The transcriptional regulators of arsenic-induced gene expression remain largely unknown. Sulfur assimilation is tightly linked with arsenic detoxification. Here, we report that mutant alleles in the SLIM1 transcription factor are substantially more sensitive to arsenic than cadmium. Arsenic treatment caused high levels of oxidative stress in the \textit{slim1} mutants, and \textit{slim1} alleles were impaired in both thiol accumulation and sulfate accumulation. We further found enhanced arsenic accumulation in roots of \textit{slim1} mutants. Transcriptome analyses indicate an important role for SLIM1 in arsenic-induced tolerance mechanisms. The present study identifies the SLIM1 transcription factor as an essential component in arsenic tolerance and arsenic-induced gene expression. Our results suggest that the severe arsenic sensitivity of the \textit{slim1} mutants is caused by altered redox status.

**Keywords:** \textit{Arabidopsis thaliana}; arsenic; sulfur limitation; transcription factor

Many advanced technologies used by modern society rely on heavy metals and arsenic. These elements are toxic and pose a significant risk to the environment and human health if consumed. However, unlike animals, plants are often partially tolerant to heavy metals and arsenic and can accumulate large amounts in diverse tissues [1]. Arsenic is a highly toxic substance commonly found in anthropogenic wastes (electronics and fertilizers) and can also be found at high levels in certain rocks, soils, and waters globally [2–5]. While this toxic metalloid has no recognized role in plant or animal nutrition, plant-based products are the main entry point for arsenic into the food chain [6]. Thus, understanding the molecular mechanisms underlying plant uptake, transport, detoxification, and accumulation of arsenic is vital for enhancing the nutritional value and safety of our food.

We previously described the development of a plant genetic reporter line that fused the promoter of a cadmium and arsenic-inducible high-affinity sulfate transporter to firefly luciferase (\textit{pSULTR1;2::LUC}) to identify mutants in signaling [7]. A major goal of this

**Abbreviations**

GS, glutathione synthetase; POD, peroxidase; ROS, reactive oxygen species; SLIM1, sulfur limitation 1; SOD, superoxide dismutase; WT, wild-type; γ-ECS, gamma-glutamylcysteine synthetase.
work was to identify the transcriptional regulators mediating rapid arsenic-induced gene expression in Arabidopsis. This approach was successful in identifying new alleles of the glutathione biosynthesis genes gamma-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS), as being required for cadmium and arsenic-induced gene expression [7]. Glutathione is necessary for the synthesis of phytochelatins, which detoxify many toxic compounds, including cadmium and arsenic, by chelation and sequestration in the vacuole [1,8–11]. Phytochelatins are short polymers of glutathione synthesized in the cytosol in response to toxic metal(loid)s. Thus, arsenic exposure can rapidly deplete glutathione levels, creating a high demand for glutathione in plant cells.

Because the tripeptide glutathione (Glu-Cys-Gly) contains the sulfur-containing amino acid cysteine, the sulfate assimilation pathway is inextricably linked to glutathione biosynthesis. Sulfate assimilation takes oxidized sulfur in the form of sulfate and, through a series of energy-dependent reducing steps, produces sulfide. Due to the toxicity of sulfide, this intermediate quickly reacts with O-acetylserine to produce the amino acid cysteine [12]. Thus, unlike animals, plants do not require exogenous sulfur-containing amino acids and proteins for survival [13]. More importantly, this creates a direct link between the sulfate assimilation pathway and the ability of plants to detoxify arsenic.

While our luciferase genetic reporter approach has not identified transcriptional regulators of arsenic-induced gene induction to date, a similar reporter gene approach successfully identified a transcriptional regulator of the sulfur deficiency response in Arabidopsis. This genetic screen used the same high-affinity sulfate transporter promoter element fused to the green fluorescent protein (pSULTR1;2::GFP) and identified four allelic mutants in an ethylene insensitive-like transcription factor called sulfur limitation 1 (SLIM1) that failed to induce the reporter construct under sulfur-limiting conditions [14]. All of the allelic slim1 mutants identified in this screen resulted in missense mutations altering single amino acid residues [14]. In slim1-1 and slim1-2, high-affinity sulfate uptake was decreased by ~60%, and sulfur-dependent microarray analyses on slim1-1 and slim1-2 showed a decrease in the induction of many sulfur limitation-induced transcripts compared with controls, suggesting that SLIM1 is a positive regulator of sulfate uptake and assimilation [14].

While the transcription factors that control arsenic-induced gene expression remain largely unknown, arsenic exposure is known to rapidly deplete cellular glutathione levels, increasing the demand for reduced sulfur compounds from the sulfur assimilation pathway [7,15,16]. A similar situation occurs under sulfur deficiency. As sulfate supply decreases, cellular levels of cysteine and glutathione become depleted. Thus, because of the similarities in glutathione depletion and subsequent upregulation of the high-affinity sulfate transporter SULTR1;2 under arsenic stress [7] and sulfur limitation [14], we investigated the hypothesis that SLIM1 plays a role in arsenic-induced transcriptional responses. Interestingly, we found that slim1-1 and slim1-2 seedlings were highly sensitive to arsenic. Here, we show that under arsenic treatment, slim1 mutants accumulate arsenic, experience high levels of oxidative stress, and fail to induce sulfate uptake and assimilation. Our results suggest that SLIM1 appears to play an important role in arsenic sensitivity due primarily to its role in regulating sulfur metabolism and the cellular redox state.

**Results**

**slim1 mutants are sensitive to arsenic in root growth assays**

In a previous screen for regulators of cadmium and arsenic-induced gene expression using a pSULTR1;2::LUC reporter construct, we identified new alleles in well-characterized glutathione biosynthesis genes that play an essential role in cadmium and arsenic detoxification [7]. Because glutathione is a significant sink of reduced sulfur in plants, we hypothesized that the transcriptional regulator of sulfur deficiency, SLIM1, might also play a role in regulating cadmium and arsenic sensitivity in plants. To test this hypothesis, we performed root growth assays to evaluate the sensitivity of the slim1-1 and slim1-2 mutant alleles [14] to cadmium and arsenic (Fig. 1A–D). The root lengths of wild-type (WT; 3.06 ± 0.09 cm, n = 22), slim1-1 (3.19 ± 0.07 cm, n = 19), and slim1-2 (3.10 ± 0.11 cm, n = 21) were not different in the control nutrient media (see Methods) [17] without addition of cadmium or arsenic (Fig. 1; P = 0.99997 slim1-1 & P = 1.0 slim1-2, one-way ANOVA). When grown on plates containing 30 μM cadmium, WT root growth was inhibited growing only 1.88 ± 0.15 cm (n = 10). This inhibition was similar to that observed for slim1-1 with a final root length of 1.86 ± 0.13 cm (n = 12) and slim1-2 having a root length of 1.82 ± 0.11 cm (P = 1.0 for slim1-1 & P = 0.99998 for slim1-2, n = 13; Fig. 1A–D, Table S1). However, when grown on minimal media plates containing 10 μM arsenite (As (III)), the root length of WT (1.72 ± 0.12 cm, n = 14) was longer than both slim1-1 (0.68 ± 0.06 cm,
P = 7 x 10^{-9}, n = 14) and slim1-2 (0.75 ± 0.06 cm, P = 1.2 x 10^{-6}, n = 10). These observations suggested that SLIM1 is involved in arsenic signaling. Thus, we further investigated possible mechanisms underlying slim1 sensitivity to arsenic.

**Arsenic accumulation and antioxidant responses of slim1 mutants**

To determine whether arsenic accumulates in the slim1 mutants, we measured root and shoot arsenic levels using ICP-MS. In As(III)-treated seedlings, we observed no significant increase in the accumulation of arsenic in the shoots of slim1-1 (174.8 ± 1.97 mg·kg^{-1} DW, n = 3) or slim1-2 (189.7 ± 3.78 mg·kg^{-1} DW, n = 3) compared with WT (173.9 ± 4.32 mg·kg^{-1} DW, n = 3, P = 1; Fig. 2A). However, in As(V)-treated seedlings, both slim1-1 (309.0 ± 47.5 mg·kg^{-1} DW, n = 3, P = 0.01) and slim1-2 (253.9 ± 19.9 mg·kg^{-1} DW, n = 3, P = 0.6) accumulated more arsenic in shoots than WT (205.6 ± 11.6 mg·kg^{-1} DW, n = 3), although the difference was only significant in slim1-1 (Fig. 2A). These results suggest an increased root-to-shoot translocation of As(V) in the slim1 mutants.

In the roots, we found arsenic accumulation in slim1-1 (1420.0 ± 281.3 mg·kg^{-1} DW, n = 3, P = 6.77E-3) and slim1-2 (1473.0 ± 187.9 mg·kg^{-1} DW, n = 3, P = 3.69E-3) compared with WT (420.1 ± 17.1 mg·kg^{-1} DW, n = 3).
arsenic accumulation in As(V)-treated seedlings (Fig. 2B, Table S3). In comparison, there was no difference in root arsenic accumulation in As(V)-treated seedlings (Fig. 2B, Table S3).

Because arsenic is known to cause oxidative stress and induce reactive oxygen species (ROS) production, we also tested the activity of the key antioxidant enzymes peroxidase (POD) and superoxide dismutase (SOD) in the slim1-1 and slim1-2 mutants. Basal SOD activity in seedlings was similar between WT (70.9 ± 9.65 units·g⁻¹ FW, n = 3), slim1-1 (77.7 ± 9.44 units·g⁻¹ FW, P = 1.0, n = 3), and slim1-2 (Fig. 2C; 75.8 ± 4.20 units·g⁻¹ FW, P = 1.0, n = 3). Similarly, arsenate (As(V)) treatment increased the SOD activity in the WT SOD increased to 234.8 ± 39.4 units·g⁻¹ FW (P = 0.0001, n = 3) and slim1-1 and P = 1.0 slim1-2) grown under control conditions. When exposed to arsenite (As(III)), WT SOD increased to 306.2 ± 9.12 units·g⁻¹ FW (n = 3) while the SOD activity in the slim1 mutants increased dramatically to 709.5 ± 4.85 units·g⁻¹ FW (P = 3.6 x 10⁻⁶, n = 3) in slim1-1 and 621.1 ± 17.7 units·g⁻¹ FW in slim1-2 (P = 2.0 x 10⁻⁸, n = 3). Similarly, arsenate (As(V)) treatment increased the WT SOD activity to 234.8 ± 27.2 units·g⁻¹ FW while the slim1-1 SOD activity increased to 543.2 ± 39.4 units·g⁻¹ FW (P = 2.9 x 10⁻⁸, n = 3) and the slim1-2 SOD activity increased to 492.1 ± 17.7 units·g⁻¹ FW (P = 4.7 x 10⁻⁷, n = 3; Fig. 2C, Table S4).

In seedlings, the peroxidase activity was higher under control conditions in slim1-1 (94.8 ± 5.95 units·g⁻¹ FW, P = 0.009, n = 3) and slim1-2 (120.6 ± 6.04 units·g⁻¹ FW, P = 5.4 x 10⁻⁵, n = 3) compared with WT (50.7 ± 1.51 units·g⁻¹ FW, n = 3; Fig. 2D). As (III) exposure increased the peroxidase activity in WT to 107.5 ± 8.91 units·g⁻¹ FW (n = 3; Fig. 2D), while the peroxidase activity in slim1-1 seedlings increased to 173.6 ± 1.79 units·g⁻¹ FW (P = 0.0001, n = 3; Fig. 2D). Similar values were observed for As (III)-treated slim1-2 seedlings (139.3 ± 4.49 units·g⁻¹ FW, P = 0.10, n = 3; Fig. 2D, Table S5).

### Decreased shoot glutathione in arsenic-treated slim1-1 and slim1-2

To determine whether thiol production might also be altered by arsenic treatment in the slim1 mutants, we measured root and shoot cysteine and glutathione levels using fluorescence HPLC of seedlings exposed to arsenite (As(III)) or arsenate (As(V)) for 48 h (Fig. 3A–D). Shoot cysteine levels were lower in slim1-1 (10.2 ± 0.5 pmol·mg⁻¹ FW, P = 0.04, n = 3) and slim1-2 (12.2 ± 1.0 pmol·mg⁻¹ FW, 0.19, n = 3) than WT (23.7 ± 2.7 pmol·mg⁻¹ FW, n = 3) in control conditions (Fig. 3A, Table S6). No clear decrease in the cysteine concentration was observed in response to As (III) or As(V) treatment (Fig. 3A, Table S6).

Root cysteine levels were statistically similar for WT, slim1-1, and slim1-2 in control conditions and were not significantly changed by As(III) or As(V) treatments (Fig. 3B, Table S7; one-way ANOVA, Tukey HSD).

**Fig. 3.** Thiol accumulation of slim1 mutants grown on arsenic. Total shoot cysteine levels in slim1-1 and slim1-2 compared with WT (A). Total root cysteine levels in slim1-1 and slim1-2 compared with WT (B). Total shoot glutathione levels for slim1-1 and slim1-2 compared with WT (C). Total root glutathione levels for WT, slim1-1, and slim1-2 (D).
Under control conditions, shoot glutathione levels were lower in \textit{slim1-1} (163.6 ± 24.2 pmol·mg\(^{-1}\) FW, \(n = 8\)) and \textit{slim1-2} (190.2 ± 34.4 pmol·mg\(^{-1}\) FW, \(n = 8\)) than in WT (382.4 ± 36.2 pmol·mg\(^{-1}\) FW, \(n = 8\); Fig. 3C; \(P = 4.99 \times 10^{-5}\) for \textit{slim1-1} and \(P = 3.9 \times 10^{-4}\) for \textit{slim1-2}). Shoot glutathione levels decreased in WT from 382.4 ± 36.2 pmol·mg\(^{-1}\) FW (\(n = 8\)) in control conditions to 278.1 ± 23.2 pmol·mg\(^{-1}\) FW (\(n = 3\)) in the As(III) treatment and 269.4 ± 12.2 pmol·mg\(^{-1}\) FW (\(n = 3\)) in the As(V) treatment (Fig. 3C). Similarly, shoot glutathione decreased in the \textit{slim1} mutants under As(III) and As(V) treatments with \textit{slim1-1} having only 110.4 ± 17.5 pmol·mg\(^{-1}\) FW of glutathione in As(III) and 31.4 ± 1.62 pmol·mg\(^{-1}\) FW of glutathione in As(V). Furthermore, \textit{slim1-2} had 83.8 ± 30.9 pmol·mg\(^{-1}\) FW (\(n = 3\)) shoot glutathione in As(III) treatment and 43.5 ± 14.7 pmol·mg\(^{-1}\) FW (\(n = 3\)) in As(V) treatment—an 80% decrease compared with control (Fig. 3C, Table S8).

Root glutathione levels decreased under both As(III) and As(V) treatments for all genotypes. However, glutathione levels in roots showed no differences between genotypes within each treatment (Fig. 3D, Table S9; one-way ANOVA, Tukey HSD). In summary, thiol measurements showed that while cysteine and glutathione levels were not dramatically decreased in the roots of the \textit{slim1} mutant alleles compared with WT (Fig. 3B,D), glutathione levels were decreased in shoots of \textit{slim1-1} and \textit{slim1-2} compared with WT plants (Fig. 3C).

**Shoot sulfate and phosphate accumulation in \textit{slim1} mutants**

Arsenic is thought to be actively taken up by phosphate transporters as As(V); however, once inside plant cells, it is reduced to As(III) and can move within plants through aquaporins [18,19]. Mutants in \textit{SLIM1} were previously shown to be impaired in root-to-shoot translocation of sulfate [14], but the translocation of other anions, including phosphate, was not reported. Thus, based on the slight arsenic accumulation in shoots of As(V)-treated plants noted by ICP-MS (Fig. 2A,B), we hypothesized that phosphate transport might also be impaired in the \textit{slim1} mutants.

To determine whether phosphate and sulfate translocation are impaired in the \textit{slim1} mutants under arsenic treatment, we measured sulfate and phosphate accumulation in both roots and shoots of plants treated with As (V) for 48 h. Interestingly, shoot phosphate accumulation was higher in \textit{slim1-1} and \textit{slim1-2} than WT in all treatments (Fig. 4A, Table S10; \(P = 5 \times 10^{-6}\) for \textit{slim1-1} and \(P = 0.004\) for \textit{slim1-2}; one-way ANOVA, Tukey HSD).

Root phosphate accumulation was similar for WT (5.03 ± 0.27 nmol·mg\(^{-1}\) FW, \(n = 5\)), \textit{slim1-1} (4.95 ± 1.07 nmol·mg\(^{-1}\) FW, \(n = 3\)), and \textit{slim1-2} (6.33 ± 0.80 nmol·mg\(^{-1}\) FW, \(n = 4\)) in control conditions and was not different under As(V) treatment (Fig. 4B). Thus, the enhanced root-vs.-shoot phosphate accumulation...
observed in slim1-1 and slim1-2 suggests an indirect role for SLIM1 in regulating phosphate and arsenate transport (Fig. 4A, Table S11).

Furthermore, sulfate accumulation in shoots was impaired in slim1-1 (0.73 ± 0.10 nmol-mg⁻¹ FW, \( P = 1.7 \times 10^{-6} \), \( n = 5 \)) and slim1-2 (0.61 ± 0.20 nmol-mg⁻¹ FW, \( P = 7.3 \times 10^{-7} \), \( n = 5 \)) relative to WT (3.09 ± 0.18 nmol-mg⁻¹ FW, \( n = 5 \)) in control conditions (Fig. 4A), consistent with previous findings [14]. WT seedlings showed a decrease in shoot sulfate upon As(V) treatment decreasing to 1.86 ± 0.44 nmol-mg⁻¹ FW (\( n = 5 \); Fig. 4C, Table S12, \( P = 0.008 \), one-way ANOVA, Tukey HSD).

Root sulfate accumulation was similar between WT (2.11 ± 0.14 nmol-mg⁻¹ FW, \( n = 5 \)), slim1-1 (1.95 ± 0.43 nmol-mg⁻¹ FW, \( n = 3 \)), and slim1-2 (1.45 ± 0.21 nmol-mg⁻¹ FW, \( n = 4 \)) in control conditions. Furthermore, WT (2.04 ± 0.16 nmol-mg⁻¹ FW, \( n = 4 \)), slim1-1 (1.72 ± 0.13 nmol-mg⁻¹ FW, \( n = 5 \)), and slim1-2 (1.85 ± 0.15 nmol-mg⁻¹ FW, \( n = 3 \)) root sulfate were not different in the As(V) treatment (Fig. 4D, Table S13).

**Microarray analyses of slim1 mutants under As treatment**

The current model for arsenic uptake and tolerance in plants suggests that arsenic is taken up from the soil in the form of arsenate (As(V)). Once it has entered the plant, it is rapidly reduced to arsenite (As(III)) by the arsenate reductase HAC1 [20]. It has been proposed that As(III) can be removed from the root by an unidentified efflux transporter [21]. In rice, the aquaporin LS1 is known to mediate As(III) efflux; however, additional efflux transporters remain elusive [21]. A recent RNA-seq experiment using a T-DNA mutant allele of SLIM1 (\( \text{slim}^{-} \)) did not find misregulation of any aquaporin genes in the roots of the slim1 mutant under control or sulfur deficiency conditions [22]. Thus, due to the observed arsenic accumulation in the roots of slim1 mutants, we hypothesized that the elusive As(III) efflux transporter, or alternatively an As(III) uptake transporter, might be disrupted in an arsenic-dependent manner in the slim1 mutant background.

To test this hypothesis and uncover genes disrupted in an arsenic-dependent manner in the slim1-1 mutant, we performed microarray analyses on WT and slim1-1 seedlings exposed to arsenite for 48 h. Raw expression values were normalized via the R “affy” package using the Robust Multi-Array Average (RMA) Expression Measure. Differential gene expression was evaluated using the R package “limma,” including a multiple test correction. We then performed a significance analysis to identify down-regulated genes under arsenic treatment and compared these to previously published putative targets of SLIM1 obtained by DNA affinity purification sequencing (DAP-seq) [23].

From the microarray analyses, we identified 11 genes significantly differentially upregulated by arsenic (WT +As vs. slim1-1 + As; Table S14). Ten of the 11 genes (AT3G49580, AT1G04770, AT1G12030, AT4G04610, AT4G21990, AT5G24660, AT5G26220, AT5G48850, AT4G20820, and AT1G36370) were identified as putative targets of SLIM1 by DNA affinity purification sequencing (DAP-Seq; Table S14). Many of the 11 upregulated genes in slim1-1 are associated with sulfur metabolism.

Genes that appear to be negatively regulated by SLIM1 include CGCT2;1 (AT5G26220), APR1 (AT4G04610), and APR3 (AT4G21990), which were upregulated in slim1-1 compared with WT in the presence of arsenic (WT +As vs. slim1-1 + As). APR1 and APR3 are involved in the reduction of sulfate into sulfide [24] and have been shown to be induced by toxic metal stress [7]. Similarly, the LOW SULFUR 1 (LSU1, AT3G49580) and LOW SULFUR 2 (LSU2, AT5G24660) genes were expressed at higher levels in slim1-1 than WT under arsenic treatment (WT +As vs. slim1-1 + As). Interestingly, six of the 11 genes (GGCT2;1, APR3, LSU1, LSU2, SD11, and SHM7) belong to a highly coregulated cluster of genes that respond to O-acetyserine treatment [25].

Microarray analyses also identified 10 significantly downregulated genes under arsenic treatment compared with WT (WT +As vs. slim1-1 + As; Fig. S1; \( P < 0.05 \), fold change > 2). Only one gene—SULTR1;2 (AT1G78000)—was identified as a putative target of SLIM1 by DAP-Seq (Table S14). Thus, our analyses confirm the reported function of SLIM1 as a transcriptional activator of SULTR1;2 and show that this role is conserved under arsenic treatment and sulfur deficiency. The remaining ten genes are involved in hormone signaling (AT1G63030, AT5G13220, and AT5G52050), redox regulation (AT3G6590 and AT1G03020), iron homeostasis (AT3G25190 and AT5G01600), glucosinolate biosynthesis (AT5G23020), ubiquitination (AT1G24330), and an uncharacterized protein (AT2G17660). Based on their putative functions, these genes encode stress response-related genes. More experiments are needed to determine whether SLIM1 is a direct transcriptional regulator of these genes under arsenic stress.

The present transcriptome data suggest that SLIM1 can function as both a transcriptional enhancer and a transcriptional repressor of specific genes in a
condition-specific manner. Furthermore, the present study provides evidence that SLIM1 plays an essential role in the regulation of sulfur metabolism gene expression in response to arsenic.

Discussion

Plant exposure to arsenic causes rapid changes in gene expression [7,26,27]. However, the transcription factors that function in arsenic-induced gene expression remain largely unknown. The few transcriptional regulators that have been identified, such as WRKY6, WRKY45, and OsARM1 (Arsenite-Responsive Myb1) [27–29], have been implicated in the regulation of arsenic transporters while regulators of arsenic detoxification remain unknown. To test the hypothesis that the SLIM1 transcription factor is involved in arsenic resistance and signaling, we evaluated the sensitivity of slim1-1 and slim1-2 to arsenic exposure. We found the slim1 mutants were more sensitive to arsenic than control plants. Arsenic treatment caused high levels of oxidative stress in the slim1 mutant alleles based on SOD and peroxidase activities. Furthermore, thiol and sulfate measurements show that slim1 mutants are impaired in both thiol accumulation and sulfate accumulation. Arsenic treatment did not further decrease sulfate levels in roots. In contrast, the concentration of the thiol GSH was greatly decreased in slim1 mutant alleles. Furthermore, peroxidase and SOD measurements show that arsenic treatments cause increased levels of oxidative stress in the slim1 mutants.

We also observed a slight increase in arsenic accumulation in the shoots of slim1 mutants treated with arsenic. This arsenic accumulation was accompanied by a significant increase in shoot phosphate translocation in the slim1 mutants. Because of the chemical similarity between phosphate and arsenic oxyanions, future research could investigate the hypothesis that the misregulation of phosphate transporters may contribute to the observed increase in shoot arsenic in the slim1 mutants. A recent study identified mutants in ethylene response factor genes (ERF34 and ERF35) that are sensitive to both arsenite (As(III)) and arsenate (As(V)) [30]. Interestingly, similar to the slim1 mutants, the double erf34erf35 mutants were far less sensitive to cadmium than arsenic, suggesting the arsenic sensitivity is not exclusively due to thiol accumulation. Furthermore, gene expression studies showed that several phosphate transporters were downregulated in erf34erf35, suggesting PHTs may play a role in both As(III) and As(V) sensitivity and/or transport [30].

Thiol measurements confirmed the role of SLIM1 in sulfate metabolism and thiol production [14,22,31], as slim1 mutants contained lower cysteine and glutathione levels in shoots than WT. We hypothesized that the weaker cadmium sensitivity of slim1 mutant alleles might be linked to thiol accumulation, but we observed no significant differences decrease in shoot GSH in the slim1 mutants under Cd treatment (Fig. S2). However, SLIM1 upregulates the root-to-shoot transport of sulfate, which restricts sulfate assimilation mainly to the roots in slim1 mutants. Root sulfate levels are maintained by the high-affinity sulfate transporter SULTR1;1, which is regulated in a SLIM1-independent manner [14]. Thus, cysteine and glutathione biosynthesis can occur in the roots. As described previously, glutathione is essential for producing phytochelatins—arsenic chelating compounds necessary for detoxification and storage. The heavy metal cadmium also binds to phytochelatins. Interestingly, recent research has shown a less dramatic effect of cadmium exposure in slim1 mutants than WT controls [31], which we have also observed (Fig. 1C,D).

Thus, the present study shows that the SLIM1 transcription factor plays a more central role in mediating arsenic resistance relative to cadmium resistance. A possible hypothesis that may contribute to this observation is that cadmium can be sequestered in vacuoles via two independent transport pathways: via phytochelatin transport [8,32] and via thiol-independent HMA3-mediated cadmium transport [33].

Sulfate measurements confirmed that SLIM1 is a major transcriptional regulator of sulfate uptake and translocation [14]. Our microarray analyses also identified 11 genes significantly differentially upregulated by arsenic (Table S14), of which ten of the 11 genes were identified as putative targets of SLIM1 by DNA affinity purification sequencing (DAP-Seq). Interestingly, nine of these genes are involved in sulfur assimilation or redox signaling. One of these genes, GGCT2;1, is involved in glutathione recycling and has also been implicated in arsenic tolerance [34–36]. Furthermore, six of these sulfur metabolism genes belong to a highly coregulated cluster of genes that respond to O-acetylsersine treatment [25]. While previous studies show these genes can regulate sulfur assimilation in a SLIM1 independent manner [25,37], results from DAP-Seq and microarray results from the current study suggest SLIM1 may act as a negative regulator of these genes during arsenic stress.

Shoot sulfate accumulation was significantly lower in the slim1 mutants under all conditions tested. Decreased shoot sulfate was accompanied by an increase in shoot phosphate in the slim1 mutants.
Similar anion compensation was noted in the arabidopsis 
phr1 mutant, which accumulates higher sulfate levels when grown under low phosphate conditions indicating crosstalk between phosphate and sulfate transport [38]. In fact, 
PHR1 has been proposed to act both positively in the regulation of root-to-shoot sulfate translocation via the sulfate transporter 
SULTR1;3, and negatively to repress other sulfate transporters under phosphate deficiency [39]. We did not identify any significantly misregulated phosphate transporters (PHTs) in the presence of arsenic in our microarray analyses. One possible explanation is that PHTs belong to a large gene family and demonstrate a high degree of genetic redundancy. Thus, a small decrease in the expression of several PHTs may result in measurable changes in phosphate accumulation without any individual transcript misregulation meeting the stringent criteria used in our microarray analyses. Xie et al. [30] identified an artificial microRNA mutant targeting three high-affinity phosphate transporters showing a similar sensitivity to arsenic. A recent study showing that sulfate deficiency increases phosphate accumulation in arabidopsis further supports this hypothesis [40].

In summary, we show here that the SLIM1 transcription factor plays an important role in mediating arsenic resistance and in arsenic-induced gene expression. Our results suggest that the arsenic sensitivity of slim1 mutants can be explained by decreased thiol production resulting in increased oxidative stress and in increased arsenic accumulation. Interestingly, we found that the slim1 mutant alleles do not show a strong cadmium sensitivity, consistent with a recent study [31] indicating a difference in the rate-limiting functions of the thiol synthesis pathway in processing arsenic and cadmium that we discuss here. We also identify a number of genes regulated by SLIM1 in an arsenic-dependent manner with DAP-seq data set analyses indicating direct binding of SLIM1 to arsenic-dependent differentially expressed genes. Taken together, our data support a model in which SLIM1 is both a positive regulator and a negative regulator of gene expression in response to arsenic.

Experimental procedures

Arabidopsis accessions

The WT 
Arabidopsis thaliana ecotype used in this study is Columbia (Col-0). The slim1-1 and slim1-2 mutants were generated in the Col-0 genetic background and were kindly provided by Dr. Akiko Maruyama-Nakashita [14].

Plant growth media and conditions

Seeds were surface sterilized by briefly soaking in 70% ethanol before allowing them to dry in a sterile hood. For root growth experiments and enzymatic assay experiments, surface-sterilized seeds were plated on minimal media containing 1/10-strength Hoagland solution, 1% phytoagar (Duchefa, http://www.duchefa.com), pH 5.6. For the microarray experiments, seeds were plated on 1/2-strength MS standard medium (M5519; Sigma-Aldrich, Miamisburg, OH, USA, http://www.sigmaaldrich.com) buffered with 1 mm 2-(N-morpholino)-ethanesulfonic acid (MES), 1% phytoagar (Duchefa, http://www.duchefa.com) and the pH was adjusted to 5.6 with 1.0 m KOH. Seeds were then stratified with cold treatment at 4 °C for 48 h and grown under controlled conditions (150 µmol·m⁻²·s⁻¹, 70% humidity, 16-h light at 21 °C/8-h dark at 18 °C) for the specified time. For toxic metal(loid) treatments, the specified amounts of either cadmium or arsenic were added to the autoclaved base media in a sterile hood prior to pouring the plates. Concentrated stock solutions of cadmium and arsenic were filter-sterilized prior to use.

Statistical analyses

The root growth, thiol, peroxidase, superoxide, and anion data were all analyzed using one-way ANOVA followed by a Tukey post hoc test to determine significance. Significance groups are indicated in the figures, and key P-values are stated in the text.

Root length measurements

For root growth experiments, surface-sterilized seeds of WT, slim1-1, and slim1-2 were plated on minimal media (2.5 mM H₃PO₄, 5 mM KNO₃, 2 mM MgSO₄, 1 mM (CaNO₃)₂, 1 mM MES, 1% phytoagar pH 5.7) supplemented with 30 µM Cd or 10 µM As (III) [17]. Plates were placed in the dark two days at 4 for vernalization and then transferred to a growth chamber. After 7 days of growth, seedlings were photographed, and root length was measured using ImageJ.

Antioxidant enzyme assays

Seedling samples were weighed and pulverized in liquid nitrogen after treatment. The powder was dissolved in precooled 50 mM phosphate buffer (pH 7.8) to extract the SOD. The extract was then centrifuged at 12 000 g for 10 min, resulting in a crude enzyme supernatant solution. In a separate 10-ml tube, 1.9 mL reaction
buffer (50 mM phosphate buffer, pH 7.8, 9.9 mM 1-
methionine, 57 µM NBT solution, 1 mM EDTA-Na2
solution, 0.0044% (w/v) riboflavin) and 0.1 mL
enzyme solution were mixed and placed into
250 µmol-m⁻²-s⁻¹ light for 20 min. Additionally,
another separate 10-mL tube was procured, where
the enzyme solution was replaced with water as a control.
The reagent was added according to the above steps,
where one tube was placed in the light together with
the sample, and the other was placed in the dark
where the reaction was allowed to complete. The
control tube that was placed in the dark was blanked,
and the absorbance of each tube was measured at 560 nm.
Peroxidase (POD) was extracted in 50 mM phosphate
buffer (pH 7.0), 30 µL of enzyme solution was mixed
with reaction buffer containing 1.77 mL of 50 mM
sodium phosphate buffer (pH 7.0), 0.1 mL of 4% gua-
iacol, and 0.1 mL of 1% (v/v) H2O2. Increased absorb-
bance was recorded at 470 nm for 1 min. All reported
enzyme activities are means of 3–5 biologically inde-
pendent samples, and error bars indicate the standard
error of the mean (SEM).

Arsenic determination by ICP-MS
Plant material was harvested, dried at 70 °C for at
least 48 h before being aliquoted, and weighed.
Approximately 10 mg of dried plant material was
mixed with 1 mL of concentrated nitric acid and
digested by heating at 100 °C for approximately
30 min or until the solution became transparent and
particle-free. These digests were diluted with deionized
water and measured by ICP-MS for total arsenic
concentrations at the University of Cologne Biocenter
Mass Spectrometry Platform. All reported ion quanti-
ties are means of 3–5 biologically independent samples,
and error bars indicate the standard error of the mean
(SEM).

Anion extraction and measurement by ion
chromatography
To quantify the water-soluble anion concentrations
(phosphate and sulfate) in plant tissues, 10–30 mg of
fresh tissue was harvested and flash-frozen in liquid
nitrogen. Frozen tissue was then pulverized using a
bead mill (make and model), and anions were
extracted by addition of 1000 µL of sterile Milli-Q-
water and incubating for 60 min at 4 °C while shaking
at 1500 rpm. The extraction process was stopped by
incubating at 95 °C for 15 min. Cell debris was
removed by centrifugation at 4 °C for 15 min, and
100–200 µL of supernatant was used for anion
exchange chromatography. An automatic ion analyzer
(DX 120, Dionex Corporation, Sunnyvale, CA, USA)
equipped with an IonPac® column (AS9-SC, 4 × 250 mm; Dionex, Thermo Fisher Scientific
GmbH; Waltham, MA, USA) was used to separate and quantify the anions. Anions were eluted with an elution buffer of 2.0 mM Na2CO3 and 0.75 mM
NaHCO3. Ion concentrations were detected using a
conductivity detector module (CDM, Dionex Corpora-
tion, CA, USA). All reported anion quantities are
means of 3–5 biologically independent samples of tis-
sue pooled from 4 to 6 individual seedlings (12–30
seedlings in total), and error bars indicate the standard
error of the mean (SEM).

Thiol detection by fluorescence HPLC
The thiol-containing compounds cysteine and GSH
were analyzed using fluorescence detection HPLC as
described by [41]. To analyze the levels of these thiol
compounds, plants were grown on minimal growth
media plates for 12 days then transferred to fresh
media plates containing either 20 µM cadmium,
100 µM arsenate, or control minimal media. To mini-
mize the oxidation of thiol compounds during the
extraction, plant seedlings were flash-frozen in liquid
nitrogen immediately after harvesting and then pulv-
erized using a bead mill and extracted as described by
[42]. Thiols were extracted from homogenized plant
material with 1 mL 0.1 M HCl for 40 min at 25 °C.
After centrifugation for 5 min at 14 000 g and 4 °C,
thiols in the supernatant were reduced by mixing 60
µL of the supernatant with 100 µL 2-(cyclohexylamino)ethanesulfonic acid (0.25 M, pH 9.4)
and 35 µL DTT (10 mM, freshly prepared). The mix-
ture was incubated at 25 °C for 40 min. Thiols were
derivatized by adding 5 µL (25 mm) monobromobi-
mancine (Sigma-Aldrich, Cat#B4380). Derivatization
was stopped by adding 110 µL methane sulfonic acid
(100 mm) and clarified by centrifugation for 15 min at
14 000 g and 4 °C. Forty microliters of the derivatiza-
tion mix were used for HPLC analysis using the Dio-
nex Ultimate 3000 HPLC System. Derivatized thiols
were separated in a Eurosphere 100-3 C18, 150 × 4 mm column (Knauer) and were detected by
fluorescence detection with an excitation of 380 nm
and emission detection at 480 nm. The peaks of thiol
compounds were identified and quantified by compar-
ison with cysteine and glutathione standards purchased
from Sigma-Aldrich. All reported thiol quantities are
statistical means of 4–5 biologically independent exper-
iments (16–30 seedlings per experiment). Error bars
indicate the standard error of the mean (SEM).
Microarray analyses

To evaluate transcriptional differences in the slim1 mutants under cadmium and arsenic stress, we performed microarray analyses. To obtain tissue for the microarray analysis, plants were grown on ¼ MS plates for 12 days then transferred to fresh media plates containing either 100 µM cadmium or 20 µM arsenite. Whole seedlings were then harvested in 2-mL Eppendorf tubes, flash-frozen in liquid nitrogen, and stored at −80 °C until further processing. The tissue was subsequently pulverized using a bead mill by adding three 2.5-mm glass beads to each tube and grinding for 15 s. RNA was extracted using the Qiagen RNEasy Mini Kit (Hilden, Germany, Cat#74104) per the manufacturer’s instructions (www.qiagen.com). RNA quality was assessed by spectrophotometer and gel electrophoresis before submission to the University of California, San Diego Gene Expression Core facility for processing. Results were analyzed using R and the Bioconductor suite of microarray analytical packages as indicated in the text.

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Conflict of interest

We certify that the submission is original work and is not under review at any other publication.

Author contributions

TOJ, QY, QX, YM, TM collected and analyzed data and cared for plants. FH assisted with microarray analyses and data interpretation. SK assisted with data interpretation and manuscript preparation. TOJ and JIS conceived the study and prepared the manuscript.

Data accessibility

Research data pertaining to this article are made available through preprint at bioRxiv: https://doi.org/10.1101/2021.01.12.426316

The data that support the findings of this study are openly available in NCBI GEO at https://www.ncbi.nlm.nih.gov/geo/, reference number GSE138943.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Supplementary Material**