

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

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## 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 phytochelatase, and *ABCC1* and *ABCC2*, which encode the major vacuolar phytochelatase 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

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## INTRODUCTION

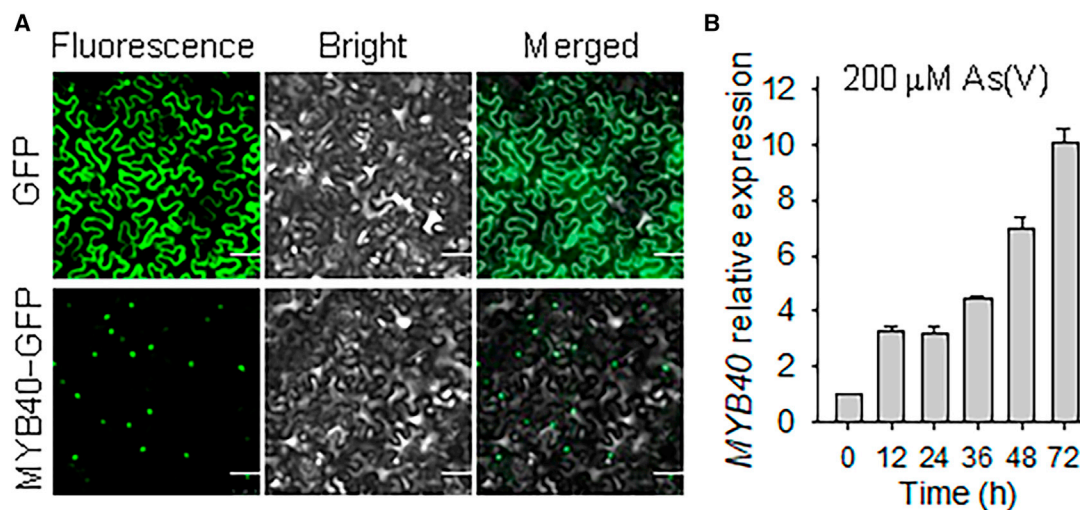
Arsenic (As) is ubiquitous in the environment and is one of the most toxic metalloids found in soils (Zhao et al., 2010; Kumar et al., 2015). People experience As poisoning by drinking As-contaminated water and ingesting crops cultivated in As-polluted soils (Zhao et al., 2010). Arsenic is nonessential 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 (Catarcha 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; Catarcha 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;1Δ4Δ* 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;1Δ4Δ* 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* (Catarcha et al., 2007). The *pht1;1-3* mutant showed an As(V)-tolerant phenotype and enhanced As accumulation (Catarcha 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

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**Figure 1. MYB40 is an As(V) response transcription factor.**

**(A)** Subcellular localization of MYB40–GFP fusion protein in *Nicotiana benthamiana* leaves. GFP alone was used as the control. Scale bar corresponds to 50  $\mu\text{m}$ .

**(B)** qRT–PCR analysis of *MYB40* in wild-type plants under As(V) stress. Seven-d-old wild-type seedlings were transferred to  $\frac{1}{2}$  MS medium containing 200  $\mu\text{M}$  As(V), then harvested at the indicated time for qRT–PCR analysis. The qRT–PCR was performed with three technical replicates, and the experiment was repeated at least three times with similar results.

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-Bermejo 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 transcription of *ACR3*, which encodes an arsenite transporter (Bobrowicz 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.

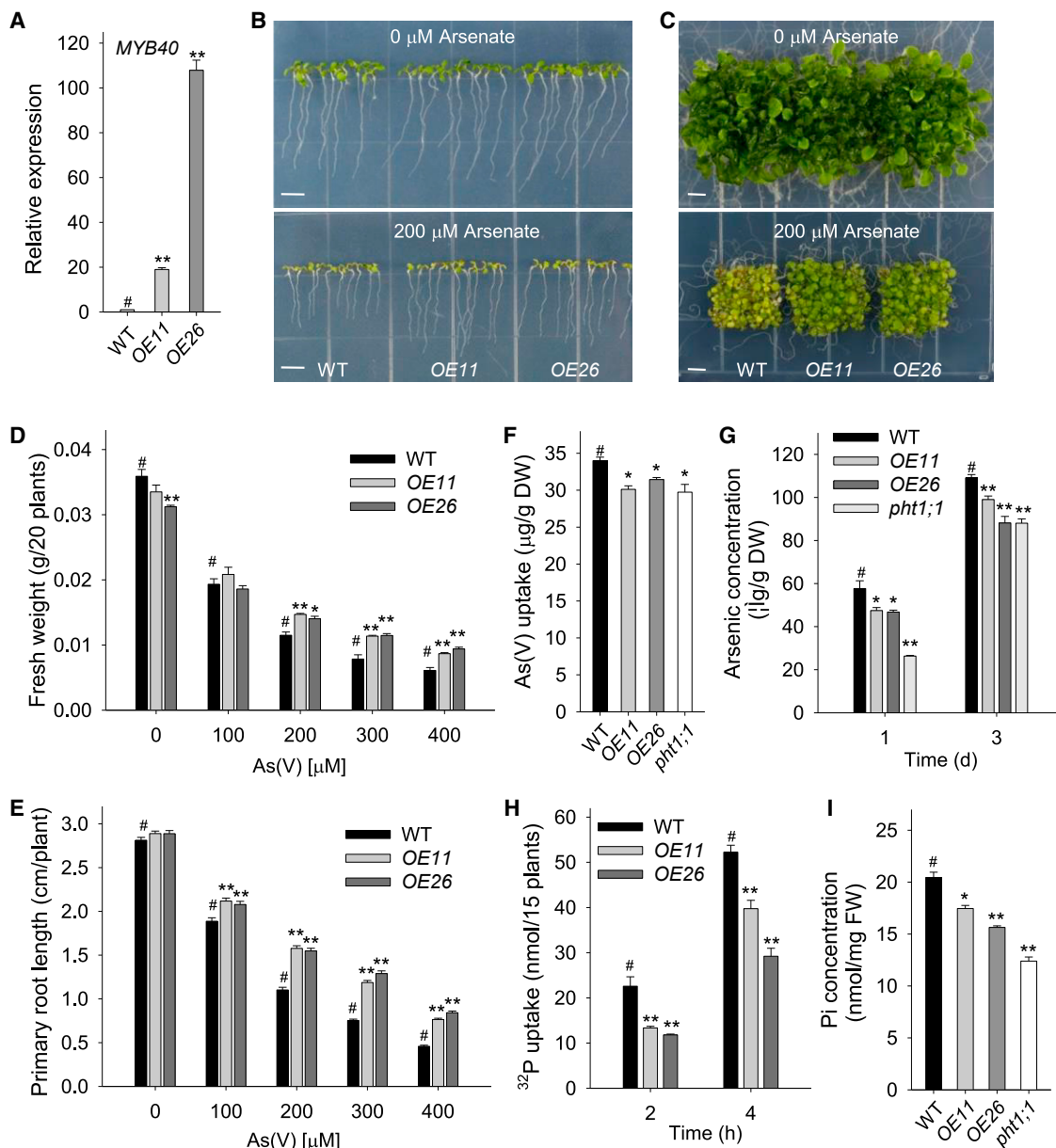
## RESULTS

### 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 and GFP alone were 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



**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  $\frac{1}{2}$  MS medium containing 0 or 200  $\mu$ M 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  $\frac{1}{2}$  MS medium containing 0, 100, 200, 300, and 400  $\mu$ M As(V) for 7 d, and then the fresh weight (D) and primary root length (E) were measured. The values are means  $\pm$  SE;  $n = 3$  in (D) and ( $n \geq 40$ ) in (E).

(F) As(V) uptake analysis. Seven-d-old plants were transferred to  $\frac{1}{2}$  MS medium with 200  $\mu$ M As(V) for 6 h, then harvested for As(V) uptake analysis. Data are shown as mean  $\pm$  SE;  $n = 3$ .

(G) As content measurement. Seven-d-old plants were transferred to  $\frac{1}{2}$  MS medium with 200  $\mu$ M As(V) for 1 or 3 d, then harvested for As content measurement. Data are shown as mean  $\pm$  SE;  $n \geq 5$ .

(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  $\pm$  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  $\pm$  SE;  $n = 3$ .

Asterisks in (A, D, E, F, G, H, and I) indicate significant differences compared with WT plants (#) 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  $\frac{1}{2}$  Murashige and Skoog (MS) medium with 0  $\mu$ M As(V)

(Figure 2B and 2C, top panel). When germinated and grown on  $\frac{1}{2}$  MS medium with 200  $\mu$ M 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  $\mu$ M 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  $\mu$ M 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).

## AtMYB40 modulates arsenate uptake and detoxification

### MYB40 negatively regulates *PHT1;1* expression

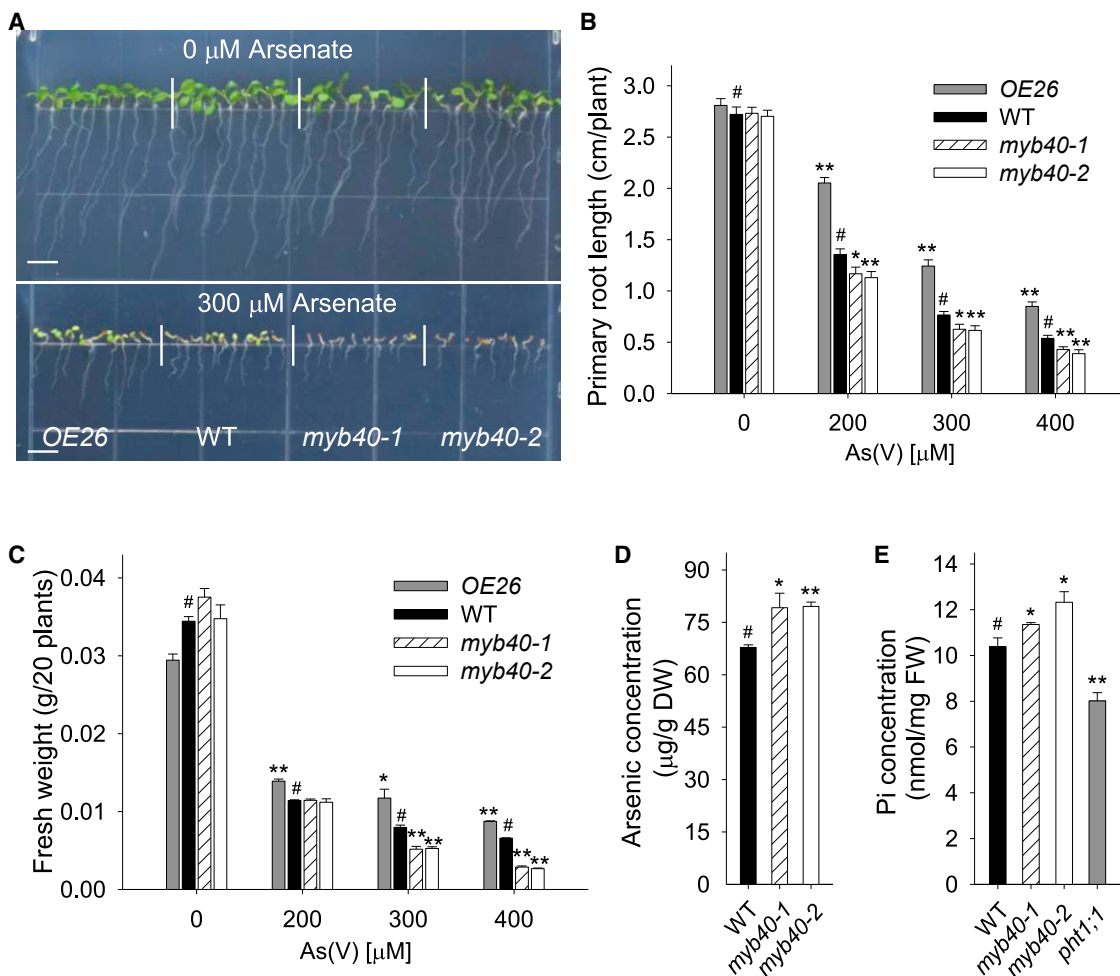
Arsenate is a structural homolog of Pi and is transported into plant cells mainly through the Pi transporters *PHT1;1* and *PHT1;4* in *Arabidopsis* (Shin et al., 2004; Catarecha et al., 2007). The *pht1;1* mutant is insensitive to As(V) (Shin et al., 2004), and the *PHT1;1*-overexpressing line shows 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  $\mu$ M 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* bound 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  $\mu$ M 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 *Super:PHT1;1* (Wang et al., 2014) to obtain the *OE26 Super:PHT1;1* co-overexpression line (Figure 4G). When germinated and grown on  $1/2$  MS medium without As(V) (0  $\mu$ M arsenate), there were no obvious phenotypic differences among the genotypes (Figure 4H, left panel). In the presence of 200  $\mu$ M As(V), the *OE26 Super:PHT1;1* co-overexpression line showed As(V)-hypersensitive phenotypes, similar to *Super:PHT1;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



**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  $\frac{1}{2}$  MS medium with or without 300  $\mu$ M 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  $\frac{1}{2}$  MS medium containing 0, 200, 300, and 400  $\mu$ M As(V) for 7 d, and then the primary root length (B) and fresh weight (C) were measured. The values are means  $\pm$  SE;  $n \geq 20$  in (B) and  $n = 3$  in (C).

(D and E) As and Pi content measurement. Seven-d-old seedlings were transferred to  $\frac{1}{2}$  MS medium with 300  $\mu$ M As(V) for 3 d, then harvested for measurement of As content (D) and Pi content (E). Data are shown as mean  $\pm$  SE;  $n = 3$ .

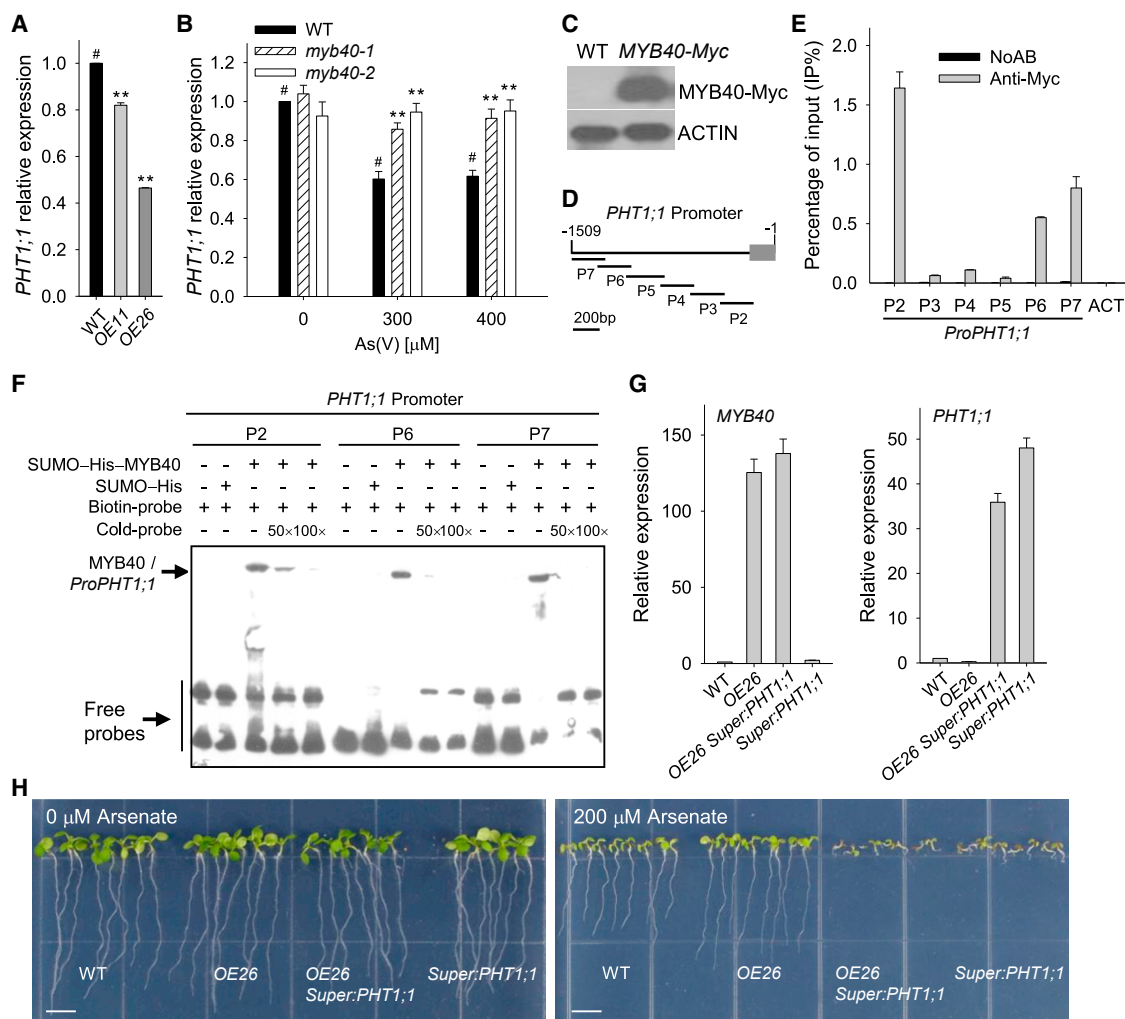
Asterisks in (B, C, D, and E) indicate significant differences compared with WT plants (#) by Student's *t*-test: \* $P < 0.05$ , \*\* $P < 0.01$ .

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  $\frac{1}{2}$  MS medium containing 200  $\mu$ M As(V) for 7 d, then harvested for measurement of PCs and GSH. Using GSH, PC<sub>2</sub>, PC<sub>3</sub>, and PC<sub>4</sub> standards, fluorescent HPLC analysis clearly identified the mBBr-labeled peptides in different genotypes (Figure 5A). The level of PC<sub>2</sub> 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



**Figure 4. MYB40 represses *PHT1;1* expression in the *Arabidopsis* response to As stress.**

(A) qRT-PCR analysis of *PHT1;1* expression in *MYB40*-overexpressing lines and WT plants. The qRT-PCR was performed with three technical replicates, and the experiment was repeated at least three times with similar results.

(B) qRT-PCR analysis of *PHT1;1* in the *myb40* mutants under As(V) stress. Seven-d-old seedlings were transferred to  $\frac{1}{2}$  MS medium with 0, 300, and 400  $\mu\text{M}$  As(V) for 2 d, then harvested for RNA extraction. The qRT-PCR was performed with three technical replicates, and the experiment was repeated at least three times with similar results. Asterisks in (A and B) indicate significant differences compared with WT plants ( $\#$ ) by Student's *t*-test:  $**P < 0.01$ .

(C) Immunoblot analysis of MYB40 protein in the *MYB40-Myc* transgenic line. Protein extract was analyzed by immunoblotting using anti-Myc antibody. Actin was used as the loading control.

(D) Diagram of the *PHT1;1* promoter showing relative positions and sizes of different fragments. The adenine residue of the translational start codon ATG was assigned position +1, and the numbers flanking the sequences of the *PHT1;1* promoter fragments are based on this number. The gray box indicates the untranslated region (UTR).

(E) ChIP-qPCR assay of MYB40 binding to the *PHT1;1* promoter *in vivo*. Seven-d-old *MYB40-Myc* seedlings were transferred to  $\frac{1}{2}$  MS medium containing 200  $\mu\text{M}$  As(V) for 2 d, then harvested for ChIP-qPCR using anti-Myc antibody. Data are shown as mean  $\pm$  SE;  $n = 3$ .

(F) EMSA of MYB40 binding to the *PHT1;1* promoter *in vitro*. Each biotin-labeled DNA probe was incubated with SUMO-His-MYB40 protein. An excess of unlabeled probe with the same sequence was added to compete with the labeled probe.

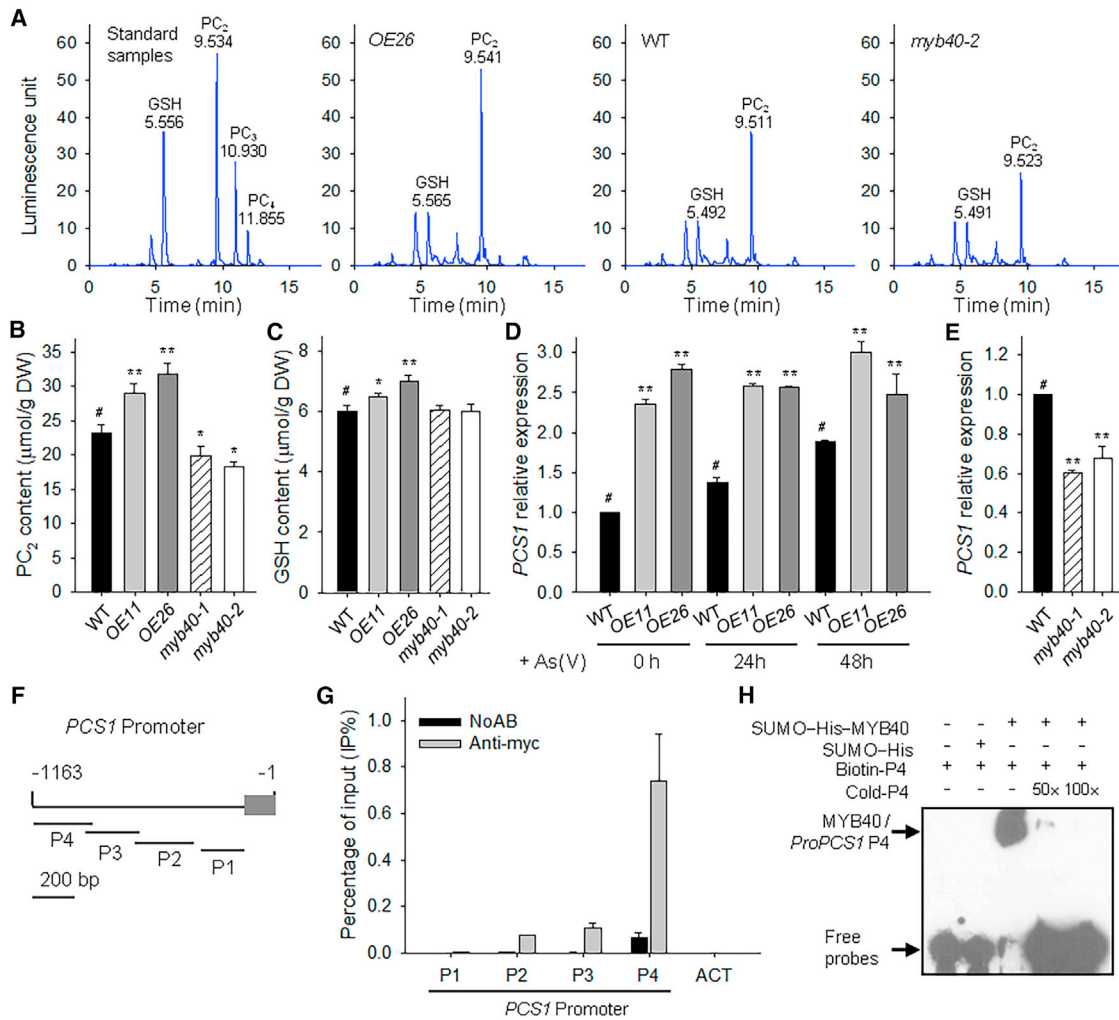
(G) The expression of *MYB40* and *PHT1;1* was tested by qRT-PCR in *OE26*, *OE26 Super:PHT1;1* co-overexpression, *Super:PHT1;1*, and WT plants. The qRT-PCR was performed with three technical replicates, and the experiment was repeated at least three times with similar results.

(H) Phenotypic comparison. Plants were germinated and grown on  $\frac{1}{2}$  MS medium containing 0 or 200  $\mu\text{M}$  arsenate for 7 d, and then representative photographs were taken. Scale bar corresponds to 0.5 cm.

lower than those in wild-type plants under As(V) stress (Figure 5E). These data indicate that MYB40 positively modulates *PCS1* expression under As(V) stress.

The *PCS1* promoter was separated into four fragments (named P1–P4) (Figure 5F), and a ChIP assay was performed with

*MYB40-Myc* seedlings treated with 200  $\mu\text{M}$  As(V) for 2 d. Chromatin immunoprecipitated with anti-Myc antibody was enriched in the P4 fragment of the *PCS1* promoter (Figure 5G), 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 SUMO-His-MYB40



**Figure 5. MYB40 modulates thiol-peptide accumulation and PCS1 expression in the Arabidopsis response to As stress.**

**(A)** HPLC-MS chromatograms of OE26, *myb40-2* mutant, and WT plants exposed to 200  $\mu\text{M}$  As(V) for 7 d.

**(B and C)** Thiol-peptide measurements. Seven-d-old seedlings of different genotypes were transferred to  $\frac{1}{2}$  MS medium containing 200  $\mu\text{M}$  As(V) for 7 d, then harvested for thiol-peptide measurement. The levels of PC<sub>2</sub> **(B)** and GSH **(C)** in whole seedlings were measured by fluorescent HPLC. Data are shown as mean  $\pm$  SE;  $n = 3$ .

**(D)** qRT-PCR analysis of PCS1 expression in MYB40-overexpressing lines. Seven-d-old plants were transferred to  $\frac{1}{2}$  MS medium containing 200  $\mu\text{M}$  As(V) and harvested at the indicated time. The qRT-PCR was performed with three technical replicates, and the experiment was repeated at least three times with similar results.

**(E)** qRT-PCR analysis of PCS1 in the *myb40* mutants under As(V) stress. The 7-d-old seedlings were transferred to  $\frac{1}{2}$  MS medium with 200  $\mu\text{M}$  As(V) for 2 d, then harvested for RNA extraction. The qRT-PCR was performed with three technical replicates, and the experiment was repeated at least three times with similar results.

**(F)** Diagram of the PCS1 promoter showing relative positions and sizes of different fragments. The adenine residue of the translational start codon ATG was assigned position +1, and the numbers flanking the sequences of the PCS1 promoter fragments are based on this number. The gray box indicates the UTR.

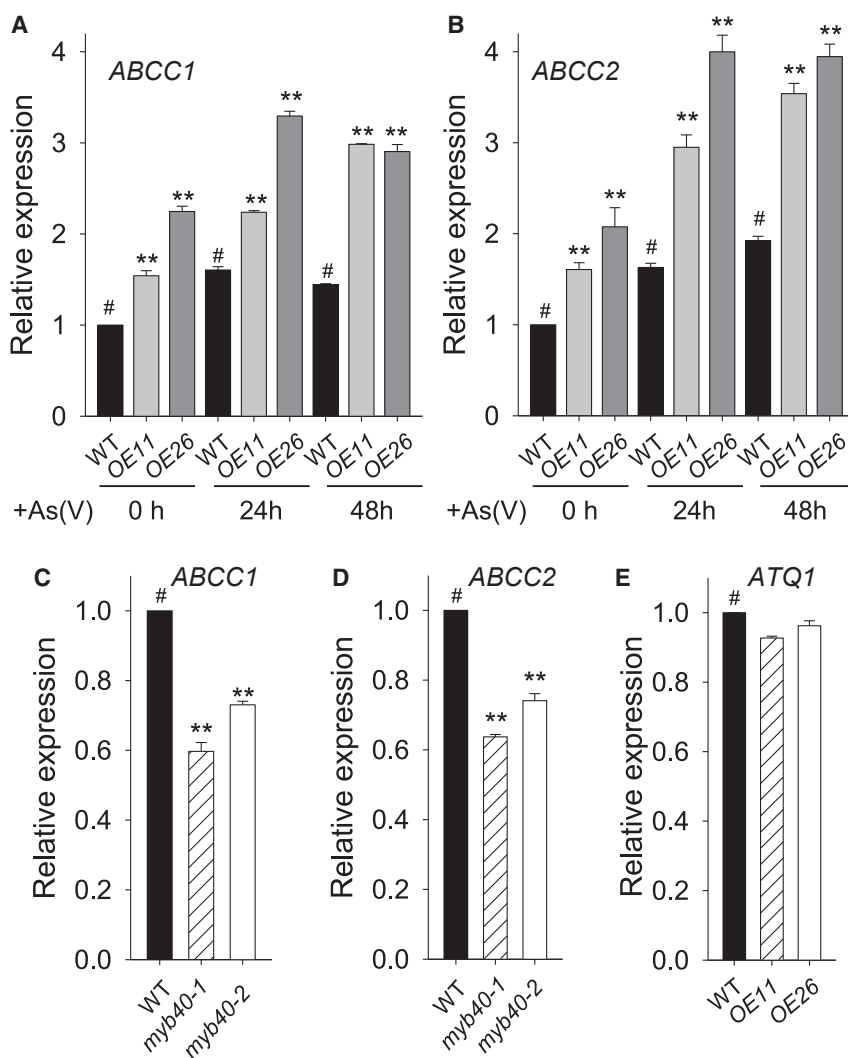
**(G)** ChIP-qPCR assay of MYB40 binding to the PCS1 promoter *in vivo*. Seven-d-old MYB40-Myc seedlings were transferred to  $\frac{1}{2}$  MS medium containing 200  $\mu\text{M}$  As(V) for 2 d, then harvested for ChIP-qPCR assay using anti-Myc antibody. Data are shown as mean  $\pm$  SE;  $n = 3$ .

**(H)** EMSA of MYB40 binding to the P4 fragment of the PCS1 promoter *in vitro*. Each biotin-labeled DNA probe was incubated with SUMO-His-MYB40 protein. An excess of unlabeled probe with the same sequence was added to compete with the labeled probe. Asterisks in **(B, C, D, and E)** indicate significant differences compared with WT plants (<sup>#</sup>) by Student's *t*-test: \* $P < 0.05$ , \*\* $P < 0.01$ .

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



**Figure 6. MYB40 positively modulates ABCC1 and ABCC2 expression.**

**(A and B)** qRT-PCR analysis of *ABCC1* and *ABCC2* expression in *MYB40*-overexpressing lines and WT plants. Seven-d-old seedlings were transferred to  $\frac{1}{2}$  MS medium containing 200  $\mu$ M As(V) and harvested at the indicated time.

**(C and D)** qRT-PCR analysis of *ABCC1* and *ABCC2* in the *myb40* mutants under As(V) stress. Seven-d-old seedlings were transferred to  $\frac{1}{2}$  MS medium containing 200  $\mu$ M As(V) for 2 d, then harvested for RNA extraction.

**(E)** qRT-PCR analysis of *ATQ1* expression in the *MYB40*-overexpressing lines and WT plants.

The qRT-PCR was performed with three technical replicates, and the experiment was repeated at least three times with similar results. Asterisks in **(A, B, C, and D)** indicate significant differences compared with WT plants (#) by Student's *t*-test: \*\**P* < 0.01.

*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.

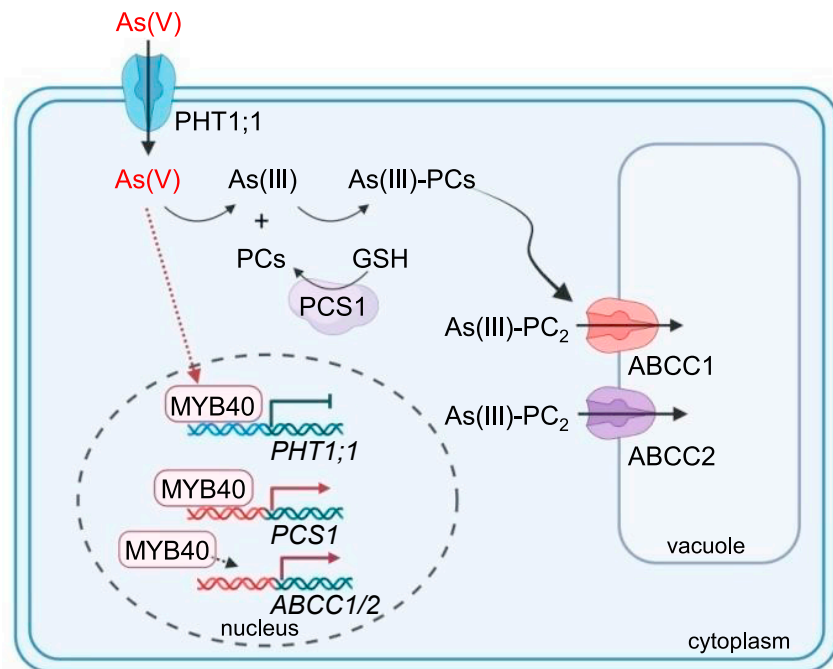
## DISCUSSION

Arsenate is a Pi mimic and toxic to plants, and it is impossible to entirely block its entry into plants. Previous reports showed that the expression of thousands of genes was modulated under As(V) stress (Abercrombie et al., 2008; Castrillo et al., 2013), but the mechanism of this transcriptional regulation is not clear. This study provides evidence that the transcription factor MYB40 plays an important role in *Arabidopsis* response to As(V) stress (Figure 7). The expression of *MYB40* was induced during As(V) stress, and MYB40 then directly repressed *PHT1;1* expression to reduce As(V) uptake into plant cells and directly upregulated *PCS1* expression to enhance the content of PCs, which formed complexes with As(III).

MYB40 also indirectly upregulated the expression of *ABCC1* and *ABCC2*, which encode two major vacuolar PC transporters that carry As(III)-PC<sub>2</sub> 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 by reducing As(V) uptake and enhancing PC synthesis 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





**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)-PC<sub>2</sub> from the cytoplasm into the vacuole. Created with [BioRender.com](https://www.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.

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

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).

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The transcript level of *PCS1* was clearly enhanced in the *MYB40*-overexpressing lines and reduced in the *myb40* mutants when exposed to As(V) (Figure 5D and 5E). Furthermore, EMSA and ChIP results showed that MYB40 could bind to the *PCS1* promoter *in vitro* and *in vivo* (Figure 5G and 5H). These data demonstrate that MYB40 elevates thiol-peptide contents by positively regulating *PCS1* expression under As(V) stress.

The plant vacuoles are the final detoxification stores for As. ABCC1 and ABCC2 are the major vacuolar PC transporters in *Arabidopsis*, and the *abcc1 abcc2* double mutant shows As(V)-hypersensitive phenotypes (Song et al., 2010). The *ABCC1*-overexpressing lines did not exhibit increased As(V) tolerance, whereas the *PCS1 ABCC1* co-overexpression lines had increased As tolerance (Song et al., 2010). The transcript levels of *ABCC1* and *ABCC2* were significantly enhanced in the *MYB40*-overexpressing lines and reduced in the *myb40* mutants under As(V) stress (Figure 6), indicating that MYB40 modulated the expression of *ABCC1* and *ABCC2*. These data demonstrate that MYB40 plays important roles in As detoxification by positively regulating the expression of *PCS1* and *ABCC1/2*, which function in a concerted way in the As detoxification pathway.

A previous report showed that the transcription factor WRKY6 downregulated *PHT1;1* expression and restricted arsenate-induced transposon activation under As(V) stress (Castrillo et al., 2013). A *WRKY6-GFP*-overexpressing line showed markedly enhanced root length, whereas the *wrky6-TNDA* insertion line showed root length similar to that of wild-type plants under As(V) stress (Castrillo et al., 2013). Both MYB40 and WRKY6 directly downregulated *PHT1;1* expression under As(V) stress. Both *MYB40*-overexpressing lines and *WRKY6-GFP*-overexpressing lines showed obvious As(V)-resistant tolerant phenotypes (Castrillo et al., 2013; Figures 2 and 4), and their mutants showed mild or no As(V)-sensitive phenotypes, probably owing to functional redundancy between MYB40 and WRKY6.

Transcription factor binding of *cis*-elements is often associated with accessible chromatin regions. Lu et al. (2017) reported that more than 90% of putative accessible chromatin regions were found within 3 kb upstream of a transcription start site (TSS), more than 75% of accessible chromatin regions were found within 1 kb upstream of the TSS, and accessible chromatin regions were mostly enriched around the TSS. In addition, the accessible chromatin region of *PCS1* was within 500 bp upstream of the TSS of *PCS1* (Lu et al., 2017). These results were obtained from whole seedlings or roots of *Arabidopsis* grown under normal conditions on  $\frac{1}{2}$  LS medium with 1% sugar under long day light conditions (16 h light/8 h dark) (Lu et al., 2017). In this work, under As(V) stress, MYB40 bound mainly to the 718–993 bp upstream of the TSS of *PCS1* *in vivo* (Figure 5G). These data suggest that *Arabidopsis PCS1* can be regulated by the binding of different transcription factors to specific DNA regions in response to environmental stimuli.

## METHODS

### Plant materials and growth conditions

The wild-type plant used in this study was *Arabidopsis thaliana* Col-0. The *PHT1;1* T-DNA insertion line Salk\_088586c, named *pht1;1*, was ordered

### AtMYB40 modulates arsenate uptake and detoxification

from the ABRC. The *Super:PHT1;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 pCXS vector under the 35S promoter (Chen et al., 2009a). The *myb40* mutants were generated by CRISPR/Cas9 technology. A pair of sgRNA targets (C1: AACCGTGCTGTGACAAAATTGG; and C2: TCACTCTCAACTTGGC AACCG) in the *MYB40* gene was selected and cloned into the pHEE2A-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  $\frac{1}{2}$  MS medium containing 1.5% (w/v) sucrose and 0.8% (w/v) agar and grown at 22°C with 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of illumination for a 16-h daily light period, unless otherwise indicated.

For the As(V) treatment, sodium arsenate was added to the  $\frac{1}{2}$  MS medium at the described concentrations.

### As content and As(V) uptake assay

*Arabidopsis* plants were germinated and grown on  $\frac{1}{2}$  MS medium with different concentrations of As(V) as described. Plants were dried, then mineralized with  $\text{HNO}_3$  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  $\frac{1}{2}$  MS medium were transferred to  $\frac{1}{2}$  MS with 200  $\mu\text{M}$  As(V) for 6 h, and their As contents were measured.

### Quantification of thiol-peptide content

Seven-d-old seedlings were transferred to  $\frac{1}{2}$  MS medium containing 200  $\mu\text{M}$  As(V) for 7 d, then harvested for thiol-peptide content measurements. Thiol-peptide compounds, including GSH,  $\text{PC}_2$ ,  $\text{PC}_3$ , and  $\text{PC}_4$ , 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  $\mu\text{M}$  Pi supplemented with 0.2  $\mu\text{Ci}$   $^{32}\text{P}$  orthophosphate. 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.

**ChIP-qPCR assay**

Seven-d-old *MYB40-Myc* seedlings were transferred to  $1/2$  MS medium containing 200  $\mu$ M As(V) for 2 d, then harvested for the ChIP-qPCR assay. The ChIP-qPCR assay was conducted as described previously (Chen et al., 2009b), and the primers used are listed in Supplemental Table 1. Three independent experiments were performed with similar results. Data are mean values of three replicates  $\pm$  SE from one experiment.

**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 vector was also introduced into *E. coli* strain BL21, and protein with a SUMO-His tag was purified. This purified protein was named SUMO-His and was used as a control in EMSA.

The EMSA assay was conducted using a LightShift Chemiluminescent EMSA Kit (Pierce) following the manufacturer's protocol. The recombinant SUMO-His-MYB40 protein and SUMO-His protein were purified from *E. coli*. 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 competitors, and the SUMO-His protein alone was used as the negative control.

**ACCESSION NUMBERS**

Sequence data from this article can be found in the EMBL and GenBank data libraries under the following accession numbers: *MYB40* (TAIR: AT5G14340), *PHT1;1* (TAIR: AT5G43350), *PCS1* (TAIR: AT5G44070), *ABCC1* (TAIR: AT1G30400), *ABCC2* (TAIR: AT2G34660), and *ATQ1* (TAIR: AT1G492252G21045).

**SUPPLEMENTAL INFORMATION**

Supplemental information is available at *Plant Communications Online*.

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**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.

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