ARS5 is a component of the 26S proteasome complex and negatively regulates thiol biosynthesis and arsenic tolerance in *Arabidopsis*

Dong-Yul Sung[1], **Tae-Houn Kim**[1], **Elizabeth A. Komives**[2], **David G. Mendoza-Cózatl**[1], and **Julian I. Schroeder**[1,*]

[1]Division of Biological Sciences, Cell and Developmental Biology Section, and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0116, USA.

[2]Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093-0378, USA

Summary

A forward genetic screen in *Arabidopsis* led to the isolation of several arsenic tolerance mutants. *ars5* is the strongest arsenate and arsenite resistant mutant identified in this genetic screen. Here, we report the characterization and cloning of the *ars5* mutant gene. *ars5* is shown to exhibit an increased accumulation of arsenic and thiol compounds during arsenic stress. Rough mapping together with microarray-based expression mapping identified the *ars5* mutation in the alpha subunit F (PAF1) of the 26S proteasome complex. Characterization of an independent *paf1* T-DNA insertion allele and complementation by PAF1 confirmed that *paf1* mutation is responsible for the enhanced thiol accumulation and the arsenic tolerance phenotypes. Arsenic tolerance was not observed in a knockout mutant of the highly homologous *PAF2* gene. However, genetic complementation of *ars5* by over expression of *PAF2* suggests that the PAF2 protein is functionally equivalent to PAF1 when expressed at high levels. No detectable difference was observed in total ubiquitinylated protein profiles between *ars5* and wild type Arabidopsis, suggesting that the arsenic tolerance observed in *ars5* is not derived from a general impairment in proteasome-mediated protein degradation. Quantitative RT-PCR showed that arsenic induces enhanced transcriptional activation of several key genes that function in glutathione and phytochelatin biosynthesis in wild type and this arsenic-induction of gene expression is more dramatic in *ars5*. The enhanced transcriptional response to arsenic and the increased accumulation of thiol compounds in *ars5* compared to WT suggest the presence of a positive regulation pathway for thiol biosynthesis that is enhanced in the *ars5* background.

Introduction

Arsenic (As) is ubiquitous in the environment and found primarily in inorganic forms, with As^{5+} (arsenate) and As^{3+} (arsenite) as the most common As oxidation states (Brown et al., 2002). Inorganic arsenic has been identified as the main cause of cancers in humans, including

*Corresponding author Julian I. Schroeder, University of California, San Diego, Division of Biological Sciences, 9500 Gilman Drive, La Jolla, CA 92093-0116, USA, Tel: +1 (858) 534 7432, Fax: +1 (858) 534 7108.

urinary bladder, lung and non melanoma skin cancers (Lubin et al., 2007). Therefore, inorganic arsenic contamination in the environment poses a serious threat to human health. Arsenic is listed as the most toxic compound found at US Superfund sites (http://epa.gov/superfund/policy/cercla.htm). Irrigation of vegetables and crop plants with arsenic-contaminated water and accumulation of As by plants causes arsenic exposure to humans through daily diet (Alam et al., 2003). Thus, the identification of the processes driving arsenic accumulation in plants, as well as removal of arsenic from contaminated environments are important priorities to reduce As intake in humans. Arsenic removal methods using plant systems (phytoremediation) offer possible non-invasive and inexpensive alternatives to conventional excavation methods (Cobbett and Meagher, 2002; Pilon-Smits and Pilon, 2002; Tripathi et al., 2007).

Understanding the mechanisms that plants employ to respond to and detoxify arsenic is essential for optimal and efficient removal of arsenic from contaminated environments through the use of plants. Arsenic is present as either arsenate (AsO_4^{-3}) or arsenite (AsO_3^{-3}) depending on pH, redox potential, drainage conditions, and phosphorus content in soil (Marin et al., 1993). Arsenate, being similar to phosphate, mainly enters plants through phosphate transporters (Meharg and Macnair, 1992; Wang et al., 2002; Catarecha et al., 2007) and interferes with glycolysis and oxidative phosphorylation, inhibiting the synthesis of ATP (Carbonell et al., 1998; Hindmarsh, 2000).

Arsenic hyper-accumulation in the fern *Pteris vittata* decreased when the phosphate level was increased in the medium (Wang et al., 2002). Similarly, a mutation in a high affinity phosphate transporter (PHT1;1) in Arabidopsis resulted in a slower rate of arsenic uptake compared to wild type and conferred arsenic tolerance to the mutant (*pht1;1–3)* (Catarecha et al., 2007). Another major arsenic tolerance mechanism is thiolmediated chelation and sequestration of arsenic, in the form of arsenite. Arsenate is reduced to arsenite in plants by an arsenate reductase and this reduced form of arsenate is able to bind thiols with high affinity (Schmöger et al., 2000). Arsenite is also able to induce the synthesis of phytochelatins (PCs) and it is thought that PC-As complexes are transported and stored in vacuoles (Dhankher et al., 2002). Thiol compounds also function in the long distance transport of heavy metals between shoots and roots (Gong et al., 2003; Chen et al., 2006; Li et al., 2006; Mendoza-Cózatl et al., 2008). In addition, and in contrast to the results obtained with cadmium (Lee et al., 2003b; Li et al., 2004; Li et al., 2005), over expression of proteins that synthesize glutathione and phytochelatins resulted in an increased arsenic tolerance (Dhankher et al., 2002; Li et al., 2004, 2005, 2006; Gasic and Korban, 2007).

Only few genetic mutants are known in plants that cause arsenic resistance. We have developed a screen to isolate arsenic tolerant mutants (Lee et al., 2003a). The strongest arsenate and arsenite tolerant mutant isolated from this screen is *ars5* (Sung et al., 2007). The *ars5* mutant was identified as a double mutant in the *ARS4* and *ARS5* loci. The *ARS4* locus encodes phytochrome A (Sung et al., 2007), however, the gene and mechanism underlying the *ars5* phenotype have remained unknown even though the *ars5* mutation causes a stronger degree of arsenic tolerance than *ars4 (phyA)* (Sung et al., 2007).

Here, we report the presence of a novel arsenic tolerance mechanism by the cloning and characterization of *ars5*, a null mutant defective in the gene encoding a subunit of the 20S proteasome core particle. *ARS5* encodes the proteasome alpha subunit F1 protein (PAF1) of the 26S proteasome complex. 26S proteasome-mediated protein degradation has been implicated in regulation of signaling mechanisms important for plant responses to various environmental stresses (Smalle et al., 2003; Kurepa et al., 2008; Book et al., 2009). In addition, the present study shows that PAF1 is also a negative regulator of thiol-mediated arsenic tolerance.

Results

Arsenic tolerance in *ars5*

We have pursued screens for mutants with increased arsenic tolerance (Lee et al., 2003a; Sung et al 2007). One particular mutant, *ars4ars5* contained two independent mutations and each mutation contributed to the overall arsenic tolerance. *ars5* was isolated from the *ars4ars5* double mutant by back-crossing the *ars4ars5* double mutant with wild type Col-0 (Sung et al., 2007). The single *ars5* mutant retained the major portion of arsenic tolerance from the *ars4ars5* double mutant (Figure 1A). No major phenotypic differences were found between wild type and *ars5-1* in the absence of arsenate under the imposed conditions. Wild type and *ars5* (named *ars*5-1 from here on) seeds were germinated for one week on ¼ MS growth medium and exposed to a range of arsenate concentrations (Figure 1A), while germination and growth of wild type plants was inhibited by arsenate treatment, *ars5-1* seedlings showed enhanced tolerance to arsenate at concentrations ranging from 500 µM to 1000 µM (Figure 1A). *ars5-1* also showed an increased tolerance to arsenite in root growth assays in the presence of 10 µM As^{III} (Figure 1B).

Arsenic accumulation is increased in *ars5*

In some plants, arsenic tolerance has been related to a decreased rate of arsenic uptake. To test whether this is the case for arsenic tolerance in *ars5-1*, we analyzed the accumulation of arsenic and other elements in WT and *ars5-1* seedlings exposed to 100 µM arsenate for 0, 24, 48, 96 hrs. *ars5-1* plants exhibited a slight average increase in arsenic accumulation at 24, 48 and 96 hrs after arsenic exposure (Figure 2A, WT *vs ars5-1* at 24 h P = 0.008), showing that the arsenic tolerance of *ars5* did not result from a decreased arsenic accumulation. Phosphorus accumulation between *ars5-1* and wild type did not show any statistically significant increase (Figure 2B), suggesting that phosphate metabolism is not related to the increased arsenic tolerance *of ars5-1*.

Thiol synthesis is increased in *ars5* mutants

Fluorescence HPLC combined with tandem mass spectrometry analyses of thiols in WT and *ars5-1* mutants (Figure 3A–B) showed that, during arsenic stress, *ars5-1* contained more organic thiols (glutathione and phytochelatins) compared to WT seedlings (Figure 3C–F). These compounds are well-known arsenic chelating agents that have been described as key metabolites for arsenic tolerance in plants. Increased accumulation of these thiol-compounds could explain, at least in part, the increased arsenic tolerance phenotype of *ars5-1* and also suggests that thiol biosynthesis is up-regulated in this mutant.

ARS5 encodes an alpha subunit F (*PAF1*) of the 20S proteasome complex

ars5-1 was isolated as an arsenic tolerant recessive mutant from the activation tagged *ars4ars5* double mutant. To identify the *ARS5* gene, a combined approach of genetic map-based cloning and microarray expression analysis was taken. Rough mapping of *ars5-1* positioned the *ARS5* locus in the region of the CIW9 marker on the lower arm of Chromosome 5 (Figure 4A). In order to identify genes with an altered expression in *ars5-1*, microarray expression data of *ars4ars5* double mutant plants was investigated (Supplementary File 1). Including the *ARS4/PHYA* gene, we identified 110 genes whose expression was strongly reduced in the *ars4ars5* double mutant compared to WT regardless of the presence or absence of any arsenic stress (Figure 4B; Supplementary File 1). Of the 110 genes, 15 genes are located on Chromosome 5 and only one candidate gene (At5g42790; Figure 4B), close to the CIW9 marker, contained a genetic mutation (deletion) within the gene (Figure 5A). PCR sequence analysis of the genomic region of At5g42790 revealed a deletion of a 1.4 kb genomic DNA

fragment, encompassing part of the promoter and the entirety of the first exon of At5g42790 (Figure 5A).

The 26S proteasome complex is composed of two sub-particles, the 20S core particle and the19S regulatory particle. The 20S core particle consists of two rings formed by alpha subunits and two additional rings formed by beta subunits. Each ring is composed by seven subunits, named A to G. The At5g42790 gene encodes an alpha subunit F (PAF) of the 20S core particle and could be encoded by two paralogous genes: *PAF1* (At5g42790) and *PAF2* (At1g47250). We searched the public T-DNA insertional mutant populations and isolated a mutant allele of *paf1* (GABI_419_H03) (Figure 5B). RT-PCR analysis showed no detectible *PAF1* transcript in either *ars5-1* or in the *paf1* mutant (*ars5-2*, see below) (Figure 5B and Figure S1). The *paf1* insertional mutant also showed an enhanced arsenic tolerance in germination assays (Figure 5C), an increased content of arsenic and elevated levels of GSH when compared to wild type (see below). Both *ars5-1* and *ars5-2* showed 80 % germination by the 4th day in growth medium containing 1 mM arsenate, compared to a 20 % germination rate of WT Col (Figure 5C). The wild type IC_{50} for arsenate was determined to be 742 ± 19 µM (n = 5, $\pm$ SD) while at an arsenate concentration of 1250 µM, more than 60% of *ars5-1* and *paf1* seeds were still able to germinate (Figure 5). These findings confirm that the arsenic resistance of *ars5* is due to disruption of the *PAF1* gene. Therefore, the *paf1* mutant was named *ars5-2*.

To further explore the role of PAF proteins in arsenic tolerance in Arabidopsis, a *paf2* T-DNA insertion mutant was isolated (SALK_098236). RT-PCR analysis showed no *PAF2* transcript, indicating that *paf2* is also a null mutant (data not shown). However, the *paf2* mutant did not show arsenic tolerance under any of the conditions tested. To investigate the consequences of a *paf1paf2* double knockout on arsenic tolerance in Arabidopsis, *ars5-1* and *ars5-2* were crossed with *paf2* and a F2 population was screened for homozygous double mutants. Extensive PCR genotyping did not produce any homozygous double mutants indicating that the *paf1paf2* double mutant is lethal. Furthermore, the absence of *ars5-1ars5-1/paf2+* and *ars5-1 +/paf2paf2* alleles in the F2 populations indicates that gametophytes are not viable if both genes are missing in the gametophyte (Table 1).

PAF1 and *PAF2* complement *ars5*

A 7 kb genomic DNA segment containing the native *PAF1* promoter and the *PAF1* coding region (*PAF1g*) complements the original *ars5-1* mutant (Figure 6A). *ars5-1* was also complemented by over-expressing the *PAF1* or *PAF2* cDNAs under the control of the 35S promoter (*35S–PAF1c* and *35S–PAF2c*) (Figure 6A). Expression of the transgenes was confirmed by RT-PCR (Figure S2). The *ars5-2* allele also showed increased levels of arsenic and glutathione content after 96 h of arsenic exposure (Figure 6B–C). *ars5-1* complemented with either genomic or *PAF1* cDNA showed restored levels of both arsenic and glutathione content compared to WT (Figure 6 B–C). These results confirmed that the arsenic tolerant phenotype of *ars5-1* resulted from the null mutation of *PAF1* and that both *PAF1* and *PAF2* are functionally capable of complementing *ars5-1*. However, the endogenous expression of *PAF2* was not sufficient to replace the loss of *PAF1* in the *ars5-1* mutant.

Increased expression of key metabolic genes in thiol biosynthesis is *ars5*

To test whether increased accumulation of thiol compounds in *ars5-1* during arsenic exposure is the result of a transcriptional activation response, the expression of key metabolic genes involved in thiol biosythesis was analyzed. 5 day-old seedlings of WT, *ars5-1* and *ars5-2* were treated with 100 µM arsenate for 96 hrs and subjected to quantitative real-time qPCR analysis. *PAF1*, but not *PAF2*, was induced by arsenic treatment (Figure 7A–B), suggesting a role for *PAF1* and the 26S proteasome during arsenic exposure. *Sultr1;2*, a sulfate transporter known to be induced by heavy metals (Herbette et al., 2006; Nocito et al., 2006) was also strongly-

induced by arsenic (Figure 7C). qPCR also showed that three key metabolic genes that mediate the synthesis of thiol compounds, γ-glutamyl cysteine synthetase (γ-*ECS*), glutathione synthetase (*GSH2*) and interestingly also phytochelatin synthase 1 (*PCS1*), were induced under arsenic stress in wild type plants (Figure 7D–F). γ-*ECS*, *GSH2*, and in some instances *PCS1*, had been previously shown to be transcriptionally activated by cadmium (Clemens et al., 1999; Xiang and Oliver, 1998; Lee and Korban, 2002). Furthermore, the arsenic-mediated induction of these key metabolic genes was significantly enhanced in both *ars5* mutants (Figure 7D–F).

PAF1-mediated arsenic tolerance is not mediated by global protein stability

Multiple isoforms of the 26S proteasome complex exist in Arabidopsis (Yang et al., 2004; Kurepa et al., 2008). This raises the question whether the PAF1-containing isoform of the 26S proteasome is essential to global protein stability and how impairment in the proteasome-dependent protein degradation process may promote arsenic tolerance. To gain insight into these questions we followed two independent approaches. First, protein ubiquitination profiles between WT and *ars5* were investigated and, second, the effect of inhibiting the 26S proteasome activity (using the proteasome inhibitor MG132) on arsenic tolerance in WT and *ars5* mutants was evaluated. Western blot analyses, using an anti-ubiquitin antibody, did not detect any difference in the protein ubiquitination profiles between WT and *ars5*, both under normal conditions and during arsenic stress (Figure S3), which suggests that knocking out *PAF1* did not compromise the global activity of the 26S proteasome. On the other hand, MG132 treatment failed to confer arsenic tolerance in wild type at concentrations ranging from 10 to 50 µM. Moreover, *ars5-1* seeds treated with MG132 lost their arsenic tolerance (Figure 8), which suggest that inhibition of the proteasome complex in *ars5-1* compromises seed viability. These results suggest that a specific function of PAF1 in the proteasome complex is responsible for the arsenic tolerance of the *ars5* mutant, and that overall inhibition of the 26S proteasome cannot explain the arsenic resistance of *ars5*. To further evaluate this hypothesis, the arsenic tolerance of a different 26S proteasome mutant was analyzed. *RPN12* encodes a regulatory subunit in the lid particle of the 26S proteasome (Smalle et al., 2002). *rpn12* mutants, in the *Arabidopsis* C24 background, showed a slight increase in arsenic tolerance compared to WT (C24 background; Figure 9).

Discussion

ars5 reveals a novel mechanism for arsenic tolerance in *Arabidopsis*

Studies have demonstrated that arsenic tolerance can be achieved through independent mechanisms including active arsenic efflux (Rosen et al., 1988), mutations in transporters favoring the uptake of phosphate over arsenic (Catarecha et al., 2007) and through an increased synthesis of the arsenic-chelating thiol compounds, glutathione and phytochelatins (Dhankher et al., 2002; Li et al., 2004,2005, 2006; Gasic and Korban, 2007). However, it has remained unknown whether the glutathione-phytochelatin synthesis genes γ-*ECS, GSH2* and *AtPCS1* are induced by arsenic stress. Moreover, it is also not known how these arsenic tolerance mechanisms are regulated at the transcriptional level. In the present study, we cloned and characterized *ARS5*, a novel mechanism for arsenic tolerance in Arabidopsis. Enhanced arsenic tolerance in two independent PAF1 null mutants *ars5-1* and *ars5-2* (Figure 1A–B, Figure 5 A–C) and genetic complementation of *ars5* with a *PAF1* genomic DNA fragment and *PAF1* cDNA (Figure 6 A–C) suggest that PAF1-containing 26S proteasome complex may negatively regulate the arsenic-induced transcription of the genes that mediate the synthesis of thiol compounds (Figure 3B–F, Figure 7 D–F).

Arsenic tolerance mechanism in *ars5*

Changes in phosphate uptake rates have been described as a mechanism able to confer enhanced arsenate tolerance (Lee et al., 2003a; Catarecha et al., 2007). However, ICP-OES analyses showed that arsenic tolerance in *ars5* is not the result of active extrusion, decreased arsenic accumulation or an altered phosphate accumulation (Figure 2A–B). Moreover, *ars5-1* also showed increased tolerance to arsenite (Figure 1B). The high reactivity of arsenite towards thiols (Schmöger et al., 2000) suggested that thiol metabolism could be contributing to the *ars5-1* arsenic tolerance mechanism. Interestingly, *ars5* contained an increased content of organic thiol compounds including phytochelatins compared to WT (Figure 3C–F). Thiol compounds represent a major mechanism for arsenic and heavy metal tolerance and accumulation in plants (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999; Zhu et al., 1999a; Zhu et al., 1999b; Schmöger et al., 2000; Dhankher et al., 2002; Li et al., 2004, 2005,2006; Gasic and Korban, 2007; Sung et al., 2007). Thiol compounds mediate complexation, translocation, and sequestration of heavy metals (Grill, 1987; Vatamaniuk et al., 1999; Gong et al., 2003; Chen et al., 2006; Mendoza-Cózatl et al., 2008). Thus, the increased accumulation of thiol compounds likely contributes to the enhanced arsenic tolerance and the increased arsenic accumulation in *ars5* compared to wild type seedlings. Figure 7 shows that the increased thiol accumulation in *ars5* correlates with an enhanced arsenic-induction of key genes that mediate thiol biosynthesis.

ARS5 is a negative regulator of arsenic tolerance

A plausible explanation for the inability of *paf2* to produce an arsenic tolerant phenotype (Figure 5C), may be the difference in *PAF2* expression compared to *PAF1*. Expression analyses, including wild type and *ars4ars5* microarray analyses (Figure 4), as well as RT-PCR analysis indicate that *PAF1* is highly expressed while *PAF2* expression is very low. Based on the lethality of *paf1paf2* double mutants, it is likely that the disruption of the highly expressed *PAF1* resulted in the production of PAF2-containing 26S proteasomes. This isoform seems able to fulfill the 26S proteasome function in global protein stability (Figure S3) but is insufficient to repress the PAF1-dependent arsenic tolerance mechanism of *ars5*, which suggests that *PAF1* might have additional functions other than protein degradation. This idea is further supported by the fact that expression of the *PAF2* cDNA, under the control of the 35S promoter, did not fully complement the germination phenotype of *ars5-1* compared to WT (Figure 6A). A similar relationship has been demonstrated for two *RPN1* genes encoding different components of the 26S proteasome in *Arabidopsis* (Brukhin et al., 2005). When the highly expressed RPN1a is knocked out, the endogenous expression of *RPN1b* was not sufficient to counteract the embryo lethality resulting from the loss of *RPN1a*, but overexpression of *RPN1b* was able to complement a *RPN1a* null mutation (Brukhin et al., 2005). It has also been shown that Arabidopsis mutants defective in the regulatory particle (19S) of the 26S proteasome display an increased tolerance to oxidative stress and this tolerance is explained by an increased capacity to remove oxidized proteins, which is catalyzed by the 20S particle (Kurepa et al., 2008).

PAF1 negatively regulates the expression of genes involved in thiol biosynthesis

Regulation, at the transcriptional level, of arsenic tolerance mechanisms has been well documented for *Synechocystis* and yeast. De-repression/activation of the arsBCH operon (arsenic efflux mechanism) in *Synechocystis* is mediated by the transcription factor arsR (Lopez-Maury et al., 2003). In *Saccharomyces cerevisiae*, increased sulfate assimilation, directed towards an enhanced synthesis of glutathione, is mediated by Yap1p and Yap8p (AP1-like transcription factors), which also regulate the expression of other arsenic detoxification genes such as *ACR2* (arsenate reductase) and *ACR3* (arsenite transporter) (Wysocki et al., 2004; Di and Tamas, 2007; Thorsen et al., 2007). Yap8p degradation is mediated by the 26S

proteasome and yeast mutants defective in 26S proteasome activity showed increased levels of Yap8p (Di and Tamas, 2007). In *Schizosaccharomyces pombe*, activation of genes related to sulfate assimilation and cysteine biosynthesis during cadmium exposure requires the ubiquitin-dependent degradation of the transcription factor Zip1 (Harrison et al., 2005).

Cadmium has been shown to activate transcription of genes that mediate GSH and PC biosynthesis in *Arabidopsis* (Xiang and Oliver, 1998; Lee and Korban, 2002). The results shown in Figure 7 suggest that arsenic induces the expression of γ-*ECS*, *GSH2* and *PCS1*, and this arsenic-induction is even stronger in *ars5* mutants. It is conceivable that, during arsenic stress, a positive regulator of sulfate assimilation and glutathione biosynthesis is rapidly degraded by the 26S proteasome, however, this positive regulator becomes stabilized in *ars5*, which in turn enhances the transcriptional arsenic-induced response. In embryonic stem cells, the 26S proteasome mediates repression of transcription of target genes by promoting both, the turnover of transcription factors and RNA polymerase II binding to DNA (Szutorisz et al., 2006). In the absence of PAF1, removal of repression of a positive regulatory component may function in the arsenic-induced enhancement of γ-*ECS*, *GSH2*, and *AtPCS1* mRNA levels.

Experimental procedures

Arsenic tolerant germination

To analyze arsenic tolerant germination, seeds were plated on arsenate-containing minimal medium (Lee et al., 2003) with the indicated concentrations of potassium arsenate and stratified at 4°C for two days in the dark. After five days of growth at 22°C, 75% humidity, with a 16-h-light/8-h-dark photoperiod regime at approximately 75 µmol m^{-2} s^{-1} light intensity in a Conviron growth chamber (Controlled Environments Inc., Pembina, ND), germinating seeds were examined under a dissecting microscope and those seedlings for which green cotyledons had emerged from seed coats were scored as surviving.

Genetic segregation analysis of ars5-1 x paf2 F2 population

ars5-1 and *paf2* mutant plants were reciprocally crossed. F1 seeds were self-pollinated to produce F2 seeds. 72 F2 plants were grown for 2 weeks and genotyped by genomic PCR. The primer pair of 5'-atcttcttgtatgagacaatatgaact-3'(F) and 5'-gcagatgtggataaaacagctaact-3'(R) distinguishes *ars5-1* from WT by generating a 1.8 kb PCR product from the WT *ARS5* gene and a 0.4 kb PCR product from the *ars5-1* mutant gene. The primer pair 5'-ttgaagaacagaggaa-3'(F) and 5'-gatcttcttttgatgattcttgg-3'(R) generates a 1.5 kb PCR product from the WT *PAF2* gene. The primer pair of 5'-ttgaagaacagaggaa-3'(F) and a T-DNA specific primer (5'-tggttcacgtagtgggccatcg-3') distinguishes *paf2* from WT by generating a 0.5 kb PCR product from the T-DNA inserted *PAF2* gene in *paf2* GABI_KAT mutant (GABI_419_H03). Segregation results were identical in both reciprocal crosses. Table 1 shows the segregation of F2 populations derived from *ars5-1* x *paf2*.

ICP-OES measurements

To measure arsenic and phosphorus, plants were grown on 10 µm nylon mesh (Fisher Scientific, Pittsburgh, PA) on minimal medium with 1% agar for 5 days, then nylon meshes with seedlings were transferred to minimal medium plates containing 100 µM potassium arsenate. After exposure to arsenate for 24, 48, and 96 hours, seedlings were harvested and briefly rinsed twice with dH$_2$O, then dried in a drying oven at 60 °C overnight, dry weight measured, and digested by boiling in concentrated nitric acid (Trace Metal grade, Sigma-Aldrich, St. Louis, MO, USA). The metal content of the digested samples was analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; Varian Vista). Total concentrations of metals were normalized based on dry weight of plant samples as described previously (Gong et al., 2004).

Thiol measurements by fluorescence HPLC

Thiol compounds including cysteine, γ-glutamyl cysteine, glutathione (GSH) and phytochelatins (PCs) were analyzed using fluorescence detection HPLC essentially as described previously (Fahey and Newton, 1987; Mendoza-Cozatl et al., 2008). To analyze the levels of thiol compounds produced by plants in response to arsenic, plants were grown on 1/4MS media plates without sucrose for 5 days then transferred to fresh media plates containing 100 µM potassium arsenate. In order to minimize oxidation of thiol compounds during extractions, plant seedlings were flash-frozen in liquid nitrogen and ground and extracted with extraction buffer (6.3 mM Diethylenetriamine-pentaacetic acid (DTPA) in 0.1% trifluoroacetic acid (TFA)) bubbled with nitrogen gas. Thiols were then derivatized with mono-bromobimane (mBBr) as described (Sneller *et al.*, 2000; Cazalé and Clemens, 2001) except that the peptides were separated with a Sunfire C_{18} column (5 µm, 4.6×250 mm; Waters) at 20°C and fluorescence was monitored on a Thermo Finnigan Fluoromonitor III fluorescence detector. The peaks of thiol compounds were identified by coupled parallel mass spectrometry measurements at the UCSD Superfund Biochemistry core as previously described (Chen *et al.*, 2006; Mendoza-Cózatl *et al.*, 2008) and quantified using Xcalibur software (Thermo Finnigan, Waltham, MA, USA). Thiol standards such as GSH, cysteine, γ-EC, and NAC were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Western blot analyses

For Western analysis of phytochelatin synthase levels in Arabidopsis, one week-old seedlings of WT and *ars5* were treated with 100 µM potassium arsenate for 0, 24, 48, 96 hours. Total proteins were extracted into an extraction buffer (50 mM Tris-HCl, pH 8.0). 30 µg of total proteins were loaded in each well in a 4–20% gradient SDS-PAGE and total proteins were transferred onto a PVDF membrane for 1 h at 100 V and then the membrane was blocked with 3% BSA in PBS overnight at 4°C. The protein bands on the PVDF membrane were probed against an anti-ubiquitin rabbit polyclonal antibody (1: 10,000 dilution) (PW9780-0025, Biomol, Plymouth Meeting, PA). Then the PVDF membrane was washed three times with 0.1% Triton X-100 in PBS. The membrane was then incubated with the secondary antibody conjugated with horseradish peroxidase (SuperSignal® West Dura kit, Pierce Biotechnology, Inc., Rockford, IL) for 1 hour at room temperature (1: 10,000 dilution). For chemiluminescence visualization, the reagents were added according to the manufacturer's instructions and then incubated for 5 minutes in the dark (Pierce Biotechnology, Inc., Rockford, IL). Chemiluminescence signals were developed on X-ray film (Fisher Scientific, Pittsburgh, PA).

RT-PCR expression analyses

Total RNA was extracted from one week-old Arabidopsis seedlings using the TRIzol reagent (Life Technologies/Gibco-BRL). Isolated RNA samples were treated with DNA-Free kit (AMBION, Austin TX, USA) to remove any DNA contamination. cDNA was reverse transcribed from the DNA-free RNA using First Strand cDNA kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and a Not I-d(T)$_{18}$ primer. Actin 7 (*ACT7*, At5g09810) mRNA content was used as loading control. The transcript levels of *ARS5* (At5g42750), *PAF2* (At1g47290), *SULTR1;2* (At1g7000) and *ACT7* (At5g09810) were PCR amplified by corresponding primer sets. *ARS5*: 5'-tcacagccgacggtcgtgtattg-3'(F) and 5'-gatcttctctagaagaatcccca-3'(R), *PAF2:* 5'-tcactgctgatggtcgtgttctc-3'(F) and 5'-gatcttcttttgatgattcttgg-3'(R), *SULTR1;2*: 5'-tggggacgtaactacactttca-3'(F) and 5'-aaggcaaggcggagatattc-3'(R). PCR reactions were stopped at 15, 20, 25, 30, and 40 cycles and subjected to gel electrophoresis. After 30 PCR cycles DNA products showed unsaturated bands reflecting initial transcript levels as shown in Figure 5B.

Real time qPCR expression analyses

Total RNA was extracted from one week-old Arabidopsis seedlings as described above and 5 ug of each total RNA were DNase treated (DNA-free, Ambion) and used for cDNA synthesis (First strand cDNA synthesis kit, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). 65 ul of water were added to each 15 ul of total cDNA and 3 ul were used for qPCR reactions using a SYBR Green based real time qPCR kit (LightCycler FastStart DNA Master plus SYBR Green I, Roche). Quantitative expression analysis of qPCR reactions was carried out using Lightcycler 3.0 software (Roche). The genes analyzed and the primers used for each gene were as follows: *CLATHRIN* (loading control): 5'-atacgcgctgagttccc-3'(F)/ 5'-ctgactggccctgctt-3'(R), *SULTR1;2(At1g78000)*: 5'-tggggacgtaactacactttca-3'(F)/ 5'-aaggcaaggcggagatattc-3'(R), *PAF1(At5g42790)*: 5'-tcacagccgacggtcgtgtaatg-3'(F)/ 5'-gatcttctctagaagaatcccca-3'(R), *PAF2*(At1g47250): 5'-tcactgctgatggtcgtgttctc-3'(F)/ 5'-gatcttcttttgatgattcttgg-3'(R), *γ-ECS*(At4g23100): 5'-gatgtgaattgacttcagcacaa-3'(F)/ 5'-gaggagtaagagagggcatcaa-3'(R), *GSH2*(At5g27380): 5'-ataagcagcctagcattccat-3'(F)/ 5'-gcatagactctgaacgtgtaccta-3'(R), *AtPCS1*(At5g15630): 5'-cctcactgggttcctcttaaact-3'(F)/ 5'-tttcagcctagacttctcctctg-3'(R), and *AtPCS2*(At1g03980): 5'-gaaggaaagcaaatcttcaatgaa-3'(F)/ 5'-ttgctcctgaagaatgagcta-3'(R). Three independent biological samples were prepared and three independent qPCR reactions were carried out per sample.

Microarray experiments

Col-0 WT and *ars4ars5* seeds were germinated on minimal media (Lee et al., 2003a), with or without 200 µM potassium arsenate. Total RNA was extracted from 10 day-old seedlings using the TRIzol reagent (Life Technologies/Gibco-BRL), labeled and hybridized using Affymetrix ATH1 chip arrays (Santa Clara, CA), containing approximately 22 500 *Arabidopsis* probes, at the University of California, San Diego Gene Chip Core facility. Raw data in Excel® format can be downloaded from Supplementary File 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

Alam MGM, Snow ET, Tanaka A. Arsenic and heavy metal contamination of vegetables grown in Samta village, Bangladesh. Sci Total Environ 2003;308:83–96. [PubMed: 12738203]

Book AJ, Smalle J, Lee KH, Yang P, Walker JM, Casper S, Holmes JH, Russo LA, Buzzinotti ZW, Jenik PD, Vierstra RD. The RPN5 Subunit of the 26s Proteasome Is Essential for Gametogenesis, Sporophyte Development, and Complex Assembly in Arabidopsis. Plant Cell 2009;21:460–478. [PubMed: 19252082]

Brown K, Ross G. American Council on Science and Health. Arsenic, drinking water, and health: a position paper of the American Council on Science and Health. Regul Toxicol Pharmacol 2002;36:162–174. [PubMed: 12460751]

Brukhin V, Gheyselinck J, Gagliardini V, Genschik P, Grossniklaus U. The RPN1 Subunit of the 26S Proteasome in Arabidopsis Is Essential for Embryogenesis. Plant Cell 2005;17:2723–2737. [PubMed: 16169895]

Carbonell A, Aarabi M, Delaune R, Grambrell R, Patrick W. Arsenic in wetland vegetation: availability, phytotoxicity, uptake and effects on plants growth and nutrition. Sci Total Environ 1998;217:189–199.

Catarecha P, Segura MD, Franco-Zorrilla JM, Garcia-Ponce B, Lanza M, Solano R, Paz-Ares J, Leyva A. A Mutant of the *Arabidopsis* Phosphate Transporter PHT1;1 Displays Enhanced Arsenic Accumulation. Plant Cell 2007;19:1123–1133. [PubMed: 17400898]

Cazalé AC, Clemens S. *Arabidopsis* thaliana expresses a second functional phytochelatin synthase. FEBS Letters 2001;507:215–219. [PubMed: 11684101]

Chen A, Komives EA, Schroeder JI. An Improved Grafting Technique for Mature *Arabidopsis* Plants Demonstrates Long-Distance Shoot-to-Root Transport of Phytochelatins in Arabidopsis. Plant Physiol 2006;141:108–120. [PubMed: 16531489]

Clemens S, Kim EJ, Neumann D, Schroeder JI. Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J 1999;18:3325–3333. [PubMed: 10369673]

Cobbett, CS.; Meagher, RB. Arabidopsis and the Genetic Potential for the Phytoremediation of Toxic Elemental and Organic Pollutants. In: Somerville, CR.; Meyerowitz, EM., editors. The Arabidopsis Book. Rockville, MD: American Society of Plant Biologists; 2002. doi/10.1199/tab.0032

Dhankher OP, Li Y, Rosen BP, Shi J, Salt D, Senecoff JF, Sashti NA, Meagher RB. Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and gamma-glutamylcysteine synthetase expression. Nat Biotechnol 2002;20:1140–1145. [PubMed: 12368812]

Di Y, Tamas MJ. Regulation of the arsenic-responsive transcription factor Yap8p involves the ubiquitin-proteasome pathway. J Cell Sci 2007;120:256–264. [PubMed: 17200139]

Fahey RC, Newton GL. Determination of low-molecular weight thiols using monobromobimane fluorescent labeling and high performance liquid chromatography. Methods Enzymol 1987;143:85–97. [PubMed: 3657565]

Gasic K, Korban S. Transgenic Indian mustard (Brassica juncea) plants expressing an *Arabidopsis* phytochelatin synthase (AtPCS1) exhibit enhanced As and Cd tolerance. Plant Mol Biol 2007;64:361–369. [PubMed: 17390107]

Gong JM, Lee DA, Schroeder JI. Long-distance root-to-shoot transport of phytochelatins and cadmium in *Arabidopsis*. Proc Natl Acad Sci U S A 2003;100:10118–10123. [PubMed: 12909714]

Gong JM, Waner DA, Horie T, Li SL, Horie R, Abid KB, Schroeder JI. Microarray-based rapid cloning of an ion accumulation deletion mutant in Arabidopsis thaliana. Proc Natl Acad Sci U S A 2004;101:15404–15409. [PubMed: 15486089]

Grill E. Phytochelatins, the heavy metal binding peptides of plants: characterization and sequence determination. Experientia Suppl 1987;52:317–322. [PubMed: 2959522]

Ha SB, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS. Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. Plant Cell 1999;11:1153–1164. [PubMed: 10368185]

Harrison C, Katayama S, Dhut S, Chen D, Jones N, Bahler J, Toda T. SCF[Pof1]-ubiquitin and its target Zip1 transcription factor mediate cadmium response in fission yeast. EMBO J 2005;24:599–610. [PubMed: 15660136]

Herbette S, Taconnat L, Hugouvieux V, Piette L, Magniette M-LM, Cuine S, Auroy P, Richarud P, Forestier C, Bourguignon J, Renou J-P, Vavasseur A, Leonhardt N. Genome-wide transcriptome profiling of the early cadmium response of *Arabidopsis* roots and shoots. Biochimie 2006;88:1751–1765. [PubMed: 16797112]

Hindmarsh JT. Arsenic, its clinical and environmental significance. The Journal of Trace Elements in Experimental Medicine 2000;13:165–172.

Kurepa J, Toh-e A, Smalle JA. 26S proteasome regulatory particle mutants have increased oxidative stress tolerance. Plant J 2008;53:102–114. [PubMed: 17971041]

Lee S, Korban SS. Transcriptional regulation of Arabidopsis thaliana phytochelatin synthase(AtPCS1) by cadmium during early stages of plant development. Planta 2002;215:689–693. [PubMed: 12172853]

Lee DA, Chen A, Schroeder JI. *ars1*, an Arabidopsis mutant exhibiting increased tolerance to arsenate and increased phosphate uptake. Plant J 2003a;35:637–646. [PubMed: 12940956]

Lee S, Moon JS, Ko T-S, Petros D, Goldsbrough PB, Korban SS. Overexpression of *Arabidopsis* Phytochelatin Synthase Paradoxically Leads to Hypersensitivity to Cadmium Stress. Plant Physiol 2003b;131:656–663. [PubMed: 12586889]

Li Y, Dhankher OP, Carreira L, Balish RS, Meagher RB. Arsenic and mercury tolerance and cadmium sensitivity *in Arabidopsis* palnts expressing bacterial γ-glutamyl cysteine synthetase. Environ Tox Chem 2005;24:1376–1386.

Li Y, Dankher OP, Carreira L, Smith AP, Meagher RB. The Shoot-Specific Expression of γ-Glutamylcysteine Synthetase Directs the Long-Distance Transport of Thiol-Peptides to Roots Conferring Tolerance to Mercury and Arsenic. Plant Physiol 2006;141:288–298. [PubMed: 16581878]

Li Y, Dhankher OP, Carreira L, Lee D, Chen A, Schroeder JI, Balish RS, Meagher RB. Overexpression of Phytochelatin Synthase in *Arabidopsis* Leads to Enhanced Arsenic Tolerance and Cadmium Hypersensitivity. Plant Cell Physiol 2004;45:1787–1797. [PubMed: 15653797]

Lopez-Maury L, Florencio FJ, Reyes JC. Arsenic sensing and resistance system in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J Bacteriol 2003;185:5363–5371. [PubMed: 12949088]

Lubin JH, Beane Freeman LE, Cantor KP. Inorganic Arsenic in Drinking Water: An Evolving Public Health Concern. J Natl Cancer Inst 2007;99:906–907. [PubMed: 17565151]

Marin A, Pezeshki S, Masscheleyn P, Choi H. Effect of dimethylarsenic acid (DMAA) on growth, tissue arsenic and photosynthesis in rice plant. J Plant Nutr 1993;16:865–880.

Meharg AA, Macnair MR. Suppression of the High Affinity Phosphate Uptake System: A Mechanism of Arsenate Tolerance in *Holcus lanatus* L. J Exp Bot 1992;43:519–524.

Mendoza-Cózatl DG, Butko E, Springer F, Torpey JW, Komives EA, Kehr J, Schroeder JI. Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap *of Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. Plant J 2008;54:249–259. [PubMed: 18208526]

Nocito FF, Lancilli C, Crema B, Fourcroy P, Davidian J-C, Sacchi GA. Heavy Metal Stress and Sulfate Uptake in Maize Roots. Plant Physiol 2006;141:1138–1148. [PubMed: 16698905]

Pilon-Smits E, Pilon M. Phytoremediation of metals using transgenic plants. Crit Rev Plant Sci 2002;21:439–456.

Rosen BP, Weigel U, Karkaria C, Gangola P. Molecular characterization of an anion pump. The *arsA* gene product is an arsenite(antimonate)-stimulated ATPase. J Biol Chem 1988;263:3067–3070. [PubMed: 2449436]

Schmöger ME, Oven M, Gril IE. Detoxification of arsenic by phytochelatins in plants. Plant Physiol 2000;122:793–801. [PubMed: 10712543]

Smalle J, Kurepa J, Yang P, Babiychuk E, Kushnir S, Durski A, Vierstra RD. Cytokinin Growth Responses in *Arabidopsis* Involve the 26S Proteasome Subunit RPN12. Plant Cell 2002;14:17–32. [PubMed: 11826296]

Smalle J, Kurepa J, Yang P, Emborg TJ, Babiychuk E, Kushnir S, Vierstra RD. The Pleiotropic Role of the 26S Proteasome Subunit RPN10 in *Arabidopsis* Growth and Development Supports a Substrate-Specific Function in Abscisic Acid Signaling. Plant Cell 2003;15:965–980. [PubMed: 12671091]

Sneller FEC, van Heerwaarden LM, Koevoets PLM, Vooijs R, Schat H, Verkleij JAC. Derivatization of phytochelatins from *Silene vulgaris*, induced upon exposure to arsenate and cadmium: Comparison of derivatization with Ellman's reagent and monobromobimane. J Ag Food Chem 2000;48:4014–4019.

Sung D-Y, Lee D, Harris H, Raab A, Feldmann J, Meharg A, Kumabe B, Komives EA, Schroeder JI. Identification of an arsenic tolerant double mutant with a thiol-mediated component and increased arsenic tolerance in *phyA* mutants. Plant J 2007;49:1064–1075. [PubMed: 17335514]

Szutorisz H, Georgiou A, Tora L, Dillon N. The Proteasome Restricts Permissive Transcription at Tissue-Specific Gene Loci in Embryonic Stem Cells. Cell 2006;127:1375–1388. [PubMed: 17190601]

Thorsen M, Lagniel G, Kristiansson E, Junot C, Nerman O, Labarre J, Tamás MJ. Quantitative transcriptome, proteome, and sulfur metabolite profiling of the Saccharomyces cerevisiae response to arsenite. Physiol Genomics 2007;30:35–43. [PubMed: 17327492]

Tripathi RD, Srivastava S, Mishra S, Singh N, Tuli R, Gupta DK, Maathuis FJ. Arsenic hazards: strategies for tolerance and remediation by plants. Trends Biotechnol 2007;25:158–165. [PubMed: 17306392]

Vatamaniuk OK, Mari S, Lu YP, Rea PA. AtPCS1, a phytochelatin synthase from *Arabidopsis*: isolation and in vitro reconstitution. Proc Natl Acad Sci U S A 1999;96:7110–7115. [PubMed: 10359847]

Wang J, Zhao F-J, Meharg AA, Raab A, Feldmann J, McGrath SP. Mechanisms of Arsenic Hyperaccumulation in *Pteris vittata*. Uptake Kinetics, Interactions with Phosphate, and Arsenic Speciation. Plant Physiol 2002;130:1552–1561. [PubMed: 12428020]

Wysocki R, Fortier P-K, Maciaszczyk E, Thorsen M, Leduc A, Odhagen A, Owsianik G, Ulaszewski S, Ramotar D, Tamas MJ. Transcriptional Activation of Metalloid Tolerance Genes in *Saccharomyces cerevisiae* Requires the AP-1-like Proteins Yap1p and Yap8p. Mol Biol Cell 2004;15:2049–2060. [PubMed: 14978214]

Xiang C, Oliver DJ. Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 1998;10:1539–1550. [PubMed: 9724699]

Yang P, Fu H, Walker J, Papa CM, Smalle J, Ju Y-M, Vierstra RD. Purification of the Arabidopsis 26 S Proteasome: Biochemical and molecular analyses revealed the presence of multiple isoforms. J Biol Chem 2004;279:6401–6413. [PubMed: 14623884]

Zhu LY, Pilon-Smits EA, Jouanin L, Terry N. Overexpression of glutathione synthetase in indian mustard enhances cadmium accumulation and tolerance. Plant Physiol 1999a;119:73–80.

Zhu YL, Pilon-Smits EA, Tarun AS, Weber SU, Jouanin L, Terry N. Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing gamma-glutamylcysteine synthetase. Plant Physiol 1999b;121:1169–1178. [PubMed: 10594104]
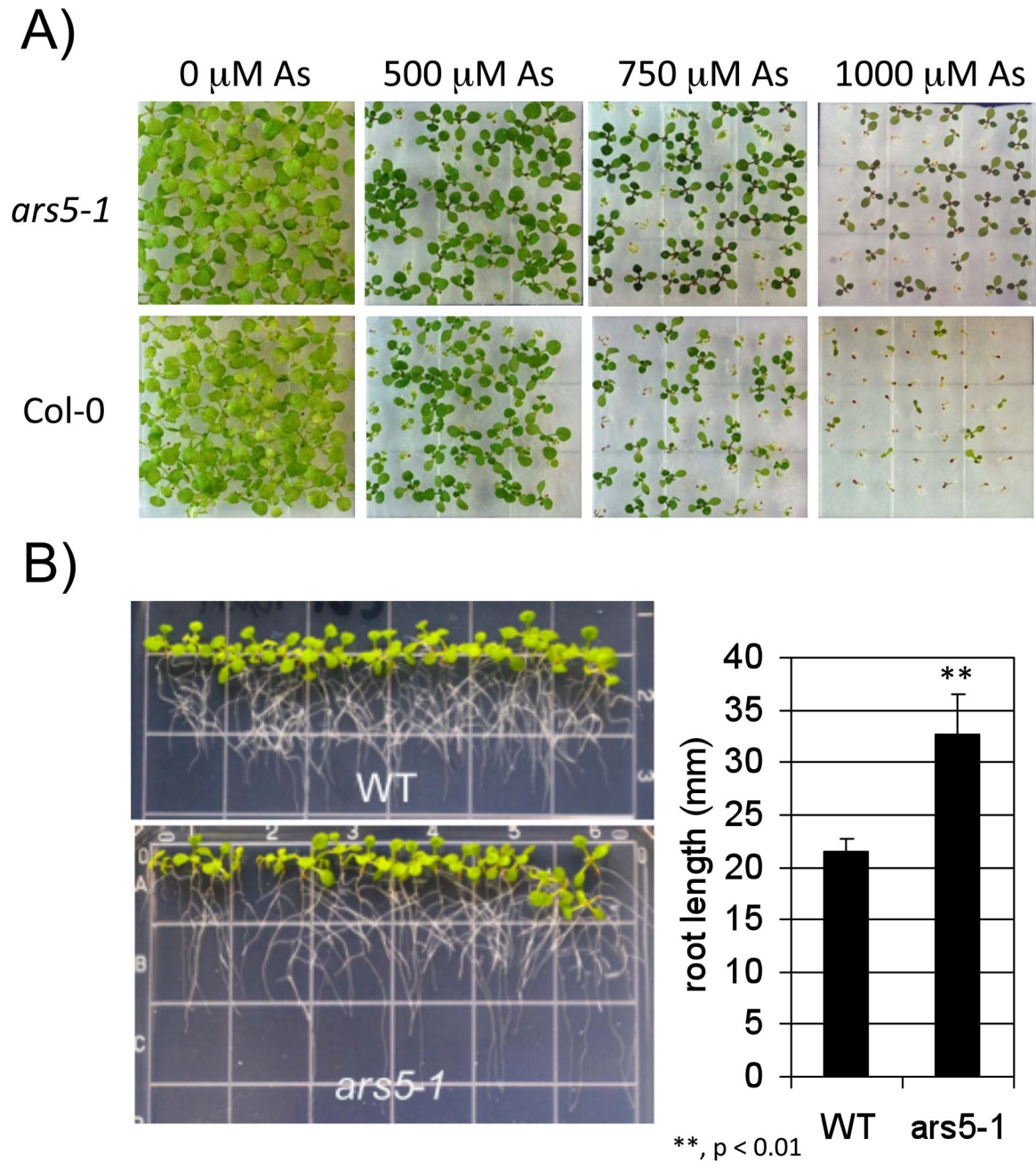
Figure 1. Arsenic tolerant seedling growth of *ars5-1*
(**A**) WT and *ars5-1* seeds were grown on minimal growth medium with 0, 500, 750, and 1000 µM arsenate (As^V). Images were photographed after one week of growth at 22 °C. (**B**) Seedlings (4 day-old) from WT and *ars5-1* were grown on minimal medium and transferred to plates containing 10 µM arsenite (As^{III}). Seedlings were grown for an additional week for root length measurements. ** denotes statistical differences ($p < 0.01$) between *ars5-1 vs* WT.
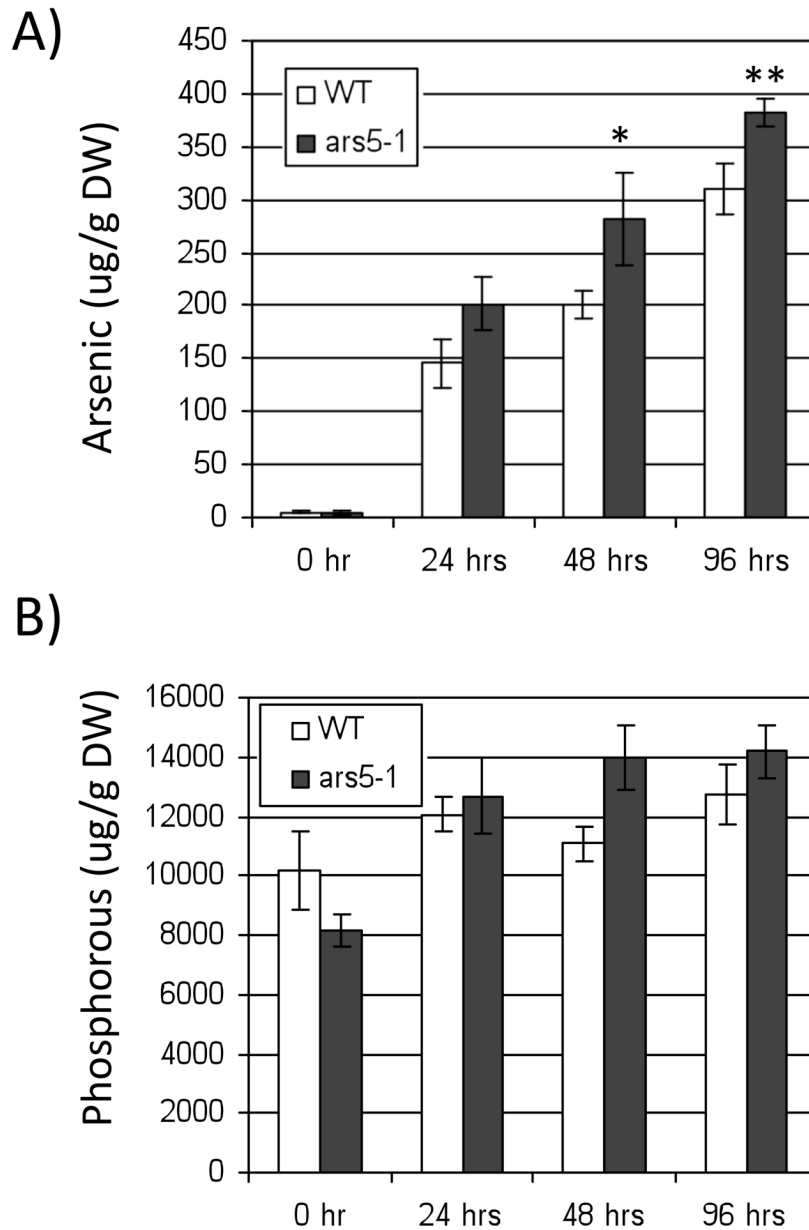
*, p < 0.05; **, p < 0.01

Figure 2. Arsenic and phosphorus accumulation in arsenate exposed wild type and *ars5-1* seedlings
One week-old seedlings were exposed to 100 µM potassium arsenate for 0, 24, 48, 96 hrs and
the levels of arsenic (**A**) and phosphorus (**B**) were analyzed by ICP-OES. Y-axis shows the
concentration of elements. Error bars represent standard error of the mean (n=4). The different
levels of statistical significance between *ars5-1* and wild type, at equal times of arsenic
exposure, are denoted with asterisks (*, $p < 0.05$; **, $p < 0.01$).
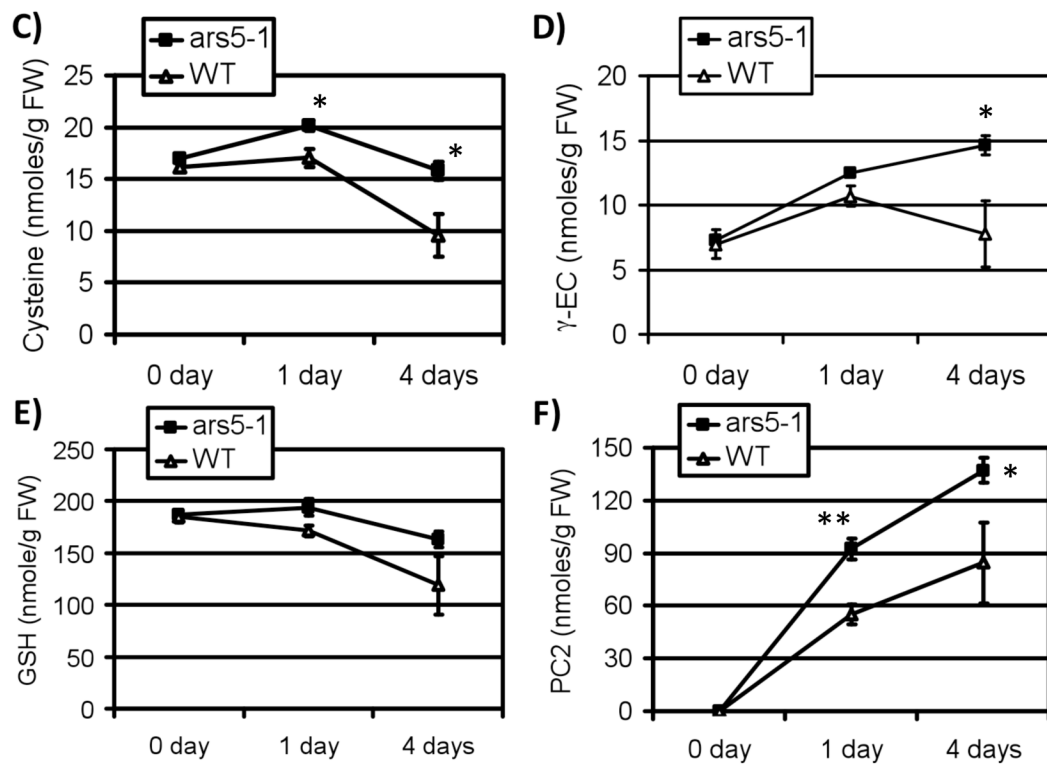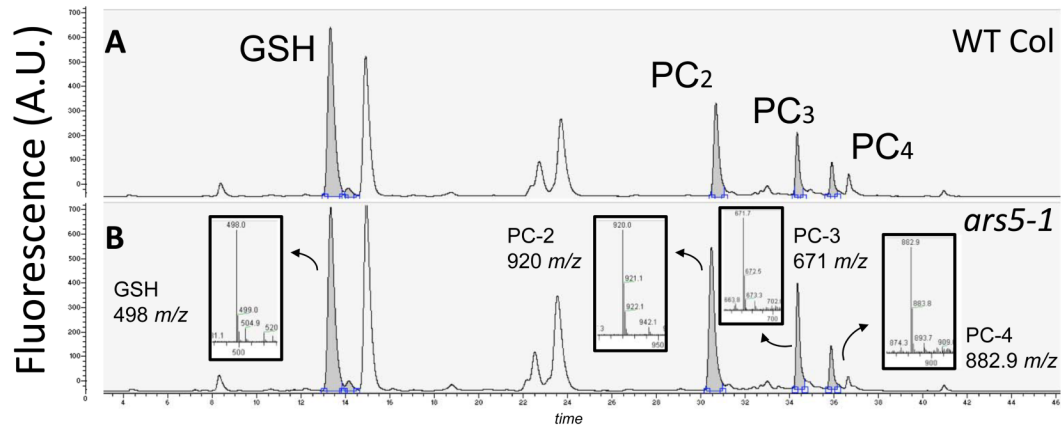
*, p < 0.05; **, p < 0.01

**Figure 3. Thiol quantification in *ars5-1* and WT**
HPLC-MS chromatograms of WT (**A**) or *ars5-1* (**B**) exposed to 100 µM potassium arsenate for 96 hrs. Thiols were separated by HPLC, identified by tandem mass spectrometry and quantified by their respective bimane-derivative fluorescence. (**C–F**) Col-0 WT and *ars5-1* seedlings (5 day-old) were treated with 100 µM arsenate for 0, 1, 4 days. The levels of (**C**) cysteine, (**D**) γ-EC, (**E**) GSH and (**F**) PC2 in whole seedlings were measured by fluorescence HPLC. Error bars represent SEM (n=3). The different levels of statistical significance between *ars5-1* and wild type, at equal times of arsenic exposure, are denoted with asterisks (*, p < 0.05; **, p < 0.01).
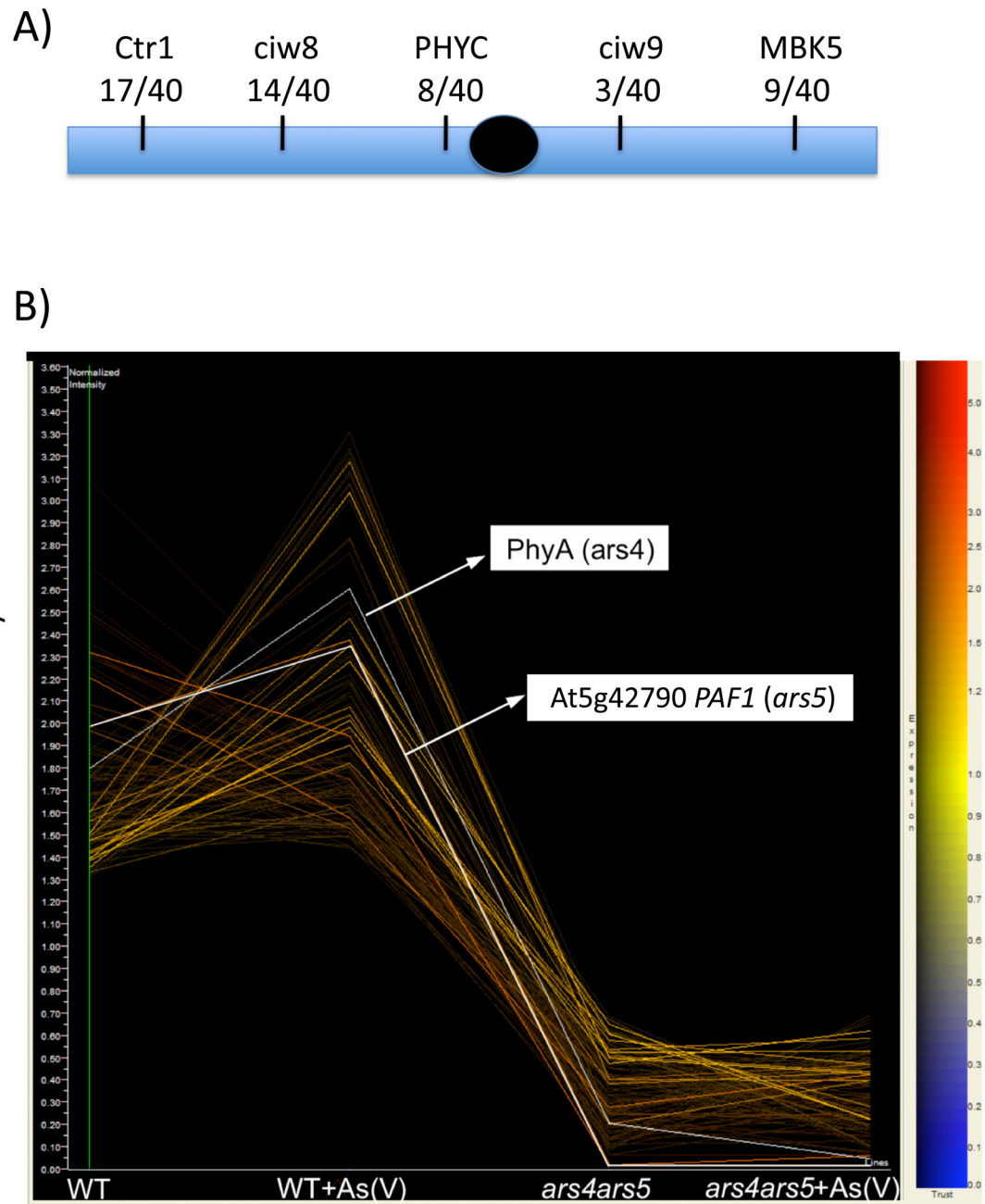
Figure 4. Microarray-based mapping of *ARS5*
(**A**) PCR-based markers on chromosome 5 are indicated at the top diagram. The number of recombination events per total number of meiotic events scored is given below each marker. (**B**) Microarray experiments of WT and *ars4ars5* double mutant plants grown with or without arsenate identified 110 strongly repressed genes in *ars4ars5* in the presence or absence of arsenic stress. Highlighted arrows show the expression values of *ARS5* (At5g42790) *and ARS4/ PHYA* (At1g09570). The vertical axis of the graph represents normalized gene expression levels.
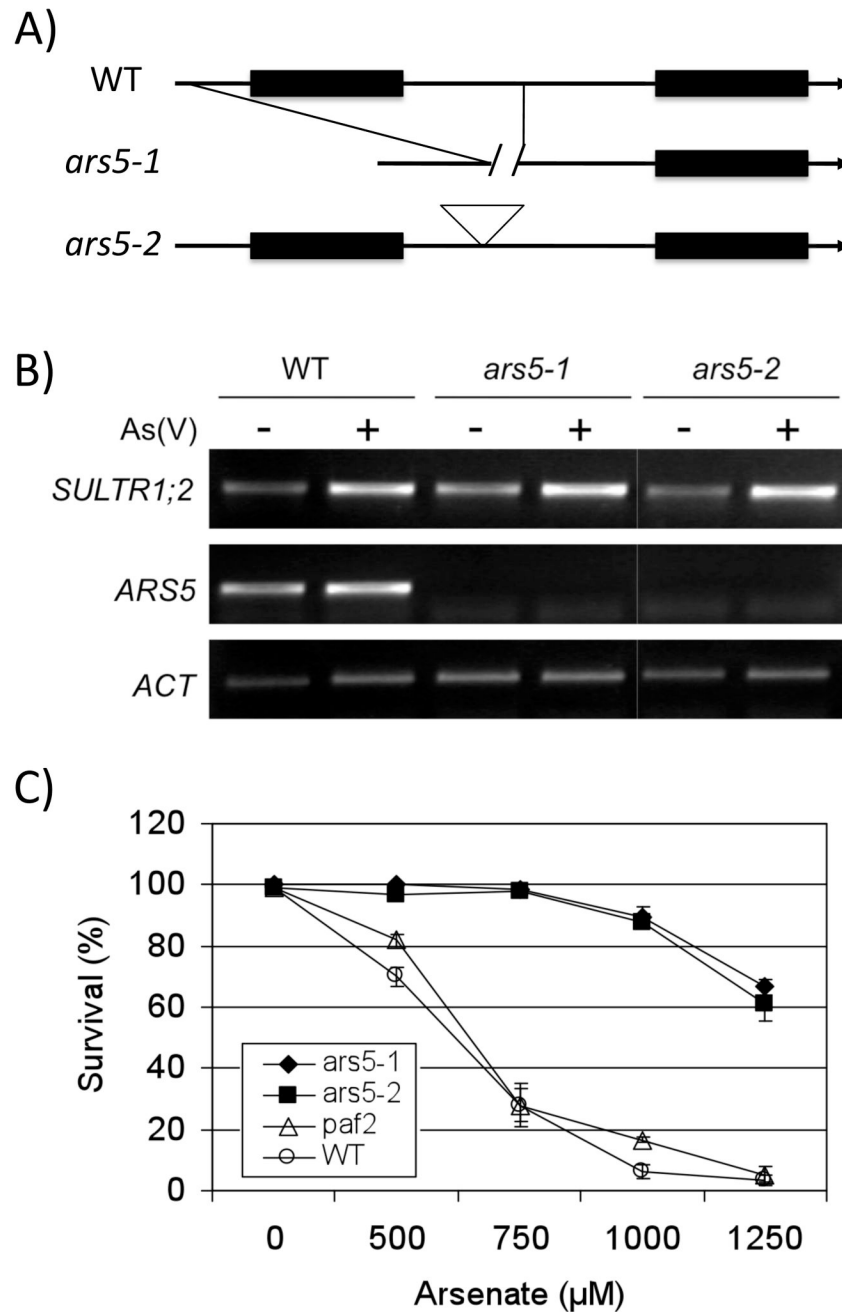
Figure 5. Identification of the *ars5* gene and analysis of arsenic tolerance of *ars5-1, ars5-2 (paf1)* and *paf2*

(**A**) Two *ars5* mutant alleles, *ars5-1* and *ars5-2 (paf1)*: *ars5-1* was isolated from the *ars4ars5* double mutant and has a 1.4Kb deletion spanning part of the promoter and the entire first exon of the *ARS5* gene. *ars5-2* is GABI T-DNA insertion allele (GABI_419_H03) and contains a T-DNA in intron 1. (**B**) RT-PCR analysis of WT, *ars5-1, ars5-2* (SALK_098236), and *paf2* (GABI_419_H03). *Sultr1;2* (At1g78000) was used as a positive control of an arsenic-induced transcript. Transcript levels were analyzed by RT-PCR of *ARS5* (At5g42790), *PAF2* (At1g47250) and *ACT7* (AT5G09810). (−): control, (+): arsenic stress (100 µM arsenate for 24 hours). (**C**) arsenic tolerant germination of *ars5* and *paf2* mutant alleles. Y-axis represents

the percent of surviving seedlings under arsenic stress after 4 days at 22 °C. Seedlings with open green cotyledons were scored as surviving. X-axis represents the concentration of arsenate (μM). Error bars represent SEM (n=3).
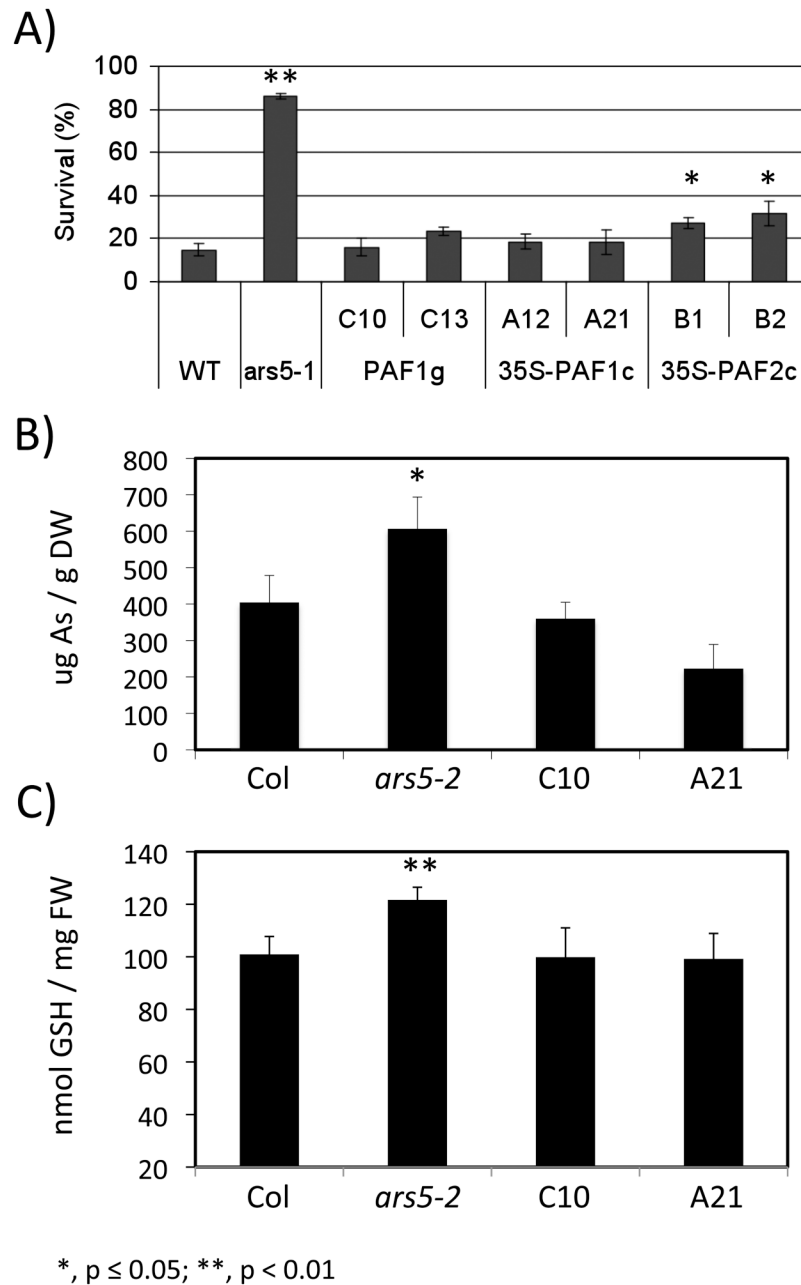
Figure 6. Genetic complementation of *ars5-1* and characterization of the ars5-2 allele
(**A**) Seeds from Col-0 WT, *ars5-1*, two lines complemented with genomic DNA (C10 and C13; promoter and the coding region of *PAF1*, PAF1g), two lines complemented *PAF1* cDNA (A12 and A21; 35S–*PAF1*c) and two lines complemented with *PAF2* cDNA (B1 and B2; 35S–*PAF2*c) were germinated on minimal medium containing 1000 µM arsenate. Seedling survival was scored after 4 days at 22 °C. Error bars represent SEM (n=3). (**B–C**) Accumulation of arsenic (**B**) and GSH content (**C**), expressed in thiol (-SH) equivalents, in *ars5-2* and two independent lines of *ars5-1* complemented with a genomic fragment of *PAF1* (C10) or with the *PAF1* cDNA under the control of the 35S promoter (A21). Statistical differences between *ars5-2 ars5-1* and *ars5-1* complemented with *PAF2 vs* wild type are denoted with asterisks (*,

p ≤ 0.05; **, p < 0.01). One week-old seedlings were exposed to 100 µM potassium arsenate for 96 hrs and the levels of arsenic were measured by ICP-OES and the glutathione content was measured by fluorescence HPLC. Error bars represent standard errors of the mean (n=3).
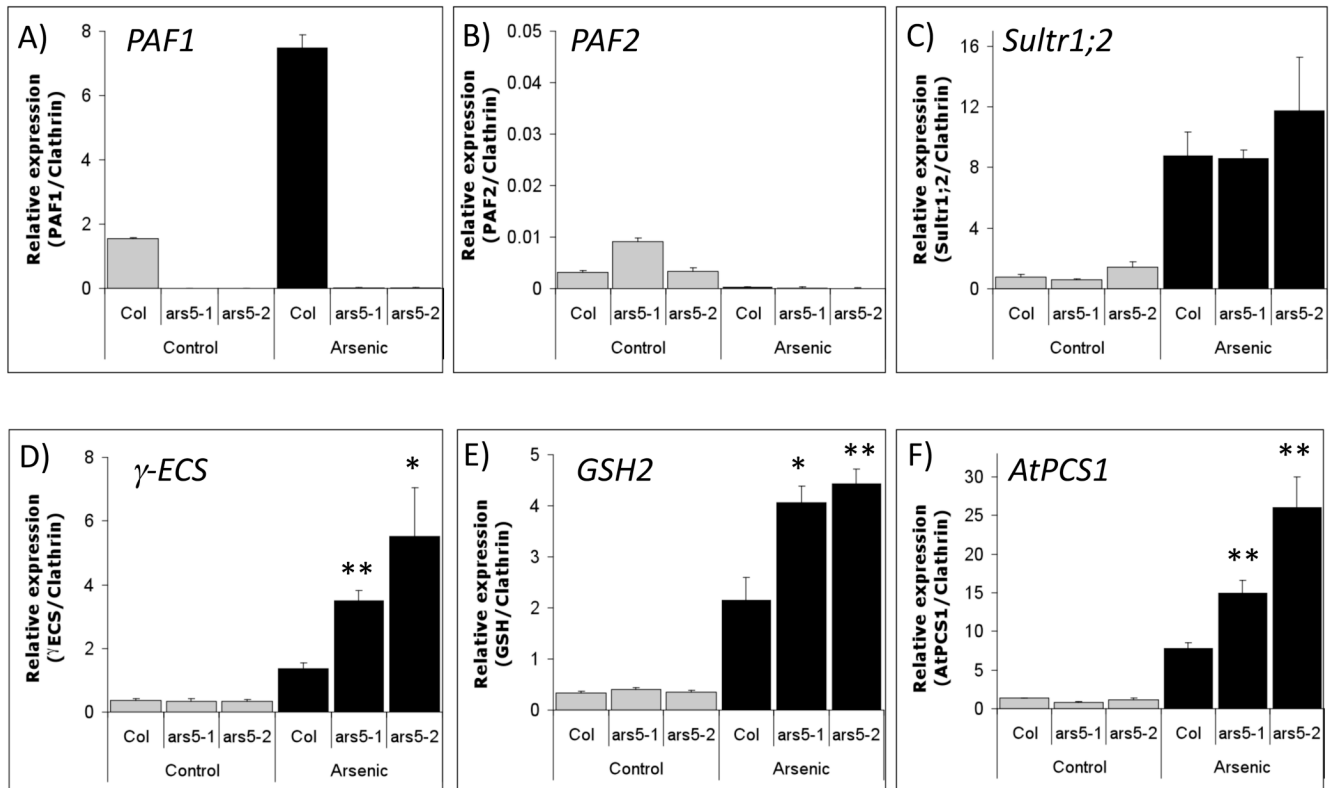
*, p < 0.05; **, p < 0.01

Figure 7. Quantitative PCR analysis of *PAF* genes and genes involved sulfate assimilation and thiol biosynthesis
Expression analysis (qPCR) of (**A**) *PAF1*, (**B**) *PAF2*, (**C**) *Sultr1;2*, (**D**) γ-*ECS*, (**E**) *GSH* synthetase and (**F**) *AtPCS1* from Col-0 WT, *ars5-1* and *ars5-2* seedlings (5 day-old) treated or not with 100 µM arsenate for 96 hours. cDNA was synthesized from RNA obtained from whole seedlings and used for qPCR analysis. Expression was normalized to Clathrin. Error bars represent SEM (n=3). Statistical differences between *ars5-1* and *ars5-2* compared to wild type, both exposed to arsenic, are denoted with asterisks (*, p < 0.05; **, p < 0.01).
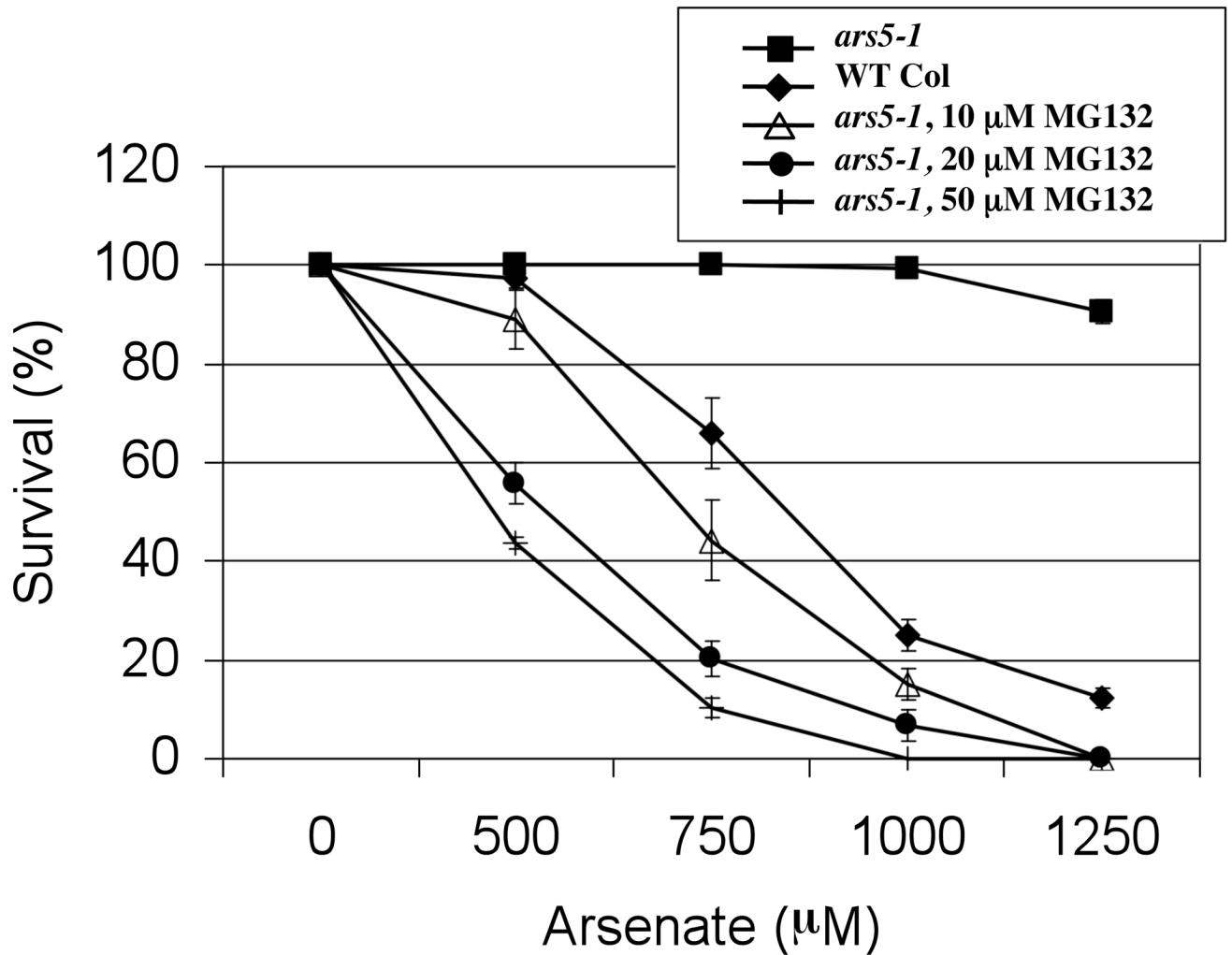
Figure 8. Proteasome inhibition reverts the arsenic tolerance phenotype of *ars5*
Col-0 WT and *ars5-1* seeds were germinated on minimal medium containing 0, 500, 750, 1000, 1250 µM arsenate. Where indicated, *ars5-1* seeds were also germinated on plates containing 10, 20 and 50 µM MG132, a potent proteasome inhibitor. Germination of *ars5* without MG132 treatment is shown as a positive control for arsenic tolerant germination. Seedling survival was scored on the 4th day based on expanded green cotyledons. Error bars represent SEM (n=3).
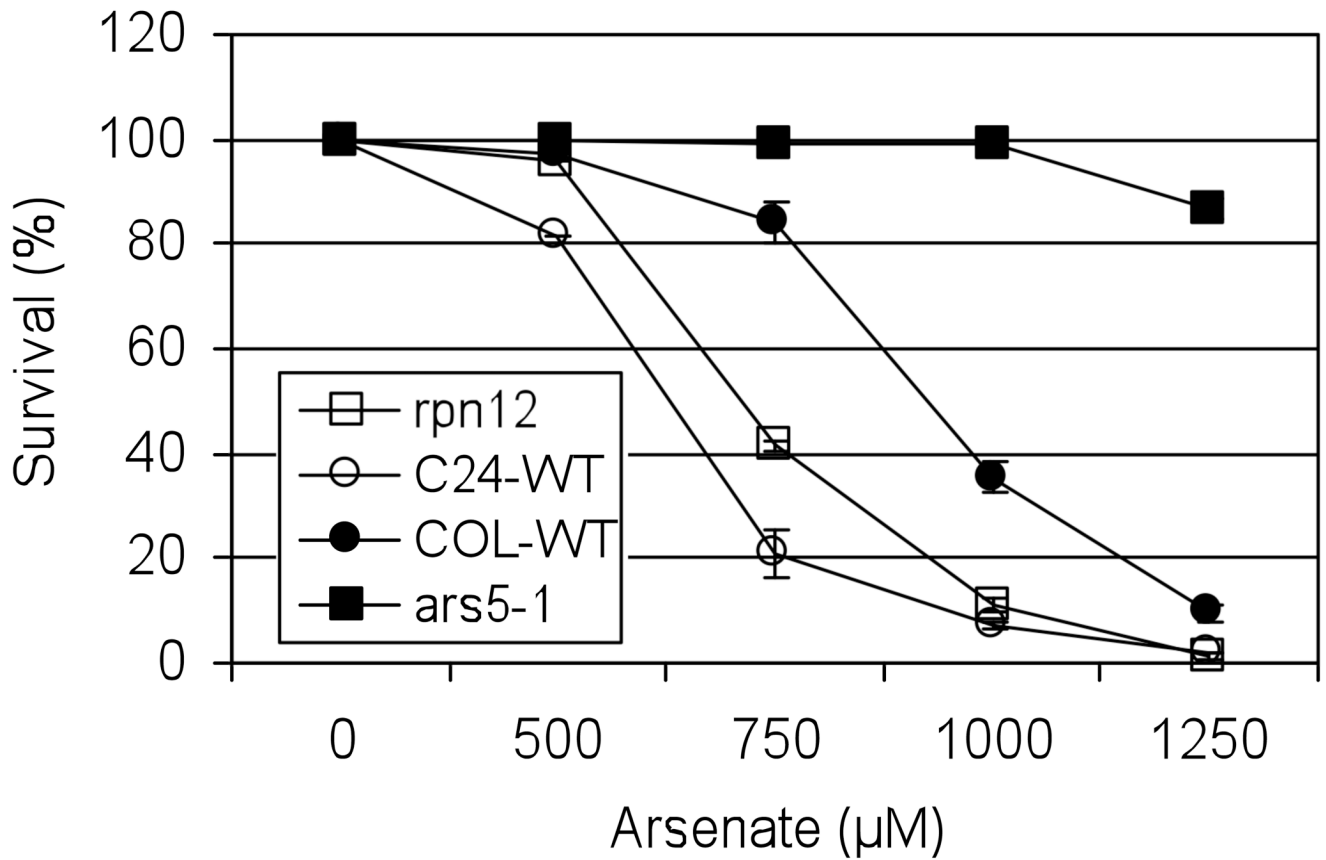
Figure 9. Arsenic tolerance in regulatory-particle mutants of the 26S proteasome *(rpn12)*
ars5-1 and *rpn12* mutant seeds were germinated on minimal medium containing 0, 500, 750, 1000, 1250 µM arsenate together with their corresponding WT accession seeds (Col-0 or C24). Seedling survival was scored on the 4th day based on expanded cotyledons. Error bars represent SEM (n=3).

Table 1

Segregation in the F2 population from *ars5-1/ars5-1* x *paf2/paf2* crosses.

	++	+*paf2*	*ars5-1+*	*ars5-1paf2*
++	++/++	++/*paf2*+	*ars5-1+/++*	*X*
+*paf2*	++/*paf2*+	++/*paf2 paf2*	*ars5-1+/paf2+*	*X*
ars5-1	*ars5-1+/++*	*ars5-1+/paf2+*	*ars5-1ars5-1/++*	*X*
ars5-1paf2	*X*	*X*	*X*	*X*

+: wild type copy of the gene

x: The offspring was lethal because gametophytes with the *ars5-1paf2* double mutation did not survive.