Coordinated cytokinin signaling and auxin biosynthesis mediates arsenate-induced root growth inhibition

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Abstract

Interactions between plant hormones and environmental signals are important for the maintenance of root growth plasticity under ever-changing environmental conditions. Here, we demonstrate that arsenate (As V), the most prevalent form of arsenic (As) in nature, restrains elongation of the primary root through transcriptional regulation of local auxin biosynthesis genes in the root tips of Arabidopsis (Arabidopsis thaliana) plants. The ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1 (ASA1) and BETA SUBUNIT 1 (ASB1) genes encode enzymes that catalyze the conversion of chorismate to anthranilate (ANT) via the tryptophan-dependent auxin biosynthesis pathway. Our results showed that As V upregulates ASA1 and ASB1 expression in root tips, and ASA1- and ASB1-mediated auxin biosynthesis is involved in As V-induced root growth inhibition. Further investigation confirmed that As V activates cytokinin signaling by stabilizing the type-B ARABIDOPSIS RESPONSE REGULATOR1 (ARR1) protein, which directly promotes the transcription of ASA1 and ASB1 genes by binding to their promoters. Genetic analysis revealed that ASA1 and ASB1 are epistatic to ARR1 in the As V-induced inhibition of primary root elongation. Overall, the results of this study illustrate a molecular framework that explains As V-induced root growth inhibition via crosstalk between two major plant growth regulators, auxin and cytokinin.

Introduction

Root architecture plays important roles in plant water and nutrient acquisition, making the plasticity of root growth an important adaptive trait (Giehl et al., 2014; Tian et al., 2014; Rellan-Alvarez et al., 2016; Ogura et al., 2019). Through changing root architecture, plants adapt to various environmental cues. Arsenic (As), a class 1 carcinogen, is widely distributed in the environment and is a major threat to all life forms worldwide (Zhao et al., 2010; Naujokas et al., 2013).
Arsenate (As\textsuperscript{V}) and arsenite (As\textsuperscript{III}) are the two major forms of As present in the environment, and As\textsuperscript{V} is the predominant form found in soil under aerobic conditions (Zhao et al., 2010). Because of its structural similarity to phosphate (Pi), As\textsuperscript{V} is easily absorbed by plant roots through Pi transporters (Shin et al., 2004; Li et al., 2016). Upon entering the root system, As\textsuperscript{V} reductases rapidly reduce As\textsuperscript{V} to As\textsuperscript{III} (Chao et al., 2014; Sanchez-Bermejo et al., 2014), which is either extruded from the cell or sequestered by phytochelatins and other related thiol-containing compounds and compartmentalized into vacuoles (Tripathi et al., 2007; Mohan et al., 2016). Excess As\textsuperscript{V} in the cell leads to toxicity, which limits plant root growth (Hartley-Whitaker et al., 2001). Tolerance to As\textsuperscript{V} stress in plants is achieved mainly through the combined action of two specific responses: restriction of As\textsuperscript{V} uptake by the suppression of ARABIDOPSIS PHOSPHATE TRANSPORTER1;1 (PHT1;1), which encodes the most active transporter of As\textsuperscript{V} and Pi (Castrillo et al., 2013), and detoxification of As\textsuperscript{III} by the formation and sequestration of As\textsuperscript{III}–thiol complexes into vacuoles (Schmoger et al., 2000; Tripathi et al., 2007; Mohan et al., 2016).

After taken up by plant roots, As accumulates in the edible parts of the plant, such as the seeds and fruits, which potentially threatens the health of humans and animals. Ingestion of As can lead to serious diseases, including cancers (Martinez et al., 2011; Bundschuh et al., 2012). In addition to being toxic, As limits the uptake of minerals essential for human health, such as iron and zinc, by plants (Duan et al., 2013; Brackhage et al., 2014). While a lot is known about the uptake, transport, and detoxification of As (Ciurli et al., 2014; Li et al., 2016; Cai et al., 2019), the mechanism responsible for As inhibition of root growth has received little or no attention, despite it being not only important for understanding root growth plasticity under various environmental cues, and also for crop design and breeding.

The phytohormone auxin plays a central role in root growth and development (Sabatini et al., 1999; Benkova and Hejatko, 2009; Vanneste and Friml, 2009; Overvoorde et al., 2010; Zhao, 2018). Auxin required for root development is partially synthesized in shoots and transported to the root tips via central vascular tissues (Grieneisen et al., 2007; Zhao, 2018). The auxin accumulated in root tips can be further transported in a basipetal manner toward the root transition and elongation zones (EZs), where it is essential for developmental processes such as cell elongation and lateral root initiation (Teale et al., 2006). These processes are dependent on auxin transport via the influx and efflux carriers of the polar auxin transport (PAT) system, directing auxin to specific cell types in the root apex (Swarup et al., 2001; Friml et al., 2002a, 2002b; Benkova et al., 2003; Biliou et al., 2005; Teale et al., 2006). The so-called fountain model of auxin transport has been used to account for root elongation and root gravitropic responses (Teale et al., 2006).

Auxin required for root development is obtained not only from the shoot but also from the root (Ljung et al., 2005; Chen et al., 2014; Zhao, 2018). Local auxin biosynthesis maintains optimal root growth in response to plant hormones and environmental signals. Mutations in the TRYPTOPHAN AMINOTRANSFERASE1 (TAA1) gene in Arabidopsis (Arabidopsis thaliana) alter the root response to gravity and 1-aminocyclopropane-1-carboxylic acid (ACC) because of the reduction in auxin level in roots (Teale et al., 2006; Stepanova et al., 2008; Tao et al., 2008). Aluminum (Al) promotes auxin biosynthesis by upregulating TAA1, YUCCA 8 (YUC8), and YUCCA 9 (YUC9) expression in the root apex transition zone (TZ), which leads to root growth inhibition under Al stress (Yang et al., 2014; Liu et al., 2016). ETHYLENE RESPONSE FACTOR1 (ERF1)- and ERF109-mediated ASA1 expression is involved in ethylene signaling- and jasmonate-signaling-induced root growth regulation, respectively (Sun et al., 2009; Cai et al., 2014; Mao et al., 2016).

The crosstalk between auxin and cytokinin signaling plays important roles in root growth regulation. The AUXIN (AUX)/INDOLE-3-ACETIC ACID (IAA) protein, SUPPRESSOR OF HYPOCOTYL2 (SHY2), regulates root meristem activity by balancing auxin and cytokinin signaling. Auxin degrades SHY2 via the SKP1-CULLIN1-F-BOX (SCF)–TRANSPORT INHIBITOR RESISTANT1 (SCF\textsuperscript{TIR1}) complex (Tian et al., 2002; Dharmasiri et al., 2003), whereas cytokinin induces SHY2 expression in the root TZ via type-B ARABIDOPSIS RESPONSE REGULATORS (ARRs), which directly activate SHY2 transcription, and SHY2 negatively regulates the transcription of PIN-FORMED (PIN) genes. In addition to modulating auxin responses, SHY2 also promotes cytokinin biosynthesis by elevating the expression of ISOPENTENYLTRANSFERASES (IPTs; Dello Ioio et al., 2008; Moubayidin et al., 2010). Cytokinin also interacts with auxin to modulate the activity of the quiescent center (QC) activity; ARR1 controls PAT by downregulating the auxin influx carrier LIKE AUXIN RESISTANT2 (LAX2), leading to cytokinin signaling, which attenuates auxin response and division in QC cells (Zhang et al., 2013). The suppression of ARR1 in the QC by SCARECROW (SCR) transcription factor affects auxin biosynthesis (Moubayidin et al., 2013). Auxin also antagonizes cytokinin signaling during zygotic embryogenesis, specifically during root stem cell specification, through the direct transcriptional activation of cytokinin signaling repressors, ARR7 and ARR15 (Muller and Sheen, 2008).

Root growth inhibition is a common symptom of As toxicity (Schmoger et al., 2000; Li et al., 2007; Shri et al., 2009; Yoon et al., 2015). Here, we show that As\textsuperscript{V}, the most prevalent As species in nature, restrains root growth by promoting ANTHRANILATE SYNTHASE A1 (ASA1) and B1 (ASB1)-mediated auxin biosynthesis in root tips. We also demonstrate that As\textsuperscript{V} activates cytokinin signaling in root tips, and ARR1, the main transcription factor in cytokinin signaling, directly modulates ASA1 and ASB1 expression in roots in response to As\textsuperscript{V} stress. Our results extend the view of how plants translate environmental stress cues into growth response, thus maintaining growth plasticity during postembryonic development.
Results

AsV inhibits elongation of the primary root

Growth of Arabidopsis ecotype Columbia (Col-0) seedlings in half-strength Murashige and Skoog (1/2 MS) medium supplemented with or without 250 μM AsV showed that AsV inhibited primary root elongation and lateral root development (Figure 1, A–D). To understand the basis of root growth inhibition by AsV, we examined the effect of different concentrations of AsV on the primary root length of Arabidopsis seedlings. The growth of Col-0 roots was inhibited by AsV in a dose-dependent manner (Figure 1, E). Because root length of Col-0 seedlings was significantly reduced over time in the presence of 250 μM AsV (Figure 1, F), based on a comparison between the two different treatments at the same time points, we used this AsV concentration in all subsequent experiments.

Next, we investigated AsV-induced cellular changes in three morphologically distinct developmental zones along the longitudinal axis of the root: differentiation zone (DZ), EZ, and meristematic zone (MZ). Final cell length in the DZ, the sizes of EZ and MZ, and cell division activity in MZ were lower in Arabidopsis plants grown in presence of AsV than in those grown in medium without AsV (Figure 1, G and H and Supplemental Figure S1, A–C). Closer observation of the DZ and EZ of AsV-treated roots indicated that AsV reduced both the number and length of cells in these regions (Supplemental Figure S1, D–G), indicating that AsV reduces
both cell proliferation and cell elongation, the two basic cellular processes affecting primary root elongation in plants (Scheres et al., 2002).

ASA1- and ASB1-mediated auxin biosynthesis is involved in As \(^{V}\)-induced root growth inhibition

To reveal the early molecular events in As \(^{V}\)-induced root growth inhibition, we carried out RNA-Seq experiments using 2-mm root tips of 5-d-old Col-0 seedlings treated with 250 μM As \(^{V}\) for 0, 1, 3, 6, 12, and 24 h. Significant differences in gene expression levels were detected between As \(^{V}\) treatments and mock (0 h) treatment. The number of differentially expressed genes (DEGs) varied between 4,000 and 9,000 at each time point (Supplemental Figure S2, A), implying that the root response to As \(^{V}\) stress is complex. Because auxin is a key player in root growth regulation (Vanneste and Friml, 2009), we mainly focused on genes involved in auxin signaling. The results showed that auxin biosynthesis-related genes were upregulated as an early response to As \(^{V}\) stress, and downregulated with 6 h onward (Supplemental Figure S2, B) which imply that –auxin biosynthesis-related genes regulated by As \(^{V}\) are tightly controlled.

Next, we examined that auxin signaling in root tips subjected to As \(^{V}\) treatment using the β-glucuronidase (GUS) reporter gene under the control of the auxin-inducible promoter DR5. In the As \(^{V}\) treatment, activity of the DR5 promoter was weakly but significantly increased in root tips at 3 h and further enhanced at 6 h compared with the 0 h time point (Figure 2, A, top panel). This suggests that auxin acts as an early As \(^{V}\) stress-responsive signal to regulate root growth inhibition.

To test whether As \(^{V}\)-induced DR5:GUS signaling maximum in root tips reflected the endogenous auxin level, we measured the concentration of IAA in 2-mm Col-0 root tips. Consistent with the increased DR5:GUS expression in root tips, the level of endogenous auxin in root tips was significantly increased in As \(^{V}\)-treated root tips at 12 h (Figure 2, B). These results indicate that As \(^{V}\)-mediated auxin biosynthesis could potentially be involved in As \(^{V}\)-mediated inhibition of primary root elongation.

The expression of several auxin biosynthetic genes was upregulated by As \(^{V}\) in RNA-Seq samples (Supplemental Figure S2, C). The transcription of ASA1 and ASB1 genes was elevated in As \(^{V}\}-treated root tips compared with the mock treatment by reverse transcription quantitative PCR (RT-qPCR; Figure 2, C). We also examined the effect of As \(^{V}\) on ASA1 and ASB1 expression by expressing the GUS reporter gene under the control of the ASA1 or ASB1 promoters (proASA1:GUS and proASB1:GUS) in 5-d-old Col-0 seedlings. The proASA1:GUS and proASB1:GUS constructs were expressed mainly in the root tips, and their expression was significantly enhanced by As \(^{V}\) (Figure 2, D). The initial upregulation of ASA1 and ASB1 expression was followed by downregulation with 6 h onward (Supplemental Figure S2, C), which imply that ASA1 and ASB1 expression mediated by As \(^{V}\) is tightly controlled by a sophisticated network. These data suggest that As \(^{V}\) promotes auxin biosynthesis by activating ASA1 and ASB1 transcription.

We also compared As \(^{V}\)-induced root growth inhibition among Col-0 (wild-type), asa1 and asb1 single mutants, and asa1 asb1 double mutant seedlings. As \(^{V}\) treatment reduced the root length of Col-0, asa1, asb1, and asa1 asb1 seedlings by 69.8%, 65.4%, 64.1%, and 16.8%, respectively, compared with the mock treatment. Compared with Col-0, root growth of asa1 and asb1 showed weak insensitivity to As \(^{V}\) whereas asa1 asb1 showed more insensitivity than asa1 or asb1 (Figure 2, E and F). Additionally, the effect of As \(^{V}\) on DR5:GUS expression and auxin biosynthesis was also greatly compromised in asa1 asb1 double mutant (Figure 2, A and B). These data imply that ASA1 and ASB1 are involved in the response of roots to As \(^{V}\) stress.

Because the double mutant asa1 asb1 showed severe developmental defects, it is reasonable to speculate that root growth insensitivity of asa1 asb1 to As \(^{V}\) is caused by the defects. To exclude this possibility, root growth inhibition by other stresses were compared between Col-0 and asa1 asb1. Root growth of asa1 asb1 showed hypersensitive phenotypes to sodium chloride, excess copper ion and phosphate deficiency stresses (Supplemental Figure S3). These data demonstrated that root growth inhibition by As \(^{V}\) does not attribute to the developmental defects of asa1 asb1.

All these lines of evidence demonstrate that As \(^{V}\)-induced root growth inhibition is partially dependent on ASA1- and ASB1-mediated auxin biosynthesis.

ARR1 promotes ASA1 and ASB1 expression through promoter binding

To identify transcription factors responsible for the As \(^{V}\)-mediated upregulation of ASA1 expression, we performed yeast one-hybrid (Y1H) screening using Arabidopsis cDNA libraries harboring the HISTIDINE3 (HIS3) reporter gene under the control of the ASA1 promoter. A total of 35 positive cDNA clones encompassing genes encoding seven transcription factors, including ARR1 and ARR10, were identified (Supplemental Figure S4). Analysis of the ASA1 promoter sequence using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) revealed several potential ARR1-binding sites (ABSs), suggesting the possibility that ARR1 is a transcriptional regulator of ASA1.

To confirm that ARR1 directly binds to the ABSs in the ASA1 promoter, electrophoretic mobility shift assays (EMSAs) were conducted using glutathione (GST)-tagged DNA-binding domain of ARR1 (ARR1-GST), and P1 and P2 regions of the ASA1 promoter harboring ABS1 (‘-CTTCGATCTT-3’) and ABS2 (‘-GAGGATGGTC-3’) motifs, respectively (Figure 3, A–C). Unlabeled DNA probes competed for binding to the ARR1-GST protein in a dose-dependent manner. Furthermore, ARR1-GST protein failed to bind to mutant probes. Parallel experiments indicated that ARR1-GST could also bind to the ASB1 promoter (proASB1, Supplemental Figure S5, A and B). These data
demonstrate that ARR1 directly binds to ASA1 and ASB1 promoters in vitro.

Next, to test the interaction of ARR1 with ASA1 and ASB1 promoters in vivo, we performed chromatin immunoprecipitation (ChIP) assays using the 35S:ARR1-Myc transgenic plants and anti-Myc antibody. DNA fragments immunoprecipitated using anti-Myc antibody were significantly enriched in the P1 and P2 regions of the ASA1 promoter and P1 region of the ASB1 promoter (Figure 3, D and Supplemental Figure S5, C). These results confirm the direct physical
association of ARR1 with the promoters of ASA1 and ASB1 in vivo.

Next, we verified the activation effect of ARR1 on ASA1 expression using the well-established Nicotiana benthamiana transient expression assay (Supplemental Figure S6). Infiltration of the construct expressing the firefly luciferase (LUC) reporter gene under the control of the ASA1 promoter (proASA1:LUC) into N. benthamiana leaves resulted in a substantial amount of LUC activity. Co-expression of proASA1:LUC with a construct expressing the ARR1 gene under the control of the constitutive 35S promoter (35S:ARR1) led to a further increase in LUC luminescence intensity,
suggesting that 35S:ARR1 promotes proASA1:LUC expression. In a parallel experiment, co-expression of the proASA1-m:LUC construct, carrying mutant variants of ASB1 and ASB2 motifs, with 35S:ARR1 in N. benthamiana leaves resulted in highly attenuated LUC activity (Supplemental Figure S6). Additionally, ARR1 also activated the expression of proASA1:LUC in N. benthamiana leaves (Supplemental Figure S5, D and E). Together, our transient expression assays confirmed that ARR1 directly activates ASA1 and ASB1 expression in vivo.

The AsV-induced expression of ASA1 and ASB1 was further analyzed in Col-0, arr1-3, arr10-5, and arr1-10 arr10-5 seedlings by RT-qPCR. AsV promoted the expression of ASA1 and ASB1 in Col-0 but not in arr1-3, arr10-5, and arr1-3 arr10-5 mutants (Figure 3, E and F). Consistent with these results, the expression of proASA1:GUS and proASA1:GUS was not induced by AsV in the arr1-3 mutant (Supplemental Figure S7).

Taken together, these lines of evidence demonstrate that ARR1 promotes ASA1 and ASB1 expression through promoter binding.

ARR proteins play an important role in AsV-induced root growth inhibition

Because ARR1 regulates the expression of ASA1 and ASB1 (as shown above), we examined AsV-induced root growth inhibition in Col-0, arr1-3, arr10-5, and arr12-1 single mutants and various double mutants (Figure 4, A). Because of the redundancy of type-B ARR transcription factors, roots of arr1-3, arr10-5, and arr12-1 single mutant showed similar root growth inhibition rate as that of Col-0 roots in the presence of AsV. However, AsV reduced the root length of Col-0, arr1-3 arr10-5, and arr1-3 arr12-1 seedlings by 73.1%, 61.2%, and 63.6%, respectively, compared with the mock treatments (Figure 4, B). The roots of arr1-3 arr10-5 and arr1-3 arr12-1 seedlings showed significantly reduced sensitivity to AsV, compared with that of Col-0 seedlings. To overcome the functional redundancy of type-B ARRs, we generated transgenic Arabidopsis lines expressing the dominant negative forms of ARR1 and ARR10 by fusing them with the SUPERMAN repression domain X (SRDX; Hiratsu et al., 2002, 2003; Du et al., 2014), which resulted in their overexpression (Supplemental Figure S8). Compared with Col-0, root lengths of ARR1-SRDX and ARR10-SRDX transgenic lines showed reduced sensitivity to AsV (Figure 4). These data indicate that AsV-mediated root growth inhibition is partially dependent on ARR1 and ARR10.

AsV promotes the accumulation of ARR proteins and activates cytokinin response

To visualize cytokinin signaling output in root tips in response to AsV stress, we employed a robust and sensitive cytokinin synthetic sensor, TWO COMPONENT SIGNALING SENSOR new (TCSn):GREEN FLUORESCENT PROTEIN (GFP), which is mainly expressed in root caps (Muller and Sheen, 2008; Zurcher et al., 2013). After a 3-h AsV treatment, an elevated TCSn:GFP signal was detected in root caps (Figure 5, A). More importantly, TCSn:GFP was activated earlier than DRISrev:GFP in the presence of AsV (Supplemental Figure S9). This demonstrates that AsV activates cytokinin signaling in root caps.

To monitor the cellular distribution patterns of ARRs proteins in root tips, we used transgenic Arabidopsis lines expressing type-B ARRs tagged with a yellow fluorescent protein, Ypet, by a recombination-based gene tagging technique which reflect the nearest endogenous expression and localization of ARRs (Zhou et al., 2011; Xie et al., 2018). The Ypet-tagged ARR1, ARR10, and ARR12 proteins showed weak signals in root tips in the absence of AsV stress. Addition of the proteasome inhibitor MG132 or AsV separately to the transgenic seedlings led to increased intensity of fluorescence compared with that of seedlings in MS medium, and the fluorescence with MG132 was much stronger than that with AsV (Figure 5, B and C). Co-treatment with MG132 and AsV showed similar fluorescence intensities with MG132 treatment (Figure 5, B and C). AsV treatment did not induce the expression of ARRs, suggesting that AsV regulates ARR expression at the post-transcriptional level (Figure 5, D). These supported the AsV-induced accumulation of ARR proteins in root tips by repressing the activity of 26S proteasome.

These data demonstrate that cytokinin signaling is activated by stabilizing ARRs proteins in response to AsV stress in root tips.

Both ASA1 and ASB1 act genetically downstream of ARR1 to regulate AsV-induced root growth inhibition

To determine the genetic relationship of ARR1 with ASA1 and ASB1, we assessed the effect of AsV on root growth inhibition in Col-0, arr1-3 arr10-5 and aka1 aka1 double mutants, and arr1-3 arr10-5 aka1 aka1 quadruple mutant. The root length of Col-0, arr1-3 arr10-5, aka1 aka1, and arr1-3 arr10-5 aka1 aka1 was reduced by 71.9%, 63.4%, 17.7%, and 2.1%, respectively, compared with the mock treatment (Figure 6, A and B). Response of the quadruple mutant to AsV resembled that of the aka1 aka1 double mutant. These data elucidate that ASA1 and ASB1 act downstream of ARR1 in the root growth inhibition by AsV.

Our results reveal that exogenous AsV treatments inhibit primary root elongation by cytokinin-mediated local auxin biosynthesis in root tips. Monitoring the expression dynamics of auxin and cytokinin signaling reporter lines in response to AsV stress revealed that AsV activates cytokinin signaling earlier than auxin signaling (Supplemental Figure S9). Auxin transporters, AUXIN RESISTANT 1 (AUX1) and PIN-FORMED 2 (PIN2), regulate the basipetal transport of auxin from root tips to the EZ along the root epidermal cells. In this study, roots of aux1 and pin2 mutant seedlings showed reduced sensitivity to AsV stress. AUX1 and PIN2 expression in root tips was not regulated by AsV which implies that basipetal auxin transport may not be affected by AsV.
stress (Supplemental Figure S10). Additionally, DII-VENUS, an auxin sensor rapidly degraded by auxin, was significantly downregulated in the root meristem and EZ by As\(^{V}\), in ARR1- and ARR10-dependent manner (Supplemental Figure S11), suggesting that auxin signaling is activated in EZ by As\(^{V}\). Based on these data, we propose a working model that explains how As\(^{V}\) inhibits primary root elongation (Figure 6, C). When plant roots sense high levels of As\(^{V}\) in the soil, cytokinin signaling is activated in the plant, which increases the accumulation of ARR proteins in root tips. Then, local auxin biosynthesis is stimulated by ARR1-mediated ASA1 and ASB1 expression in root tips. Excess auxin accumulation in the EZ due to AUX1- and PIN2-mediated basipetal transport from root tips to EZ inhibits cell elongation in the EZ, which leads to primary root growth arrest.

**Discussion**

Identification of cellular and molecular mechanisms that mediate the effect of environmental stresses on plant growth is challenging. The contamination of soil and water resources by As is a worldwide environmental problem. In recent years, studies have increased our knowledge of the absorption, transportation, and detoxification of As in plants; however, how As restrains plant growth, especially root growth, remains largely unknown. Here, we address the cellular and molecular mechanisms underlying As\(^{V}\)-induced inhibition of primary root growth in Arabidopsis. We show that As\(^{V}\) inhibits cell division activity in the root meristem, and reduces cell number and limits cell elongation in the root DZ and EZ. At the molecular level, we show that ARR1 directly activates the expression of ASA1.
and ASB1 upon As\textsuperscript{V} stress, suggesting that ARR1-mediated promotion of ASA1 expression is important for As\textsuperscript{V}-induced modulation of primary root elongation. This study demonstrates crosstalk between auxin and cytokinin in roots in response to As\textsuperscript{V}. Our results provide further insights into how plants translate stress cues into growth response, thus retaining growth plasticity during postembryonic development.

Local auxin biosynthesis is involved in the root response to As\textsuperscript{V}

Auxin is a core regulator of root cell division, cell differentiation, and overall root growth. Other plant hormones and environmental stimuli can regulate root growth by interacting with auxin. Whether auxin is involved in As\textsuperscript{V}-induced root growth inhibition is not clear. In this study, we found that auxin signaling was activated at a very early stage in
the root response to As\textsuperscript{V} stress (Figure 2, A and Supplemental Figures S2, B, S9), and that auxin biosynthesis was promoted by the root response to As\textsuperscript{V} (Figure 2, B). Together, these observations suggest that auxin serves as a regulator of the early root response to As\textsuperscript{V} stress. Genetic experiments further demonstrated that the auxin synthesis genes ASA1 and ASB1 were involved in root growth inhibition by As\textsuperscript{V} (Figure 2). Local auxin biosynthesis maintains optimal plant growth in response to environmental signals (Zhao, 2018). The increased auxin level in root tips and the changes in the expression patterns of ASA1 and ASB1 in response to As\textsuperscript{V} (Figure 2, B and D) suggest that local auxin biosynthesis is promoted by As\textsuperscript{V} in root tips, and that this facilitates root growth responses to high levels of As. ASA1 and ASB1 are mainly expressed in the root caps (Figure 2, D), and this suggested that root cap is a potential site for perception of As\textsuperscript{V} stress in root tips. The TZ in root apex is a critical site for the perception and response to both endogenous phytohormones and environmental cues (Baluška et al., 2010). In the root meristem, the antagonistic interaction of auxin and cytokinin regulates the balance between cell division in RAM and cell elongation in EZ by positioning the TZ, where mitotically active cells lose their capacity to divide and initiate their differentiation programs (Dello Ioio et al., 2008). Our data demonstrated that the sizes of EZ and RAM were reduced by As\textsuperscript{V} which means TZ in root apex moved rootward (Figure 1, G and H and Supplemental Figure S1). Expression of the auxin reporter DII-VENUS was rapidly repressed in TZ with As\textsuperscript{V} treatment for 6 h, and this demonstrated that auxin signaling or auxin synthesis was
activated by As\textsuperscript{V} in a short time in TZ (Supplemental Figure S11). Our data also showed that cytokinin signaling was promoted in RAM and TZ (Figure 5, A and B). All these lines of evidence demonstrated that the cytokinin signaling and auxin signaling in TZ were regulated by As\textsuperscript{V} stress and TZ is another potential important site for root responses to As\textsuperscript{V} stress. Whether root caps or TZ or both of them are involved in perception of As\textsuperscript{V} stress signals in root tips requires further investigation. And the mechanism of how auxin signaling and cytokinin signaling are coordinated in response to As\textsuperscript{V} stress in root tips also needs further exploration.

Double mutant asa1 asb1 seedlings still showed some root growth inhibition and increased auxin synthesis after exposure to As\textsuperscript{V} stress (Figure 2, B and F), suggesting that other auxin biosynthesis genes are also involved in this process. Our data demonstrate that ASA1 and ASB1 act as important nodes for the transmission of environmental As\textsuperscript{V} signals to the mechanism regulating root growth. Excess auxin accumulation in the EZ induced by As\textsuperscript{V} stress inhibited cell elongation (Figure 1, G and H). Cell division in the MZ and cell elongation in the EZ collaborate during primary root elongation (Scheres et al., 2002). It should be noted that not only cell elongation in the EZ but also cell division in the MZ were decreased during the root response to As\textsuperscript{V} (Supplemental Figure S1). It will be important to clarify further how cell division activity in the root MZ is regulated by As\textsuperscript{V}.

Plant roots sense As\textsuperscript{V} stress partially through cytokinin signaling

Cytokinin is a multifunctional plant hormone, which plays momentous roles not only in plant growth and development but also in plant response to environmental stimuli. Current evidence suggests that the metabolism, transport, and signaling of cytokinin are involved in the tolerance to various environmental stresses, including drought, salt, extreme temperature, and nutrient deficiencies (Pavlu et al., 2018). This study uncovers at least a part of the molecular mechanism underlying As\textsuperscript{V}-mediated primary root growth arrest, which involves cytokinin signaling. We showed that As\textsuperscript{V} activates cytokinin signaling within 10 min (Supplemental Figure S9), which suggests that cytokinin signaling serves as an early As\textsuperscript{V} stress-responsive signal. Previously, Mohan et al. (2016) reported that As\textsuperscript{V} reduces the endogenous cytokinin level in whole plants, which enhances tolerance to As\textsuperscript{V} stress because of the accumulation of thiol compounds, such as phytochelatins and GST, essential for As sequestration. In this study, ASA1 and the cytokinin sensor TCShn:GFP showed enhanced expression in root caps (Figures 2, C and D, and 5, A), and ARR protein levels also increased significantly in the root cap under As\textsuperscript{V} stress (Figure 5, B). A previous study reported that the endogenous cytokinin level in whole plants is reduced upon As\textsuperscript{V} stress (Mohan et al., 2016); however, we found that cytokinin signaling was activated. This suggests that cytokinin synthesis may not be coupled to cytokinin signaling during the root response to As\textsuperscript{V}. In Arabidopsis, the root tip exhibits a cytokinin gradient, with a concentration maximum in the lateral root cap, columella, columella initials, and QC cells (Antoniadi et al., 2015). Together, these data demonstrate that the root cap is a potential site of As stress perception. Further investigation is needed to understand whether cytokinin content in the root tip is regulated under As\textsuperscript{V} stress, and how the latter induces cytokinin signaling in root caps.

Our data demonstrated that root growth of asa1 asb1 showed strong insensitivity to As\textsuperscript{V} stress than Col-0 (Figure 2, E and F). This showed that the auxin biosynthesis genes ASA1 and ASB1 are major regulators involved in As\textsuperscript{V}-mediated root growth inhibition. We further found that the B-type transcription factors ARR1 and ARR10 were direct modulators of ASA1 and ASB1 transcription (Figure 3 and Supplemental Figures S5, S6). But the roots of arr1-3 arr10-5, arr1-3 arr12-1, ARR1-SRDX, and ARR10-SRDX seedlings showed subtle but significantly reduced sensitivity to As\textsuperscript{V}, compared with that of Col-0 and asa1 asb1 seedlings (Figure 4). This weak phenotype may be caused by the functional redundancy among the B-type ARR transcription factors. And it is also possible that there are other signaling pathways parallel to the ARR1- and ARR10-mediated cytokinin signaling involved in root responses to As\textsuperscript{V} stress. And it will be interesting to explore other regulators involved in root responses to As\textsuperscript{V} stress.

Materials and methods

Plant materials and growth conditions

Arabidopsis (A. thaliana) ecotype Columbia (Col-0) was used as the wild type in this study. Various transgenic lines used in this study have been described previously: proCYCB1:1:GUS (Colon-Carmona et al., 1999); DR5:GUS (Ulmasov et al., 1997); DR5rev:GFP (Benkova et al., 2003); DIL:VENUS (Brunoud et al., 2012); TCShn:GFP (Zurcher et al., 2013); proASA1:GUS and proASB1:GUS (Stepanova et al., 2005); asa1 (wei2-1), asb1 (wei7-1), and asa1 asb1 (wei2-1 wei7-1; Alonso et al., 2003; Stepanova et al., 2005); arr1-3, arr10-5, arr12-1, arr1-3 arr10-5, arr1-3 arr12-1, arr10-5 arr12-1, and arr1-3 arr10-5 arr12-1 (Mason et al., 2005; Yokoyama et al., 2007; Argyros et al., 2008); and aux1-22 (Swarup et al., 2007). Seeds of pin2 (SALK_122916C), ARR1-C1 × Ypet (CS71599), ARR10-C1 × Ypet (CS71600), and ARR12-C1 × Ypet (CS71601; Xie et al., 2018) lines were obtained from the Arabidopsis Biological Resource Center (ABRC, OH, USA; Alonso et al., 2003).

Arabidopsis seeds were surface-sterilized for 15 min in 10% bleach, washed four times with sterile water, and plated on 1/2 MS medium supplemented with or without 250 μM As\textsuperscript{V} (NaH\textsubscript{2}AsO\textsubscript{4}·7H\textsubscript{2}O) and 0.8% agar. Plants were stratified at 4°C for 2 d in darkness and then transferred to a growth chamber maintained at 22°C and illuminated for 16 h using white light (100 μmol m\textsuperscript{−2}s\textsuperscript{−1} light intensity).
Plasmid construction and plant transformation
To construct 35S:ARR1-Myc, the coding sequence (CDS) of 
ARR1 was amplified using ARR1-Myc-F/-R primers and 
cloned into pDONR 221 (Invitrogen). To construct 
35S:ARR1-SRDX and 35S:ARR10-SRDX plasmids, a 36-bp 
DNA sequence encoding the SRDX repression domain 
(LDDLLELRLGFA) was fused in frame to the 3'-end of 
ARR1 and ARR10 CDSs using ARR1-SRDX-F/-R and 
ARR10-SRDX-F/-R primers, respectively, and cloned into the pDONR 221 
vector. The above-mentioned pDONR vectors were subcloned 
onto pK7FWG2 using the MultiSite Gateway Three- 
Fragment Vector Construction Kit (Invitrogen) to generate 
35S:ARR1-Myc, 35S:ARR1-SRDX, and 35S:ARR10-SRDX 
constructs (Hilson et al., 2004; Karimi et al., 2007). The mutants 
and reporter lines were crossed with the asa1 asb1 double mutant and arr1-3 single mutant. All primers are listed in 
Supplemental Table S1.

The above-mentioned constructs were transformed into 
Agrobacterium tumefaciens strain GV3101, which was used for 
the transformation of Arabidopsis plants by vacuum in- 
filtration (Bechtold and Pelletier, 1998).

Phenotypic, statistical, and microscopy analyses
To analyze the root phenotype, Arabidopsis seedlings were 
grown on plates and root length was measured using Image 
(National Institutes of Health; http://rsb.info.nih.gov/ij). Root 
meristem size was analyzed using seedlings mounted in 
chloroacetaldehyde:water:glycerol (HCG; 8:3:1) solution. 
Microscopy was performed on a ZEISS Axio Imager Z2 mi- 
croscope, fitted with an Axiocam 506 color camera. Images 
were processed using Adobe Photoshop CS and ZEN 2.6 sys- 

tem. Data are presented as mean values ± standard devia- 
tion (SD) of at least three biological repeats. Statistical 
significance of the data was evaluated using Student’s t test 
for pairwise comparison and analysis of variance (ANOVA), 
followed by Fisher’s least significant difference (LSD) mean 
separation test, for multiple comparisons in SPSS at a proba- 

bility level of P < 0.01 or P < 0.05.

GFP and propidium iodide fluorescence was detected at 
excitation wavelengths of 488 and 543 nm, respectively, and 
emission wavelengths of 510 and 620 nm, respectively, using 
a ZEISS LSM880 NLO. Fluorescence in the confocal images 
acquired using the same microscope settings (Ruzicka et al., 
2007; Zhou et al., 2010) was quantified using the ZEN 2.3 
SP1 program. At least 10 seedlings per sample were exam- 
inied, and at least three independent experiments were per- 
formed. The statistical significance was evaluated using 
Student’s t test.

Whole seedlings or different tissues were cleared in HCG 
solution for several minutes before microscopy. Histochemical GUS staining was performed as described pre- 
vously (Chen et al., 2015).

Measurement of IAA levels
Five-day-old wild-type (Col-0) and asa1 asb1 double mutant 
seedlings were treated with 250 μM AsV for 0 or 12 h. Two-
millimeter root tips were excised from the seedlings and 
immediately frozen in liquid nitrogen. To extract IAA, the 
root tips were ground to a fine powder in liquid nitrogen 
and 1 mL of 80% (v/v) methanol was added to each sample 
and vortexed. The concentration of IAA in each sample was 
determined by gas chromatography–mass spectrometry 
(GC–MS), as described previously (Zhou et al., 2010). Data 
are presented as mean ± SD of at least three biological 
replicates.

Gene expression analysis by RT-qPCR
Five-day-old Arabidopsis seedlings were treated with 250 
μM AsV for different durations, and 2-mm root tips were 
harvested for RNA extraction. Total RNA was extracted using 
the OminiPlant RNA Kit (CWbiotech), and cDNA was 
synthesized from 1 μg of total RNA using the SuperMix Kit 
(Transgen). Then, qRT-PCR was performed on a CFX 
Connect Real-Time PCR Detection System (Bio-Rad) using 
the TransStart Tip Green qPCR SuperMix (Transgen), 
according to the manufacturer’s instructions. Primers for 
qRT-PCR were designed using Primer 5 and are listed in 
Supplemental Table S1. All PCR reactions were performed as 
independent biological triplicates. Expression levels of genes 
were normalized to the expression of ACTIN2. Statistical sig- 
nificance of qRT-PCR data was evaluated by Student’s t test 
for pairwise comparisons and by Fisher’s LSD means separa-
test for multiple comparisons using SPSS.

ChIP-qPCR assay
ChIP was performed using roots of 8-d-old 35S:ARR1-Myc 
seedlings (1 g), as previously described (Gendrel et al., 2005; 
Kaufmann et al., 2010). Briefly, roots were crosslinked in 1% 
(w/v) formaldehyde, followed by chromatin isolation. Anti-
Myc antibody (Abcam, ab32; 1:500) was used to immuno-
precipitate protein–DNA complexes, and the precipitated 
DNA was purified using a PCR purification kit (Qiagen). 
ChIP experiments were performed in triplicate. Chromatin 
precipitated without any antibody was used as a negative 
control, while the isolated chromatin before precipitation 
was used as the input. The enrichment of DNA fragments 
was determined by quantitative real-time PCR (qPCR) using 
primer pairs listed in Supplemental Table S1. Data are pre- 

tented as the mean ± SD of at least three biological 
replicates.

EMSA
The EMSAs were conducted using LightShift 
Chemiluminescent EMSA Kit (Thermo Scientific), according 
to the manufacturer’s protocol. DNA fragments encompassing 
ABs (236–299 amino acids) were cloned into the 
pGEX-4T-1 vector digested with BamHI and Xhol. The 
resulting construct was then expressed in Escherichia coli 
BL21 (DE3) cells to produce GST-tagged ARR1 protein. The 
recombinant fusion protein was purified using BeaverBeads 
GSH, according to the manufacturer’s instructions. Annealed 
double-stranded oligos containing putative ABs were la-

teled with biotin and detected using a Chemiluminescent 
Nucleic Acid Detection Module (Thermo Scientific).
Fragments of ASA1 and ASB1 promoters were amplified by PCR using biotin-labeled or unlabeled primers. Unlabeled fragments of the identical sequence with or without the ABs were used as competitors. Sequences of the probes and primers are listed in Supplemental Table S1.

**Y1H screening**

The Y1H assay was performed using the Matchmaker One-Hybrid System (Clontech; Mitsuda et al., 2010). To construct the reporter plasmid (bait), a 1,024-bp fragment upstream of the transcription start site (TSS) of ASA1 was amplified using proASA1-Y1H-F/-R primers and cloned into the EcoRI and XbaI sites of pHiSi-1. The resulting construct was then linearized and introduced into Saccharomyces cerevisiae strain YM4271, according to the Matchmaker One-Hybrid System user manual (Clontech Laboratories, Inc.). The cDNA library was cloned in the pDEST22 vector. To perform Y1H screening, the strain YM4271 carrying the library was cloned in the pDEST22 vector. To perform Y1H screening, the strain YM4271 carrying the proASA1-HISi-1 reporter construct was transformed with 4 µg of the cDNA library and plated on synthetic defined (SD) medium lacking His and tryptophan (SD/-His/-Trp) and containing 4 mM 3-amino-1,2,4-triazole (3-AT). More than 10,000 small colonies were recovered and the large positive colonies were selected for further analysis.

**Immunoblot assays**

Five-day-old ARR1-C1 × Ypet, ARR10-C1 × Ypet, and ARR12-C1 × Ypet seedlings were treated with or without 250 µM AsV for 6 h, and root tissues were harvested for protein extraction. The recombinant fusion proteins were visualized by immunoblotting using anti-GFP antibody (Abcam, ab290). The anti-Actin antibody was used as a loading control.

**Transient expression assays in N. benthamiana**

Transient expression assays were performed in N. benthamiana leaves as described previously (Matsui et al., 2008; Guo et al., 2015). To generate the effector construct, ARR1 CDS was cloned into the pGreenII62-SK vector downstream of the 3SS promoter. Then, 1,024- and 995-bp fragments upstream of the TSSs of ASA1 and ASB1 were amplified using proASA1/LUC-F/-R and proASB1/LUC-F/-R primers, respectively. The PCR products were sequenced and cloned into the pGreenII 0800-LUC vector digested with KpnI and BamHI to produce proASA1/LUC and proASA1/LUC constructs, respectively. To generate mutant variants of ASA1 and ASB1 promoter fragments, proASA1mu and proASB1mu were synthesized (Shanghai Sangon Biotechnology Incorporation, Shanghai, China) and cloned into the pGreenII 0800-LUC vector. The resulting proASB1mu/LUC and proASA1mu/LUC plasmids were used as reporter constructs. The pGreenII 0800-LUC vector harboring the Renilla luciferase (REN) gene under the control of the 3SS promoter was used as an internal control. These experiments were performed in three independent biological replicates, each comprising three leaves from different plants. Primer and mutant promoter sequences are listed in Supplemental Table S1.

**RNA-Seq and data analysis**

Five-day-old, uniform Col-0 seedlings cultivated under normal conditions were selected and continually treated with or without 250 µM AsV for 0, 1, 3, 6, 12, and 24 h. A total of 18 root samples were harvested for transcriptome analysis. Total RNA was extracted using the OminiPlant RNA Kit (ComWin Biotech Co., Beijing, China), according to the manufacturer’s instructions. The quality of total RNA was assessed by a spectrophotometer (ThermoNanoDrop 2000, CA, USA) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Subsequently, total RNA (3 µg) isolated from each sample (with A260/A280 > 2.0) was used for the construction of RNA-Seq libraries, which were sequenced on the Illumina HiSeq 2500 platform, according to the manufacturer’s instructions. Raw sequence reads containing adapter sequences and low-quality reads were filtered to generate clean reads, which were used for further analysis. The clean reads were mapped onto the Arabidopsis reference genome downloaded from the Ensembl Plants (http://plantsensembl.org/index.html) using TopHat (v2.0.12; Trapnell et al., 2009). Gene expression levels, expressed as Fragments Per Kilobase of transcript sequence per Millions of base pairs (FPKM), were calculated using Kallisto (v0.44.0), according to the gene length and read count (Bray et al., 2016). Genes showing significant difference in expression between treated and control groups (log2 fold-change > 1.5 and adjusted P-value < 0.05) were identified as DEGs using the DESeq R package (v1.18.0; Anders and Huber, 2012). Gene Ontology (GO) enrichment analysis was performed using AGRIGO (v2.0; http://systembiology.cau.edu.cn/agriGOv2/index.php).

**Accession numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative (AGI) under the following accession numbers: AT5G05730 (ASA1), AT1G25220 (ASB1), At3G16857 (ARR1), At4G31920 (ARR10), At2G25180 (ARR12), AT2G38120 (AUX1), AT5G57090 (PIN2), and AT3G18780 (ACTIN2).

**Supplemental data**

**Supplemental Figure S1.** Arsenate (AsV) affects the MZ, EZ, and D2 in Arabidopsis roots.

**Supplemental Figure S2.** Transcriptome profiling of Col-0 roots in response to arsenate (AsV) stress.

**Supplemental Figure S3.** Wild type (Col-0) and asa1asb1 response to different stresses.

**Supplemental Figure S4.** Identification of ASA1 promoter (proASA1)-interacting transcription factors by Y1H screening.

**Supplemental Figure S5.** ARR1 binds to ASB1 promoter.

**Supplemental Figure S6.** ARR1 promotes ASA1 expression.

**Supplemental Figure S7.** Arsenate (AsV) induces ASA1 and ASB1 expression in an ARR1-dependent manner.
**Supplemental Figure S8.** Expression analysis of ARR1 and ARR10 in 35S:ARR1-SRDX and 35S:ARR10-SRDX root tips by RT-qPCR.

**Supplemental Figure S9.** Expression dynamics of DRSrev:GFP and TCSn:GFP in Arabidopsis roots in response to arsenate (As\(^{\text{V}}\)) stress.

**Supplemental Figure S10.** Reduced sensitivity of aux1 and pin2 roots to arsenate As\(^{\text{V}}\) stress.

**Supplemental Figure S11.** Effect of arsenate As\(^{\text{V}}\) stress on the auxin sensor DII-VENUS.

**Supplemental Table S1.** List of sequence-specific primers used in this study

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