

Cytokinin Determines Thiol-Mediated Arsenic Tolerance and Accumulation¹

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The presence of arsenic in soil and water is a constant threat to plant growth in many regions of the world. Phytohormones act in the integration of growth control and stress response, but their role in plant responses to arsenic remains to be elucidated. Here, we show that arsenate [As(V)], the most prevalent arsenic chemical species in nature, causes severe depletion of endogenous cytokinins (CKs) in the model plant *Arabidopsis* (*Arabidopsis thaliana*). We found that CK signaling mutants and transgenic plants with reduced endogenous CK levels showed an As(V)-tolerant phenotype. Our data indicate that in CK-depleted plants exposed to As(V), transcript levels of As(V)/phosphate-transporters were similar or even higher than in wild-type plants. In contrast, CK depletion provoked the coordinated activation of As(V) tolerance mechanisms, leading to the accumulation of thiol compounds such as phytochelatin and glutathione, which are essential for arsenic sequestration. Transgenic CK-deficient *Arabidopsis* and tobacco lines show a marked increase in arsenic accumulation. Our findings indicate that CK is an important regulatory factor in plant adaptation to arsenic stress.

Since the inception of life, arsenic in the biosphere has been a constant challenge to the survival of life forms. Although all organisms on earth have developed strategies to cope with this extremely toxic metalloid (Rosen, 2002; Tripathi et al., 2007), arsenic currently poses a major worldwide environmental problem (Naujokas et al., 2013).

Arsenate [As(V)] is the most abundant chemical form of arsenic. Due to its structural similarity to phosphate (Pi), it is easily incorporated into plants and other organisms through Pi transporters. Once taken up by the cell, As(V) is rapidly reduced to arsenite [As(III)] by As(V) reductases. As(III) is either extruded from the cytoplasm or is sequestered by phytochelatin (PCs) and other related thiol-containing compounds and is compartmentalized into vacuoles (Tripathi et al., 2007).

In plants, when As(V) is perceived, Pi transporters are rapidly downregulated, and thiol compound accumulation increases concomitantly to cope with the metalloid. These two responses are key As(V) tolerance strategies for natural plant populations (Meharg and Macnair 1992; Bleeker et al., 2006). Accumulation of PC and other related thiol-containing compounds is widely used by plants for heavy metal detoxification. PCs are

synthesized from glutathione (GSH) by the enzyme PHYTOCHELATIN SYNTHASE1 (PCS1). As(V) induces PCS1, together with two other genes involved in GSH biosynthesis: γ -GLUTAMYL CYSTEINE SYNTHETASE and GLUTATHIONE SYNTHETASE (GSH2; Sung et al., 2009). In addition, As(III) activates GSH and PC accumulation; As(V) reductase activity is thus essential for arsenic tolerance (Bleeker et al., 2006). *Arabidopsis thaliana* ARSENATE REDUCTASE QTL1 (*AtARQ1*, *At2g21045*; also termed *HAC1*; Chao et al., 2014), an As(V) reductase-encoding gene, is also transcriptionally regulated by As(V) (Sánchez-Bermejo et al., 2014). Arsenic tolerance thus requires coordinated expression of several As(V)-responsive genes involved in As(V) reductase activity and PC accumulation.

Exposure to stress arrests plant growth. When plants perceive As(V), for example, root growth is immediately inhibited (Li et al., 2007; Shri et al., 2009; Yoon et al., 2015). Root meristem activity can be restored by removing As(V) from the medium. Root growth recovery depends on the function of an As(V) reductase that is essential for arsenic detoxification (Sánchez-Bermejo et al., 2014), which supports the idea that plants are able to integrate arsenic tolerance mechanisms

into developmental programs. In general, phytohormones are involved in reconfiguration of developmental patterns in response to abiotic stress (Peleg and Blumwald, 2011; Zwack and Rashotte, 2015), although their role in arsenic-mediated growth control is currently unknown.

Here, we characterized the involvement of the hormone cytokinin (CK) in the As(V) response. We found that As(V) provokes severe depletion of endogenous CK levels, leading to activation of As(V) tolerance mechanisms, including As(V) reductase activity and PC and GSH accumulation, all critical for arsenic detoxification. CK is therefore an essential component of plant strategies that lead to As(V) tolerance.

RESULTS

CK-Deficient Plants Have an Enhanced As(V) Tolerance Phenotype

In response to As(V), expression of the As(V)/Pi-transporter gene *PHT1;1* is quickly repressed, which provides an efficient strategy for As(V) tolerance (Castrillo et al., 2013). CK acts as a negative regulator of *PHT1;1* expression in response to Pi starvation (Martín et al., 2000; Franco-Zorrilla et al., 2002; Wang et al., 2006). To determine the effect of CK on *PHT1;1* repression in response to As(V), we performed a time course analysis of *PHT1;1* transcript accumulation in As(V)-exposed plants. We used *Arabidopsis* (*Arabidopsis thaliana*) lines that

constitutively express the *CYTOKININ OXIDASE/DEHYDROGENASE1* (*CKX1*) gene (35S:*CKX1*; Werner et al., 2003); all bioactive CK levels are strongly decreased in these plants (Werner et al., 2003, 2010; Nishiyama et al., 2011). qRT-PCR analysis showed that in the absence of As(V), CK deficiency increased *PHT1;1* expression relative to that observed in wild-type plants, in line with previous studies. After As(V) exposure, *CKX1*-overexpressing lines rapidly repressed *PHT1;1* expression (2.7-fold) to a similar degree or even more than in wild-type plants (Fig. 1A). Absolute mRNA levels for *PHT1;1* after As(V) exposure are thus similar in *CKX1*-overexpressing and wild-type plants.

As CK is defined as a negative regulator of several stress responses (Tran et al., 2007; Srivastava et al., 2009; Werner et al., 2010; Nishiyama et al., 2011; Jeon and Kim, 2013), we examined the As(V) tolerance phenotype using CK-depleted transgenic lines and mutants. Since *CKX1*-overexpressing lines and CK signaling mutants show increased root length (Werner et al., 2003, 2010; Riefler et al., 2006), we compared root growth inhibition in the presence of As(V) (Fig. 1B) relative to root length in the corresponding plants grown in As(V)-free media (Supplemental Fig. S1). The fold change in growth reduction between these conditions showed that growth arrest was lower in all mutant and transgenic lines, with lower CK levels than wild-type ecotype Columbia (Col-0) plants (Fig. 1, B and C). These lines included the 35S:*CKX1* transgenic line as well as the triple mutant *ipt 3 5 7* and the *cyp735a1 a2* double mutant (both with impaired CK biosynthesis), and the double receptor mutant *ahk2/ahk3* with reduced CK sensitivity (Fig. 1, B and C). Relative root growth inhibition in the CK mutants was <4.5-fold, while it was >6-fold in wild-type plants. Similar *PHT1;1* mRNA levels in wild-type and CK mutants, with less As(V) sensitivity in the latter, suggest that other mechanisms involved in arsenic tolerance must be under CK control.

As(V) Reduces Endogenous CK Levels

The arsenic tolerance phenotype shown by CK-depleted transgenic lines and CK signaling mutants led us to test the As(V) effect on endogenous CK levels. Quantification of biologically active CK metabolites (*iP*, *tZ*, *cZ*) and their ribosides (*iPR*, *tZR*, *cZR*) in As(V)-exposed *Arabidopsis* plants showed that concentrations of all species were significantly reduced after As(V) exposure, particularly of *tZ*-type species (Fig. 2A). The As(V) tolerance phenotype of the *cyp735a1 a2* double mutant, in which *tZ*-type CK are specifically reduced (Kiba et al., 2013), was similar to that of *CKX1*-overexpressing lines, in which the level of all CK metabolites is lower (Fig. 1, B and C). Consistent with lower CK levels, the *IPT* genes (*IPT3*, *IPT5*, and *IPT7*) responsible for biosynthesis of the bulk of CK during vegetative growth were markedly down-regulated after As(V) exposure (Fig. 2B). Expression of five *CKX*

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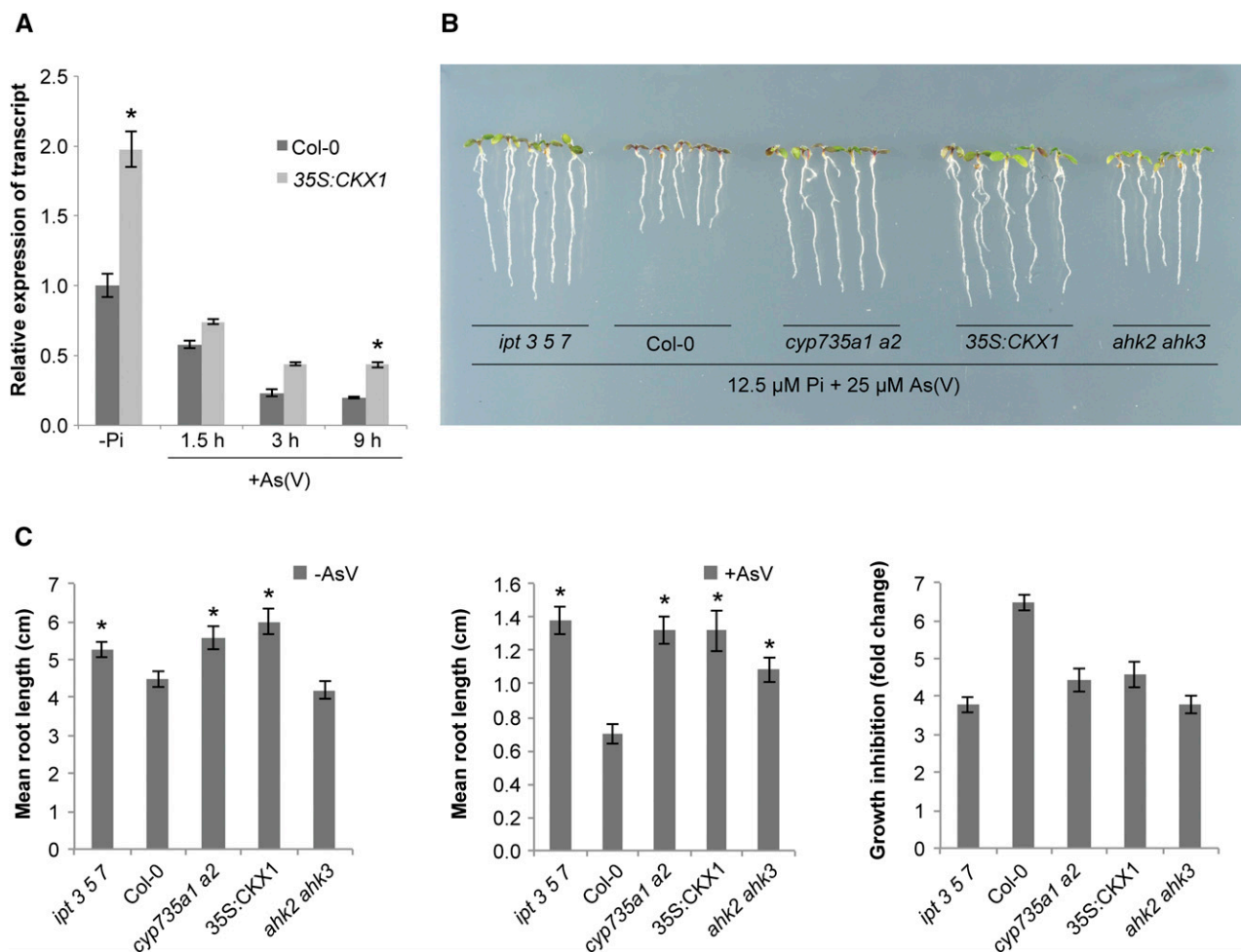


Figure 1. Reduction of endogenous CK confers As(V) tolerance. *A*, qRT-PCR analysis of *PHT1;1* expression in wild-type plants (Col-0) and in a *35S:CKX1*-overexpressing line grown in +Pi medium for 7 d, transferred to -Pi medium for 3 d, then transferred to -Pi liquid medium for the indicated time, alone (-Pi) or with $30 \mu\text{M}$ As(V). Values show mean \pm SD ($n = 3$). *B*, As(V) tolerance phenotypes of *ipt 3 5 7*, wild-type (Col-0), *cyp735a1 a2*, *35S:CKX1*, and *ahk2 ahk3* plants grown on $12.5 \mu\text{M}$ Pi supplemented with $25 \mu\text{M}$ As(V) for 10 d. *C*, Root length of indicated mutant, transgenic, and wild-type plants grown alone (left) or with As(V) (center) were measured in plants growth as described in *B*. Right, root growth inhibition (fold change) by As(V) compared to growth of corresponding plant types in the absence of As(V). Values show mean \pm SD ($n = 5$). Means are significantly different (Student's *t* test, $*P < 0.01$).

family members (*CKX1*, *CKX3*, *CKX4*, *CKX5*, and *CKX7*) was also repressed, which might be the consequence of lower CK synthesis (Fig. 2C). As(V) thus reduces CK levels, particularly of the *tZ* type, which suggests the biological relevance of CK depletion for As(V) tolerance in plants.

To further understand the basis of CK depletion in response to As(V), we compared a transcriptome profile of the As(V) response (Castrillo et al., 2013) with a meta-analysis of CK microarray data (Brenner and Schmülling, 2015). We found that As(V) down-regulates a significant number of genes that are considered CK core-inducible (36 of 65 TOP65CytK), including CK biosynthesis genes and response regulator genes (Table 1; Supplemental Table S1). As(V) repression of three CK core responsive genes was confirmed by qRT-PCR expression analysis (Supplemental Fig. S2). These data

showed that the CK receptor gene *CRE1/AHK4* was also significantly down-regulated in response to As(V), which would probably result in lower CK signaling. Indeed, reduced expression of numerous CK-responsive genes supports this idea (Supplemental Table S1; Supplemental Fig. S2).

The Plant Detoxification Machinery Is Modulated by Endogenous CK

To identify the molecular mechanism that underlies As(V) tolerance in response to CK depletion, we tested whether CK deficiency alters essential As(V) resistance mechanisms in plants. As(V) repression of the As(V)/Pi transporter gene, an essential strategy in As(V) tolerance, was very efficient in the *CKX1*-overexpressing lines; however, Pi transporter transcript levels were

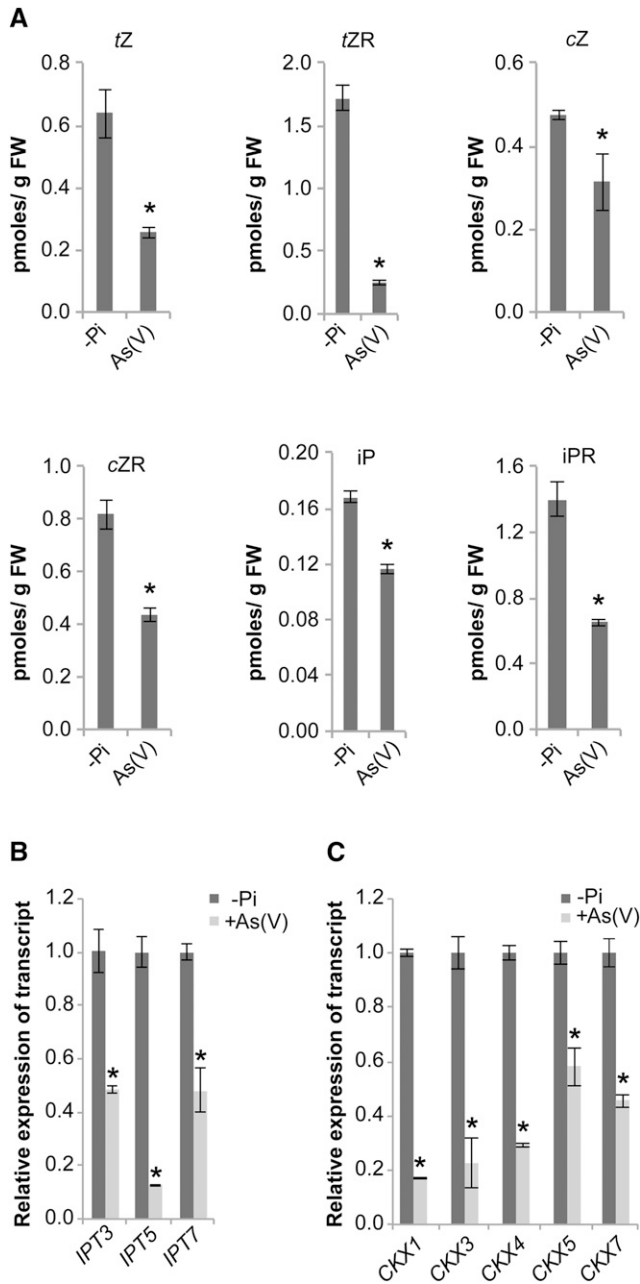


Figure 2. As(V) depletes endogenous CKs. A, Wild-type plants (Col-0) were grown in +Pi medium for 7 d, transferred to -Pi medium for 3 d, and then to -Pi liquid medium alone or with 30 μM As(V) for 6 h. CK levels were analyzed as pmol/g fresh weight (FW) of whole plants. Values show mean \pm SD ($n = 3$). Means are significantly different (ANOVA, $*P < 0.05$). B and C, As(V) inhibits expression of CK metabolism genes. qRT-PCR analysis of *IPT* (B) and *CKX* (C) gene expression. Treatment was as in A. Values show mean \pm SD ($n = 3$), t test $*P < 0.05$.

higher in the As(V)-treated *CKX1*-overexpressing line than in wild-type plants (Fig. 1A). Alternative mechanisms must therefore be invoked to explain the increased As(V) tolerance of low CK-accumulating plants. To determine whether CKs are involved in regulation of the detoxification machinery, we analyzed expression

of the biothiol biosynthetic genes γ -GLUTAMYL-CYSTEINE SYNTHETASE, *GSH2*, and *AtPCS1* in wild-type and *CKX1*-overexpressing plants (Fig. 3). Since As(III) is necessary for PC biosynthesis and accumulation, the action of As(V) reductases is essential for As(V) tolerance in plants (Bleeker et al., 2006); we thus also analyzed expression of the recently identified As(V) reductase gene *AtARQ1/HAC1* (Chao et al., 2014; Sánchez-Bermejo et al., 2014). qRT-PCR expression analysis of

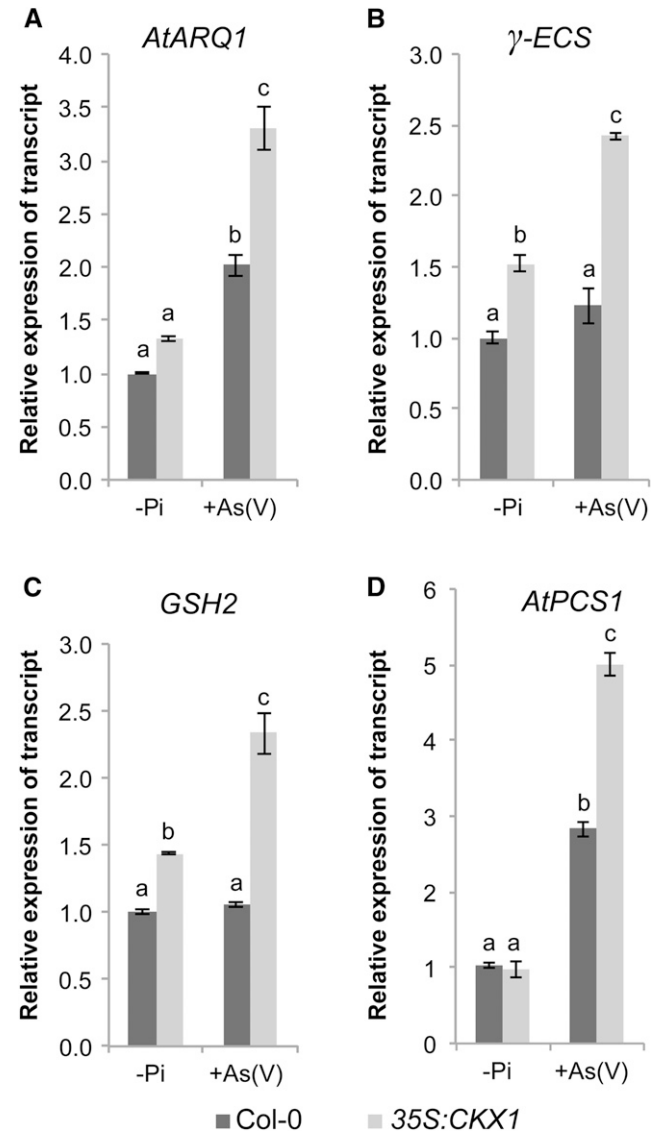


Figure 3. Genes involved in thiol compound accumulation are up-regulated in CK-depleted transgenic plants. A to D, qRT-PCR expression analysis of *AtARQ1* (A), γ -ECS (B), *GSH2* (C), and *AtPCS1* (D) in Col-0 and 35S:CKX1. Plants were grown in +Pi medium for 7 d, transferred to -Pi medium for 3 d, and then to -Pi liquid medium alone (-Pi) or with 30 μM As(V) [+As(V)] for 6 h. Expression was analyzed in whole seedlings except for *AtARQ1*, for which only roots were used. Values show mean \pm SD ($n = 3$). Means labeled with the same letter indicate no significant difference, based on Tukey's posthoc test ($\alpha = 0.05$).

wild-type plants showed that *PCS1* and *AtARQ1* transcript accumulation was induced after 6-h As(V) exposure. In the *CKX1*-overexpressing line, the As(V) responsiveness of *AtARQ1* and other PC biosynthetic genes (γ -ECS, *GSH2*, and *AtPCS1*) was increased compared to wild-type controls (Fig. 3). In accordance with these findings, microarray data for CK-treated Arabidopsis seedlings showed that, in response to this phytohormone, *AtPCS1* and *AtARQ1* are down-regulated 4.0- and 4.4-fold, respectively (Brenner et al., 2005). In the *CKX1*-overexpressing line, these genes were not up-regulated in the absence of As(V) (Fig. 3). Nonetheless, transcripts altered in response to CK do not always behave as opposites in CK-deficient plants (Brenner and Schümiling, 2012). These results thus indicate that CK negatively regulates the expression of genes involved in biothiol accumulation and that the As(V) tolerance phenotype of the *CKX1*-overexpressing plants is due at least in part to the up-regulation of genes related to the As(V) detoxification machinery.

The contribution of PC accumulation to the As(V) tolerance phenotype shown by the *CKX1*-overexpressing line was evaluated by analyzing the effect of L-buthionine sulphoximine (BSO) on As(V) sensitivity in wild-type and *CKX1*-overexpressing plants. BSO is a specific inhibitor of γ -ECS activity and thus particularly effective for PC depletion. Wild-type Arabidopsis plants exposed to this compound perfectly mimic the metal sensitivity of PC biosynthetic mutants (Howden et al., 1995). Coinciding with previous observations (Ortega-Villasante et al., 2005), we found that BSO had no effect on root growth in the absence of As(V), even at the highest concentration tested (Supplemental Fig. S3). In the presence of the metalloid, increased BSO concentrations greatly

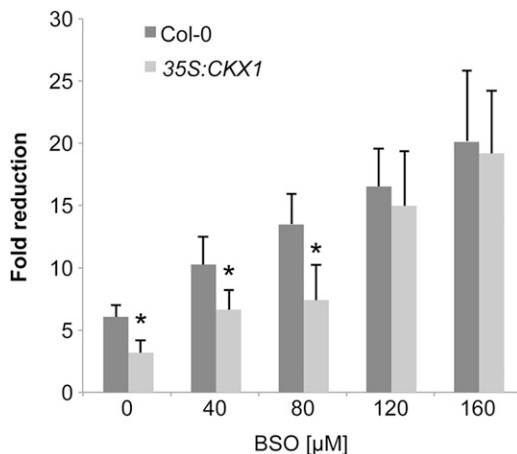


Figure 4. As(V) sensitivity to BSO increased preferentially in CK-depleted plants. Plants were grown on Johnson medium with 12.5 μ M Pi in a vertical position for 5 d and then transferred to the same medium with 20 μ M As(V) alone or with increasing concentrations of BSO. Root growth inhibition (fold change) was determined by quantifying root elongation after 3 d of As(V) exposure alone or with BSO. Bars show mean \pm SD ($n \geq 15$). * $P < 0.0001$ (Student's t test).

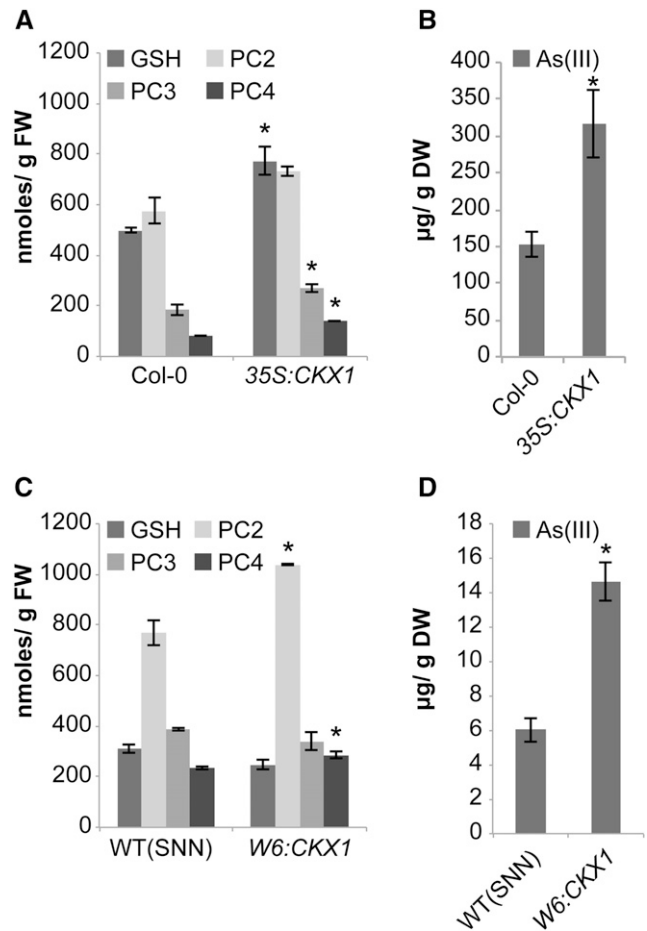


Figure 5. Phytochelatin, GSH, and As(III) accumulation increased in response to CK depletion in Arabidopsis and tobacco. GSH, PCs PC2, PC3, PC4 A, and As(III) (B) levels were determined in Col-0 and 35S: *CKX1* Arabidopsis seedlings. Quantification of thiols (C) and As(III) (D) in wild-type (SNN) and W6:: *CKX1* tobacco roots. Phytochelatin and As (III) were quantified as described in "Methods." Values show mean \pm SD ($n = 3$). * $P < 0.05$ (Student's t test). FW, fresh weight; DW, dry weight.

enhanced As(V) sensitivity; we therefore analyzed root growth inhibition after brief exposure to As(V). In plants exposed to BSO, As(V) sensitivity increased preferentially in the *CKX1*-overexpressing lines compared to wild-type plants (Fig. 4; Supplemental Fig. S3). In the presence of As(V) and at relatively high BSO concentrations, *CKX1*-overexpressing and wild-type plants showed similar root growth. As(V) exposure in wild-type and the *CKX1*-overexpressing line showed that CK depletion led to a clear increase in PC2, PC3, and PC4 and their precursor, GSH (Fig. 5A), and that these plants accumulated more than twice as much As (III) as wild-type plants (Fig. 5B). These observations strongly support the concept that severe depletion of endogenous CK leads to enhanced PC accumulation, which suggests that the higher arsenic tolerance in the *CKX1*-overexpressing line is caused to a great extent by the higher PC levels in these plants. Once arsenic is perceived, CK depletion modulates Arabidopsis

detoxification capacity and is thus a key factor in plant tolerance and adaptation to As(V).

Various strategies have been used to enhance arsenic tolerance and accumulation in plants (Dhankher et al., 2006; Zhu and Rosen, 2009; Song et al., 2010). Our data suggest CK depletion as an alternative approach to increase the efficiency of arsenic accumulation. CK reduction also leads to a notable increase in root biomass, which could contribute to better arsenic extraction from soil (Werner et al., 2010). A CK decrease thus confers two important characteristics for arsenic hyperaccumulation: increased levels of thiol-containing compounds and enhancement of total root area. To analyze potential applications of CK reduction for arsenic phytoaccumulation in other plant species, we measured PC levels and As(III) accumulation in *CKX1*-expressing tobacco roots (Fig. 5, C and D). Since constitutive *CKX1* expression provokes pleiotropic effects in the aerial part of the plant, we analyzed tobacco plants that express the *CKX1* gene under the control of the *WRKY6* promoter (Werner et al., 2010). These plants are particularly suitable for such experiments, as this promoter is mainly root specific and As(V) responsive (Castrillo et al., 2013). Similar to *Arabidopsis*, root-specific *CKX1* expression in response to As(V) provoked an increase in total PC levels in tobacco roots, particularly of PC2 (Fig. 5C). As(III) quantification in *CKX1*-expressing tobacco plants showed that root-specific CK depletion led to a 2-fold As(III) increase compared to wild-type plants (Fig. 5D). These results suggest CK depletion as a suitable strategy to enhance arsenic accumulation in different plant species.

DISCUSSION

The biological role of phytohormones in plant tolerance to arsenic is currently unknown. Transcriptome profiling of a few plant species indicates that several phytohormones might be involved in plant growth regulation in response to arsenic (Yu et al., 2012; Fu et al., 2014), although downstream targets remain to be determined. Here, we found that CK modulates arsenic detoxification in plants. *CKX1*-overexpressing lines depleted of endogenous CK, plants with reduced CK

synthesis, and CK signaling mutants showed a clear arsenic tolerance phenotype. CK acts as a negative regulator of plant responses to several forms of stress. The arsenic tolerance phenotype of the CK-deficient plants and signaling mutants nonetheless suggests that CK coordinates arsenic detoxification mechanisms. As(V) tolerance is achieved mainly through the combined action of two specific responses, prevention of As(V) uptake by suppression of *PHT1;1*, the most active As(V)/Pi-transporter (Castrillo et al., 2013), and through detoxification by As(III) complex formation with thiol compounds and sequestration into the vacuoles (Schmöger et al., 2000). Although CK negatively regulates the As(V)/Pi transporter (Martín et al., 2000), As(V)-mediated down-regulation of *PHT1;1* was somewhat more efficient in the CK-depleted lines than in wild-type plants. *PHT1;1* transcript levels after As(V) exposure were similar in CK-depleted transgenic and wild-type plants, however, which implies that the effect of CK depletion on *PHT1;1* expression is unlikely to be responsible for the increased As(V) tolerance of *CKX1*-overexpressing lines. Repression of As(V)/Pi transporters in response to As(V) is controlled by the WRKY6 transcription factor (Castrillo et al., 2013). Our results support the idea that As(V) down-regulation of the Pi transporter is controlled preferentially by WRKY6 action. In contrast, As(V) reductase gene expression and thiol compound accumulation was increased in CK-depleted plants, which suggests that CK controls activation of the detoxification machinery responsible for As(V) tolerance.

As(V) caused a significant reduction in endogenous CK levels, which coincides with reduced expression of CK synthesis genes (Fig. 2B). Our analyses showed that As(V) reduces the expression of CK-inducible genes (Table I). As(V) efficiently represses the *CYP735A* gene, responsible for biosynthesis of *tZ*-type CK in roots (Kiba et al., 2013; Ramireddy et al., 2014). In addition, the arsenic-tolerant phenotype of the *Arabidopsis cyp735a1 a2* double mutant was identical to that of the *CKX1*-overexpressing line (Fig. 1, B and C). *tZ*-type CKs therefore appear to have a major role in the repression of arsenic tolerance mechanisms, probably because this CK species is synthesized in roots (Kiba et al., 2013), where biothiols are essential for As(III) sequestration.

Table 1. As(V) down-regulates CK core response genes. The total number of up- and down-regulated genes after As(V) exposure (1.5 or 8 h; Castrillo et al., 2013) was compared with 65 genes identified as CK core response genes (TOP65CytK) from a meta-analysis of microarray data (Brenner and Schmölling, 2015). Numbers for observed/expected indicate As(V)-regulated genes that overlap with CK core response genes. The expected number of overlapping genes was calculated assuming random distribution. Enrichment was assessed using a hypergeometric test with Bonferroni correction; significant overrepresentation is in italics ($P < 0.05$).

Array	Gene Expression	TOP65CytK			P-Value
		No. of Genes	Observed	Expected	
As(V) -1.5 h	Up	1936	3	3.39	1
	Down	1518	14	2.66	<i>1.19E-06</i>
As(V) -8 h	Up	1263	3	2.62	1
	Down	926	32	1.92	<i>3.93E-31</i>

Endogenous CK depletion in response to As(V) alters the expression of genes involved in arsenic detoxification. CK negative regulation is not limited to genes involved in GSH and PC biosynthesis, but also affects expression of the As(V) reductase gene *AtARQ1*. As(III) and As(V) increase the intracellular thiol content in arsenic hyperaccumulator plants (Bleeker et al., 2006); As(V) reductase activity is therefore a prerequisite for arsenic tolerance and accumulation. CK also represses sulfur transporters (Maruyama-Nakashita et al., 2004), and As(III) causes severe sulfur starvation and induces expression of sulfur transporters (Reid et al., 2013). These data support the idea that sulfur is critical for increased biosynthesis of PC and other thiol-related compounds, which leads to arsenic accumulation. The use of an inhibitor of GSH and PC biosynthesis showed that the As(V) tolerance phenotype in CK-depleted Arabidopsis plants is due in great measure to increased accumulation of thiol compounds. Although these compounds have a limited role in some arsenic hyperaccumulator plants (Webb et al., 2003), As(III) is the major arsenic species that accumulates in hyper- and nonaccumulator plant vacuoles. Enhanced As(V) reductase gene expression in response to CK depletion is therefore a critical prerequisite for arsenic accumulation in both plant types. Reduction of endogenous CK levels in response to As(V) is thus a central factor in arsenic tolerance and accumulation in plants.

Hitherto only a few transgenic approaches have been used to coordinate expression of key genes for arsenic phytoremediation (Dhankher et al., 2002; Zhu and Rosen, 2009). Efficient use of a system-wide approach that integrates all responses to increase arsenic accumulation would require identification of the master regulatory genes that underlie the response to arsenic. The Arabidopsis and tobacco *CKX1*-overexpressing plants used in this study accumulate more cadmium as well as nutrient elements, associated with their increased root biomass and possibly with deregulated expression of the respective transporter genes (Werner et al., 2010). Our results show that CK reduction not only increases root biomass but also promotes activation of the metal(loid) detoxification machinery, a major cause of As(III) accumulation. We suggest CK depletion as a strategy to increase arsenic accumulation in non-hyperaccumulator plants.

Our findings show that CK acts as a negative regulator of the response to As(V). Reduction of CK levels in response to the metalloid stimulates the detoxification machinery that leads to As(III) accumulation. Further experiments are needed to determine how plants sense the amount of biothiol elicited by CK depletion to coordinate As(V) uptake and detoxification. Identification of CK as an integral part of the As(V) response offers a new understanding of how plants integrate phytohormone signaling pathways with adaptation to stress and provides an additional molecular component with which to engineer arsenic tolerance in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

We used Arabidopsis (*Arabidopsis thaliana*) seeds of Col-0, 35S:CKX1 (Werner et al., 2003), the CK receptor mutant *ahk2 ahk3 (ahk2-5/ahk3-7)*; Riefler et al., 2006), and the CK biosynthetic mutants *ipt3 5 7 (ipt3-2/ipt5-3/ipt7-1)*; Miyawaki et al., 2006) and *cyp735a1 a2 (cyp735a1-1/cyp735a2-2)*; Kiba et al., 2013). The last two lines were kindly provided by Tatsuo Kakimoto (Laboratory of Plant Growth and Development, Osaka University Japan) and Hitoshi Sakakibara (RIKEN Center for Sustainable Resource Science, Yokohama, Japan), respectively. For tobacco (*Nicotiana tabacum*) experiments, we used cultivar SNN as the wild type and transgenic line *WRKY6:CKX1#29* (Werner et al., 2010). Arabidopsis seedlings were grown on Johnson medium (Johnson et al., 1957) without Pi or supplemented with Pi (KH_2PO_4) alone or with As(V) ($\text{NaH}_2\text{AsO}_4 \cdot 7\text{H}_2\text{O}$) at distinct concentrations, depending on the experiment. For all molecular, phenotypic, and biochemical analyses, Arabidopsis plants were grown in a growth chamber on a 16-h-light/8-h-dark regime (24°C/21°C).

Tobacco seeds were germinated on Murashige and Skoog (MS) with 3% Suc plates and cultivated in long-day conditions (16-h light/8-h dark) at 22°C for 10 d. Seedlings at a similar developmental stage were then transferred to a hydroponic system containing 0.1-strength Hoagland solution (Krämer et al., 1996) and cultivated for another 4 weeks. Medium was aerated and renewed every five days.

Phenotypic Analysis

For As(V) tolerance analysis, Arabidopsis plants were grown on Johnson medium with 12.5 μM Pi alone or with 25 μM As(V) in a vertical position for 10 d. To determine root growth retardation in response to As(V), main root length was measured in these plants using ImageJ software (Schneider et al., 2012).

To analyze the effect of BSO on As(V) tolerance, Arabidopsis plants were grown on Johnson medium with 12.5 μM Pi in a vertical position for 5 d. Plants were then transferred to the same medium with 20 μM As(V) alone or with increasing BSO concentrations. Root growth inhibition (fold change) was determined by quantifying root elongation after 3 d of As(V) exposure, alone or with BSO, and comparing it with roots of plants grown at corresponding BSO concentrations without As(V). Root elongation was measured using ImageJ software.

Arsenic Quantification

For arsenic quantification experiments in Arabidopsis, plants were grown and treated using a method that accurately determines arsenic uptake kinetics and accumulation (Narang et al., 2000). Arabidopsis plants were grown on plates containing Johnson media 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, after which 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) for 24 h. Plants were dried (60°C, 3 d) prior to arsenic quantification.

To determine arsenic accumulation in tobacco plants were grown in hydroponic conditions (4 weeks), washed with deionized water, grown for another 2 d in Pi-deficient conditions, then exposed to As(V) in medium containing 0.1 mM CaCl_2 , 5 μM As(V), 5 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.75 (24 h). Plants were placed in ice-cold buffer [0.1 mM CaCl_2 , 1 mM KH_2PO_4 , 5 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.75] for 1 h. Shoot and root samples were harvested separately and dried (60°C, 3 d).

Arsenic species from Arabidopsis and tobacco samples were extracted with 1:1 methanol:water using an ultrasonic homogenizer SONOPULS HD 2200 (30%, 60 s). Extracts were centrifuged (5500g, 10 min) and supernatant filtered through a 22- μm nylon syringe before injection into the HPLC for arsenic speciation. Diluted samples were injected onto an anion-exchange column PRP-X100 (250 \times 4.1 mm, particle size 10 μm ; Hamilton, Reno, NV). Arsenic species were eluted in 10 mM $\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^{-}$, 2% (v/v) MeOH mobile phase, at a 1.5 mL min^{-1} flow rate. To test the efficiency of the method, three replicates of the extracts were digested individually with $\text{HNO}_3\text{-H}_2\text{O}_2$ to compare total arsenic content (ICP-MS) with the concentration sum of all arsenic species (LC-ICP-MS) in the initial extracts. As(III) was quantified on an As(III) calibration curve; As(V) in the sample was used as an internal normalization standard.

Analytical quality control was used for total arsenic content and arsenic species were validated by analyzing certified reference material (ref. numbers

SRM 1568a; NIST and CRM-627; BCR, Belgium). Good agreement was found for both reference materials (<10% uncertainty considering a 95% confidence level). For extraction recovery, samples were spiked with known amounts of distinct arsenic species, with recoveries of 80% to 100%.

Quantification of Biothiols

For biothiol quantification, Arabidopsis and tobacco plants were grown as described for arsenic quantification. An alkaline-reducing procedure was used to extract biothiols from Arabidopsis seedlings exposed to As(V) using the procedure described by LeBlanc et al. (2013) with minor modifications. Frozen samples (0.1 mg) were ground with 30 μ L 50 mM N-acetyl Cys as an internal standard and homogenized with 270 μ L reducing reagent (2 mg mL⁻¹ NaBH₄ in 0.1 M NaOH). The mixture was transferred to 1.5-mL Eppendorf tubes, acidified with 50 μ L 10 M HCl, centrifuged (12,000g, 15 min, 4°C), and supernatant transferred to chromatographic vials under dim light for HPLC analysis.

For tobacco samples, we used an acidic extraction procedure (Sobrinho-Plata et al., 2014). Frozen root tissue (100 mg) was homogenized in 0.125 N HCl (270 μ L) with 30 μ L 50 mM N-acetyl-Cys as an internal standard. Homogenized samples were centrifuged (12,000g, 15 min, 4°C) and supernatant used for HPLC analysis.

Biothiol profiles were analyzed by HPLC (Sobrinho-Plata et al., 2009). Extracts (100 μ L) were injected into a Mediterranean SEA18 column (250 x 4.6 mm; Teknokroma, Sant Cugat del Vallés, Spain), using an Agilent Technologies 1200 series HPLC system (Santa Clara, CA). Thiols were detected after postcolumn derivatization with Ellman reagent and quantified against the N-acetyl Cys internal standard. Biothiols were identified by comparing retention times of peaks from each sample with those of commercially available standards; Cys and GSH were purchased from Sigma-Aldrich (St. Louis, MO) and PC2, PC3 and PC4 from AnaSpec (Fremont, CA).

CK Quantification

For quantification of CKs, Arabidopsis seedlings were grown in Johnson medium (+Pi) for 7 d and transferred to Pi-lacking medium for 3 d to activate expression of the As(V)/Pi transporters. Pi-starved plants were then exposed to 30 μ M As(V) in liquid medium (-Pi) for 6 h. Fresh samples were collected and frozen in liquid N₂. Frozen samples (250 mg) were ground in a mortar with liquid N₂ and homogenized with 4 mL precooled (-20°C) methanol:water:formic acid (15:4:1, v/v/v). Deuterium-labeled CK internal standards ([²H₅]Z, [²H₅]ZR, [²H₆]iP, [²H₆]iPR, [²H₇]BA, and [²H₇]BAR; Olchemim Ltd, Olomouc, Czech Republic) were added (40 μ L stock solution of 50 ng/mL of each standard in methanol) and extracted as described (Sudre et al., 2013). CKs were quantified using a High performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) system as described (Sudre et al., 2013). Significant differences ($P < 0.05$) among treatments were calculated using one-way ANOVA and the Least Significant Difference Fisher post hoc test. Statistical test were performed using the statistical package Statistica 6.0 (StatSoft, Tulsa, OK).

Gene Expression Analysis by qRT-PCR

Gene expression analysis was performed with plants grown as for CK quantification. Quantitative real-time reverse transcription-PCR was performed on three independent biological samples as described (Aguilar-Martínez et al., 2007). RNA extracted with TRI Reagent (Sigma) was used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. cDNA was diluted ten times in water and qRT-PCR performed using SYBR Green reagent. Each sample was normalized using *EF1 α* (for primer sequences, see Supplemental Table S2).

Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. TOP65CytK genes that overlap with As(V)-down-regulated genes.

Supplemental Table S2. Primers used for qRT-PCR analysis.

Supplemental Figure S1. Phenotypes of *ipt 3 5 7*, wild-type (Col-0), *cyp735a1 a2*, *35S:CKX1*, and *ahk2 ahk3* plants grown on 12.5 μ M Pi for 10 d.

Supplemental Figure S2. CK-responsive genes are down-regulated in response to As(V). qRT-PCR expression analysis of *ARR9*, *AHK4*, and *CYP82F1* in Col-0.

Supplemental Figure S3. As(V) sensitivity to BSO increased preferentially in CK-depleted plants.

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