Phosphorylation of ARF2 Relieves Its Repression of Transcription of the K⁺ Transporter Gene HAK5 in Response to Low Potassium Stress

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Potassium (K⁺) plays crucial roles in plant growth and development. In natural environments, K⁺ availability in soils is relatively low and fluctuating. Transcriptional regulation of K⁺ transporter genes is one of the most important mechanisms in the plant’s response to K⁺ deficiency. In this study, we demonstrated that the transcription factor ARF2 (Auxin Response Factor 2) modulates the expression of the K⁺ transporter gene HAK5 (High Affinity K⁺ transporter 5) in Arabidopsis thaliana. The arf2 mutant plants showed a tolerant phenotype similar to the HAK5-overexpressing lines on low-K⁺ medium, whose primary root lengths were longer than those of wild-type plants. High-affinity K⁺ uptake was significantly increased in these plants. ARF2-overexpressing lines and the haks5 mutant were both sensitive to low-K⁺ stress. Disruption of HAK5 in the arf2 mutant abolished the low-K⁺-tolerant phenotype of arf2. As a transcriptional repressor, ARF2 directly bound to the HAK5 promoter and repressed HAK5 expression under K⁺ sufficient conditions. ARF2 can be phosphorylated after low-K⁺ treatment, which abolished its DNA binding activity to the HAK5 promoter and relieved the inhibition on HAK5 transcription. Therefore, HAK5 transcript could be induced, and HAK5-mediated high-affinity K⁺ uptake was enhanced under K⁺ deficient conditions. The presented results demonstrate that ARF2 plays important roles in the response to external K⁺ supply in Arabidopsis and regulates HAK5 transcription accordingly.

INTRODUCTION

Potassium (K⁺) is an essential macronutrient for plant growth and development. It participates in many physiological processes in living plant cells, e.g., electrical neutralization, enzyme activation, stomata movement, membrane potential maintenance, and osmotic regulation (Clarkson and Hanson, 1980). It also promotes photosynthesis, starch synthesis, and transport of assimilation products, which determines crop yield and quality in agricultural production (Pettigrew, 2008; Zörb et al., 2014). As a major nutrient ion, K⁺ constitutes 2 to 10% of the plant's dry weight, whose concentration in the cytoplasm is relatively stable at ~100 mM (Leigh and Wyn Jones, 1984; Walker et al., 1996). However, K⁺ concentration in soils is relatively low and fluctuating, varying from micro- to millimolar (Schroeder et al., 1994; Maathuis, 2009). Thus, plants must possess multiple K⁺ transport systems with different K⁺ affinities and transport activities to absorb adequate amounts of K⁺ from the soil. An early investigation revealed that dual mechanisms of K⁺ absorption exist in plants (Epstein et al., 1963). Mechanism 1 is responsible for high-affinity K⁺ uptake under low K⁺ concentrations (below 0.2 mM), while mechanism 2 contributes to low-affinity K⁺ uptake at higher K⁺ concentrations (over 0.5 mM). Plants can sense the fluctuation of external K⁺ concentrations and switch between these two mechanisms to absorb K⁺ more efficiently (Maathuis and Sanders, 1997; Ashley et al., 2006; Wang and Wu, 2013; Chérel et al., 2014).

Over the past decade, an increased number of plant K⁺ transporters and K⁺ channels have been identified, which have different K⁺ affinities and operate in different K⁺ uptake mechanisms (Véry and Sentenac, 2003; Véry et al., 2014). In general, K⁺ channels mediate low-affinity K⁺ uptake, and K⁺ transporters conduct high-affinity K⁺ uptake. Plants employ K⁺ channels or K⁺ transporters in response to external K⁺ concentrations and regulate K⁺ absorption in root cells (Maathuis and Sanders, 1997). In Arabidopsis, the K⁺ channel AKT1 from the Shaker family has been identified as one of the most important K⁺ uptake components in roots (Hirsch et al., 1998). AKT1 is involved in not only low-affinity ([K⁺]ext > 1 mM) but also high-affinity ([K⁺]ext<100 μM) K⁺ uptake (Hirsch et al., 1998; Spalding et al., 1999; Xu et al., 2006). Another important component, High Affinity K⁺ transporter 5 (HAK5), belonging to KUP/HAK/KT family, has been characterized as a high-affinity K⁺ transporter (Ahn et al., 2004; Gierth et al., 2005). AKT1 and HAK5 constitute the main K⁺ absorption systems in Arabidopsis roots (Gierth et al., 2005; Pyo et al., 2010).

Transcriptional regulation is an important mechanism in the plant’s response to low-K⁺ stress (Ashley et al., 2006; Wang and Wu, 2013; Chérel et al., 2014). In Arabidopsis thaliana, the HAK5 transcript is remarkably induced by K⁺ deficiency in order to enhance high-affinity K⁺ uptake (Shin and Schachtman, 2004; Gierth et al., 2005). HAK5 is considered as a marker gene in the response to K⁺ deficiency in Arabidopsis. A previous
A transcription factor, RAP2.11 (Related to AP2 11), positively regulates HAK5 transcription under low-K⁺ stress (Kim et al., 2012). It was also suggested that HAK5 may be modulated by multiple transcription factors (Hong et al., 2013).

Auxin response factors (ARFs) are important transcription factors that play crucial roles in regulating the expression of auxin response genes (Tiwari et al., 2003; Guilfoyle and Hagen, 2007). Twenty-three members constitute the ARF gene family in Arabidopsis. According to their functions, these ARFs are divided into two classes: transcriptional activators and transcriptional repressors (Guilfoyle and Hagen, 2007). Many investigations have revealed the essential roles of ARFs in plant growth and development as well as in stress adaptation (Hardtke and Berleth, 1998; Nemhauser et al., 2000; Ellis et al., 2005; Okushima et al., 2005a, 2005b; Wang et al., 2011; Yang et al., 2013). In this study, we demonstrate that ARF2 participates in the regulation of K⁺ uptake. It functions as a transcriptional repressor and modulates HAK5 transcription in the response to low-K⁺ stress in Arabidopsis.

RESULTS

The arf2 Mutant Is Tolerant to Low-K⁺ Stress

AKT1 and HAK5 are the two major components mediating high-affinity K⁺ uptake in Arabidopsis roots (Gierth et al., 2005; Pyo et al., 2010). Their single mutant and double mutant exhibit root growth defects at extremely low-K⁺ concentrations (10 μM K⁺).

Previous reports showed that CIPK23 (CBL-interacting protein kinase 23) and KC1 (K⁺ rectifying channel 1) are two important regulators of AKT1; the transcription of both CIPK23 and KC1 are induced after low-K⁺ treatment (Shin and Schachtman, 2004; Gierth et al., 2005; Xu et al., 2006). In addition, HAK5 transcription is also elevated after K⁺ starvation (Shin and Schachtman, 2004; Gierth et al., 2005). To investigate the mechanism of transcriptional regulation in the response to low-K⁺ stress in Arabidopsis, we analyzed the promoter regions of these genes and identified some phytohormone-related cis-elements. Then, 15 mutants of phytohormone-related transcription factors, whose low-K⁺ phenotypes were determined, were collected from the ABRC, NASC (Nottingham Arabidopsis Stock Centre), and other research groups. We found that the mutant with a defect in transcription factor ARF2 showed a root growth advantage under low-K⁺ conditions (Figure 1A). The primary root lengths of two allelic (COM1 and COM2). Seedlings were germinated and grown on low-K⁺ (10 and 20 μM) medium or K⁺-sufficient (1 mM) medium for 7 d.

(B) Primary root length of various plants tested in (A). Data are means ± se (n = 15). Student’s t test (**P < 0.01) was used to analyze statistical significance, and “#” represents control.

(C) Diagram of ARF2. Filled boxes and lines represent exons and introns, respectively. Triangles indicate the T-DNA insertion sites in arf2-7 and arf2-8.

(D) RT-PCR analyses of ARF2 expression in various plants. EF1α was used as a control.

(E) Immunoblot analyses of ARF2 protein levels in various plants. ACTIN was used as a control.
mutants of ARF2 (arf2-7 and arf2-8) were longer than those of wild-type plants (Col), when they were directly germinated on low-K⁺ medium (10 and 20 μM K⁺) for 7 d (Figures 1A and 1B). However, the root growth advantage of arf2 did not appear under K⁺ sufficient conditions (1 mM K⁺) (Figures 1A and 1B). The ARF2 transcripts in these two T-DNA insertion mutants were both disrupted (Figures 1C and 1D), although a truncated ARF2 protein accumulated in arf2-7 (Figure 1E). ARF2 contains three domains, a DNA binding domain (160 to 293 amino acids), a repression domain (290 to 372 amino acids), and a C-terminal dimerization domain (CTD; 716 to 818 amino acids) (http://www.ebi.ac.uk/interpro/protein/Q94JM3). The truncated proteins in arf2-7 and arf2-8 may contain DNA binding domains, but lose CTD domains (Figures 1G). A previous study has revealed that the CTD domain stabilizes the dimerization of ARF DNA binding domains and facilitates ARF DNA binding activity in vivo (Boer et al., 2014). Therefore, both arf2-7 and arf2-8 used in this study should be loss-of-function mutants.

We constructed the complementation lines arf2-7/ProARF2: ARF2-Flag (COM1 and COM2), in which the transcription and protein levels of ARF2 were both restored (Figures 1D and 1E). The primary root lengths of these two independent transgenic lines (COM1 and COM2) were both reverted to wild-type lengths (Figures 1A and 1B).

The results of K⁺ content measurement indicated that the K⁺ contents in arf2 mutants was significantly increased compared with the wild-type and complementation lines, on low-K⁺ (10 and 20 μM) medium but not on K⁺ sufficient (1 mM) medium (Figure 2A). We then performed a ⁸⁶Rb⁺ uptake assay to test the high-affinity K⁺ uptake capacity among various plants. Seven-day-old seedlings were starved of K⁺ for 3 d prior to the addition of ⁸⁶Rb⁺, so that high-affinity K⁺ uptake was activated. Then, the ⁸⁶Rb⁺ uptake rate was measured at 0.5 and 1 h. The results confirmed that the high-affinity [K⁺] uptake capacity of arf2 mutants was markedly enhanced compared with wild-type plants (Figure 2B). These results suggest that ARF2 regulates primary root growth under low-K⁺ conditions by modulating high-affinity K⁺ uptake in Arabidopsis roots.

**ARF2-Overexpressing Lines Are Sensitive to Low-K⁺ Stress**

ARF2-overexpressing lines were also constructed, in which the ARF2 coding sequence was driven by the 35S promoter. We obtained two independent transgenic lines (ARF2 OE1 and OE2). An RT-qPCR assay confirmed that the ARF2 transcripts were increased in both lines (Supplemental Figure 1A). Phenotype analysis showed that ARF2 OE1 and OE2 were sensitive to low-K⁺ stress, exhibiting primary roots that were shorter than those of wild-type plants (Supplemental Figures 1B and 1C). However, these two overexpressing lines had shorter primary roots and more lateral roots and root hairs compared with wild-type plants under K⁺ sufficient conditions (Supplemental Figure 1B). This abnormal root architecture might be due to the constitutive overexpression of ARF2.

We thus constructed inducible ARF2-overexpressing lines (ARF2 OE3 and OE4) driven by a β-estradiol (E2) inducible promoter (Schlücking et al., 2013). A GFP tag was also fused to the C terminus of ARF2 in ARF2 OE3 and OE4 to monitor the expression and localization of ARF2. As shown in Figure 3A, without E2 in the medium, ARF2 OE3 and OE4 did not show any phenotype difference compared with wild-type plants, both on low-K⁺ and K⁺-sufficient medium (Figures 3A and 3C). Upon application of E2 (10 μM), the ARF2 transcript was induced (Figure 3B), and ARF2-GFP proteins were expressed and targeted to the nucleus of the root epidermis and root hairs (Supplemental Figure 2). After application of E2 in the medium, ARF2 OE3 and OE4 exhibited shorter primary roots than did wild-type plants, only under low-K⁺ conditions (Figures 3A and 3C). Although the primary root lengths of all plants became shorter on K⁺-sufficient medium after application of E2, there was no phenotype difference between wild-type and ARF2-overexpressing plants (Figures 3A and 3C). Furthermore, the K⁺ contents in ARF2 OE3 and OE4 were significantly reduced on

![Figure 2. High-Affinity K⁺ Uptake Is Enhanced in arf2 Mutants.](image)

The K⁺ content (A) and ⁸⁶Rb⁺ uptake rate (B) of the wild type, arf2 mutants, and complementation lines. Data are means ± se (n = 3). Student’s t test (**P < 0.01) was used to analyze statistical significance, and “#” represents control.

(A) Seedlings were germinated and grown on low-K⁺ medium or K⁺-sufficient medium for 7 d.

(B) Seven-day-old seedlings were starved of K⁺ for 3 d. Then, ⁸⁶Rb⁺ uptake rates were measured at the indicated times (0.5 and 1 h).
low-K\textsuperscript{+} medium, only when E2 was added in the medium (Figure 3D). However, ARF2 OE3 and OE4 did not show any difference in K\textsuperscript{+} content with wild-type plants under K\textsuperscript{+} sufficient conditions (Figure 3E). Together, these results demonstrated that ARF2 is a negative regulator involved in the response to low-K\textsuperscript{+} stress in Arabidopsis.

We noticed that the induced ARF2-GFP signal was only observed in the root epidermis and root hairs (Supplemental Figure 2). This is because E2 was added in the medium and can easily enter the outer layer of root tissues (epidermis and root hairs), but barely enters the inner layer (cortex and endodermis), where ARF2-GFP is thus not induced.

**ARF2 Negatively Regulates HAK5 Expression**

Phenotype analysis and K\textsuperscript{+} content measurement suggested that ARF2 negatively regulates high-affinity K\textsuperscript{+} uptake in Arabidopsis roots. Since ARF2 has been reported as a transcription factor that represses the transcription of target genes (Guilfoyle and Hagen, 2007; Wang et al., 2011), we hypothesized that ARF2 inhibits

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**Figure 3. ARF2-Overexpressing Lines Are Sensitive to Low-K\textsuperscript{+} Stress.**

(A) Phenotype comparison between wild-type (Col) and inducible ARF2-overexpressing lines (OE3 and OE4). Seedlings were germinated and grown on low-K\textsuperscript{+} medium or K\textsuperscript{+}-sufficient medium with or without 10 \textmu M \beta-estradiol (E2) for 7 d.

(B) RT-qPCR analyses of ARF2 expression in various plants treated with or without \beta-estradiol (E2). Data are means ± se (n = 3). Student’s t-test (*P < 0.01) was used to analyze statistical significance, and “#” represents control.

(C) to (E) Primary root length (C) and K\textsuperscript{+} content (D) and (E) of various plants tested in (A). Data are shown as means ± se. Student’s t-test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance, and “#” represents control. Sample number (n) was 15 in (C) and 3 in (D) and (E).
the expression of genes that control high-affinity K⁺ uptake in Arabidopsis roots, such as \( HAK5, \) AKT1, KC1, CIPK23, and CBL1/9/10. After detecting the expression of these relevant genes, we found that only \( HAK5 \) showed a difference in expression between the wild type and \( arf2 \) mutants (Figure 4A; Supplemental Figure 3). The transcript of \( HAK5 \) was markedly increased in \( arf2 \) mutants, especially under low-K⁺ conditions (Figure 4A), while \( HAK5 \) expression was significantly repressed in ARF2 OE3 and OE4 after application of E2 (Figure 4B). In addition, \( HAK5 \) expression was reduced in ARF2 OE1 and OE2 (Supplemental Figure 1D).

To further test whether ARF2 directly regulated \( HAK5 \) expression, we performed transient expression experiments in tobacco (\( Nicotiana benthamiana \)) leaves. When Pro35S:ARF2 and ProHAK5:GUS were coexpressed in tobacco leaves, the GUS activity was significantly reduced (Figure 4C). This suggests that ARF2 inhibits \( HAK5 \) promoter activity. Using the ProHAK5:LUC Arabidopsis transgenic line, we also tested the expression level of \( HAK5 \) in vivo. As shown in Figures 4D and 4E, the \( arf2 \) mutant emitted stronger luminescence than control plants, especially under low-K⁺ conditions. These results demonstrate that ARF2 negatively modulates \( HAK5 \) expression in response to low-K⁺ stress in Arabidopsis.

**ARF2 Directly Binds to the HAK5 Promoter**

As a transcription factor, ARF2 contains a conserved DNA binding domain at the N terminus that can bind to auxin-responsive elements (AuxREs) (Ulmasov et al., 1997; Guilfoyle and Hagen, 2007). Promoter sequence analysis showed that several AuxREs are located within the \( HAK5 \) promoter region. According to their positions, we designated three fragments (P1 to P3) within the \( HAK5 \) promoter, each of which contains at least one AuxRE (Figure 5A). Using an ARF2 antibody, we conducted a chromatin immunoprecipitation (ChIP) assay to detect the DNA binding activity of ARF2 to the \( HAK5 \) promoter in vivo. The \( arf2-8 \) mutant was used as a negative control. ChIP-qPCR results indicated that ARF2 binds to the P1 and P3 fragments but not to the P2 fragment (Figure 5B).

Furthermore, ARF2 DNA binding activity was also validated in vitro using an EMSA (electrophoresis mobility shift assay). The N terminus of ARF2 (ARF2-N), which contains a DNA binding domain, was fused with CKS (CTP:CMP-3-deoxy-D-manno-octulosonate cytidylyltransferase synthetase) protein and His tag. The CKS-ARF2-N-His protein was expressed in \( Escherichia coli \) and purified from the soluble fraction using...
labeled competitive probes (Figure 5C). In addition, when the binding activity was gradually reduced by the addition of unlabeled probes, free labeled probes are indicated with arrows. Chromatin was isolated from 7-d-old seedlings grown on K+-sufficient medium. Chromatin was immunoprecipitated with ARF2 antibody (anti-ARF2). Then, the three fragments (P1 to P3) were tested using qPCR. The positions of hysteresis bands were indicated with arrows. CKS-ARF2-N-His protein was used as a negative control. Data are means ± SE (n = 3).

Figure 5. ARF2 Directly Binds to the HAK5 Promoter.
(A) Diagram of the HAK5 promoter region. Gray lines represent the positions of AuxREs in the HAK5 promoter. Black lines indicate three fragments (P1 to P3) containing AuxREs in the HAK5 promoter that were used in the ChIP-qPCR assay.
(B) ChIP-qPCR analyses of ARF2 DNA binding activity to the HAK5 promoter. Chromatin was isolated from 7-d-old seedlings grown on K⁺-sufficient medium. Chromatin was immunoprecipitated with ARF2 antibody (anti-ARF2). Then, the three fragments (P1 to P3) were tested using qPCR. The ratios of immunoprecipitated DNA over the input DNA were calculated and presented as percentage of input (% IP). Chromatin without ARF2 antibody was used as a negative control. Data are means ± SE (n = 3).
(C) EMSA to analyze the DNA binding activity of ARF2 N-terminal to P1 and P3 fragments in the HAK5 promoter. The purified CKS-ARF2-N-His proteins were incubated with P1 or P3 probes labeled with biotin. An excess of unlabeled probes was added to compete with biotin-labeled probes. ARF2-N can bind to P1 and P3 fragments, but not interact with the fragments with mutated AuxREs. The AuxREs were labeled in red. CKS-His protein was used as a negative control. The positions of hysteresis bands and free labeled probes are indicated with arrows.

His antibody. EMSA results showed that ARF2-N can directly bind to P1 and P3 probes labeled with biotin (Figure 5C). This binding activity was gradually reduced by the addition of unlabeled competitive probes (Figure 5C). In addition, when the AUXREs within P1 and P3 were mutated, ARF2-N no longer interacted with these two fragments (Figure 5C). As a negative control, CKS-His protein alone did not bind to the HAK5 promoter (Figure 5C). Taking all these data together, we demonstrated that ARF2 directly binds to the AuxREs within the HAK5 promoter region, suggesting that ARF2 regulates HAK5 transcription.

ARF2 Participates in K⁺ Uptake by Regulating HAK5 Expression

Since ARF2 can bind to the HAK5 promoter and repress HAK5 expression, we further investigated this regulation in planta by constructing arf2 hak5 double mutant and HAK5-overexpressing lines. The arf2-7 single mutant was crossed with the hak5 single mutant to generate the arf2-7 hak5 double mutant, in which the transcripts of both ARF2 and HAK5 are disrupted (Figure 6A). Phenotype analysis indicated that the low-K⁺-tollerton phenotype of arf2-7 was entirely abolished in the arf2-7 hak5 double mutant, whose primary root length was as short as that of the hak5 single mutant under low-K⁺ conditions (Figures 6B and 6C). These results suggest that HAK5 is epistatic to ARF2. The K⁺ content of the arf2-7 hak5 double mutant was also as low as that of the hak5 single mutant on low-K⁺ medium, even though the arf2-7 single mutant showed a high K⁺ content (Figure 6D). These results indicate that the low-K⁺-tolerant phenotype of arf2 relies on HAK5 function.

In addition, we determined the phenotype and K⁺ content of HAK5-overexpressing lines. These overexpressing lines were generated by transformation of the HAK5 coding sequence driven by the HAK5 native promoter into the hak5 mutant. Two independent transgenic lines (HAK5 OE1 and OE2) were selected by detecting the HAK5 expression level (Figure 6E). They exhibited a low-K⁺-tolerant phenotype similar to that of the arf2-7 single mutant, whose primary root lengths were longer than those of wild-type plants under low-K⁺ conditions (Figures 6F and 6G). Furthermore, the arf2-7 mutant and HAK5-overexpressing lines accumulated more K⁺ ions in plants compared with wild-type plants under low-K⁺ conditions (Figure 6H). These results demonstrate that the low-K⁺-tolerant phenotype of arf2 is due to the elevation of HAK5 expression.

The K⁺ content measurement was consistent with the results of the 86Rb⁺ uptake assay. As shown in Figure 7, both the arf2-7 mutant and the HAK5-overexpressing lines had higher Rb⁺ (K⁺) uptake rates than did wild-type plants within the high-affinity range. By contrast, the Rb⁺ (K⁺) uptake rates in the hak5 mutant and arf2-7 hak5 double mutant were significantly reduced (Figure 7). These data indicate that ARF2 modulates HAK5-mediated high-affinity K⁺ uptake.

Overexpression of HAK5 Stimulates Root Hair Elongation under Low-K⁺ Conditions

A previous report showed that low-K⁺ treatment induces root hair elongation (Jung et al., 2009). In this study, we observed that both root hair length and density were increased in wild-type plants, when plants were directly germinated on low-K⁺ medium (Figure 8). This increase in root hair length and density
Figure 6. ARF2 Participates in K⁺ Uptake by Regulating HAK5 Expression.

(A) RT-qPCR verification of ARF2 and HAK5 expression in various plants as indicated. Seedlings were germinated and grown on K⁺-sufficient (1 mM) medium for 7 d, and then total RNA of plants was extracted and used for RT-qPCR analysis.

(B) to (D) Root phenotype (B), primary root length (C), and K⁺ content (D) of various plants as indicated. Seedlings were germinated and grown on low-K⁺ medium or K⁺-sufficient medium for 7 d.

(E) RT-qPCR analysis of HAK5 expression in the arf2 mutant and HAK5-overexpressing lines.

(F) to (H) Root phenotype (F), primary root length (G), and K⁺ content (H) of arf2 mutant and HAK5-overexpressing lines. Data are means ± se. Sample numbers (n) were 15 in (C) and (G) and 3 in (D) and (H). Student’s t test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance, and “#” represents control.
that phosphorylation can block ARF2 DNA binding activity (Vert et al., 2008). In this study, the arf2-7/ProARF2/ARF2-Flag transgenic line (COM1) was used to detect the ARF2 phosphorylation level in planta using the Phos-biotin method (Kinoshita-Kikuta et al., 2007). As shown in Figure 9B, low-K⁺ treatment triggers the rapid phosphorylation of ARF2 within several hours. After addition of CIAP (calf intestine alkaline phosphatase), this low-K⁺-induced ARF2 phosphorylation vanished (Figure 9B). These results indicate that low-K⁺ treatment leads to ARF2 phosphorylation and then may remove ARF2 repression from the HAK5 promoter.

To further investigate the role of ARF2 phosphorylation in the regulation of HAK5 transcription, we tried to identify the phosphorylation sites on the ARF2 sequence. Phosphoproteomics analysis revealed three Ser residues (Ser-533, Ser-689, and Ser-840) on ARF2 that are phosphorylated in the response to abiotic stresses in Arabidopsis (http://phosphat.uni-hohenheim.de/). Then, we examined the roles of these Ser residues in HAK5 regulation. These three Ser residues were mutated to Ala or Asp in order to mimic the dephosphorylation or phosphorylation status, respectively, of ARF2. Then, the activities of mutated ARF2 were determined in tobacco leaves using a GUS/LUC assay. The results indicated that mutations on Ser-533 and Ser-840 did not affect ARF2 activity in repressing HAK5 transcription (Supplemental Figure 5). However, ARF2S689D significantly relieves the repression on HAK5 transcription (Figure 9C), suggesting that Ser-689 may be crucial for ARF2 DNA binding activity.

Using the Phos-tag method, we detected the phosphorylation status of Ser-689 in the response to low-K⁺ stress in Arabidopsis. As shown in Figure 9D, the ARF2 peptide (ARF2501-700) containing Ser-689 showed low phosphorylation levels under K⁺ sufficient conditions, while those phosphorylation levels were markedly enhanced after low-K⁺ treatment. However, the mutated ARF2S533D and ARF2S840D can hardly be phosphorylated under low-K⁺ conditions (Figure 9D). These results indicate that Ser-689 is an important phosphorylation site on ARF2 that responds to low-K⁺ stress.

Furthermore, we tested the DNA binding activity of ARF2S533D and ARF2S840D to the HAK5 promoter using the EMSA method. Here, the full-length ARF2 protein was expressed and purified. EMSA results indicated that full-length ARF2 can also bind to the P1 and P3 fragments within the HAK5 promoter (Figure 9E). This binding activity was reduced by the addition of unlabeled competitive probes (Figure 9E). The S689A mutation did not affect ARF2 binding activity to P1 and P3 (Figure 9E). However, ARF2S533D no longer interacted with P1, whose binding activity to P3 was also significantly impaired (Figure 9E). All these data demonstrated that low-K⁺-induced phosphorylation of ARF2 abolishes its DNA binding activity to the HAK5 promoter and removes the repression of HAK5 transcription. Therefore, HAK5 could be rapidly induced under low-K⁺ stress.

Conversely, the phosphorylated ARF2 induced by low-K⁺ stress can be rapidly dephosphorylated again when K⁺ was resupplied (Figure 10A). We also found that the phosphorylation status of ARF2 corresponded to HAK5 expression levels (Figure 10). The dephosphorylated ARF2 repressed HAK5 transcription again after K⁺ was resupplied (Figure 10B). Disruption of ARF2 relieved this repression of HAK5 transcription (Figure 10B). These results indicated that the regulation of phosphorylation status of ARF2 is

Phosphorylation of ARF2 Relieves the Repression of HAK5 Transcription under low-K⁺ Conditions

Consistent with previous reports (Shin and Schachtman, 2004; Gierth et al., 2005; Rubio et al., 2014), we also observed that HAK5 expression was induced by low-K⁺ treatment (Supplemental Figure 4A). Since ARF2 is a negative regulator of HAK5, we hypothesized that low-K⁺ treatment might relieve the repression of HAK5 transcription by reducing ARF2 transcription or ARF2 activity. RT-qPCR analysis revealed that ARF2 transcription was not affected after low-K⁺ treatment (Supplemental Figure 4B). Therefore, it is possible that the DNA binding activity of ARF2 declines under low-K⁺ conditions. A ChIP-qPCR assay confirmed that ARF2 no longer bound to the P1 and P3 fragments within the HAK5 promoter after low-K⁺ treatment (Figure 9A). How does this binding activity disappear? A previous report showed

Figure 7. Rb⁺ Uptake Rates Are Increased in arf2 Mutant and HAK5-Overexpressing Lines.

Seven-day-old seedlings were starved of K⁺ for 3 d. Then Rb⁺ uptake rates were measured at the indicated times (0.5 and 1 h). Data are means ± se (n = 3). Student’s t test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance, and “#” represents control.
dependent on the external K⁺ level, which directly determines the expression level of $HAK5$.

**DISCUSSION**

**Transcriptional Regulation of $HAK5$ by ARF2 May Not Rely on Auxin Signaling**

ARFs are an important family of transcription factors that regulate the expression of auxin response genes (Tiwari et al., 2003; Guilfoyle and Hagen, 2007). Some $arf$ mutants exhibit obvious auxin-related phenotypes, such as $arf3$, $arf5$, $arf7$, and $arf7 arf19$ (Hardtke and Berleth, 1998; Nemhauser et al., 2000; Okushima et al., 2005b; Schruff et al., 2006). In this study, we also wondered if the transcriptional regulation of $HAK5$ by ARF2 is dependent on auxin signaling. Phenotype analysis indicated that the $arf2$ mutant did not show any phenotypic difference in root growth compared with the wild type under K⁺-sufficient conditions (Supplemental Figure 6). ARF2 has been found to be involved in many phytohormone signaling pathways. It can function as a negative regulator in ABA-mediated seed germination and primary root growth (Wang et al., 2011). ARF2 is also regulated by BIN2 (BRASSINOSTEROID-INSENSITIVE2) kinase and involved in brassinosteroid (BR) signaling (Vert et al., 2008). In addition, ARF2 proteins are degraded by ethylene signaling in a HLS1-dependent manner, which suggests that ARF2 participates in ethylene and light signaling pathways that control hypocotyl bending (Li et al., 2004). All these reports demonstrated that, as an auxin response factor, ARF2 has diverse roles in multiple phytohormone signaling pathways and may function as an important connecting node in the plant’s response to different stresses or stimuli. As a transcriptional repressor, ARF2 might function as a general “brake” that controls plant growth and development.

ARF2 Maintains the Low Expression Level of $HAK5$ under K⁺-Sufficient Conditions

$HAK5$ transcription is induced by low-K⁺ stress, which is one of the most important mechanisms in the adaptation to low-K⁺ stress in

![Image of the figure](image_url)
Figure 9. Phosphorylation of ARF2 Relieves the Repression of HAK5 Transcription under Low-K⁺ Conditions.

(A) ChIP-qPCR analyses of ARF2 DNA binding activity to P1 and P3 fragments in the HAK5 promoter with or without low-K⁺ treatment. Wild-type seedlings were germinated and grown on K⁺-sufficient medium for 5 d and then transferred to K⁺-sufficient medium or low-K⁺ medium for 6 h. Data are means ± se (n = 3).

(B) ARF2 can be phosphorylated after low-K⁺ treatment. arf2-7/ProARF2:ARF2-Flag (COM1) transgenic plants were germinated and grown on K⁺-sufficient medium for 5 d and then transferred to K⁺-sufficient medium or low-K⁺ medium for 2, 4, and 6 h. Total proteins of the plants at different time points were extracted. ARF2 proteins were immunoprecipitated using anti-Flag mAb agarose. Phosphorylated ARF2 proteins were detected by immunoblotting (IB) using biotinylated Phos-tag. CIAP can dephosphorylate ARF2, which was used as a control.

(C) GUS activity measurement in tobacco leaves after transient expression of ProHAK5:GUS and Pro35S:ARF2 with point mutations (S689A and S689D). ARF2S689D can relieve the repression of HAK5 transcription. Data are means ± se (n = 6). Student’s t test (**P < 0.01) was used to analyze statistical significance, and “#” represents control.

(D) Ser-689 on ARF2 is phosphorylated after low-K⁺ treatment. Wild-type ARF2 peptide (ARF2501-700) and mutated peptide (ARF2501-700 S689A) were expressed in E. coli and purified, respectively. Wild-type plants were germinated and grown on K⁺-sufficient medium for 5 d and then transferred to K⁺-sufficient medium or low-K⁺ medium for 2, 4, and 6 h. Total proteins were extracted from wild-type plants at the indicated time points. The purified ARF2 peptides were incubated with the total proteins for 40 min. Then the phosphorylation status of ARF2 peptides was tested using Phos-tag SDS-PAGE. ARF2 peptides were detected by immunoblot analysis using His antibody. CIAP can dephosphorylate ARF2 peptides, which were used as a control.

(E) EMSA to analyze the DNA binding activity of ARF2S689A (SA) and ARF2S689D (SD) to P1 and P3 fragments. The purified CKS-ARF2-His proteins were incubated with P1 or P3 probes labeled with biotin. An excess of unlabeled probes was added to compete with biotin-labeled probes. The CKS-His protein was used as a negative control. The positions of hysteresis bands and free labeled probes are indicated with arrows.
Arabidopsis (Ahn et al., 2004; Armengaud et al., 2004; Shin and Schachtman, 2004; Gierth et al., 2005). In this study, we demonstrated that ARF2 is a negative regulator of \( HAK5 \) transcription. Why do plants need negative regulation of \( HAK5 \)? \( HAK5 \) is a high-affinity \( K^+ \) transporter (\( K_m \); 15 to 24 \( \mu \)M) and conducts \( K^+ \) uptake only at the low \( K^+ \) concentration range (Gierth et al., 2005; Pyo et al., 2010). \( HAK5 \)-mediated \( K^+ \) uptake constitutes a high energetic cost (Maathuis and Sanders, 1997). By contrast, AKT1 has a lower affinity than \( HAK5 \) for \( K^+ \) uptake (Gierth et al., 2005; Pyo et al., 2010). AKT1-mediated \( K^+ \) uptake is more energy efficient at a range of higher \( K^+ \) concentrations (Maathuis and Sanders, 1997; Hirsch et al., 1998; Spalding et al., 1999). Under \( K^+ \)-sufficient conditions (millimolar level), \( HAK5 \)-mediated \( K^+ \) uptake would be energetically expensive and wasteful, which should be shut down and replaced by the low-affinity \( K^+ \) uptake system mainly mediated by AKT1 (Maathuis and Sanders, 1997). Therefore, the \( HAK5 \) transcript should be maintained at extremely low levels under \( K^+ \) sufficient conditions, and ARF2 plays a crucial role in the process.

In this study, we found that \( HAK5 \) repression was impaired in the \( arf2 \) mutant under \( K^+ \)-sufficient conditions (Figures 4A and 10B). Furthermore, when plants were transferred from low-\( K^+ \) medium to \( K^+ \)-sufficient medium, \( HAK5 \) transcript was rapidly reduced in wild-type plants. However, \( HAK5 \) expression in the \( arf2 \) mutant cannot decline as rapidly as in the wild type (Figure 10B). It has been demonstrated that the physiological role of ARF2 is maintaining the low expression level of \( HAK5 \) under \( K^+ \)-sufficient conditions in order to save energy. Since \( HAK5 \) transcription is induced by low-\( K^+ \) stress, there must exist other transcription factors that positively regulate \( HAK5 \) expression under low-\( K^+ \) conditions (Kim et al., 2012; Hong et al., 2013). These transcription factors are also important regulators of \( HAK5 \) expression and thus merit further investigation.

After resupply of \( K^+ \), \( HAK5 \) transcript levels steadily decreased in \( arf2 \) mutants, although the expression levels were still higher than in wild-type plants (Figure 10B). Some other negative regulators that repress \( HAK5 \) transcription might exist. Alternatively, the positive regulators may be removed from \( HAK5 \) promoter after \( K^+ \) resupply, thereby reducing \( HAK5 \) expression.

Phosphorylation Is an Important Regulatory Mechanism for ARF2 Function

In plants, phosphorylation-mediated regulation of transcription factors is a common mechanism, which has been revealed in many signaling pathways. In the BR signaling pathway, the protein kinase BIN2 phosphorylates two transcription factors, BZR1

![Figure 10. Potassium Level Controls ARF2 Phosphorylation Status and \( HAK5 \) Expression.](image)

(A) ARF2 phosphorylation status in wild-type plants under different \( K^+ \) conditions. \( arf2-7/ProARF2:ARF2-Flag \) (COM1) transgenic plants were germinated and grown on \( K^+ \)-sufficient medium (1 mM \( K^+ \)) for 5 d and then transferred to low-\( K^+ \) medium (10 \( \mu \)M \( K^+ \)) for 6 h. After that, the seedlings were transferred back to \( K^+ \)-sufficient medium (1 mM \( K^+ \)) for 4 and 6 h. Total proteins of the plants at different time points were extracted. ARF2 proteins were immunoprecipitated using anti-Flag mAb agarose. Phosphorylated ARF2 proteins were detected by immunoblotting (IB) using biotinylated Phos-tag. CIAP can dephosphorylate ARF2, which was used as a control. (B) \( HAK5 \) expression in wild-type plants and \( arf2 \) mutants under different \( K^+ \) conditions described in (A). Total RNA of the plants at different time points was extracted and used for RT-qPCR analyses. Transcript level of \( HAK5 \) was quantified.

![Figure 11. Working Model of \( HAK5 \) Transcriptional Regulation by ARF2.](image)

Under \( K^+ \)-sufficient conditions, ARF2 binds to AuxREs within the \( HAK5 \) promoter and represses \( HAK5 \) transcription. When plants are subjected to low-\( K^+ \) stress, ARF2 is rapidly phosphorylated and removed from the \( HAK5 \) promoter, which relieves the repression on \( HAK5 \) transcription. Then some other transcription factors (such as RAP2.11) bind to the \( HAK5 \) promoter and activate \( HAK5 \) transcription. The T-bar represents transcriptional repression, and the arrow represents transcriptional activation.
(BRASSINAZOLE-RESISTANT1) and BZR2/BRI1-EMS-SUPPRESSOR1, when BR levels are low. Phosphorylated BZR1/2 lose DNA binding activity and cannot regulate target genes. In the presence of BR, BZR1/2 are dephosphorylated and then bind to BR response elements in the promoters of BR target genes to regulate their expression (Wang et al., 2012). In theABA signaling pathway, transcription factor RAV1 represses the expression of ABI3, ABI4, and ABI5. Protein kinases SnRK2s are activated by the ABA signal and then phosphorylate RAV1, which removes RAV1 repression on ABI5 transcription (Feng et al., 2014). Here, we demonstrated that low-K⁺ stress triggers a rapid phosphorylation of ARF2 (Figure 9B). Conversely, phosphorylated ARF2 can be dephosphorylated again when K⁺ is resupplied (Figure 10A). The phosphorylation status of ARF2 directly determines the expression level of HAK5 (Figure 10B). In addition, a previous report has shown that the protein kinase BIN2 from the BR signaling pathway can phosphorylate ARF2, which also results in loss of ARF2 DNA binding and repression activity (Vert et al., 2008). Phosphorylation might be a universal mechanism in ARF2-mediated stress responses. Future studies should aim to identify the protein kinases and protein phosphatases that modulate ARF2.

The results presented in this study reveal a regulatory mechanism of HAK5 transcription that is controlled by the transcription factor ARF2. As shown in Figure 11, we propose a working model to illustrate this mechanism. Under K⁺-sufficient conditions, ARF2 binds to the AuxREs within the HAK5 promoter and represses HAK5 transcription to maintain a low expression level of HAK5. When plants are subjected to low-K⁺ stress, ARF2 is rapidly phosphorylated and loses DNA binding activity. ARF2 is removed from the HAK5 promoter, which relieves the repression on HAK5 transcription. Then some other transcription factors (such as RAP2.11) bind to the HAK5 promoter and activate HAK5 transcription. Finally, the elevated HAK5 expression enhances HAK5-mediated high-affinity K⁺ uptake, which helps plants survive under K⁺-deficient conditions.

METHODS

Plant Materials and Growth Conditions

The Arabidopsis thaliana mutants (Columbia ecotype) used in this study, including arf2-7 (CS24601), arf2-8 (SALK_108995), and hak5 (SALK_005604), were obtained from the ABRC or the NASC. For plant hybridization and seed harvesting, Arabidopsis plants were grown in a potting soil mixture (rich soil:vermiculite = 2:1, v/v) and kept in growth chambers at 22°C with illumination (fluorescent lamps) at 120 μmol m⁻² s⁻¹ under long-day (16 h light/8 h darkness) conditions. The relative humidity was ~70% (±5%). For the low-K⁺ phenotype assay, seeds were surface sterilized and treated at 4°C in darkness for 4 d. Then, the seeds were plated on K⁺-sufficient (1 mM) or low-K⁺ (10 and 20 μM) medium containing 0.5% (w/v) sucrose and 0.7% (w/v) agarose (USB) and grown at 22°C with illumination at 100 μmol m⁻² s⁻¹ under long-day conditions, unless otherwise indicated.

Generation of Arabidopsis Transgenic Plants

The ARF2 coding sequence fused with the Flag tag was cloned into the pCAMBIA1305 vector driven by an ARF2 native promoter and was transformed into the arf2-7 mutant to obtain complementation lines (COM1 and COM2). The ARF2 coding sequence was cloned into the pCAMBIA1300 vector driven by the 3SS promoter and transformed into wild-type (Columbia ecotype) plants to obtain constitutive ARF2-overexpressing lines (ARF2 OE1 and OE2). The ARF2 coding sequence was cloned into the pCAMBIA1391 vector driven by the HAK5 native promoter. This construct was transformed into the hak5 mutant to obtain the HAK5-overexpressing lines (HAK5 OE1 and OE2). Arabidopsis plants were transformed with Agrobacterium tumefaciens (strain GV3101) using the floral dip method (Clough and Bent, 1998). The T4 homozygous transgenic plants were used for the phenotype test.

Root Phenotype Observation and K⁺ Content Measurement

The K⁺-sufficient medium used for phenotype analysis was prepared by modifying the Murashige and Skoog medium. NH₄NO₃ and KNO₃ were removed, and KH₂PO₄ was replaced by NH₄H₂PO₄. The medium contained 1.5 mM MgSO₄·7H₂O, 1.25 mM NH₄H₂PO₄, 2.99 mM Ca(NO₃)₂·4H₂O, 0.1 mM MnSO₄·4H₂O, 5 μM KI, 0.1 μM CuSO₄·5H₂O, 0.1 mM Mn₂B₂O₇, 0.1 μM CoSO₄·6H₂O, 0.03 mM ZnSO₄·7H₂O, 10 μM Na₂MoO₄·2H₂O, 0.1 mM FeSO₄·7H₂O, and 0.1 mM Na₂EDTA. For low-K⁺ medium, the NH₄H₂PO₄ was replaced by H₂PO₄. Different K⁺ concentrations were supplemented with KCl. Seeds were germinated and grown on K⁺-sufficient (1 mM) or low-K⁺ (10 and 20 μM) medium for 4 or 7 d and then photographs were taken. ImageJ software was used to measure root hair length and primary root length. The 7-d-old seedlings grown on K⁺-sufficient or low-K⁺ medium were harvested and used for K⁺ content measurement. The dry weight was measured as biomass. Samples were treated in a muffle furnace at 300°C for 1 h and 575°C for 5 h. The ashes were dissolved and diluted in 0.1 M HCl. The K⁺ concentrations were measured using 4100-MP AES (Agilent).

GUS/LUC Assay

The transient GUS/LUC assay was performed as described previously (Feng et al., 2014). The constructs Pro3SS:ARF2 and ProHAK5:GUS in the pCAMBIA1381 vector were transfected into Agrobacterium (strain GV3101) separately. Then, the bacterial cell suspension was injected into tobacco (Nicotiana benthamiana) leaves using syringes. Three days later, the GUS activity was measured using methyl umbelliferyl glucuronide (Sigma-Aldrich), and luciferase (LUC) activity was used as an internal control. The GUS/LUC ratio was used to determine the ARF2 binding activity to the HAK5 promoter.

EMSA

EMSA was conducted using a LightShift Chemiluminescent EMSA Kit (Pierce) following the manufacturer’s protocol. The recombinant proteins CKS-ARF2-N-His, CKS-ARF2-His, and CKS-His were expressed and purified from Escherichia coli. The QuickChange Lightning site-directed mutagenesis kit (Agilent Technologies) was used to generate CKS-ARF2Δ589-D-His and CKS-ARF2Δ588-D-His constructs. Three fragments (P1 to P3) in the HAK5 promoter were amplified by PCR and labeled with or without 5′-biotin. The unlabeled fragments were used as competitors, and CKS-His protein was used as a negative control.

ChiP-qPCR Assay

The ChiP-qPCR assay was performed as described previously (Feng et al., 2014). Mouse polyclonal ARF2 antibody was generated against
ARF2 Modulates HAK5 Expression

His-tagged recombinant ARF2 peptides (residues 1 to 60 and 395 to 627) purified from E. coli. The 5-d-old seedlings were transferred to K+-sufficient (1 mM) or low-K+ (0.1 mM) medium for 6 h. Then the plants were harvested and used for ChIP assays.

**86Rb+ Uptake Assay**

86Rb+ isotope was used as a tracer to analyze K+ uptake. Seven-day-old His-tagged recombinant ARF2 peptides (residues 1 to 60 and 395 to 627) were expressed in E. coli and purified. Arabidopsis wild-type plants were germinated and grown on K+-sufficient medium for 5 d and then transferred to K+-sufficient medium or low-K+ medium for 2, 4, and 6 h. Total proteins were extracted from wild-type plants at the indicated time points. Then, 100 µg of the total proteins was incubated with 2 µg of ARF2 peptides in phosphorylation buffer with 10 µM ATP for 40 min at 22°C. The phosphorylated and dephosphorylated ARF2 peptides were separated using 8% Phos-tag SDS-PAGE (NARD AAL-107) following the manufacturer’s protocol. The peptides were detected by immunoblotting using His antibody. CIAP (Takara) was used to dephosphorylate ARF2 peptides.

**Accession Numbers**

Sequence data for the genes described in this article can be found in the Arabidopsis TAIR database (https://www.arabidopsis.org/index.jsp) under the following accession numbers: At1g30270 for ARF2, At4g32650 for HAK5, At2g26650 for AKT1, At1g30270 for CIPK23, At4g32650 for KC1, At4g17615 for CBL1, At5g47100 for CBL9, and At4g33000 for CBL10.

**Supplemental Data**

- **Supplemental Figure 1.** Phenotype Analysis of ARF2-Overexpressing Lines.
- **Supplemental Figure 2.** ARF2 Proteins Are Targeted to the Nucleus in the Root Epidermis and Root Hairs.
- **Supplemental Figure 3.** Expression Analyses of Potassium Relevant Genes in arf2 Mutants.
- **Supplemental Figure 4.** Expression Analyses of HAK5 and ARF2 in Response to Low-K+ Stress.
- **Supplemental Figure 5.** GUS/LUC Assay in Tobacco Leaves to Test the Activity of Mutant ARF2 Proteins.
- **Supplemental Figure 6.** Phenotype Test of arf2 Mutant in Response to NAA or NPA.
- **Supplemental Table 1.** Primer Sequences and Enzyme Sites Used in This Study.

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**Author Contributions**

S.Z. and Y.W. designed the research. S.Z., M.-L.Z., and T.-L.M. performed research and analyzed data. S.Z. and Y.W. wrote and revised the article.
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Phosphorylation of ARF2 Relieves Its Repression of Transcription of the K⁺ Transporter Gene HAK5 in Response to Low Potassium Stress
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