The transcription factor MYB40 is a central regulator in arsenic resistance in Arabidopsis

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https://doi.org/10.1016/j.xplc.2021.100234

ABSTRACT
Arsenic is a metalloid that is toxic to plants. Arsenate (As(V)), the prevalent chemical form of arsenic, is a phosphate (Pi) analog and is incorporated into plant cells via Pi transporters. Here, we found that the MYB40 transcription factor played important roles in the control of Arabidopsis As(V) resistance. The expression of MYB40 was induced by As(V) stress. MYB40-overexpressing lines had an obvious As(V)-resistant phenotype and a reduced As(V)/Pi uptake rate, whereas myb40 mutants were sensitive to As(V) stress. Upon exposure to As(V), MYB40 directly repressed the expression of PHT1;1, which encodes a main Pi transporter. The As(V)-resistant phenotypes of MYB40-overexpressing lines were impaired by overexpression of PHT1;1, demonstrating an epistatic genetic relationship between MYB40 and PHT1;1. Moreover, overexpression of MYB40 enhanced, and disruption of MYB40 reduced, thiol-peptide contents. Upon exposure to As(V), MYB40 positively regulated the expression of PCS1, which encodes a phytochelatin synthase, and ABCC1 and ABCC2, which encode the major vacuolar phytochelatin transporters. Together, our data demonstrate that AtMYB40 acts as a central regulator of As(V) responses, providing a genetic strategy for enhancing plant As(V) tolerance and reducing As(V) uptake to improve food safety.

Key words: AtMYB40, arsenate, uptake, detoxification, transcription

Chen Y., Wang H.-Y., and Chen Y.-F. (2021). The transcription factor MYB40 is a central regulator in arsenic resistance in Arabidopsis. Plant Comm. 2, 100234.

INTRODUCTION
Arsenic (As) is ubiquitous in the environment and is one of the most toxic metalloids found in soils (Zhao et al., 2010; Kumar et al., 2015). People experience As poisoning by drinking As-contaminated water and ingesting crops cultivated in As-polluted soils (Zhao et al., 2010). Arsenic is nonessential and toxic to plants, but it is impossible to entirely block its entry into plants because it shares transporters with essential or beneficial elements, such as phosphate (Pi) and silicon (Catarecha et al., 2007; Ma et al., 2008).

Arsenic is found primarily in inorganic forms, and arsenate (As(V)) and arsenite (As(III)) are the most common oxidation forms (Chao et al., 2014). In aerobic soils, As(V) is the predominant species and is taken up mainly via Pi transporters (Shin et al., 2004; Catarecha et al., 2007; Wu et al., 2011; Remy et al., 2012; Castrillo et al., 2013; DiTusa et al., 2016). In Arabidopsis thaliana, PHT1;1 and PHT1;4 are the main Pi transporters, and the pht1;1ΔΔ4Δ double mutant shows a 75% reduction in Pi uptake capacity relative to wild-type plants (Shin et al., 2004). Upon exposure to As(V), both the pht1;1 mutant and the pht1;1ΔΔ4Δ double mutant displayed significant As(V)-resistant phenotypes (Shin et al., 2004). Later, based on As(V) toxicity screening, the pht1;1-3 mutant was identified, which harbors a missense mutation in PHT1;1 (Catarecha et al., 2007). The pht1;1-3 mutant showed an As(V)-tolerant phenotype and enhanced As accumulation (Catarecha et al., 2007). As(V) represses the expression of PHT1;1 and influences the relocalization of PHT1;1 (Castrillo et al., 2013). In fact, some proteins that modulate the expression or localization of PHT1;1 are also involved in responses to As(V) stress (González et al., 2005; Castrillo et al., 2013; Wang et al., 2014; Su et al., 2015).

After being taken up into the roots, As(V) is rapidly reduced to As(III) (Pickering et al., 2000). Several arsenate reductases have been identified in plants, such as ATQ1 (also known as HAC1) from Arabidopsis (Chao et al., 2014; Sánchez-Bermejo et al., 2014), PvACR2 from Pteris vittata (Ellis et al., 2006), and OsACR2.1 and OsACR2.3 from Oryza sativa (Duan et al., 2007). Loss of ATQ1 leads to a lack of As(III) efflux ability in roots and...
a significant increase in As accumulation in shoots (Chao et al., 2014). As(III) is either extruded from the roots or detoxified through complexation and vacuolar sequestration (Zhao et al., 2010). The complexation of As(III) by phytochelatins (PCs) is an important mechanism of As detoxification. Upon exposure to As stress, synthesis and accumulation of PCs are induced (Grill et al., 1987; Sneller et al., 1999; Schmöger et al., 2000). The Arabidopsis phytochelatin synthase PCS1-defective mutant cad1-3, which lacks the function of PC synthase, produces few PCs and shows As(V)-hypersensitive phenotypes (Howden et al., 1995; Ha et al., 1999). Overexpression of AtPCS1 leads to enhanced As(V) tolerance and increased PC accumulation (Lee et al., 2003; Li et al., 2004). Plant vacuoles are the final detoxification stores for As, and PC–As(III) complexes are transported into the vacuoles by ABCC-type transporters (Song et al., 2010, 2014). The Arabidopsis abcc1 abcc2 double mutant is sensitive to As(V) stress, and AtPCS1 AtABCC1 co-overexpression lines show As(V)-tolerant phenotypes (Lee et al., 2003). As is translocated through the xylem to the shoots and is then loaded from the xylem into the phloem and seeds. A recent report showed that the inositol transporters AtINT2 and AtINT4 contribute to As loading into the phloem (Duan et al., 2015).

Upon exposure to As stress, the transcript levels of numerous genes, including key genes involved in As uptake, PC biosynthesis, and As translocation, undergo significant changes that help plants to survive sub-optimal growth conditions (Li et al., 2004; Abercrombie et al., 2008; Sung et al., 2009; Castrillo et al., 2013; Chao et al., 2014; Sánchez-Bermúdez et al., 2014; Kumar et al., 2015). Several articles have reported the relevant regulatory mechanisms. Disruption of ACR1 in yeast results in As(III) and As(V) hypersensitivity, and ACR1 encodes a transcription factor that regulates transcriptions of ACR3, which encodes an arsenite transporter (Sobrowicz et al., 1997; Ghosh et al., 1999). Arabidopsis WRKY6 is an arsenate-responsive transcription factor that mediates arsenate/phosphate transporter gene expression and arsenate-induced transposon activation (Castrillo et al., 2013).

In this study, we found that the transcription factor AtMYB40 played an important role in As resistance. MYB40 was induced by As(V) stress, and MYB40-overexpressing lines were As(V) resistant. Upon exposure to As(V), MYB40 repressed the expression of the As(V)/Pi transporter gene PHT1;1, thereby decreasing As(V)/Pi uptake, and positively regulated the expression of PCS1, ABCC1, and ABCC2 to enhance As detoxification ability.

**RESULTS**

**MYB40 is an As(V)-responsive transcription factor**

Arabidopsis MYB40 belongs to the R2R3 MYB sub-family (Stracke et al., 2001). To test the subcellular location of MYB40, the coding region of MYB40 was fused with the 3’ end of the GFP reporter gene and expressed under the Super promoter. The GFP gene alone under the Super promoter was used as a control. MYB40-GFP alone was transiently expressed in Nicotiana benthamiana leaves. The MYB40-GFP fusion protein was localized in the nucleus and GFP alone was localized in the cytoplasm and nucleus (Figure 1A). When exposed to As(V) stress, the transcript level of MYB40 was clearly elevated (Figure 1B). These data indicate that MYB40 is an As(V)-responsive MYB transcription factor.

Overexpression of MYB40 enhances Arabidopsis As(V) resistance

To test the function of MYB40 on As(V) tolerance, MYB40-overexpression lines were generated. The coding sequence of MYB40 was expressed under the cauliflower mosaic virus 35S promoter, and two homozygous single-copy MYB40-overexpressing lines
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Figure 2. Overexpression of MYB40 increases Arabidopsis As(V) resistance and represses As(V)/Pi uptake.

(A) qRT–PCR analysis of MYB40 expression in the MYB40-overexpressing lines (OE11 and OE26) and wild-type plants (WT). The qRT–PCR was performed with three technical replicates, and the experiment was repeated at least three times with similar results.

(B and C) Phenotypic comparison. MYB40-overexpressing lines and WT plants were germinated and grown on 1/2 MS medium containing 0 or 200 μM As(V) for 7 d (B) and 15 d (C), and then representative photographs were taken. Scale bar corresponds to 0.5 cm.

(D and E) Fresh weight and primary root length analysis. The MYB40-overexpressing lines and WT plants were germinated and grown on 1/2 MS medium containing 0, 100, 200, 300, and 400 μM As(V) for 7 d, and then the fresh weight (D) and primary root length (E) were measured. The values are means ± SE; n = 3 in (D) and (n ≥ 40) in (E).

(F) As(V) uptake analysis. Seven-d-old plants were transferred to 1/2 MS medium with 200 μM As(V) for 6 h, then harvested for As(V) uptake analysis. Data are shown as mean ± SE; n = 3.

(G) As content measurement. Seven-d-old plants were transferred to 1/2 MS medium with 200 μM As(V) for 1 or 3 d, then harvested for As content measurement. Data are shown as mean ± SE; n ≥ 5.

(H) Pi uptake was monitored over a 4-h period in 7-d-old MYB40-overexpressing lines and WT plants. Data are shown as mean ± SE; n = 3.

(I) Pi concentration of 7-d-old MYB40-overexpressing lines and WT plants grown on MS medium. Data are shown as mean ± SE; n = 3.

(Asterisks in (A, D, E, F, G, H, and I) indicate significant differences compared with WT plants (⁴) by Student’s t-test: *P < 0.05, **P < 0.01.

(OE11 and OE26) were obtained (Figure 2A). There were no obvious differences among the tested genotypes when grown on 1/2 Murashige and Skoog (MS) medium with 0 μM As(V) (Figure 2B and 2C, top panel). When germinated and grown on 1/2 MS medium with 200 μM As(V) for 7 d, the MYB40-overexpressing lines (OE11 and OE26) showed As(V)-tolerant
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phenotypes with longer primary roots than wild-type seedlings (Figure 2B, bottom panel). When grown for 15 d, wild-type plants displayed obvious As(V) toxicosis symptoms, with stunted growth and yellow leaves, whereas the OE11 and OE26 lines remained healthy, with larger, green leaves (Figure 2C, bottom panel). Fresh weight and root length were also measured under As(V) stress. Upon exposure to different concentrations of As(V), the OE11 and OE26 lines had much higher fresh weights and longer primary roots than wild-type plants (Figure 2D and 2E), indicating that overexpression of MYB40 enhances Arabidopsis As(V) tolerance.

As MYB40-overexpressing lines displayed As(V)-resistant phenotypes (Figure 2B and 2C), and As(V) is taken up via Pi transporters from the environment (Shin et al., 2004; Catarecha et al., 2007; Remy et al., 2012), we hypothesized that MYB40 may repress As(V) uptake. First, the As(V) uptake rate was measured. After exposure to 200 μM As(V) for 6 h, the OE11 and OE26 lines had lower As(V) uptake rates than the wild-type plants, similar to the pht1;1 mutant, which also showed an As(V)-tolerant phenotype (Shin et al., 2004) (Figure 2F). We further hypothesized that MYB40 represses Pi uptake and, as a result, the As(V) uptake is reduced. We therefore measured Pi uptake over a 4-h period. The Pi uptake ability of OE11 and OE26 was significantly lower than that of wild-type plants (Figure 2G). These data indicate that MYB40 enhances Arabidopsis As(V) tolerance at least in part by repressing As(V)/Pi uptake.

Disruption of MYB40 reduces Arabidopsis As(V) resistance

In an attempt to determine the roles of MYB40 in Arabidopsis As(V) resistance, we used CRISPR/Cas9 technology to generate the myb40 mutant, as there was no T-DNA insertion mutant at the ABRC. A pair of sgRNA targets in the MYB40 gene was selected (Supplemental Figure 1A and 1B). The CRISPR construct was transformed into wild-type Arabidopsis, and two homozygous myb40 mutants, named myb40-1 and myb40-2, were obtained. The myb40-1 mutant contained a 323-bp deletion in the MYB40 gene, resulting in the deletion of most of the first two exons and the first intron (Supplemental Figure 1C). The myb40-2 mutant had a nucleotide deletion in the C1 site and a nucleotide insertion in the C2 site (Supplemental Figure 1D), which led to a frameshift mutation. Both myb40-1 and myb40-2 were null mutants.

When germinated and grown on 1/2 MS medium without As(V), the myb40-1 and myb40-2 mutants had no obvious differences compared with wild-type plants (Figure 3A, top panel). When germinated and grown on 1/2 MS medium containing 300 μM As(V) for 7 d, the growth of myb40-1 and myb40-2 mutants was dramatically impaired relative to wild-type plants (Figure 3A, bottom panel). In the presence of As(V), the myb40-1 and myb40-2 mutants had significantly shorter primary roots and reduced fresh weights relative to wild-type plants (Figure 3B and 3C). In contrast to MYB40-overexpressing lines, the myb40-1 and myb40-2 mutants had higher As accumulation and Pi contents than wild-type plants when exposed to As(V) (Figure 3D and 3E). These data demonstrate that disruption of MYB40 results in an increased sensitivity to As(V).

MYB40 negatively regulates PHT1;1 expression

Arsenate is a structural homolog of Pi and is transported into plant cells mainly through the Pi transporters PHT1;1 and PHT1;4 in Arabidopsis (Shin et al., 2004; Catarecha et al., 2007). The pht1;1 mutant is insensitive to As(V) (Shin et al., 2004), and the PHT1;1-overexpressing line shows an As(V)-hypersensitive phenotype (Wang et al., 2014). The MYB40-overexpressing lines displayed As(V)-resistant phenotypes and had lower Pi uptake rates (Figure 2), similar to the pht1;1 mutant (Shin et al., 2004; Catarecha et al., 2007). We then hypothesized that MYB40, as an MYB transcription factor, downregulates PHT1;1 expression. The qRT–PCR results showed that the transcript levels of PHT1;1 were much lower in MYB40-overexpressing lines than in wild-type plants (Figure 4A). PHT1;1 expression was also tested in the myb40 mutants. After treatment with 300 or 400 μM As(V) for 2 d, PHT1;1 expression was repressed in the wild-type plants. By contrast, transcript levels of PHT1;1 in the myb40 mutants were similar to those in wild-type plants without As(V) treatment (Figure 4B). These data indicate that MYB40 downregulates PHT1;1 expression.

Because MYB40 is a transcription factor (Figure 1), we investigated whether MYB40 binds to the PHT1;1 promoter. To test this binding, an MYB40-Myc transgenic line (Figure 4C) was generated for chromatin immunoprecipitation (ChIP). The PHT1;1 promoter was separated into six fragments (named P2–P7) (Figure 4D) and amplified by PCR using the primers listed in Supplemental Table 1. The 7-d-old MYB40-Myc seedlings were transferred to 200 μM As(V) for 2 d, then harvested for the ChIP assay. Chromatin immunoprecipitated with anti-Myc antibody was enriched in P2, P6, and P7 of the PHT1;1 promoter (Figure 4E), indicating that MYB40 could bind to the PHT1;1 promoter in vivo. An electrophoretic mobility shift assay (EMSA) was also conducted. The recombinant SUMO–His–MYB40 protein bound to the P2, P6, and P7 fragments of the PHT1;1 promoter, and this binding was effectively reduced by the addition of unlabeled competitors with the same sequence; the SUMO–His protein did not bind to the PHT1;1 promoter (Figure 4F), indicating that MYB40 bound to the PHT1;1 promoter in vitro. Together, these data demonstrate that MYB40 directly represses PHT1;1 expression by binding to the PHT1;1 promoter.

The MYB40-overexpressing line (OE26) was crossed with the PHT1;1-overexpressing line SuperPHT1;1 (Wang et al., 2014) to obtain the OE26 SuperPHT1;1 co-overexpression line (Figure 4G). When germinated and grown on 1/2 MS medium without As(V) (0 μM arsenate), there were no obvious phenotypic differences among the genotypes (Figure 4H, left panel). In the presence of 200 μM As(V), the OE26 SuperPHT1;1 co-overexpression line showed As(V)-hypersensitive phenotypes, similar to SuperPHT1;1 (Figure 4H, right panel), demonstrating that overexpression of PHT1;1 abolished the As(V)-tolerant phenotypes of the MYB40-overexpressing line.

Overexpression of MYB40 enhances, and disruption of MYB40 reduces, thiol-peptide contents

After being transported into plant cells, As(V) is reduced to As(III) by a reductase and is detoxified through complex formation with
PCs and glutathione (GSH) (Zhao et al., 2010; Kumar et al., 2015). The PCS1 gene (also named CAD1) encodes a PC synthase in Arabidopsis (Ha et al., 1999; Vatamaniuk et al., 1999). The cad1 mutant is hypersensitive to As(V) stress (Ha et al., 1999), and overexpression of PCS1 increases thiol-peptide accumulation and As tolerance (Lee et al., 2003; Li et al., 2004). This led us to investigate whether MYB40 was involved in As detoxification. Seven-d-old MYB40-overexpressing lines, myb40 mutants, and wild-type seedlings were transferred to 1/2 MS medium containing 0, 200, 300, and 400 µM As(V) for 7 d, and then harvested for measurement of PCs and GSH. Using GSH, PC2, PC3, and PC4 standards, fluorescent HPLC analysis clearly identified the mBBr-labeled peptides in different genotypes (Figure 5A). The level of PC2 was significantly increased in OE11 and OE26, and decreased in the myb40-1 and myb40-2 mutants, relative to wild-type seedlings (Figure 5A and 5B). GSH is a substrate for PC synthesis and plays roles in As detoxification (Zhao et al., 2010; Kumar et al., 2015). The GSH contents were higher in the MYB40-overexpressing lines (OE11 and OE26) than in wild-type plants when exposed to As(V) (Figure 5C).

**MYB40 positively regulates PCS1 expression**

PCS1 expression is induced under As(V) stress (Sung et al., 2009), and MYB40-overexpressing lines had higher thiol-peptide contents (Figure 5A–5C). We further hypothesized that MYB40 modulates PCS1 expression. Upon exposure to As(V), the expression of PCS1 was induced in wild-type seedlings, but it did not change in OE11 and OE26 (Figure 5D). The transcript levels of PCS1 in OE11 and OE26 were much higher than those in wild-type plants, and they were also higher than those of wild-type seedlings treated with As(V) for 48 h (Figure 5D). In contrast to MYB40-overexpressing lines, the transcript levels of PCS1 in the myb40-1 and myb40-2 mutants were obviously

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**Figure 3. Disruption of MYB40 results in Arabidopsis sensitivity to As(V).**

(A) Phenotypic comparison. The myb40 mutants, OE26, and WT plants were germinated and grown on 1/2 MS medium with or without 300 µM As(V) for 7 d, and then representative photographs were taken. Scale bar corresponds to 0.5 cm.

(B and C) Primary root length and fresh weight analysis. The myb40 mutants, OE26, and WT plants were germinated and grown on 1/2 MS medium containing 0, 200, 300, and 400 µM As(V) for 7 d, and then the primary root length (B) and fresh weight (C) were measured. The values are means ± SE; n ≥ 20 in (B) and n = 3 in (C).

(D and E) As and Pi content measurement. Seven-d-old seedlings were transferred to 1/2 MS medium with 300 µM As(V) for 3 d, then harvested for measurement of As content (D) and Pi content (E). Data are shown as mean ± SE; n = 3.

Asterisks in (B, C, D, and E) indicate significant differences compared with WT plants (*) by Student’s t-test: *P < 0.05, **P < 0.01.
rendering is not applicable for this text. The text is a scientific paper discussing the role of MYB40 in modulating arsenate uptake and detoxification in Arabidopsis. The key points include:

- MYB40 positively modulates PCS1 expression under As(V) stress.
- The PCS1 promoter was separated into four fragments (named P1–P4) and a ChIP assay was performed with MYB40-Myc seedlings treated with 200 μM As(V) for 2 d. Chromatin immunoprecipitated with anti-Myc antibody was enriched in the P4 fragment of the PCS1 promoter, indicating that MYB40 bound to the PCS1 promoter in vivo.
- EMSA was also conducted to detect the binding of MYB40 to the P4 fragment of the PCS1 promoter.

The text also includes several figures and graphs, each with a legend and axis labels, illustrating the experimental results and molecular interactions involved in MYB40's role in arsenate modulation.
protein bound to the P4 fragment of the PCS1 promoter, and this binding was effectively reduced by an unlabeled competitor with the same sequence (Figure 5H), indicating that MYB40 bound to the P4 fragment of the PCS1 promoter in vitro. Together, these data demonstrate that MYB40 is a major transcription factor that positively modulates PCS1 expression under As(V) stress.

### MYB40 modulates ABCC1 and ABCC2 expression

The PC–As(III) complexes are transported into vacuoles, which act as final detoxification stores for As (Zhao et al., 2010; Kumar et al., 2015). Arabidopsis ABCC1 and ABCC2 are two major vacuolar PC transporters (Song et al., 2010). The abcc1 abcc2 double mutant is hypersensitive to As(V) stress, and the
ABCC1 PCS1 co-overexpression line shows As(V)-tolerant phenotypes (Song et al., 2010). We therefore tested the expression of ABCC1 and ABCC2. Upon exposure to As(V), expression of ABCC1 and ABCC2 was induced, and their transcript levels were significantly elevated in OE11 and OE26 (Figure 6A and 6B) and reduced in the myb40-1 and myb40-2 mutants (Figure 6C and 6D) relative to wild-type plants, indicating that MYB40 positively modulates the expression of ABCC1 and ABCC2.

Intracellular As(III) can be effluxed from plant cells (Zhao et al., 2010; Kumar et al., 2015). Previous reports show that ATQ1 (also named HAC1) encodes an As(V) reductase that plays important roles in As(III) efflux from roots and limitation of As loading into the xylem (Chao et al., 2014; Sánchez-Bermejo et al., 2014). ATQ1 is induced by As(V) stress (Chao et al., 2014), and ATQ1 expression was therefore tested in MYB40-overexpressing lines. The qRT–PCR results showed that the transcript level of ATQ1 in MYB40-overexpressing lines was similar to that in wild-type plants (Figure 6E), indicating that MYB40 does not modulate ATQ1 expression.

MYB40 also indirectly upregulated the expression of ABCC1 and ABCC2, which encode two major vacuolar PC transporters that carry As(III)-PC2 from the cytoplasm into the vacuole. Previous reports showed that, under As(V) stress, PHT1;1 expression was repressed (Castrillo et al., 2013), and PCS1 expression was induced (Sung et al., 2009). Repressing PHT1;1 expression or increasing PCS1 expression can enhance Arabidopsis As(V) resistance (Li et al., 2004; Su et al., 2015). These data suggest that MYB40 functions as a positive regulator in Arabidopsis resistance to As(V) stress through direct repression of PHT1;1 expression and upregulation of PCS1 expression.

MYB40 was localized in the nucleus and had transcriptional activity (Figure 1A and 1B), confirming that it was a transcription factor. The expression of MYB40 was significantly induced by As(V) stress, and the induction level of MYB40 was related to the duration of As(V) stress exposure (Figure 1B). Overexpression of MYB40 enhanced Arabidopsis As(V) resistance (Figure 2), and myb40 mutants showed As(V)-sensitive phenotypes.
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Figure 7. A proposed working model for MYB40 in the Arabidopsis response to As stress.

During As(V) stress, the expression of MYB40 is induced, and MYB40 then directly represses PHT1;1 expression to reduce As(V) uptake into plant cells. It also directly upregulates PCS1 expression to enhance the contents of PCs, which form complexes with As(III). MYB40 indirectly upregulates the expression of ABCC1 and ABCC2, which encode two major vacuolar PC transporters that carry As(III)-PCs from the cytoplasm into the vacuole. Created with BioRender.com.

MYB40 can enhance Arabidopsis As(V) resistance at least in part through regulation of PHT1;1-dependent As(V)/Pi uptake.

A previous report demonstrated that the WRKY6 transcription factor restricts As(V) uptake in Arabidopsis (Castrillo et al., 2013). When grown on 15 µM Pi supplemented with 15 µM As(V) for 7 d, a WRKY6-overexpressing line displayed As(V)-resistant phenotypes, and no visible differences were observed in the wrky6 mutant relative to wild-type plants (Castrillo et al., 2013). WRKY6 can bind to the PHT1;1 promoter to repress PHT1;1 expression (Castrillo et al., 2013). When germinated and grown on 1/2 MS medium with 200 µM As(V), the WRKY6-overexpressing line and the wrky6 mutant showed no obvious differences compared with wild-type plants (data not shown), whereas the MYB40-overexpressing lines were markedly tolerant, and the myb40 mutants sensitive, to As(V) stress (Figures 2 and 3). This suggested that MYB40 and WRKY6 have different functions in Arabidopsis responses to As(V) stress. WRKY6 expression was clearly induced by As(V), and WRKY6 transcripts accumulated to their highest level after exposure to As(V) for 3 h (Castrillo et al., 2013). MYB40 was also induced by As(V) and, unlike WRKY6, its transcript level was still increased, even after exposure to As(V) for 72 h (Figure 1B). These data indicate that MYB40 and WRKY6 modulate Arabidopsis arsenate tolerance at different times.

After uptake into plant cells, As(V) is readily reduced to As(III), which is then detoxified by complexation with thiol-rich peptides and sequestered in the vacuoles (Zhao et al., 2010). PCS1 (also named CAD1) is the main PC synthase in Arabidopsis (Ha et al., 1999; Vatamaniuk et al., 1999), and the expression of PCS1 was clearly induced by As(V) (Sung et al., 2009). The cad1 mutant shows As(V)-hypersensitive phenotypes (Ha et al., 1999), and overexpression of PCS1 increases thiol-peptide accumulation and As tolerance (Lee et al., 2003; Li et al., 2004), indicating that transcriptional regulation of PCS1 is important for plant As(V) resistance. However, until now the mechanism has been unclear. Our results demonstrate that the MYB40 transcription factor positively regulates PCS1 by binding to the PCS1 promoter under As(V) stress. When plants were grown on 1/2 MS medium with added As(V), thiol-peptide contents were enhanced in the MYB40-overexpressing lines and reduced in the myb40 mutant relative to wild-type plants (Figure 5A–5C).

As a structural homolog of Pi, As(V) is taken up into plant cells through Pi transporters (Shin et al., 2004; Catarecha et al., 2007; Wu et al., 2011; Remy et al., 2012; Castrillo et al., 2013; DiTusa et al., 2016). The MYB40-overexpressing lines showed As(V)-tolerant phenotypes and had reduced As(V)/Pi uptake rates (Figure 2), suggesting that MYB40 enhanced Arabidopsis As(V) tolerance in part by repressing As(V)/Pi uptake. There are at least nine Pi transporters in Arabidopsis, and PHT1;1 is most highly expressed in roots of plants grown on Pi-sufficient medium (Mudge et al., 2002). During As(V) stress, the expression of PHT1;1 is clearly repressed (Castrillo et al., 2013). The pht1;1 mutant shows As(V)-tolerant phenotypes (Shin et al., 2004; Catarecha et al., 2007), and overexpression of PHT1;1 results in As(V) hypersensitivity (Wang et al., 2014), indicating that uptake of As(V) occurs mainly through the Pi transporter PHT1;1 in Arabidopsis. The expression of PHT1;1 was clearly repressed in the MYB40-overexpressing lines, and the repression level of PHT1;1 was closely related to MYB40 expression levels (Figure 4A). Upon exposure to As(V), PHT1;1 expression was repressed in wild-type plants, and this repression was abolished in the myb40 mutants (Figure 4B). Further EMSA and ChIP results showed that MYB40 could bind to the PHT1;1 promoter in vitro and in vivo (Figure 4E and 4F). All these data demonstrate that
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from the ABRC. The SuperPHT1;1 line used in the study was described previously (Wang et al., 2014). The MYB40-overexpressing lines were generated by cloning the coding sequence of MYB40 into the pCXSN vector under the 3SS promoter (Chen et al., 2009a). The myb40 mutants were generated by CRISPR/Cas9 technology. A pair of sgRNA targets (C1: AACCGTGCTGTGACAAAATTGG; and C2: TCACTCCTACCTTGC AACC) in the MYB40 gene was selected and cloned into the pHIE2A-TRI vector (Wang et al., 2015). The MYB40-Myc transgenic line

was generated by cloning the coding sequence of MYB40 into the pCAMBIA1300-Myc vector. The constructs were introduced into Arabidopsis by Agrobacterium-mediated transformation (Agrobacterium strain GV3101) using the floral-dip method (Clough and Bent, 1998), and homozygous transgenic lines were obtained.

Arabidopsis seeds were surface sterilized and kept at 4°C for 72 h in darkness before germination. Then, the seeds were plated on 1/2 MS medium containing 1.5% (w/v) sucrose and 0.8% (w/v) agar and grown at 22°C for 100 μmol m⁻² s⁻¹ of illumination for a 16-h daily light period, unless otherwise indicated.

For the As(V) treatment, sodium arsenate was added to the 1/2 MS medium at the described concentrations.

As content and As(V) uptake assay

Arabidopsis plants were germinated and grown on 1/2 MS medium with different concentrations of As(V) as described. Plants were dried, then mineralized with HNO₃ in a pressure digester, and the As contents were determined by atomic fluorescence spectrometry.

For the As(V) uptake assay, 7-d-old seedlings grown on 1/2 MS medium were transferred to 1/2 MS with 200 μM As(V) for 6 h, and their As contents were measured.

Quantification of thiol-peptide content

Seven-d-old seedlings were transferred to 1/2 MS medium containing 200 μM As(V) for 7 d, then harvested for thiol-peptide content measurements. Thiol-peptide compounds, including GSH, PC2, PC3, and PC4, were analyzed using fluorescence-detection HPLC, essentially as described previously (Li et al., 2004).

Pi concentration and Pi uptake assay

Arabidopsis plants were germinated and grown on MS medium for 7 d, then harvested for Pi concentration measurement as described previously (Chen et al., 2009b).

For the Pi uptake assay, 7-d-old Arabidopsis seedlings grown on MS medium were transferred to Pi uptake solution containing 500 μM Pi supplemented with 0.2 μCi ³²Porthophosphate. A group of 15 seedlings was used as one biological sample.

Subcellular localization

MYB40 fused to GFP was cloned into the pCAMBIA1300:GFP vector, named GFP, to create a MYB40-GFP construct. The plasmids (MYB40-GFP and GFP) were transformed into Agrobacterium GV3101, and transient expression assays were conducted as described previously (Chen et al., 2009b). GFP fluorescence in the transformed leaves was imaged using a confocal laser scanning microscope (Leica TCS SP5II).

qRT-PCR assay

qRT-PCR was performed using SYBR Green PCR Master Mix (Life Technologies) on a 7500 Real-Time PCR System (Applied Biosystems) following the manufacturer’s protocol. Actin2/8 expression was used as an internal control. The qPCR analysis was performed with three technical replicates, and each experiment was repeated at least three times with similar results. The primers used are listed in Supplemental Table 1.
PCR using biotin-labeled or unlabeled primers (Supplemental Table 1).

control.

Biotin-unlabeled fragments with the same sequences were used as com-

Polymerase chain reaction.

coli
tor was also introduced into

was determined by the Bio-Rad protein assay. The pET-28a-SUMO vec-

SUMO–His–MYB40 protein and SUMO–His protein were purified from

EMSA Kit (Pierce) following the manufacturer's protocol. The recombinant

The EMSA assay was conducted using a LightShift Chemiluminescent

EMSA assay

The coding sequence of MYB40 was amplified and cloned into the pET-28a-SUMO vector (Novagen). The recombinant plasmid was introduced into E. coli strain BL21. The SUMO–His–MYB40 protein was purified using Ni-Sepharose 6 Fast Flow (GE Healthcare), and the protein concentration was determined by the Bio-Rad protein assay. The pET-28a-SUMO vec-

Fragments of the PHT1:1 and PCS1 promoters were obtained by PCR using biotin-labeled or unlabeled primers (Supplemental Table 1). Biotin-unlabeled fragments with the same sequences were used as com-

The EMSA assay was conducted using a LightShift Chemiluminescent

ACCESSION NUMBERS

Sequence data from this article can be found in the EMBL and GenBank data libraries under the following accession numbers: MYB40 (TAIR: AT5G44070), MYB40 (TAIR: AT5G44070), ABC1 (TAIR: AT1G30400), ABC2 (TAIR: AT2G34660), and ATQ1 (TAIR: AT1G492252G21045).

SUPPLEMENTAL INFORMATION

Supplemental information is available at Plant Communications Online.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (nos. 31970273 and 31670245) and the Beijing Outstanding University Discipline Program.

AUTHOR CONTRIBUTIONS

Y.C. and H.-Y.W. conducted the experiments. Y.-F.C. designed the experiments. Y.C., H.-Y.W., and Y.-F.C. analyzed the data and wrote the article.

ACKNOWLEDGMENTS

We thank Dr. Zhen Li for assistance with thiol-peptide content measure-

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