The Plant Cell, Vol. 25: 2944–2957, August 2013, www.plantcell.org

WRKY6 Transcription Factor Restricts Arsenate Uptake and Transposon Activation in *Arabidopsis*[W]

Gabriel Castrillo,[a] Eduardo Sánchez-Bermejo,[a] Laura de Lorenzo,[a] Pedro Crevillén,[b] Ana Fraile-Escanciano,[b] Mohan TC,[a] Alfonso Mouriz,[b] Pablo Catarecha,[a] Juan Sobrino-Plata,[c] Sanna Olsson,[a] Yolanda Leo del Puerto,[a] Isabel Mateos,[a] Enrique Rojo,[a] Luis E. Hernández,[c] Jose A. Jarillo,[b] Manuel Piñeiro,[b] Javier Paz-Ares,[a] and Antonio Leyva[a,1]

[a] Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología–Consejo Superior de Investigaciones Cientificas, Campus de Cantoblanco, 28049 Madrid, Spain

[b] Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid-Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Parque Científico y Tecnológico de la Universidad Politécnica de Madrid, 28223 Pozuelo de Alarcón, Madrid, Spain

[c] Laboratorio de Fisiología Vegetal, Departamento de Biología, Universidad Autónoma de Madrid, 28049 Madrid, Spain

ORCID IDs: 0000-0003-1276-9792 (P.C.); 0000-0001-8425-251X (M.TC.); 0000-0003-3492-5813 (L.E.H.); 0000-0001-7617-0418 (A.L.).

Stress constantly challenges plant adaptation to the environment. Of all stress types, arsenic was a major threat during the early evolution of plants. The most prevalent chemical form of arsenic is arsenate, whose similarity to phosphate renders it easily incorporated into cells via the phosphate transporters. Here, we found that arsenate stress provokes a notable transposon burst in plants, in coordination with arsenate/phosphate transporter repression, which immediately restricts arsenate uptake. This repression was accompanied by delocalization of the phosphate transporter from the plasma membrane. When arsenate was removed, the system rapidly restored transcriptional expression and membrane localization of the transporter. We identify WRKY6 as an arsenate-responsive transcription factor that mediates arsenate/phosphate transporter gene expression and restricts arsenate-induced transposon activation. Plants therefore have a dual WRKY-dependent signaling mechanism that modulates arsenate uptake and transposon expression, providing a coordinated strategy for arsenate tolerance and transposon gene silencing.

INTRODUCTION

Environmental stress is a driving force in evolution. Plants have evolved sophisticated mechanisms to perceive different environmental stresses and activate specific tolerance mechanisms. Early in the earth's history, volcanic emissions of arsenic to the biosphere comprised a major threat to incipient life forms (Oremland et al., 2009; Dani, 2010); indeed, all life forms have strategies to cope with this metalloid (Rosen, 2002; Tripathi et al., 2007; Mendoza-Cózatl et al., 2011; Ye et al., 2012). When the oxygen concentration increased, arsenate [As(V)] became the most prevalent form of arsenic in the biosphere (Oremland et al., 2009; Dahl et al., 2010; Dani, 2010). This chemical threat was particularly critical for sessile organisms, such as plants, which were forced to evolve rapid tolerance responses when As(V) was detected. An additional challenge related to As(V) in the biosphere is its similarity to the macronutrient Pi; when Pi is limited, Pi transporters are induced and As(V) is incorporated preferentially into plant cells (Raghothama, 1999; Catarecha et al., 2007; Wu et al., 2011).

Modulation of Pi transporter activity could be an efficient strategy for As(V) tolerance. The *Arabidopsis thaliana PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1* mutant, in which general Pi transporter trafficking to the plasma membrane is altered and As(V) uptake is impaired, shows remarkable As(V) tolerance (González et al., 2005). Of all transporters, the high affinity Pi transporters PHOSPHATE TRANSPORTER1;1 (PHT1;1) and PHOSPHATE TRANSPORTER1;4 (PHT1;4) in *Arabidopsis* have a major role in As(V) uptake (Shin et al., 2004). The *pht1;1 pht1;4* double mutant has a notable As(V) tolerance phenotype, indicating that these two members of the Pi transporter family contribute to As(V) uptake in *Arabidopsis* (Shin et al., 2004). Some species restrict phosphate uptake as an adaptive response in As(V) tolerance (Meharg and Macnair, 1990; Murota et al., 2012). In the reference plant *Arabidopsis*, we identified an As(V)-tolerant mutant that harbors a semidominant allele of the Pi transporter PHT1;1 (Catarecha et al., 2007). This mutant has a slow rate of As(V) uptake that allows the arsenic detoxification machinery to cope more efficiently with the metalloid leading to enhanced arsenic accumulation in the plant; nonetheless, any strategy that interrupts As(V) uptake to protect plants from its toxic effects also compromises Pi acquisition and, thus, plant growth in natural soils.

There are few descriptions of the molecular mechanisms involved in plant As(V) perception, although recent efforts have been made to understand the systems underlying As(V) tolerance (Sung et al., 2009; Song et al., 2010; Mendoza-Cózatl et al., 2011;

[1] Address correspondence to aleyva@cnb.csic.es.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Antonio Leyva (aleyva@cnb.csic.es).
[W] Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.113.114009

Jobe et al., 2012). In As(V)- or Pi-exposed *Arabidopsis*, As(V) downregulates expression of the high affinity Pi transporter gene *PHT1;1* more efficiently than does Pi, even when the Pi uptake rate is 2 times greater than that of As(V) (Catarecha et al., 2007). By contrast, another set of genes that respond to Pi starvation (such as *IPS1*) are repressed more efficiently by Pi than by As(V) (Catarecha et al., 2007). Although As(V) is rapidly reduced to arsenite once incorporated into cells, *PHT1;1* expression is not repressed by this form or by other heavy metals or even by short-term exposure to Pi (Catarecha et al., 2007), suggesting that *PHT1;1* repression is As(V) specific.

Here, we found that As(V) uptake was modulated by the As(V)-responsive transcription factor WRKY6, which rapidly repressed expression of the As(V)/Pi transporter *PHT1;1*. This repression was accompanied by *PHT1;1* delocalization from the plasma membrane. Once As(V) was removed, the system rapidly restored *PHT1;1* transporter expression and membrane relocalization. In addition, As(V) induced transcriptional activation of transposons,

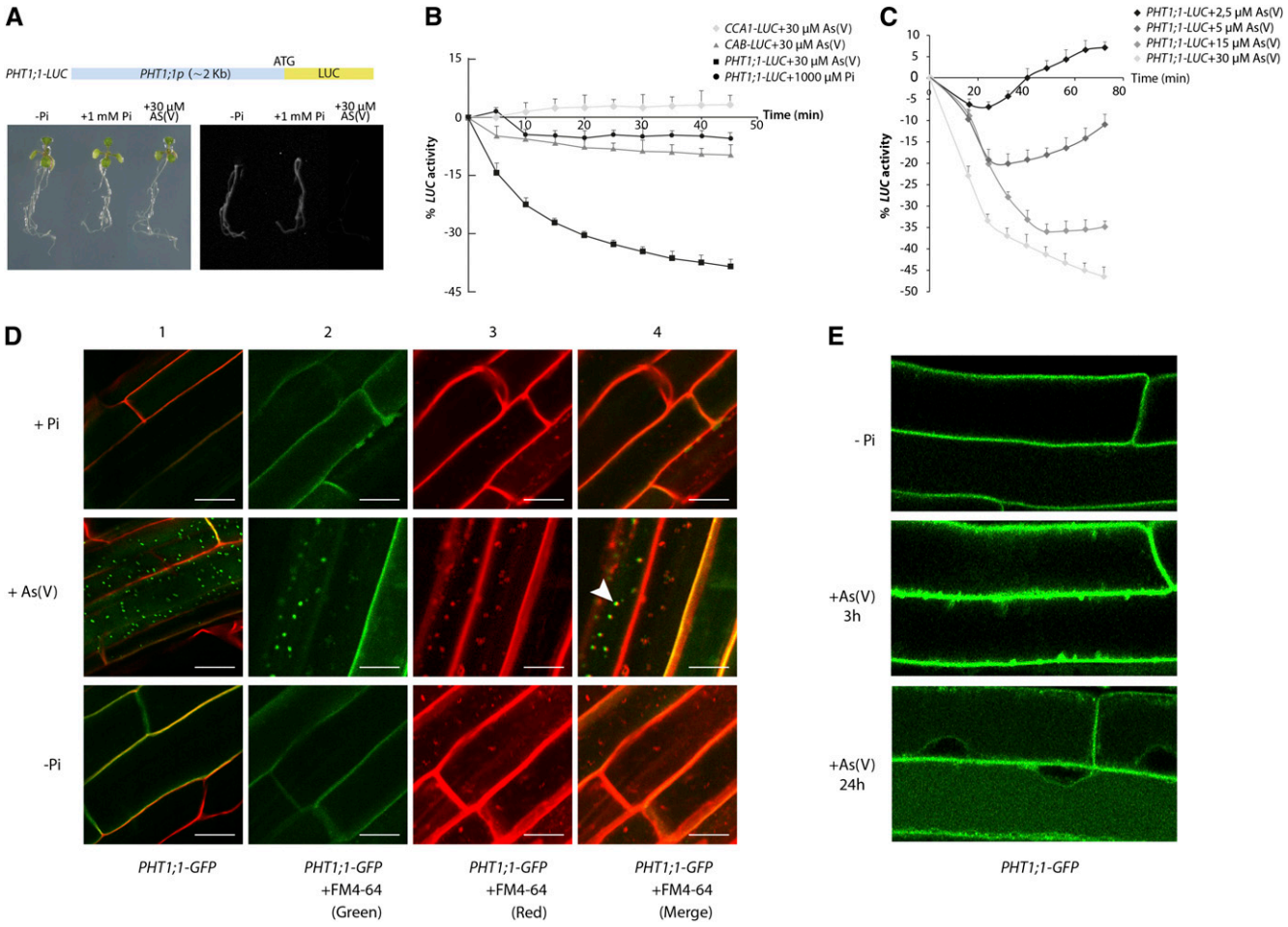


Figure 1. As(V) Represses and Delocalizes the Pi Transporter PHT1;1.

(A) Diagram of the 2-kb *PHT1;1* promoter region fused to the luciferase reporter gene (*PHT1;1-LUC*; top). Analysis of LUC activity (right panel) in transgenic plants expressing *PHT1;1-LUC* grown on 1 mM phosphate medium for 7 d, transferred to -Pi medium for 2 d, and finally to -Pi medium supplemented with 30 µM As(V) or 1 mM Pi for 16 h (left panel).

(B) Kinetic study of LUC activity in response to 30 µM As(V) or 1 mM Pi in *PHT1;1-LUC*–expressing plants and in control transgenic lines *CCA1-LUC* and *CAB-LUC*. Values show mean ± SD.

(C) Kinetic study of LUC activity in response to different As(V) concentrations in *PHT1;1-LUC*–expressing plants. Values show mean ± SD.

(D) and **(E)** Confocal analysis of *PHT1;1-GFP*–expressing *Arabidopsis* root epidermal cells.

(D) Five-day-old plants grown in -Pi medium were transferred to fresh -Pi medium or to medium containing 1 mM Pi (+Pi) or 30 µM As(V) [+As(V)] for 1.5 h in the dark. Roots were stained with propidium iodide (column 1) or with the endocytic tracer FM4-64 (columns 2 to 4). Arrowhead indicates colocalization of FM4-64 with *PHT1;1-GFP* in endosomes. Bars = 10 µm.

(E) Five-day-old *PHT1;1-GFP*–expressing plants grown in -Pi medium were incubated (24 h) (-Pi) or exposed to 30 µM As(V) for 3 h [+As(V) 3 h] and 24 h [+As(V) 24 h] in the dark.

also restricted by WRKY6. Plants thus have a regulatory mechanism that controls As(V) uptake and transposon expression in an integrated strategy for As(V) tolerance and transposon gene silencing.

RESULTS

As(V) Is Responsible for Rapid Pi Transporter Repression and Its Relocalization

To define the molecular mechanisms that underlie *PHT1;1* repression in response to As(V), we generated a transcriptional reporter line that expresses the luciferase (*LUC*) gene under the control of the *PHT1;1* promoter region (*PHT1;1-LUC*). This line stably expresses luciferase activity in response to Pi starvation. *PHT1;1-LUC* activity decreased more rapidly in response to As(V) than to Pi (Figure 1A). Time-course analysis showed that the LUC activity of the *PHT1;1-LUC* construct was reduced by >40% after 45-min exposure to As(V), at which time no repression was observed in response to Pi, even at a 33-fold higher concentration (Figure 1B). As(V) had no effect on unrelated genes such as *CCA1-LUC* and *CAB-LUC* promoter fusions. Dose–response experiments using decreasing As(V) doses showed that *PHT1;1-LUC* repression occurred at low As(V) concentrations (Figure 1C). *PHT1;1* repression and dose–response experiments were validated by quantitative RT-PCR (qRT-PCR) (see Supplemental Figure 1 online), indicating that this construct serves as a suitable readout for As(V) repression of *PHT1;1*. These observations indicate the operation of a sensitive As(V) detection mechanism in *Arabidopsis* plants.

PHT1;1 repression in response to As(V) is also accompanied by endocytosis of the As(V)/Pi transporter from the plasma membrane (Figure 1D). Shortly after plant exposure to As(V), we found PHT1;1-GFP (for green fluorescent protein) fluorescence in punctate cytosolic structures that colocalized with the styryl endocytic tracer FM4-64, indicating transporter internalization into endosomes (Figure 1D). After longer incubation with As(V), GFP fluorescence labeled the vacuole lumen (Figure 1E), indicating PHT1;1-GFP internalization from the plasma membrane and transport through the endocytic pathway for turnover in the

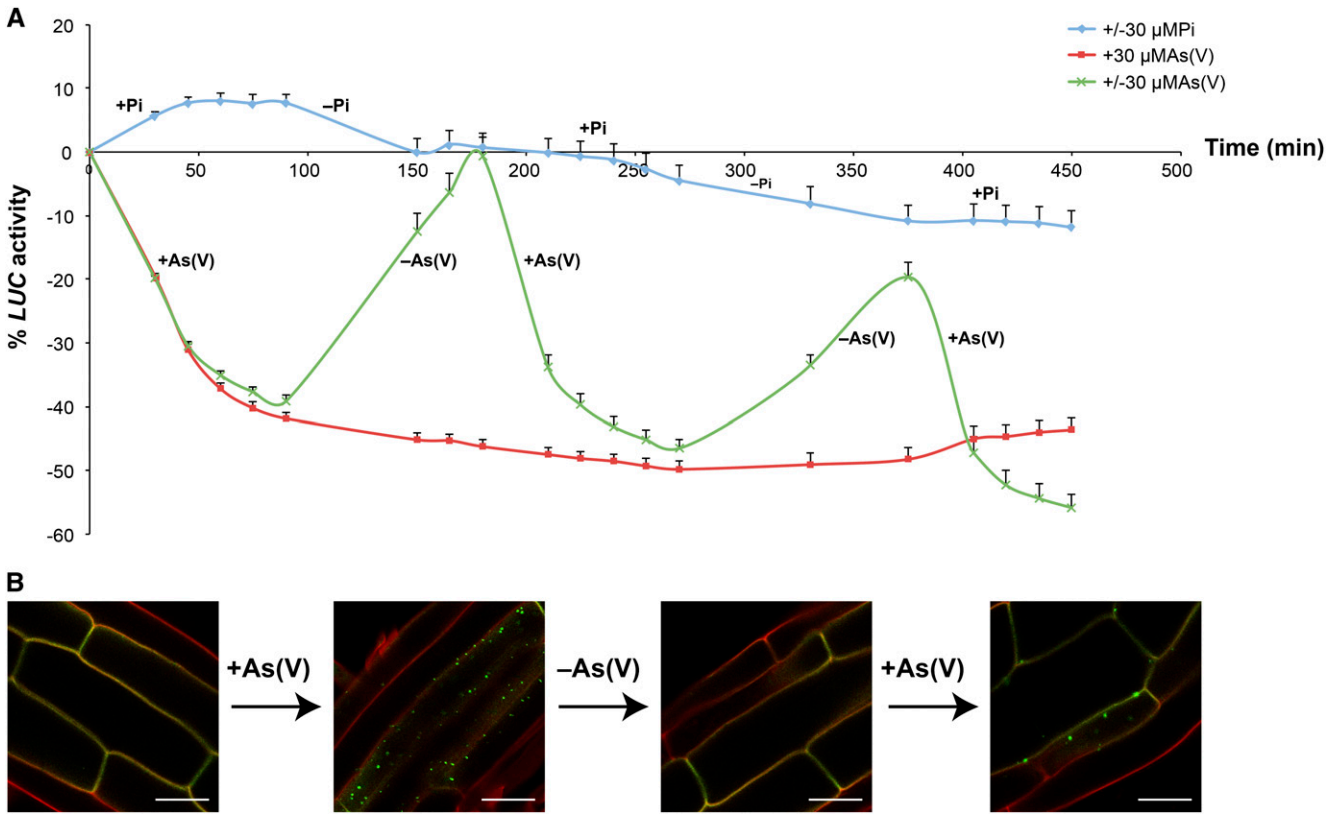


Figure 2. *PHT1;1* Expression and Its Membrane Localization Depend on As(V) Concentration.

(A) Kinetic study of LUC activity in response to pulses of 30 μM Pi (±Pi; blue line), 30 μM As(V) [±As(V); green line], or to a continuous concentration of 30 μM As(V) (red line) in *PHT1;1-LUC*–expressing plants. Duration of each pulse and gaps between them were 1.5 h; during the gap, samples were washed with buffer to remove Pi and As(V) from the medium. Values show mean ± SD.

(B) Analysis of *PHT1;1-GFP* localization after two pulses of 30 μM As(V) [±As(V)] in *PHT1;1-GFP*–expressing *Arabidopsis* root cells. Duration of each pulse and gaps between them were 1.5 h in the conditions as in **(A)**. Bars = 10 μm.

vacuole. By contrast, As(V) did not alter PIP1;4-YFP control protein localization at the plasma membrane, showing that Pi transporter relocalization is not a nonspecific response to As(V) toxicity (see Supplemental Figure 2A online). After As(V) exposure, there was an intracellular arsenic accumulation arrest that correlated with PHT1;1 relocalization (see Supplemental Figures 2B and 2C online). We thus propose the existence of a mechanism in plants that restricts As(V) uptake via two parallel responses: downregulation and relocalization of PHT1;1. As(V) removal led to restoration of correct *PHT1;1* expression, with kinetics similar to that of repression (Figure 2A). After a second round of high/low As(V) exposure, plants again showed the transcriptional repression/reactivation pattern (Figure 2A) closely linked to As(V)/Pi transporter delocalization/relocalization at the plasma membrane (Figure 2B). qRT-PCR experiments again confirmed *PHT1;1-LUC* expression mimicry of *PHT1;1* transcript accumulation (see Supplemental Figure 3 online). The rapid recovery of the system under Pi-free conditions indicated that both *PHT1;1* transcriptional repression and membrane relocalization depend exclusively on As(V).

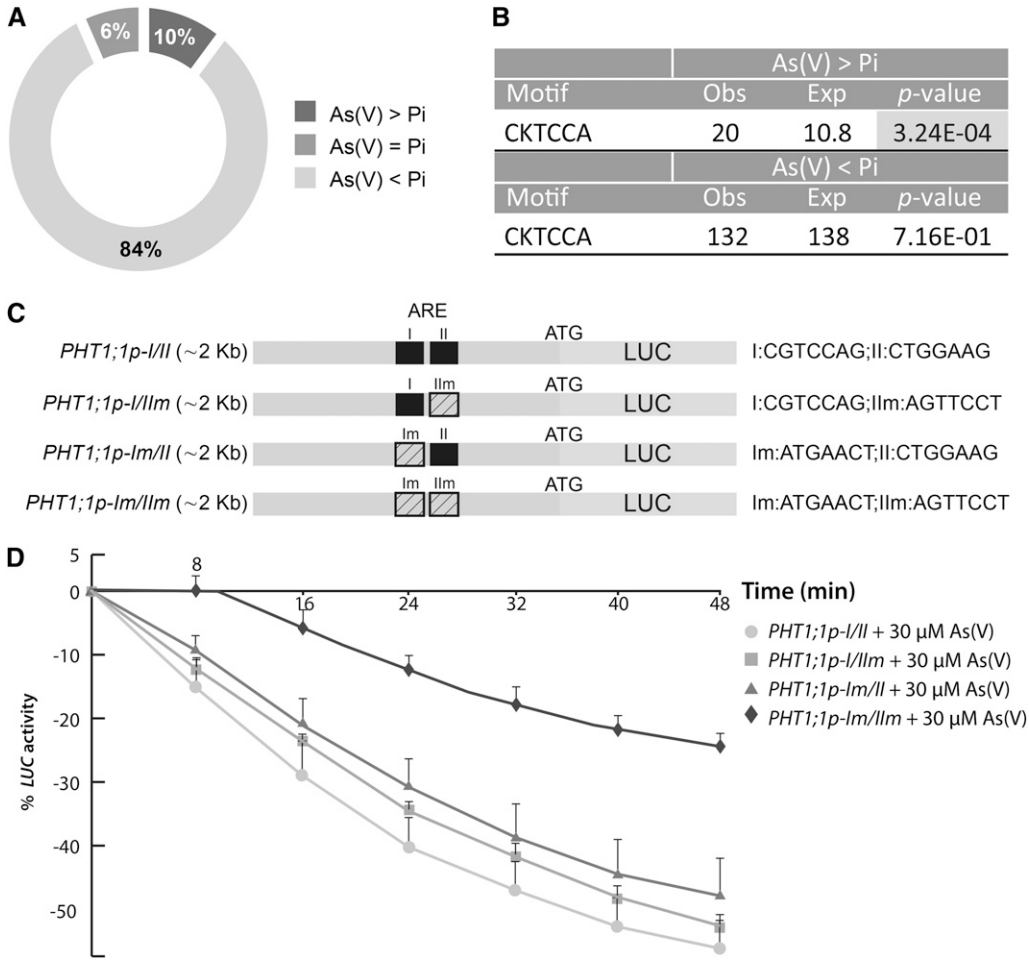


Figure 3. The ARE Contributes to Downregulation of the Pi Transporter in Response to As(V).

(A) Diagram showing the relative sizes of the three classes of downregulated genes in response to 30 μM As(V) and 30 μM Pi identified in a microarray analysis. As(V) > Pi, genes preferentially downregulated by As(V); As(V) < Pi, genes preferentially downregulated by Pi; As(V) = Pi, genes downregulated equally in response to both. For microarray analysis, wild-type plants were grown on Johnson medium with 1 mM Pi for 7 d, transferred to -Pi for 2 d, and finally to -Pi medium supplemented with 30 μM As(V) or 30 μM Pi (8 h).

(B) Analysis of ARE frequency in 0.5 kb of the promoter regions of genes in the As(V)>Pi and As(V)<Pi classes. The table shows observed and expected ARE numbers assuming random distribution. Significant overrepresentation is highlighted (χ^2 test, $P < 0.05$).

(C) Diagram showing wild-type (*PHT1;1p-I/II*) and mutated versions (*PHT1;1p-I/IIm*, *PHT1;1p-Im/II*, and *PHT1;1p-Im/IIm*) of the *PHT1;1* promoter region fused to luciferase (*PHT1;1-LUC*). The ARE in the *PHT1;1* promoter (I and II; black) was mutated sequentially (Im and IIm; diagonal stripes) by PCR site-directed mutagenesis.

(D) Kinetic analysis of LUC activity in transgenic plants expressing the *PHT1;1p-I/II*, *PHT1;1p-I/IIm*, *PHT1;1p-Im/II*, or *PHT1;1p-Im/IIm* constructs, in response to 30 μM As(V) (1.5 h). Values represent data from analysis of 12 independent lines of each construct; mean ± SD.

WRKY6 Transcription Factor Mediates *PHT1;1* Repression in Response to As(V)

To further study the role of As(V) in the repression of Pi starvation-induced genes similar to *PHT1;1*, we performed a transcriptomic analysis of Pi-starved plants exposed to As(V) or Pi for 8 h. Only 10% of Pi starvation-upregulated genes were repressed more efficiently by As(V) than by Pi, indicating that few genes show the transcriptional repression pattern of the Pi transporters in response to As(V) (Figure 3A; see Supplemental Data Set 1 online). In silico analysis of enriched regulatory elements in the promoter regions of these genes allowed identification of a *cis* As(V) repression element (ARE; KTCCAG, K:G/T) also found in the *PHT1;1* promoter region (Figures 3B and 3C). Analysis of luciferase activity in transgenic plants expressing ARE-mutated versions of *PHT1;1-LUC* constructs showed that this element contributes to repression of the Pi transporter in response to As(V) (Figure 3D). This ARE box resembles the TTTTCCAC (WK-box) core motif that, in addition to the classical W-box, is bound by plant WRKY transcription factors (Rushton et al., 2010). As(V) transcriptome profile analysis identified a member of the WRKY family, *WRKY6*, as an As

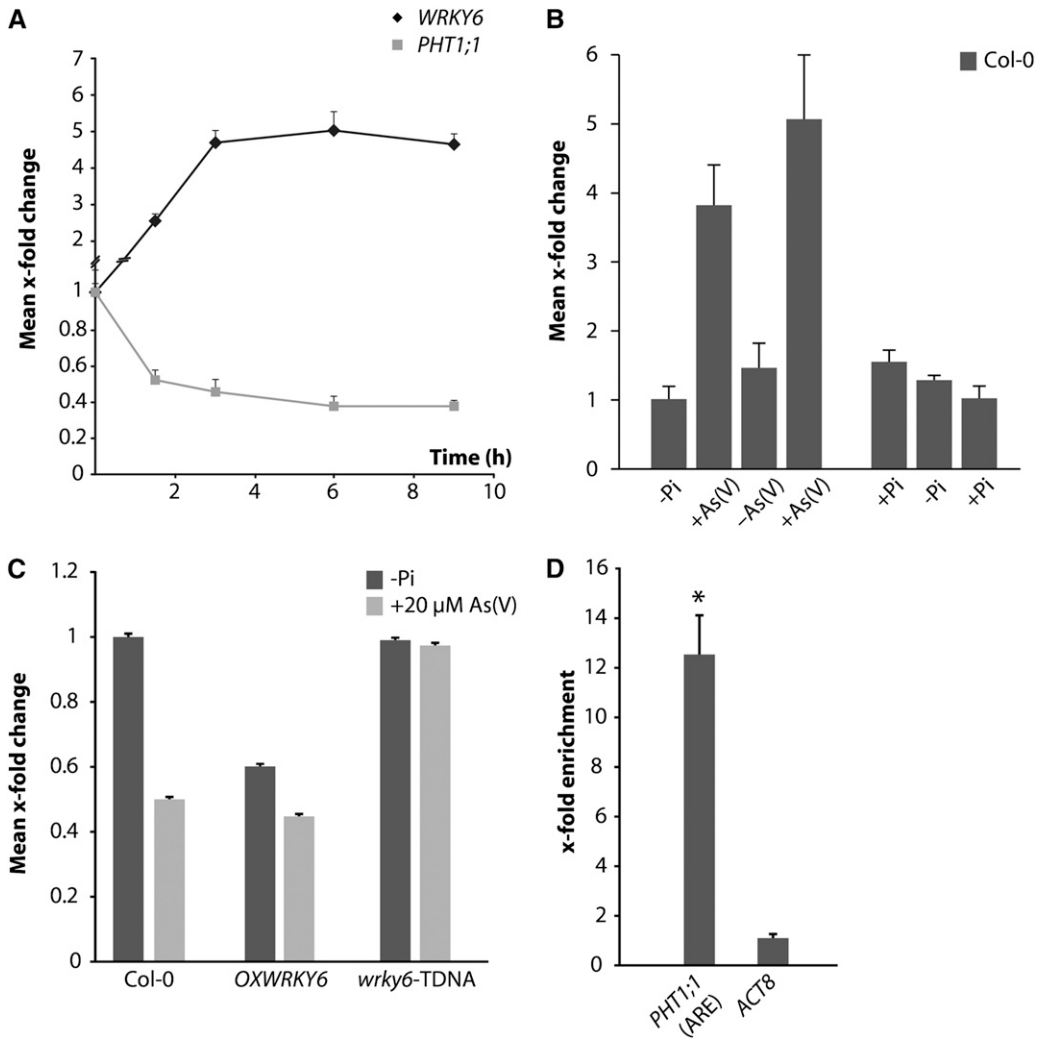


Figure 4. *WRKY6* Responds to As(V) and Represses the Pi Transporter *PHT1;1*.

(A) Kinetic study of *PHT1;1* and *WRKY6* expression by qRT-PCR in wild-type plants exposed to 30 μM As(V). Values show mean ± SD.
(B) qRT-PCR expression analysis of *WRKY6* in wild-type plants in response to 30 μM As(V) pulses [±As(V)] or in response to 30 μM Pi pulses (±Pi); duration of each pulse and gap was 1.5 h. Values show mean ± SD.
(C) qRT-PCR of *PHT1;1* transcript in wild-type plants (Col-0), in the *WRKY6-GFP*–overexpressing line (*OXWRKY6*), and in *wrky6*-TDNA line grown in +Pi medium for 7 d, transferred to -Pi for 2 d and then to -Pi medium alone or with 20 μM As(V) (1.5 h). In the case of *WRKY6*-overexpressing lines, values show data from analysis of 10 independent lines. Values show mean ± SD.
(D) ChIP assay of *WRKY6-GFP* seedlings and *PHT1;1* promoter PCR amplification analysis. qPCR of ARE-containing fragments of the *PHT1;1* promoter. Enrichment was calculated relative to wild-type plants. *ACT8* was used as negative control. Values show mean ± SD. *$P < 0.05$ (Student's *t* test).

(V)-responsive gene (see Supplemental Data Set 1 online). WRKY6 is involved in the control of several plant responses, including nutrient starvation (Robatzek and Somssich, 2002; Kasajima et al., 2010), and negatively regulates PHO1, a plasma membrane protein involved in xylem Pi loading (Chen et al., 2009). These observations prompted us to consider *WRKY6* involvement in *PHT1;1* As(V) repression.

To test whether WRKY6 is responsible for *PHT1;1* repression following As(V) stress, we first evaluated *WRKY6* kinetic responsiveness to As(V). The kinetics of *WRKY6* transcript accumulation in response to As(V) was inverse to that of As(V)/Pi transporter *PHT1;1* repression by As(V) (Figure 4A). In response to As(V) pulses, the *WRKY6* activation/repression pattern was again inverse to that of *PHT1;1*, as predicted for a transport repressor-mediated mechanism, whereas *WRKY6* transcript accumulation was unaltered in response to Pi pulses (Figure 4B).

In a transgenic *Arabidopsis* line expressing *WRKY6-GFP* at a level similar to that of endogenous *WRKY6* in response to As(V) (see Supplemental Figure 4A online; WRKY6-GFP-L10), *PHT1;1* expression was reduced in As(V)-exposed wild-type plants (Figure 4C). By contrast, expression of the Pi starvation-induced gene *SQD1*, which is not preferentially repressed by As(V), was unaltered in the *WRKY6-GFP*–overexpressing line (see Supplemental Figure 4B online). To test whether WRKY6 interacts directly with the *PHT1;1* promoter region in vivo, we used chromatin immunoprecipitation (ChIP) assays in the

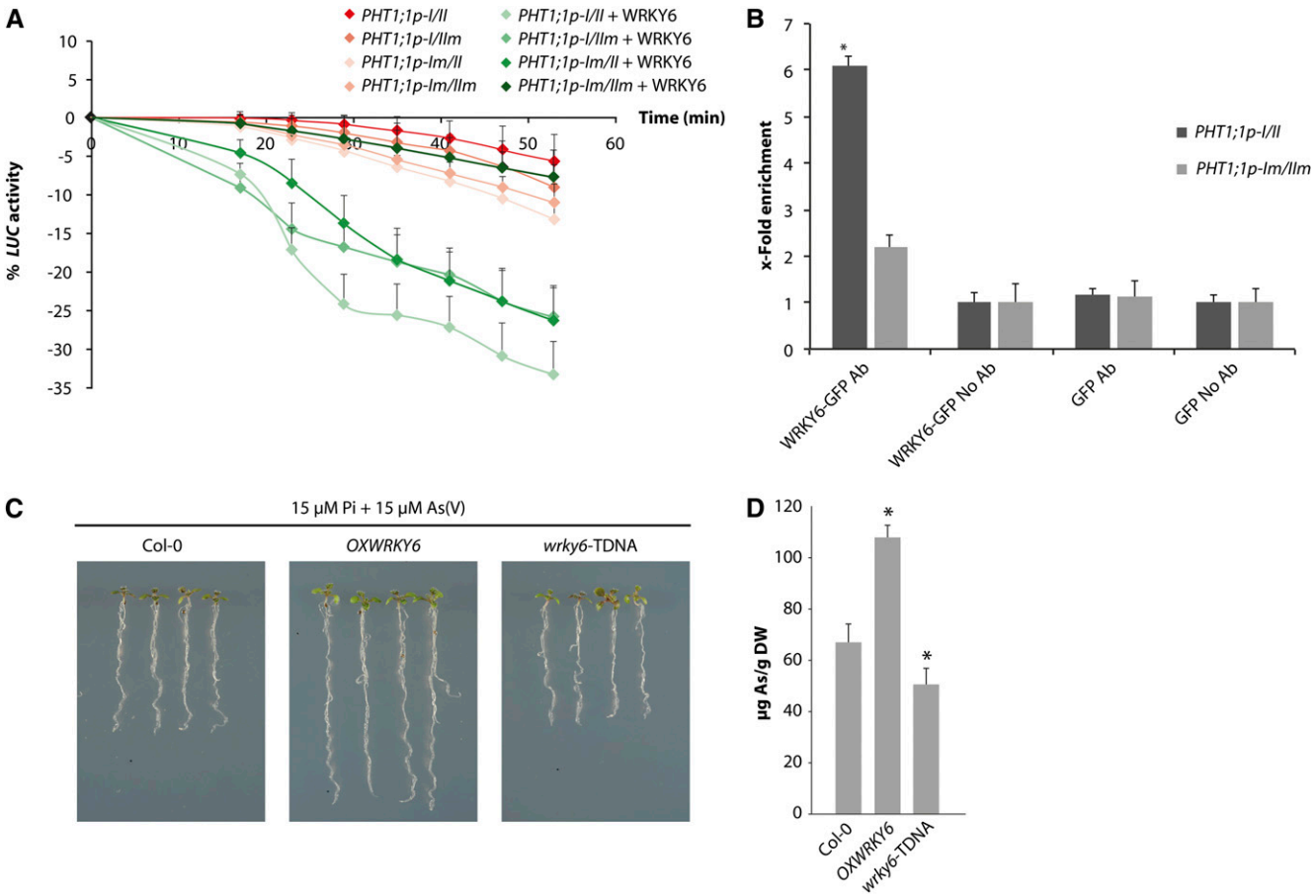


Figure 5. The ARE Mediates *WRKY6* Repression of the Pi Transporter *PHT1;1* and Confers the As(V) Tolerance Phenotype.

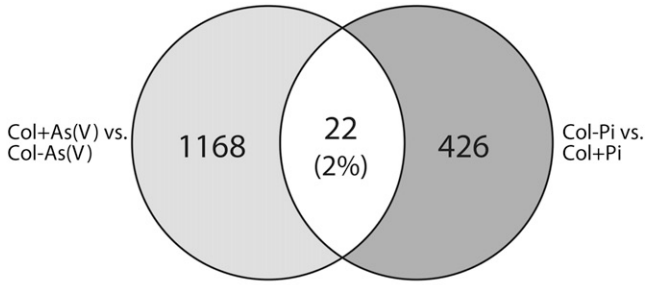
(A) Kinetic analysis of transient LUC activity in *N. benthamiana* leaf discs agroinfiltrated with *PHT1;1-LUC* wild type or the mutated versions alone or with a *WRKY6-GFP*–overexpressing construct. Leaf discs were incubated in medium with 30 µM As(V) (1.5 h). Values show mean ± SD.

(B) ChIP assay of *WRKY6-GFP* followed by qPCR of the *PHT1;1* promoter. ChIP assays were performed in *N. benthamiana* leaf discs agroinfiltrated with *PHT1;1-LUC* wild type and the *PHT1;1-LUC* mutated version (PHT1;1p-Im/IIm), with *WRKY6-GFP* or GFP-overexpressing constructs. Values represent the *x*-fold enrichment of WRKY6-bound DNA of the *PHT1;1* promoter in immunoprecipitated samples relative to total input DNA. ARE (*PHT1;1p-I/II-LUC*) or mutated ARE-containing fragments (*PHT1;1p-Im/IIm-LUC*) in the *PHT1;1* promoter were amplified by qPCR using specific primers. Values show mean ± SD. *P < 0.01 (Student's *t* test). Values show mean ± SD.

(C) As(V) tolerance phenotype of wild-type (Col-0), *OXWRKY6-GFP*–overexpressing line (*OXWRKY6*), and *wrky6*-TDNA plants grown on 15 µM Pi supplemented with 15 µM As(V) for 7 d.

(D) Intracellular arsenic concentration in Col-0, *OXWRKY6*, and *wrky6*-TDNA plants exposed to 5 µM As(V) (1 h). Values show mean ± SD. *p < 0.05 (Student's *t* test). DW, dry weight.

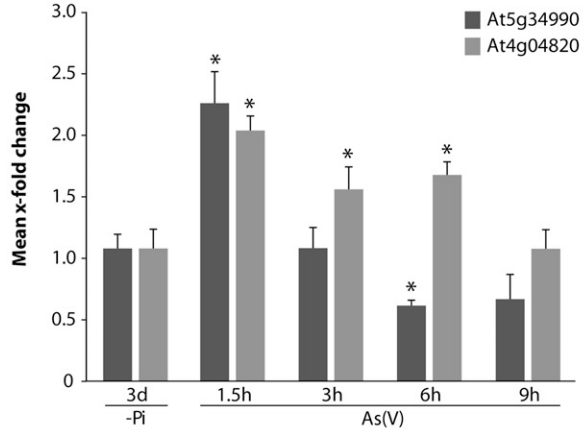
A



B

x-Fold expression in Col+As(V) *vs.* Col-As(V) (2x;FDR<0.1)	Transposable Elements		*x*-Fold expression in *OXWRKY6*+As(V) *vs.* Col+As(V) (1.5x;FDR<0.2) Upregulated (362)	Downregulated (335)
			1	**164**
Increased expression	**869**	Observed (%)	0 (0)	157 (95.7)
		Expected	0.2	37
		p-value	0.8	**2.6E-91**
Reduced expression	**4**	Observed (%)	0 (0)	0
		Expected	0	0.2
		p-value	1	0.8

C



D

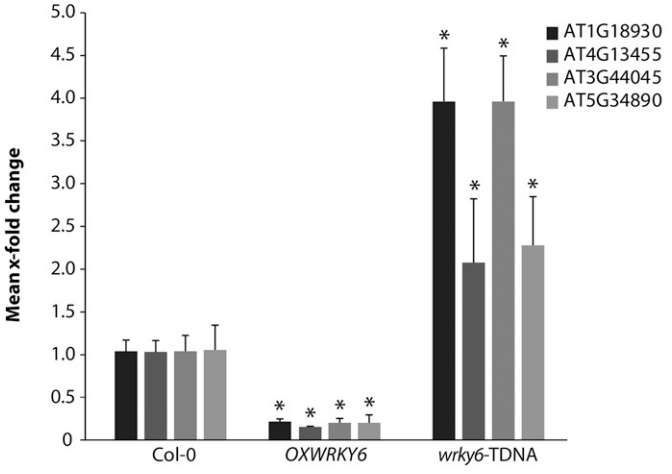


Figure 6. WRKY6 Restricts Transposon Expression.

(A) Venn diagram showing relative proportion of As(V)-downregulated genes [Col+As(V) versus Col-As(V)] and Pi starvation-upregulated genes (Col-Pi versus Col+Pi) and degree of overlap. For comparison, only genes of the 12x135K platform (NimbleGen) that coincide with those in the ATH1 platform (Affymetrix) were considered (twofold [2x], FDR < 0.1, and twofold [2x], FDR <0.05, respectively).

(B) Comparative transcriptome analysis of As(V)-responsive transposons in wild-type plants and in the *WRKY6-GFP*–overexpressing line (*OXWRKY6*). Total number and percentage is indicated of transposons induced and repressed in wild-type [Col+As(V) versus Col-As(V)] and in *WRKY6-GFP*–overexpressing plants [*OXWRKY6*+As(V) versus Col+As(V)] in response to As(V). Cutoff values for analysis of As(V)-responsive transposons were

WRKY6-GFP–overexpressing line. Quantitative PCR (qPCR) of chromatin fragments from this line immunoprecipitated with anti-GFP showed enrichment in ARE-bearing fragments of the *PHT1;1* promoter compared with wild-type plants and the *ACTIN8* (*ACT8*) control (Figure 4D). Transient LUC activity expression assays in *Nicotiana benthamiana* leaf discs agroinfiltrated with *PHT1;1-LUC* or *PHT1;1-LUC* ARE mutants showed that this *cis* motif mediates WRKY6 repression of the Pi transporter (Figure 5A). ChIP assays using *N. benthamiana* plants infiltrated with wild-type and *PHT1;1* ARE mutant promoters showed that WRKY6 requires an intact ARE to bind the *PHT1;1* promoter region (Figure 5B). In our experiments, we included a *wrky6*-TDNA insertion line that behaves as a null mutant, with no detectable *WRKY6* expression (see Supplemental Figure 4A online). The *wrky6*-TDNA line showed no *PHT1;1* repression in response to As(V) (Figure 4C). These results reinforce the idea that WRKY6 mediates *PHT1;1* transcriptional repression.

In the absence of As(V), the *WRKY6-GFP*–overexpressing line and *wrky6*-TDNA insertion line showed no visible differences in root length compared with wild-type plants (see Supplemental Figures 4C and 4D online). By contrast, As(V) tolerance was markedly enhanced in the *WRKY6-GFP*–overexpressing line (Figure 5C); quantification of root length showed a significant increase compared with wild-type plants in the presence of As(V) (see Supplemental Figure 4D online). No differences in root length were observed in the *wrky6*-TDNA insertion line relative to wild-type plants, probably due to functional redundancy among *WRKY* gene family members (Figure 5C; see Supplemental Figure 4D online). As noted above, however, *PHT1;1* expression was not repressed in response to As(V) in the *wrky6*-TDNA background, indicating incomplete functional redundancy.

Arsenic accumulation was increased in the *WRKY6*-overexpressing line compared with wild-type controls, whereas levels in the *wrky6*-TDNA mutant were significantly lower (Figure 5D). Reduction in As(V) uptake is associated with enhanced arsenic accumulation (Catarecha et al., 2007). These findings thus indicate that *WRKY6* modulates As(V) uptake through transcriptional repression of the As(V)/Pi transporters.

As(V) Causes a Transposon Burst Modulated by WRKY6

In our experiments, >80% of the *PHT1;1* repression in response to As(V) was observed as early as 1.5 h after treatment (Figure 1B). For detailed study of the expression profile of the As(V) response, we thus performed transcriptome analysis on plants exposed to As(V) for 1.5 h. As(V) induces marked transcriptional repression of 1519 genes (see Supplemental Figure 5 and Supplemental Data Set 2 online), similar to a previous report (Abercrombie et al., 2008). Less than 2% of the As(V)-downregulated transcripts overlapped with Pi starvation-inducible genes, based on data from the 8-h array experiment (Figure 6A; see Supplemental Figure 5 online). As(V) repression of the As(V)/Pi transporters is thus a specific short-term response.

This gene expression analysis also identified 1936 genes upregulated in response to As(V) (see Supplemental Figure 5 and Supplemental Data Set 2 online); of these, 869 (44.9%) encoded transposons (Figure 6B). qRT-PCR analysis confirmed transposon induction (see Supplemental Figure 6A online); As(V) stress therefore induced a notable transposon burst (Figure 6B; see Supplemental Data Set 3 online). As(V) induction of transposons was transient, with the highest expression at 1.5 h after As(V) exposure (Figure 6C). Maximum *WRKY6* transcript accumulation was delayed compared with that observed for transposons; however, its expression peak correlated with intense transposon downregulation (Figures 4A and 6C), in accordance with a possible role of WRKY6 in transposon repression. Transcriptome analysis of the As(V)-exposed *WRKY6-GFP*–overexpressing line showed that close to 50% of transcripts downregulated in this line encoded transposons (164 of 335; see Supplemental Data Set 4 online) and nearly all (95.7%) corresponded to As(V)-induced transposons (Figure 6B; see Supplemental Data Set 4 online). qRT-PCR experiments confirmed that *WRKY6* overexpression downregulated transposon expression, which was enhanced in *wrky6*-TDNA (Figure 6D; see Supplemental Figure 6B online). *WRKY6* activation thus has an important function in limiting As(V)-induced transposon activation.

Transposon downregulation by WRKY6 could be, at least in part, an indirect effect of reduced As(V) uptake due to *PHT1;1* repression. To explore this possibility, we tested whether Pi starvation was able to activate transposon expression independently of As(V)/Pi transport. Five of 12 As(V)-responsive transposons were induced by Pi starvation (see Supplemental Figure 6C online); in all cases, *WRKY6* overexpression reduced transposon induction in response to Pi starvation (see Supplemental Figure 6D online). Transposon downregulation in the *WRKY6*-overexpressing lines is thus not merely a consequence of stress alleviation due to the arrest of intracellular arsenic accumulation.

We found *WRKY* canonical *cis*-acting elements (W-box) in the promoter regions of As(V)-responsive transposons. To study W-box function, we performed kinetic analyses of luciferase activity in *N. benthamiana* leaves agroinfiltrated with *WRKY6-GFP* and the luciferase-fused promoter region of the transposable element At5g35030 or of the At5g35030 mutated W-box version; *WRKY6-GFP* repressed luciferase activity efficiently in the former (Figure 7A), but not in the latter (Figure 7B). Downregulation of transposon expression thus depends on an intact W-box at the promoter region. ChIP assays on *N. benthamiana* plants using At5g35030 transposon promoters and the mutated version of the

Figure 6. (continued).

twofold (2x), FDR < 0.1, and for *OXWRKY6*-responsive transposons, 1.5-fold (1.5x), FDR < 0.2. The table shows the size of observed overlap and expected size, assuming random distribution. Significant overlaps are in bold (χ^2 test, $P < 0.05$).

(C) qRT-PCR time-course analysis of transposon expression in wild-type plants exposed to 30 μM As(V).

(D) qRT-PCR analysis of transposon expression in wild-type (Col-0), *OXWRKY6*, and *wrky6*-TDNA plants in response to 30 μM As(V) (1.5 h). Values show mean ± SD; *$P < 0.05$ (Student's *t* test).

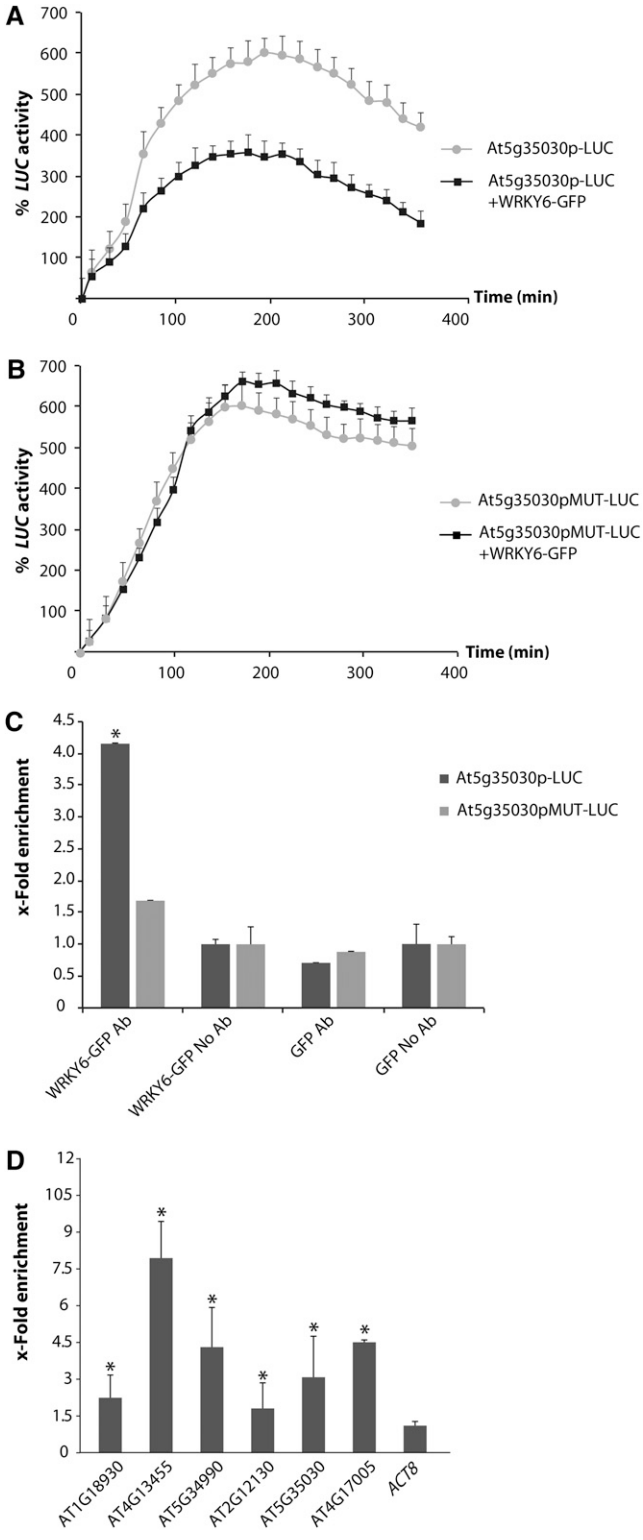


Figure 7. WRKY6 Interacts with the W-Box in the Promoter Region of As(V)-Responsive Transposable Elements.

(A) and **(B)** Kinetic analysis of LUC activity in *N. benthamiana* leaves agroinfiltrated with the promoter region of a transposable element At5g35030 fused to LUC (*At5g35030p-LUC*) **(A)** or *At5g35030p-LUC* in which the W-box was mutated (*At5g35030pMUT-LUC*) **(B)**, alone or with a *WRKY6-GFP*–overexpressing construct. Leaf discs were incubated in medium with 30 μM As(V) (1.5 h).

(C) ChIP assay of *WRKY6-GFP* followed by qPCR of the *At5g35030* promoter. ChIP assays were performed in *N. benthamiana* leaf discs agroinfiltrated with *At5g35030p-LUC* and the *At5g35030pMUT-LUC* version, with *WRKY6-GFP*– or GFP-overexpressing constructs. Values represent *x*-fold enrichment of *WRKY6*-bound DNA of the *At5g35030* promoter in immunoprecipitated samples relative to total input DNA. W-box–containing fragments (*At5g35030p-LUC*) or mutated W-box–containing fragments (*At5g35030pMUT-LUC*) of the *At5g35030* promoter were qPCR amplified using specific primers. *P < 0.01 (Student's *t* test).

(D) ChIP assay of *WRKY6-GFP*–overexpressing plants, followed by qPCR of the W-box–bearing fragments of transposon promoters. Enrichment was calculated relative to wild-type plants. *ACT8* was used as negative control. *P < 0.05 (Student's *t* test). Values show mean ± SD.

At5g35030 W-box confirmed that WRKY6 repression of transposable elements operates via direct WRKY6 interaction with its *cis*-acting binding site (Figure 7C). We also performed ChIP assays in the *WRKY6-GFP* transgenic plants. qPCR of chromatin fragments from *WRKY6-GFP*–overexpressing lines immunoprecipitated with anti-GFP showed enrichment in fragments of six transposon promoters with the W-box, compared with wild-type plants and the *ACT8* control (Figure 7D). WRKY6 therefore interacts in vivo with these six promoter regions, as also observed for *PHT1;1*. These experiments clearly indicate that WRKY6 acts directly as a transcriptional repressor of both the Pi transporter *PHT1;1* and As(V)-responsive transposable elements.

Transposable elements of *Arabidopsis* are under epigenetic regulation (Tsukahara et al., 2009; Mirouze and Paszkowski, 2011; Bucher et al., 2012). We thus assessed whether transcriptional activation of transposons in response to As(V) occurs through changes in histone modifications. We used ChIP to analyze chromatin in the genomic regions of six As(V)-responsive transposable elements in As(V)-treated plants, to determine histone H3 dimethylation levels at Lys-9 (H3K9me2), a hallmark of heterochromatin and transposable element repression (Johnson et al., 2002), and H3Ac, a histone modification associated with transcriptional activation (Kouzarides, 2007). As predicted, we found high H3K9me2 and low H3Ac levels in these transposons (see Supplemental Figure 7 online). This pattern is similar to that of the *Ta3* transposon, a classical target of epigenetic silencing (Johnson et al., 2002), and differs from the constitutively expressed *TUB8* (see Supplemental Figure 7 online). There were no differences in the amount of immunoprecipitated H3K9me2 between As(V)-treated and untreated plants, suggesting that at least in the short term, transposons are activated without modifications of this epigenetic mark, commonly associated with transposon silencing (Figure 8A). H3Ac levels were also unchanged, indicating that transcriptional activation of As(V)-responsive transposable elements is independent of changes in this histone mark (Figure 8B).

DISCUSSION

In this study, we show that in response to As(V), WRKY6 is involved in the rapid repression of the As(V)/Pi transporter *PHT1;1*,

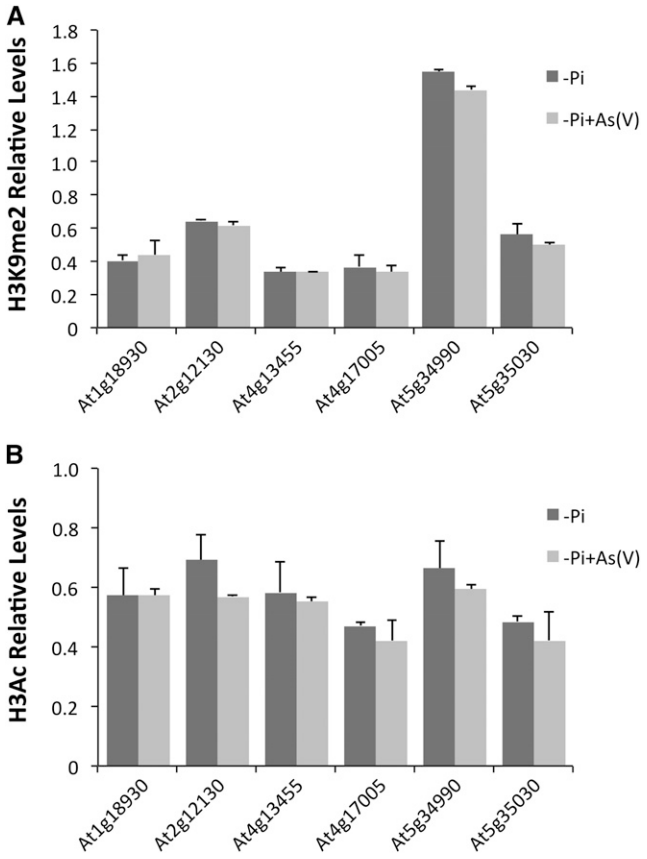


Figure 8. Activation of As(V)-Responsive Transposable Elements Is Independent of Changes in H3K9me2 and H3Ac Levels.

ChIP analysis of As(V)-induced transposable elements in *Arabidopsis* Col-0 plants grown on 1 mM Pi medium for 7 d, transferred to -Pi medium for 2 d and finally to liquid -Pi medium, alone or supplemented with 30 μM As(V). The assay was performed using antibodies specific for H3K9me2 **(A)** or H3Ac **(B)**, histone marks associated with repressive and active transcription, respectively. Levels of histone modifications in the genomic regions of As(V)-responsive transposable elements are represented relative to *Ta3* in the case of H3K9me2 or to *TUB8* for H3Ac. Values show mean ± SD.

which is coordinated with its delocalization from the plasma membrane. When As(V) was removed, the system quickly restored *PHT1;1* expression and membrane localization. We show that As(V) provoked a notable transposon burst, which was also counteracted by WRKY6 action. A classical repressor is therefore involved in a dual protection mechanism that restricts As(V) uptake and transposon expression.

Arabidopsis Plants Have a Sensitive As(V) Perception Mechanism

In plants, the expression of genes that encode transporters for nutrients, including Pi, is altered in response to nutrient deficiencies (Maruyama-Nakashita et al., 2004; Bustos et al., 2010; Kiba et al., 2012). The regulation of Pi transporter expression in response to nutrient starvation and the underlying components have been studied in detail (Bustos et al., 2010; Bayle et al., 2011); by contrast, the mechanisms involved in Pi perception are mostly unknown. Phosphate transporters are precisely regulated by Pi amount. In plants and yeast, exposure to Pi or nonmetabolized phosphate analogs triggers Pi transporter degradation (Mouillon and Persson, 2005; Bayle et al., 2011). Here we show that in response to As(V), Pi transporters are also delocalized from the plasma membrane to the vacuole, in parallel with the arrest of As(V) uptake. The kinetics of transcriptional repression and plasma membrane delocalization is nonetheless more rapid in response to As(V) than in response to resupply of Pi (or Pi analogs). Furthermore, Pi and its analogs cause broad repression of the Pi starvation response (Carswell et al., 1996, 1997; Rubio et al., 2001; Ticconi et al., 2001; Varadarajan et al., 2002; Bustos et al., 2010), whereas As(V) induced short-term repression specific mainly for the Pi transporter, with no marked alteration in the overall phosphate starvation response. Both As(V) and Pi thus act as signals in the control of Pi transporter expression, but specific regulatory elements must be involved for each of these environmental cues.

WRKY6 Mediates As(V) Repression of As(V)/Pi Transporters

Here, we found that *WRKY6* expression is activated in response to As(V) and is an essential component of the As(V) repression of As(V)/Pi transporters. WRKY transcription factors are involved in plant responses to a wide variety of environmental stresses (Rushton and Somssich, 1998; Robatzek and Somssich, 2002; Rushton et al., 2010). WRKY6 was initially implicated in the control of gene expression during plant senescence and more recently in Pi and boron starvation (Robatzek and Somssich, 2002; Chen et al., 2009; Kasajima et al., 2010). Members of the WRKY family can act as activators or repressors of distinct biological functions including Pi homeostasis. WRKY6 is described as a repressor of *PHO1*, which encodes a plasma membrane protein involved in Pi loading into xylem (Chen et al., 2009). The ability of WRKY6 to repress both extracellular Pi uptake and vascular Pi loading suggests that it operates in an integral strategy to prevent arsenic accumulation in the plant. Once As(V) is incorporated into cells, however, it is rapidly reduced to As(III) and is transported to the aerial part of the plant (Dhankher et al., 2006). In addition, arsenic amount in *pho1* mutant shoots is similar to or slightly higher than that observed in wild-type shoots (Quaghebeur and Rengel, 2004), suggesting that arsenic content in the aerial part of the plant is at least partially independent of *PHO1* regulation and, thus, of WRKY6. WRKY6 transcriptional repression of the Pi transporter nonetheless leads to a clear As(V) tolerance phenotype. These findings reinforce the importance of WRKY6 as an essential component in the regulation of As(V)/Pi transporters in response to As(V) and dependent on its concentration.

WRKY6 Restricts Transposon Activation in Response to As(V)

Our studies show that As(V) induces a rapid transposon burst in plants. Transposon activation in response to stress can be responsible for deleterious effects, such as gene deletion or insertion, chromosome rearrangement, and alterations in gene

expression (Ma and Bennetzen, 2006; Ito, 2012). Of the many stress factors in the early environment, high arsenic concentration was a serious impediment to the establishment of life on earth (Oremland et al., 2009; Dani, 2010); suppression of transposon bursts might thus be a valuable plant strategy for survival in the presence of arsenic. Stress-induced activation of transposons is usually suppressed by host epigenetic mechanisms, including methylation and histone tail modifications (Tittel-Elmer et al., 2010; Ito, 2012; Saze et al., 2012). It is thus assumed that stress-induced transposon activation takes place through changes in these epigenetic marks. Our data indicate that WRKY6 contributes to the restriction of transposon-induced activation in response to As(V). Although there is evidence that WRKY transcriptional activity can be regulated by interaction with histone modifiers (Kim et al., 2008), we show here that WRKY6 acts directly as a transcriptional repressor of As(V)-responsive transposable elements. More than 90% of transposons repressed in the WRKY6-overexpressing L10 line are As(V) induced, indicating that transcriptional repression contributes to transposon silencing in response to As(V). We also show that transposons are upregulated with no modification of two classical histone marks commonly associated with transposon silencing release. Our results coincide with data for the heat stress response, in which silencing is released with no obvious changes in these marks; this suggests that other mechanisms operate in transposon silencing (Pecinka et al., 2010; Tittel-Elmer et al., 2010; Saze et al., 2012). Although we cannot rule out the involvement of other epigenetic mechanisms in the activation of As(V)-responsive transposable elements, our results show that classical repression contributes to transposon silencing in response to As(V).

In this study, we found that *Arabidopsis* plants have a rapid detection mechanism that restricts As(V) uptake and transposon expression. When As(V) was removed, plants recovered *PHT1;1* expression and membrane localization, indicating a sensitive mechanism for perception of As(V). In addition, we show that the WRKY6 transcription factor is involved in *PHT1;1* repression and in preventing transposon expression in response to As(V). Both responses are coordinated temporally with the delocalization of the As(V)/Pi transporter PHT1;1, although it is currently not known whether PHT1;1 relocalization is under WRKY6 control. The coordination of these two responses under the control of a single repressor is a unique strategy that allows the plant to overcome As(V) toxicity and transposon activation. These findings provide evidence of a major role for classical transcription repressors in restoring transposon silencing after stress-induced activation. We identify a dual As(V) signaling mechanism that, depending on As(V) amounts, regulates stress-induced transposon expression and As(V) uptake.

METHODS

Materials and Growth Conditions

We used the T-DNA mutant of gene *WRKY6* SALK_012997C that bears an insertion in the third exon, as well as several *Arabidopsis thaliana* transgenic lines, including *35S:PHT1;1-GFP* (González et al., 2005), *CCA1-LUC*, *CAB-LUC* (a kind gift of Salomé Prat [Centro Nacional de Biotecnología–Consejo Superior de Investigaciones Cientificas]), and *PIP1;4-YFP* (wave 138) (Geldner et al., 2009).

Seedlings were grown on Johnson medium supplemented with distinct Pi (KH_2PO_4) concentrations depending on the experiment; 1 mM Pi was used for complete medium (+Pi), 30 μM for low-Pi medium, and 5 μM for phosphate starvation (-Pi). In general, for treatment with As(V) ($NaH_2AsO_4 \cdot 7H_2O$), plants were grown on Johnson medium +Pi for 7 d, then transferred to -Pi medium for 2 d, and finally to liquid -Pi medium alone or supplemented with 30 μM As(V). For all molecular and biochemical analyses, *Arabidopsis* plants were grown in a culture chamber in a 16-h-light/8-h-dark regime (24°C/21°C).

Physiological Measurements

For As(V) tolerance analysis, plants were grown on Johnson medium with 15 μM Pi, alone or with 15 μM As(V), in a horizontal position for 10 d. Main root length was then measured using ImageJ software (Barboriak et al., 2005). For arsenic accumulation experiments, plants were grown on plates containing half-strength Bates and Lynch medium solidified with 0.4% bacto-agar with 30 μM Pi and covered with 0.4-mm-pore nylon mesh. Seeds were sown onto the mesh and cultured for 7 d. Plants were treated with PI and PII buffers (Narang et al., 2000). Arsenic accumulation experiments were performed in 50-mL pots using 5 μM As(V). Plants were dried (60°C, 5 d), mineralized with HNO_4-H_2O_2 in a pressure digester, and analyzed for total arsenic content by inductively coupled plasma–mass spectrometry at the Servicio Interdepartamental de Investigación (Universidad Autónoma de Madrid, Spain).

Binary Construct and Plant Transformation

To generate the *WRKY6*-overexpressing lines, full-size *WRKY6* cDNA was amplified with primers Wrky6-GWF and Wrky6-GWR (see Supplemental Table 1 online), cloned into the pDNR207 vector, and inserted into the pGWB5 binary vector containing the constitutive 35S promoter, using the LR recombination reaction (Invitrogen). This binary construct was introduced into *Agrobacterium tumefaciens* strain C58C1 and transformed in wild-type Columbia-0 (Col-0) plants using the floral dip transformation method (Clough and Bent, 1998). Transformants were selected on 40 mg/L hygromycin-containing medium. Homozygous lines were selected for strong expression.

Approximately 2 kb of the *PHT1;1* and 1.3 kb of the transposable element (At5g35030) promoter regions were amplified using their respective primer pairs, PHT1;1p-I/II-F, PHT1;1p-I/II-R and At5g35030p-I-F, At5g35030p-I-R (see Supplemental Table 1 online). The products were excised with *Hin*dIII and *Bam*HI and cloned into the pLUC vector (Ulm et al., 2004). Using these constructs as templates, three versions of the *PHT1;1* and one of the At5g35030 promoters were generated by PCR site-directed mutagenesis to alter the sequence of the ARE and W-box, respectively; primers used are listed in Supplemental Table 1 online. All binary constructs were introduced into *Agrobacterium* strain C58C1. *Agrobacterium*-mediated transient expression assays in *Nicotiana benthamiana* plants were performed as described (Jiménez et al., 2006). The *PHT1;1* binary constructs were transformed in wild-type Col-0 plants by floral dip transformation. Transformants were selected on 50 mg/L kanamycin-containing medium. Homozygous lines were selected for strong expression.

Confocal Imaging

For intracellular fluorescence, we used a confocal multispectral system TCS SP5 with LAS AF v.2.3.6 software (Leica Microsystems) and a ×63.0 1.20 water-immersion objective. To avoid emission spectra overlap, the sequential mode was used. GFP, propidium iodide, and FM4-64 were excited with a 488-nm argon laser line. Fluorescence was detected using a photomultiplier (GFP 505 to 546 nm, propidium iodide, and FM4-64 625 to 670 nm).

Determination of Luciferase Activity

Luciferase activity (LA) levels were detected using the LB 960 microplate luminometer center system (Berthold Technologies) with MikronWin 2000 software. Plants were grown in +Pi medium for 7 d and transferred to -Pi medium for 2 d, and single plants were transferred to a microplate (Costar) containing Johnson medium. D-luciferin substrate (Sigma-Aldrich) was added to each well and the plate incubated (1 h) before As(V) supplementation. The plate was covered with optical film. The percentage of luciferase activity (%*LUC* activity) was calculated as (%*LUC* activity = $[(LA_n - LA_0)/LA_0)*100]$. Luciferase activity was determined in *N. benthamiana* using 1-cm discs of agroinfiltrated leaves transferred to a microplate as above.

qRT-PCR Expression Analysis

qRT-PCR was performed on three independent biological samples as described (Aguilar-Martínez et al., 2007). Each sample was normalized using EF1α. The primer pairs used are described in Supplemental Table 1 online.

ChIP Assay

ChIP assays in the *WRKY6-GFP*–overexpressing line and *N. benthamiana* were as described (Lee et al., 2007). *OXWRKY6-GFP* seedlings were grown on +Pi medium for 7 d and transferred to -Pi medium for 2 d; plants were then treated with 30 μM As(V) for 1.5 h. Leaves of *N. benthamiana* were agroinfiltrated with *PHT1;1p-LUC* wild type, a mutated version of *PHT1;1p-Im/IIm-LUC* and *WRKY6-GFP*–overexpressing construct or with *At5g35030p-LUC*, a mutated version of *At5g35030pMUT-LUC* and a *WRKY6-GFP*–overexpressing construct. As a negative control in both cases, we used leaves of *N. benthamiana* agroinfiltrated as above, replacing the *WRKY6-GFP*–overexpressing construct with a *GFP*-overexpressing construct.

Extracts of *WRKY6-GFP*–overexpressing seedlings (~2 g) or *N. benthamiana* leaves (~4 g) were immunoprecipitated with 40 μL anti-GFP Affinity Matrix (Abcam). Precipitated DNA was dissolved in 40 μL water, and 1 μL was used for qPCR. *ACT2* was used as internal control for the *OXWRKY6-GFP*–overexpressing line experiment.

Histone modification was analyzed by ChIP using antidimethyl-histone H3 Lys 9 (CS200550; Millipore) and antiacetyl-histone H3 Lys 9 and 14 (06-599; Millipore) antibodies as described (De Lucia et al., 2008). Briefly, *Arabidopsis* Col-0 plants were grown on 1 mM Pi medium for 7 d, transferred to -Pi medium for 2 d and then to liquid -Pi medium alone or supplemented with 30 μM As(V). Tissue was fixed, and immunoprecipitated DNA was recovered using Chelex 100 resin (Bio-Rad; 10 g/100 mL double distilled water). All ChIP experiments were quantified by qPCR. *Ta3* (Johnson et al., 2002) or *TUB8* (Tittel-Elmer et al., 2010) was used as an internal control for H3K9me2 or H3Ac ChIP experiments, respectively. Primers used to amplify the target gene fragments are shown in Supplemental Table 1 online.

Transcriptome Analyses of As(V) Response

Transcriptomic analyses were performed using the Affymetrix ATH1 and NimbleGen Gene Expression 12x135K platforms. For the Affymetrix ATH1 array, wild-type plants were grown on Johnson medium with 1 mM Pi for 7 d, transferred to -Pi medium for 2 d and then to medium supplemented with 30 μM As(V) or 30 μM Pi (8 h). RNA was isolated from three independent biological samples for each condition using the RNeasy plant mini kit (Qiagen). RNA amplification, microarray hybridization, and scanning were as reported (Bustos et al., 2010).

Data were analyzed using GeneChip Operating Software and the affylmGUIR package (Wettenhall et al., 2006). Background correction and normalization of expression data were as reported (Bustos et al., 2010). Differential expression analysis was performed with the Bayes t-statistics from the linear models for Microarray data (limma). P values were corrected for multiple testing using the Benjamini-Hochberg method (false discovery rate) (Reiner et al., 2003).

For the NimbleGen Gene Expression 12x135K platform (Roche), wild-type plants and the *WRKY6-GFP*–overexpressing line were grown in +Pi solid medium for 7 d, transferred to -Pi medium for 2 d and then to liquid medium supplemented with 30 μM As(V) for 1.5 h. RNA from three independent biological samples was isolated as above. cDNA was synthesized with 10 μg RNA using the double-stranded cDNA synthesis kit (Invitrogen), and cDNA was Cy3-labeled with the NimbleGen one-color DNA labeling kit (Roche). Three replicates for each condition were hybridized independently to the 100718:Athal_TAIR9_exp_H12 genome array (Roche). Each microarray was hybridized with the NimbleGen hybridization kit, washed using the NimbleGen wash buffer kit (both from Roche), and scanned at 2-μm double-pass at 16-bit resolution. Data were analyzed through robust multiarray analysis with NimbleGen Scan software (Roche). Differential expression analysis and P value correction were performed as above.

Identification of *cis*-Regulatory Elements by in Silico Analysis

To identify putative *cis*-acting regulatory elements, we identified promoter sequences (0.5 kb) of genes preferentially downregulated by As(V) in The Arabidopsis Information Resource (http://www.Arabidopsis.org/). This group of promoter sequences was submitted for overrepresented motifs analysis to Element (http://element.mocklerlab.org/). The motifs identified with $P < 0.05$ were used in further analysis.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: WRKY6, AT1G62300; PHT1;1, AT5G43350. Array data from this article can be found in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) database under accession number GSE49037.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. qRT-PCR Assays of *PHT1;1* Expression Kinetics and Dose Response to As(V).

Supplemental Figure 2. Relocalization of the Pi Transporter Is an As(V)-Specific Response That Correlates with Arrest of Intracellular Arsenic Accumulation.

Supplemental Figure 3. qRT-PCR Kinetics Expression Analysis of *PHT1;1* Gene in Response to As(V) and Pi Pulses.

Supplemental Figure 4. WRKY6 Mediates *PHT1;1* Transcriptional Repression and Confers As(V) Tolerance.

Supplemental Figure 5. Comparative Transcriptome Analysis of As(V) Response and Pi Starvation-Upregulated Genes.

Supplemental Figure 6. *WRKY6* Overexpression Restricts As(V) Induction of Transposons.

Supplemental Figure 7. Levels of H3K9me2 and H3Ac Immunoprecipitated from the Genomic Regions of As(V)-Induced Transposable Elements.

Supplemental Table 1. Primers Used for Binary Constructs, qRT-PCR, and Chromatin Immunoprecipitation Assays.

Supplemental Data Set 1. Data set of ATH1 Probes with Significantly Altered Expression in Wild-Type Plants Grown on 1 mM Pi versus -Pi Plants, 30 μM As(V)-Treated versus -Pi Plants, and 30 μM Pi-Treated versus -Pi Plants.

Supplemental Data Set 2. Data Set of NimbleGen Probes with Significantly Altered Expression in Wild-Type Plants Exposed to As(V) versus Untreated Plants.

Supplemental Data Set 3. Data Set of As(V)-Induced Transposable Elements, Ordered by Families.

Supplemental Data Set 4. Data Set of NimbleGen Probes with Significantly Altered Expression in *WRKY6*-Overexpressing Plants Exposed to As(V) versus Wild-Type Plants Treated with As(V).

ACKNOWLEDGMENTS

We thank Salomé Prat, Roberto Solano, Cathie Martin, and Carmen L. Torán for critical reading of the article. We appreciate the support of Alonso Rodriguez-Navarro (Centro de Biotecnología y Genómica de Plantas Universidad Politécnica de Madrid-Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria). We also thank Sylvia Gutiérrez for her help with confocal microscopy studies, José Manuel Franco and the Centro Nacional de Biotecnología Genomics Facility for microarray hybridization and analysis, Cristihan González and Luis Calderón for technical assistance, and Catherine Mark for editorial assistance. This work was supported by fellowships (to L.D., P.C., and E.S.-B.) from the Spanish Ministry of Science and Innovation and from La-Caixa/Centro Nacional de Biotecnología International PhD program (to M.T.C.) as well as research grants (BIO2004-03759, BIO2007-66104, BIO2010-16687, AGL2007-61705, AGL2010-15151, CSD2007-00057, EUI2009-03993, and BIO2011-29085) from the Spanish Ministry of Science and Innovation.

AUTHOR CONTRIBUTIONS

G.C. performed the research, was responsible for most work reported in the article, and contributed to experimental design. E.S.-B. performed RT-PCR and phenotypic analyses. L.D. performed all ChIP experiments. P.C. did arsenic quantification. A.F.-E. collaborated in phenotypic analyses. M.T.C. performed most RT-PCR experiments. P.C. performed the 8h-As(V) transcriptomic analysis. A.M. and J.S.-P. performed histone modification analyses. S.O. did RT-PCR and phenotypic analyses. Y.L.D. collaborated in transcriptomic analysis and confocal microscopy. I.M. performed 1.5h-As(V) transcriptomic analysis. E.R. did all confocal analyses. L.E.H. carried out arsenic quantification. J.A.J. and M.P. did histone modification and data analysis. J.P.-A. analyzed the data. A.L. designed experiments and wrote the article.

Received May 28, 2013; revised July 8, 2013; accepted July 19, 2013; published August 12, 2013.

REFERENCES

Abercrombie, J.M., Halfhill, M.D., Ranjan, P., Rao, M.R., Saxton, A.M., Yuan, J.S., and Stewart, C.N., Jr. (2008). Transcriptional responses of *Arabidopsis thaliana* plants to As(V) stress. BMC Plant Biol. **8:** 87.

Aguilar-Martínez, J.A., Poza-Carrión, C., and Cubas, P. (2007). *Arabidopsis* BRANCHED1 acts as an integrator of branching signals within axillary buds. Plant Cell **19:** 458–472.

Barboriak, D.P., Padua, A.O., York, G.E., and Macfall, J.R. (2005). Creation of DICOM—aware applications using ImageJ. J. Digit. Imaging **18:** 91–99.

Bayle, V., Arrighi, J.F., Creff, A., Nespoulous, C., Vialaret, J., Rossignol, M., Gonzalez, E., Paz-Ares, J., and Nussaume, L. (2011). *Arabidopsis thaliana* high-affinity phosphate transporters exhibit multiple levels of posttranslational regulation. Plant Cell **23:** 1523–1535.

Bucher, E., Reinders, J., and Mirouze, M. (2012). Epigenetic control of transposon transcription and mobility in *Arabidopsis*. Curr. Opin. Plant Biol. **15:** 503–510.

Bustos, R., Castrillo, G., Linhares, F., Puga, M.I., Rubio, V., Pérez-Pérez, J., Solano, R., Leyva, A., and Paz-Ares, J. (2010). A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in *Arabidopsis*. PLoS Genet. **6:** e1001102.

Carswell, C., Grant, B.R., Theodorou, M.E., Harris, J., Niere, J.O., and Plaxton, W.C. (1996). The fungicide phosphonate disrupts the phosphate-starvation response in *Brassica nigra* seedlings. Plant Physiol. **110:** 105–110.

Carswell, M.C., Grant, B.R., and Plaxton, W.C. (1997). Disruption of the phosphate-starvation response of oilseed rape suspension cells by the fungicide phosphonate. Planta **203:** 67–74.

Catarecha, P., Segura, M.D., Franco-Zorrilla, J.M., García-Ponce, B., Lanza, M., Solano, R., Paz-Ares, J., and Leyva, A. (2007). A mutant of the *Arabidopsis* phosphate transporter PHT1;1 displays enhanced arsenic accumulation. Plant Cell **19:** 1123–1133.

Chen, Y.F., Li, L.Q., Xu, Q., Kong, Y.H., Wang, H., and Wu, W.H. (2009). The WRKY6 transcription factor modulates PHOSPHATE1 expression in response to low Pi stress in *Arabidopsis*. Plant Cell **21:** 3554–3566.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Dahl, T.W., Hammarlund, E.U., Anbar, A.D., Bond, D.P., Gill, B.C., Gordon, G.W., Knoll, A.H., Nielsen, A.T., Schovsbo, N.H., and Canfield, D.E. (2010). Devonian rise in atmospheric oxygen correlated to the radiations of terrestrial plants and large predatory fish. Proc. Natl. Acad. Sci. USA **107:** 17911–17915.

Dani, S.U. (2010). Gold, coal and oil. Med. Hypotheses **74:** 534–541.

De Lucia, F., Crevillen, P., Jones, A.M., Greb, T., and Dean, C. (2008). A PHD-polycomb repressive complex 2 triggers the epigenetic silencing of FLC during vernalization. Proc. Natl. Acad. Sci. USA **105:** 16831–16836.

Dhankher, O.P., Rosen, B.P., McKinney, E.C., and Meagher, R.B. (2006). Hyperaccumulation of arsenic in the shoots of *Arabidopsis* silenced for arsenate reductase (ACR2). Proc. Natl. Acad. Sci. USA **103:** 5413–5418.

Geldner, N., Dénervaud-Tendon, V., Hyman, D.L., Mayer, U., Stierhof, Y.D., and Chory, J. (2009). Rapid, combinatorial analysis of membrane compartments in intact plants with a multicolor marker set. Plant J. **59:** 169–178.

González, E., Solano, R., Rubio, V., Leyva, A., and Paz-Ares, J. (2005). PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 is a plant-specific SEC12-related protein that enables the endoplasmic reticulum exit of a high-affinity phosphate transporter in *Arabidopsis*. Plant Cell **17:** 3500–3512.

Ito, H. (2012). Small RNAs and transposon silencing in plants. Dev. Growth Differ. **54:** 100–107.

Jiménez, I., López, L., Alamillo, J.M., Valli, A., and García, J.A. (2006). Identification of a plum pox virus CI-interacting protein from chloroplast that has a negative effect in virus infection. Mol. Plant Microbe Interact. **19:** 350–358.

Jobe, T.O., Sung, D.Y., Akmakjian, G., Pham, A., Komives, E.A., Mendoza-Cózatl, D.G., and Schroeder, J.I. (2012). Feedback

inhibition by thiols outranks glutathione depletion: A luciferase-based screen reveals glutathione-deficient γ-ECS and glutathione synthetase mutants impaired in cadmium-induced sulfate assimilation. Plant J. **70:** 783–795.

Johnson, L., Cao, X., and Jacobsen, S. (2002). Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. Curr. Biol. **12:** 1360–1367.

Kasajima, I., Ide, Y., Yokota Hirai, M., and Fujiwara, T. (2010). WRKY6 is involved in the response to boron deficiency in *Arabidopsis thaliana*. Physiol. Plant. **139:** 80–92.

Kiba, T., Feria-Bourrellier, A.B., Lafouge, F., Lezhneva, L., Boutet-Mercey, S., Orsel, M., Bréhaut, V., Miller, A., Daniel-Vedele, F., Sakakibara, H., and Krapp, A. (2012). The *Arabidopsis* nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants. Plant Cell **24:** 245–258.

Kim, K.C., Lai, Z., Fan, B., and Chen, Z. (2008). *Arabidopsis* WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. Plant Cell **20:** 2357–2371.

Kouzarides, T. (2007). Chromatin modifications and their function. Cell **128:** 693–705.

Lee, J., He, K., Stolc, V., Lee, H., Figueroa, P., Gao, Y., Tongprasit, W., Zhao, H., Lee, I., and Deng, X.W. (2007). Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. Plant Cell **19:** 731–749.

Ma, J., and Bennetzen, J.L. (2006). Recombination, rearrangement, reshuffling, and divergence in a centromeric region of rice. Proc. Natl. Acad. Sci. USA **103:** 383–388.

Maruyama-Nakashita, A., Nakamura, Y., Yamaya, T., and Takahashi, H. (2004). Regulation of high-affinity sulphate transporters in plants: Towards systematic analysis of sulphur signalling and regulation. J. Exp. Bot. **55:** 1843–1849.

Meharg, A.A., and Macnair, M.R. (1990). An altered phosphate uptake system in arsenate-tolerant *Holcus lanatus* L. New Phytol. **116:** 29–35.

Mendoza-Cózatl, D.G., Jobe, T.O., Hauser, F., and Schroeder, J.I. (2011). Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. Curr. Opin. Plant Biol. **14:** 554–562.

Mirouze, M., and Paszkowski, J. (2011). Epigenetic contribution to stress adaptation in plants. Curr. Opin. Plant Biol. **14:** 267–274.

Mouillon, J.M., and Persson, B.L. (2005). Inhibition of the protein kinase A alters the degradation of the high-affinity phosphate transporter Pho84 in *Saccharomyces cerevisiae*. Curr. Genet. **48:** 226–234.

Murota, C., Matsumoto, H., Fujiwara, S., Hiruta, Y., Miyashita, S., Shimoya, M., Kobayashi, I., Hudock, M.O., Togasaki, R.K., Sato, N., and Tsuzuki, M. (2012). Arsenic tolerance in a *Chlamydomonas* photosynthetic mutant is due to reduced arsenic uptake even in light conditions. Planta **236:** 1395–1403.

Narang, R.A., Bruene, A., and Altmann, T. (2000). Analysis of phosphate acquisition efficiency in different *Arabidopsis* accessions. Plant Physiol. **124:** 1786–1799.

Oremland, R.S., Saltkov, C.W., Wolfe-Simon, F., and Stolz, J.F. (2009). Arsenic in the evolution of earth and extraterrestrial ecosystems. Geomicrobiol. J. **26:** 522–536.

Pecinka, A., Dinh, H.Q., Baubec, T., Rosa, M., Lettner, N., and Mittelsten Scheid, O. (2010). Epigenetic regulation of repetitive elements is attenuated by prolonged heat stress in *Arabidopsis*. Plant Cell **22:** 3118–3129.

Quaghebeur, M., and Rengel, Z. (2004). Arsenic uptake, translocation and speciation in pho1 and pho2 mutants of *Arabidopsis thaliana*. Physiol. Plant. **120:** 280–286.

Raghothama, K.G. (1999). Phosphate acquisition. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50:** 665–693.

Reiner, A., Yekutieli, D., and Benjamini, Y. (2003). Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics **19:** 368–375.

Robatzek, S., and Somssich, I.E. (2002). Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes Dev. **16:** 1139–1149.

Rosen, B.P. (2002). Biochemistry of arsenic detoxification. FEBS Lett. **529:** 86–92.

Rubio, V., Linhares, F., Solano, R., Martín, A.C., Iglesias, J., Leyva, A., and Paz-Ares, J. (2001). A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Genes Dev. **15:** 2122–2133.

Rushton, P.J., and Somssich, I.E. (1998). Transcriptional control of plant genes responsive to pathogens. Curr. Opin. Plant Biol. **1:** 311–315.

Rushton, P.J., Somssich, I.E., Ringler, P., and Shen, Q.J. (2010). WRKY transcription factors. Trends Plant Sci. **15:** 247–258.

Saze, H., Tsugane, K., Kanno, T., and Nishimura, T. (2012). DNA methylation in plants: Relationship to small RNAs and histone modifications, and functions in transposon inactivation. Plant Cell Physiol. **53:** 766–784.

Shin, H., Shin, H.S., Dewbre, G.R., and Harrison, M.J. (2004). Phosphate transport in *Arabidopsis*: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J. **39:** 629–642.

Song, W.Y., et al. (2010). Arsenic tolerance in *Arabidopsis* is mediated by two ABCC-type phytochelatin transporters. Proc. Natl. Acad. Sci. USA **107:** 21187–21192.

Sung, D.Y., Kim, T.H., Komives, E.A., Mendoza-Cózatl, D.G., and Schroeder, J.I. (2009). ARS5 is a component of the 26S proteasome complex, and negatively regulates thiol biosynthesis and arsenic tolerance in *Arabidopsis*. Plant J. **59:** 802–813.

Ticconi, C.A., Delatorre, C.A., and Abel, S. (2001). Attenuation of phosphate starvation responses by phosphite in *Arabidopsis*. Plant Physiol. **127:** 963–972.

Tittel-Elmer, M., Bucher, E., Broger, L., Mathieu, O., Paszkowski, J., and Vaillant, I. (2010). Stress-induced activation of heterochromatic transcription. PLoS Genet. **6:** e1001175.

Tripathi, R.D., Srivastava, S., Mishra, S., Singh, N., Tuli, R., Gupta, D.K., and Maathuis, F.J. (2007). Arsenic hazards: Strategies for tolerance and remediation by plants. Trends Biotechnol. **25:** 158–165.

Tsukahara, S., Kobayashi, A., Kawabe, A., Mathieu, O., Miura, A., and Kakutani, T. (2009). Bursts of retrotransposition reproduced in *Arabidopsis*. Nature **461:** 423–426.

Ulm, R., Baumann, A., Oravecz, A., Máté, Z., Adám, E., Oakeley, E.J., Schäfer, E., and Nagy, F. (2004). Genome-wide analysis of gene expression reveals function of the bZIP transcription factor HY5 in the UV-B response of *Arabidopsis*. Proc. Natl. Acad. Sci. USA **101:** 1397–1402.

Varadarajan, D.K., Karthikeyan, A.S., Matilda, P.D., and Raghothama, K.G. (2002). Phosphite, an analog of phosphate, suppresses the coordinated expression of genes under phosphate starvation. Plant Physiol. **129:** 1232–1240.

Wettenhall, J.M., Simpson, K.M., Satterley, K., and Smyth, G.K. (2006). affylmGUI: A graphical user interface for linear modeling of single channel microarray data. Bioinformatics **22:** 897–899.

Wu, Z., Ren, H., McGrath, S.P., Wu, P., and Zhao, F.J. (2011). Investigating the contribution of the phosphate transport pathway to arsenic accumulation in rice. Plant Physiol. **157:** 498–508.

Ye, J., Rensing, C., Rosen, B.P., and Zhu, Y.G. (2012). Arsenic biomethylation by photosynthetic organisms. Trends Plant Sci. **17:** 155–162.