongly suggests that such limitation is biologically relevant and underpins the importance of tight control of sulfate transport over the chloroplast membrane via SULTRs of group 3.

Very recently, cysteine biosynthesis has been established as a relevant trigger for abscisic acid (ABA) production and stomatal closure (Batool et al., 2018). The decrease of ABA levels in all sultr3 mutants suggests that sulfate uptake into chloroplasts does also limit ABA production via modulation of cysteine supply. As a consequence of perturbed ABA biosynthesis, germination of the higher-order sultr3 mutants (double, triple, quadruple, and quintuple) is strongly impaired amidst ABA and high salt stress (Figure 5). In concordance with the hypothesis that sulfate reduction perturbs ABA biosynthesis during germination, the sir1-1 and apr2 mutants are also hypersensitive to ABA and high salt stress (Cao et al., 2014).

Drought stress strongly impairs sulfur metabolism in an organ-specific manner and is highly connected at multiple levels with sulfate transport, sulfate assimilation, and sulfate use in sulfation reactions (Estavillo et al., 2011; Chan et al., 2013; Ahmad et al., 2016). Remarkably, sulfate has been identified as an early accumulating signal in the xylem sap of drought-stressed maize, common hop, and poplar (Ernst et al., 2010; Korovetska et al., 2014; Malcheska et al., 2017). Since petiole feeding of sulfate is sufficient to close stomata in poplar and Arabidopsis, when sulfate reduction and...
cysteine biosynthesis are functional, we tested if active sulfate transport into the plastids is mandatory for sulfate-induced stomatal closure. Indeed, loss-of all SULTR3 isoforms in the SULTR3 quintuple mutant abolishes sulfate-induced stomatal closure (Figure 6). These results demonstrate that active transport of sulfate into the plastids by SULTR3-type transporters is a prerequisite for sulfate-induced ABA biosynthesis. Indeed transcription of SULTR3;1, SULTR3;2, SULTR3;3, and SULTR3;5 is strongly enriched in guard cells (Bauer et al., 2013), supporting the hypothesis that active sulfate metabolism in the guard cells contributes to ABA production in response to petiole-fed sulfate or drought-induced root-to-shoot transport in the xylem (Ernst et al., 2010; Korovetska et al., 2014; Malcheska et al., 2017). Indeed drought stress and high salt stress differentially affect transcription of SULTR3 members in leaves (Gallardo et al., 2014), supporting a role of the SULTR3s during abiotic stresses that affects water management of the plant.

In conclusion, our findings in this study using the multi-gene defective mutants of SULTR3 subfamily members unambiguously show their functional redundancy in chloroplast sulfate uptake and consequent influence on cysteine, glutathione, and ABA biosynthesis, which upon mutation result in growth retardation and altered stress responses. We also demonstrate a critical role of SULTR3s for dynamic transport of sulfate into the chloroplasts to promote stress-induced synthesis of cysteine, which in turn triggers biosynthesis of the phytohormone ABA to regulate stomatal closure. The highly positive correlations between chloroplast sulfate availability and cysteine and ABA levels implies that they are highly coordinated and tightly regulated.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (ecotype Columbia, Col-0, and mutants with Col-0 background) was grown on half-strength MS solid medium (Sigma) that contained 1%
(w/v) sucrose at 22°C under 16-h-light/8-h-dark cycles. The plants for isolation of chloroplasts and protoplasts were grown on soil for 4 weeks under the same light regime.

Identification of the multi-gene knockout mutants

The single knockout mutants *sultr3;1, sultr3;2, sultr3;3, sultr3;4*, and *sultr3;5* were T-DNA insertion lines (SALK_023190, SALK_023980, SALK_000822C, CS859766, and SALK_127024, respectively) ordered from the Arabidopsis Biological Resource Center (ABRC). The single mutants were crossed to obtain the double, triple, and quadruple mutants. Quintuple mutants were created by knocking out *SULTR3;4* expression in *sultr3;1 sultr3;2 sultr3;3 sultr3;5* quadruple mutant using the *pYAO*-based CRISPR/Cas9 system (Yan et al., 2015). The *SULTR3;4* genomic region targeted by sgRNA was TCCCTGATGATCCGTTACAG with AGG as the PAM sequence, following the recommendation from MIT online software (http://crispr.mit.edu).

Double-stranded DNA of the targeted region was created by annealing sgRNA-P1 and sgRNA-P2, and introduced into the *pAtU6-26-sgRNA-SK* cassette before fusion into the pCAMBIA1300::*pYAO-Cas9* plasmid.

The homozygote of each *SULTR3* gene was identified by polymerase chain reaction (PCR) using a common primer LBb1.3 and gene-specific primer pairs 3;1-F/R for *SULTR3;1*, 3;2-F/R for *SULTR3;2*, 3;3-F/R for *SULTR3;3*, 3;4-F/R for *SULTR3;4*, and 3;5-F/R for *SULTR3;5*, respectively. Homozygote lines were further confirmed by RT-qPCR, with primer pairs 3;1-qF/qR for *SULTR3;1*, 3;2-qF/qR for *SULTR3;2*, 3;3-qF/qR for *SULTR3;3*, 3;4-qF/qR for *SULTR3;4*, and 3;5-qF/qR for *SULTR3;5*, respectively. Two homozygotes of *SULTR3;4* in the quintuple mutants were screened and confirmed by sequencing using primer pairs seq-LP1/RP1 for genome and seq-LP2/RP2 for cDNA amplification. All primer sequences are listed in Supplemental Table S2.

Localization of *SULTR3;1-GFP* fusion protein

The full-length coding regions of *SULTR3;2 to SULTR3;5* were amplified by PCR...
and fused into GFP fusion binary vector pGWB5 (Invitrogen). The SULTR3-GFP construct was transferred into the wild type and the T2 generation was used for fluorescence imaging. Fluorescence in hypocotyl of SULTR3-GFP transgenic plants was observed using a confocal microscope (Carl Zeiss LSM510) under 488 nm excitation. The emission wavelength was restricted to 530 nm for green fluorescence and 650 nm for red autofluorescence of chlorophyll.

**Reverse transcription quantitative PCR (RT-qPCR)**

Total RNA was extracted from primary root material of 4-day-old wild type seedlings using TRIzol reagent (Invitrogen) and reverse transcribed with the Takara RT kit (Invitrogen) in accordance with the manufacturer’s instructions. All RT-qPCR assays were performed using a SYBR kit (Transgene) in a One Step real-time PCR system (Applied Biosystem) as described in the manufacturer’s protocol. Each assay consisted of three biological replicates and was performed twice. UBQ5 was used as the control in RT-qPCR assays with primer pairs UBQ5-qF/qR (Supplemental Table S2).

**Isolation of intact chloroplasts**

Plants were grown under day-neutral conditions at 22°C and sampled in the early morning to avoid starch accumulation in chloroplasts. Crude chloroplasts were obtained from the rosette leaves of 4-week-old plants using isolation buffer (pH 8.0) and purified using Percoll gradients and activated with high light as described (Kunst, 1998). Hill reaction was carried out to determine integrity and photosynthetic activity of chloroplasts using DCIP dye as described (Bregman, 1990).

**Sulfate uptake by purified chloroplasts and measurement of chloroplast sulfate levels**

Chloroplast sulfate uptake assay was carried out as described previously (Cao et al., 2013). Three exogenous sulfate concentrations (21.25, 42.5 and 170 μM) were used in the uptake assay. The reaction buffer (0.3 M sorbitol, 50 mM HEPES, 10 mM...
NaCl, 2 mM MgCl$_2$, 2 mM EDTA, 0.5 mM KH$_2$PO$_4$, pH 7.0) containing 42.5, 85 or 340 μM Na$_2$$^{35}$SO$_4$ and 1 mM ATP was mixed with same volume of chloroplast suspension to initiate the uptake assay.

To measure chloroplast sulfate levels, chloroplasts were purified as described above and dried in a 70°C oven, then ground to powder. Sulfate concentrations were measured using a turbidimetry method described previously (Tabatabai, 1974). Briefly, the samples were boiled in hydrochloric acid and filtered. The filtrate was reacted with barium chloride as a turbidifier. The absorbance was measured using a spectrophotometer at 410 nm. A series of potassium sulfate dilutions were used to generate the standard curve. The sulfate content in each sample was determined using the standard curve.

**Determination of ABA, Cysteine, and GSH content**

Wild type and SULTR3 knockout mutants were germinated and grown on half-strength MS medium for eight days before being sampled. ABA was extracted and quantified by enzyme linked immunosorbent assay (ELISA; Sigma) as described previously (Yang et al., 2001). Cysteine and GSH were quantified as described elsewhere (Xiang and Oliver, 1998).

**Germination response to exogenous ABA and salt stress and rescue of the mutant phenotypes with sulfide**

Sterilized seeds were vernalized for three days and then plated on half-strength MS medium that contained 1% sucrose and 0 or 3 μM ABA or 180 mM NaCl in the presence or absence of 1 mM NaHS. Three replicate plates (45 seeds per plate) were used for each wild type and mutant line. The plates were kept at 22°C under long-day conditions. Seed germination was evaluated from day to day and seeds were considered germinated when the radicles broke the seed coat.

**Stomatal aperture bioassay**

The assay was conducted as recently described (Batool et al., 2018). Epidermal
strips were peeled off the abaxial side of wild-type and quintuple mutant leaves and floated on distilled water for two hours under constant light. The strips were then transferred to distilled water (pH 5.5) supplemented without (control) or with effectors (15 mM MgSO$_4$, 0.5 mM Cys, 0.5 mM Gly, and 50 μM ABA) for three hours. Stomata were imaged before and after treatment with a conventional wide-angle microscope (Leica DMIRB). Each experiment was performed at least in triplicate and showed the same results.

**Statistical Analysis**

Statistically significant differences were determined with one-way repeated measure ANOVA. Lower case alphabetical letters indicate significant difference (P< 0.05) between two neighboring letters.

**Accession numbers**

SULTR3;1 (AT3G51895), SULTR3;2 (AT4G02700), SULTR3;3 (AT1G23090), SULTR3;4 (AT3G15990), and SULTR3;5 (AT5G19600).

**Supplemental Data**

The following supplemental materials are available.

- **Supplemental Figure S1.** Schematic of pGWB5::SULTR3-GFP fusion construct.
- **Supplemental Figure S2.** Activity determination of isolated chloroplasts by Hill Reaction.
- **Supplemental Figure S3.** Sulfate and GSH levels.
- **Supplemental Figure S4.** Rescuing mutant phenotype by sulfide.
- **Supplemental Table S1.** Mutants constructed and used in the experiments.
- **Supplemental Table S2.** Primer pairs used in the experiments.

**Acknowledgement**

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**Figure legends**

**Figure 1.** Group 3 sulfate transporters are all localized on plastid envelope.

Confocal images of hypocotyl cells of SULTR3-GFP transgenic plants. The panels from left to right represent GFP fluorescence of the SULTR3-GFP fusion protein, bright field, autofluorescence of chlorophyll, and the merged images, respectively. Scale bars represent 100 μm.

**Figure 2.** Creation and identification of quintuple mutant of group 3 sulfate transporters.

(a) Relative expression level of SULTR3;1, SULTR3;2, SULTR3;3, SULTR3;4, and SULTR3;5 in Col-0 wild type, sultr3;1 sultr3;2 sultr3;3 sultr3;5 and sultr3;2 sultr3;3 sultr3;4 sultr3;5 T-DNA insertion quadruple mutants. The expression of SULTR3;4 in sultr3;2 sultr3;3 sultr3;4 sultr3;5 quadruple mutant was knockdown rather than knockout as for the other SULTR3 genes.

(b) T-DNA insertion identification of SULTR3;1, SULTR3;2, SULTR3;3, and SULTR3;5 in two quintuple mutant lines 1-2 and 26-19. The results confirm that 1-2 and 26-19 are homozygote lines of SULTR3;1, SULTR3;2, SULTR3;3, and SULTR3;5.

(c-d) Sequencing results of genomic DNA (c) and cDNA (d) of SULTR3;4 in two quintuple-mutant lines 1-2 and 26-19. Both results demonstrate that 1-2 and 26-19 are homozygote lines with a single thymidine (T) nucleotide insertion in the SULTR3;4 coding region.

(e) Schematic illustration of early termination of a 58 amino acid SULTR3;4 translation fragment in two quintuple mutant lines 1-2 and 26-19 due to the frameshift mutation by a T nucleotide insertion in the sgRNA targeted region.

**Figure 3.** Multi-gene defective mutants of SULTR3 show various degrees of growth retardation.

(a-b) Phenotype of aerial part (a) and whole plant (b) of SULTR3 knockout mutants.
and wild type plants (Col-0). Plants were photographed 29 days post germination.

Scale bars represent 1 cm.

c) Time-course change of rosette diameters. Values are means ± SD (n = 20 plants for each line).

d) Fresh weight of rosette leaves. Values are means ± SD (n = 20 plants for each line).

Letters indicate statistically significant differences between groups determined with one-way repeated measure ANOVA (P<0.001).

e) Length of primary roots. Values are means ± SD (n = 20 plants for each line).

Letters indicate statistically significant differences between groups determined with one-way repeated measure ANOVA (P<0.001).

Figure 4. Loss of SULTR3 genes decreases sulfate uptake rate of chloroplasts, cysteine levels, and ABA content.

(a) Sulfate uptake by isolated chloroplasts of the SULTR3 knockout mutants and wild type plants (Col-0) under three different sulfate conditions. Values are means ± SD (n = three experiments for each line). Letters indicate statistically significant differences between groups determined with one-way repeated measure ANOVA (P<0.001).

(b) Cysteine content in SULTR3 knockout mutants and wild type plants (Col-0) under normal conditions. Values are means ± SD (n = three experiments for each line). Letters indicate statistically significant differences between groups determined with one-way repeated measure ANOVA (P<0.001).

c) ABA content in eight-day-old SULTR3 knockout mutants and wild type plants (Col-0). Values are means ± SD (n = three experiments for each line). Letters indicate statistically significant differences between groups determined with one-way repeated measure ANOVA (P<0.001).

(d) Correlation between chloroplast sulfate uptake and growth. A simple regression was made with the chloroplast sulfate uptake (as a percentage of the wild type) at 170 µM in (a) and rosette fresh weight (as a percentage of the wild type) in Figure 3d.

(e) Correlation between chloroplast sulfate uptake and cysteine content. A simple regression was made with the chloroplast sulfate uptake (as a percentage of the wild type) at 170 µM in (a) and cysteine content (as a percentage of the wild type) in Figure 3d.
Figure 5. Loss of SULTR3 genes alters seed germination response to ABA and salt stress.

(a) SULTR3 knockout mutants are hypersensitive to exogenous ABA and salt stress during germination. Plants were photographed eight days post germination. Scale bar represents 1 cm.

(b-g) Germination rates of SULTR3 knockout mutants and wild type seeds (Col-0) under normal, ABA, and salt conditions in (a). Seeds were evaluated at five (5D) and eight (8D) days after sowing and were considered germinated when the radicles penetrated the seed coats. Values are means \( \pm \) SD (n = three experiments with 45 seeds for each line). Letters indicate statistically significant differences between groups determined with one-way repeated measure ANOVA (\( P < 0.001 \)).

Figure 6. Sulfate-induced stomatal closure requires sulfate import into chloroplast mediated by SULTR3.

(a) Stomatal images for each treatment. Impact of sulfate (white, 15 mM MgSO\(_4\)), cysteine (yellow, 500 \( \mu \)M), glycine (green, 500 \( \mu \)M), and ABA (grey, 50 \( \mu \)M) on stomatal aperture of the wild type and the SULTR3 quintuple mutant suffering from decreased sulfate uptake into chloroplasts. Control refers to water adjusted to pH 5.5 (black). Epidermal peels were obtained from five-week-old wild type and SULTR3 quintuple plants grown on soil under short-day conditions and treated for 180 min with the effectors dissolved in water at pH 5.5. Scale bar represents 10 \( \mu \)m.

(b) Stomatal aperture measured for the treatments in (a). Values are means \( \pm \) SE
(n ≥ 60 stomata, derived from ≥ 6 individual plants). Letters indicate statistically significant differences between groups determined with the one-way repeated measure ANOVA (P<0.001).

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Figures and Tables:

**a** Relative expression levels of SULTR3 genes in Col-0 and sultr3 mutants.

**b** Gel electrophoresis images of SULTR3 genes for Col-0 and mutants.

**c** Sequencing analysis showing sultr3 quintuple mutants 1-2 and 26-19.

**d** Expanded sequencing view of SgRNA target region for 1-2 and 26-19 cDNAs.

**e** Sequence alignments of Col-0, 1-2, and 26-19 showing the SgRNA target region and stop codon.
Wild-type Stomatal aperture (µm)

sultr3 quintuple

Control
Sulfate
Cysteine
Glycine
ABA

Wild type sultr3

Stomatal aperture (µm)

Wild-type sultr3 quintuple

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