RESEARCH ARTICLE

The Transcription Factor MYB59 Regulates K⁺/NO₃⁻ Translocation in the Arabidopsis Response to Low K⁺ Stress

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Short title: MYB59 regulates K⁺/NO₃⁻ transport

One Sentence Summary: In response to low K⁺ stress, the MYB59 transcription factor regulates both the expression of transporter gene NRT1.5/NPF7.3 in Arabidopsis roots and the rate of root-to-shoot K⁺/NO₃⁻ transport.

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ABSTRACT:

Potassium and nitrogen are essential nutrients for plant growth and development. Plants can sense K⁺/NO₃⁻ levels in soils, and accordingly they adjust root-to-shoot K⁺/NO₃⁻ transport to balance the distribution of these ions between roots and shoots. In this study, we show that the transcription factor MYB59 maintains this balance by regulating the transcription of the Arabidopsis transporter NRT1.5/NPF7.3 in response to low K⁺ (LK) stress. The myb59 mutant showed a yellow-shoot sensitive phenotype when grown on LK medium. Both the transcript and protein levels of NPF7.3 were remarkably reduced in the myb59 mutant. LK stress repressed transcript levels of both MYB59 and NPF7.3. The npf7.3 and myb59 mutants, as well as the npf7.3 myb59 double mutant, showed similar LK-sensitive phenotypes. Ion content analyses indicated that root-to-shoot K⁺/NO₃⁻ transport was significantly reduced in these mutants under LK conditions. Moreover, ChIP and EMSA assays confirmed that MYB59 bound directly to the NPF7.3 promoter. Expression of NPF7.3 in root vasculature driven by the PHO1 promoter rescued the sensitive phenotype of both npf7.3 and myb59 mutants. Together, these data demonstrate that MYB59 responds to LK stress and directs root-to-shoot K⁺/NO₃⁻ transport by regulating the expression of NPF7.3 in Arabidopsis roots.

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INTRODUCTION

Potassium (K) and nitrogen (N) are essential macronutrients for plant growth and development. In plants, K⁺ is the most abundant cation, which constitutes 2 to 10% of the plant’s dry weight (Leigh and Wyn Jones, 1984). Its functions include enzyme activation, osmotic regulation, and electrical neutralization (Clarkson and Hanson, 1980). Besides, it can facilitate photosynthesis, starch synthesis, and transport of assimilation products (Pettigrew, 2008; Zörb et al., 2014).

Nitrogen is the macronutrient that plants require in the greatest amounts. It is part of numerous organic compounds and is an essential component of amino acids, proteins, and nucleic acids (Mengel and Kirkby, 2001). Therefore, sufficient K and N supplies are necessary to promote crop yield and quality, as well as to enhance crop resistance to biotic and abiotic stresses.

For most terrestrial plants, K⁺ and NO₃⁻ are the major forms of potassium and nitrogen that are absorbed by plant roots and transported within plants. They represent the most abundant inorganic cation and anion, respectively, in plant cells, and their absorption and transport have to be coordinated (Blevins et al., 1978; Triplett et al., 1980; White, 2012) for proper growth and development. However, in agricultural production, excessive application of nitrogen fertilizer with insufficient potassium fertilizer disturbs the N/K balance. This reduces the efficiency of fertilizer utilization and results in environmental pollution (Guo et al., 2010; Zhang, 2017). Therefore, understanding the mechanisms that coordinate N and K uptake and transport are critical for both the improvement of crop nutrient efficiency and protection of our environment from excess fertilizer runoff.

K⁺ and NO₃⁻ are absorbed into plant root cells by K⁺ transporters and NO₃⁻ transporters, respectively. In Arabidopsis, the K⁺ channel AKT1 (ARABIDOPSIS K⁺ TRANSPORTER1) and the K⁺ transporters HAK5 (HIGH AFFINITY K⁺ TRANSPORTER5) and KUP7 (K⁺ UPTAKE PERMEASE7) are major components involved in K⁺ uptake (Hirsch et al., 1998; Rubio et al., 2000; Gierth et al., 2005; Pyo et al., 2010; Adams and Shin, 2014; Han et al., 2016). The absorption of NO₃⁻ is mediated by transporters of the NRT1/PTR (Nitrate Transporter 1/Peptide Transporter) and NRT2 families (Chen et al., 2008; Wang et al., 2012; Léran et al., 2014; O'Brien
et al., 2016). After absorption into root cells, K\(^+\) and NO\(_3^-\) are released into xylem vessels and subsequently transported towards the shoot. In *Arabidopsis*, the K\(^+\) channel SKOR (STELAR K\(^+\) OUTWARD RECTIFIER) and the K\(^+\) transporter KUP7 are responsible for root-to-shoot K\(^+\) transport (Gaymard et al., 1998). The NO\(_3^-\) transporter NRT1.5 is involved in root-to-shoot NO\(_3^-\) transport (Lin et al., 2008), and is now known as NPF7.3 (in the NRT1/PTR family) (Léran et al., 2014). Our recent study revealed that NPF7.3 can also function as a proton-coupled H\(^+\)/K\(^+\) antiporter, which also mediates root-to-shoot K\(^+\) translocation (Li et al., 2017). Therefore, NPF7.3 plays essential roles in regulating the coordinated transport of K\(^+\)/NO\(_3^-\) from root to shoot in *Arabidopsis* (Lin et al., 2008; Drechsler et al., 2015; Meng et al., 2016; Li et al., 2017).

Plants can sense K\(^+\)/NO\(_3^-\) levels in soils, and accordingly can regulate K\(^+\)/NO\(_3^-\) uptake as well as root-to-shoot translocation to balance the distribution of these ions between roots and shoots. Under nutrient sufficient conditions, the root-to-shoot K\(^+\)/NO\(_3^-\) transport is enhanced. Conversely, this transport is restricted in plants under nutrient-limited conditions. Previous studies have revealed that SKOR and NPF7.3 are regulated at the transcriptional level in response to external K\(^+\)/NO\(_3^-\) levels. The transcripts of both SKOR and NPF7.3 are upregulated by the NO\(_3^-\) supply (Wang et al., 2004; Lin et al., 2008). During low K\(^+\) stress, the NPF7.3 transcript is downregulated to inhibit root-to-shoot K\(^+\)/NO\(_3^-\) transport (Lin et al., 2008; Li et al., 2017). It has been suggested that the coordination of root-to-shoot K\(^+\)/NO\(_3^-\) transport may be achieved via the transcriptional regulation of SKOR and NPF7.3. However, the underlying molecular mechanisms have remained unclear.

MYB-type transcription factors are members of a large family of important regulators in plants. There are approximately 339 and 230 MYB proteins in *Arabidopsis* and rice, respectively (Feller et al., 2011). They play crucial roles in various physiological processes (Dubos et al., 2010) such as hormone response (Li et al., 2009; Peng, 2009), abiotic stress tolerance (Agarwal et al., 2006; Jung et al., 2008), plant development (Baumann et al., 2007; Wang et al., 2009; Zhang et al., 2013), and metabolic regulation (Zhong et al., 2008; Zhou et al., 2009). Here, we showed that the MYB transcription factor MYB59 directly bind to the NPF7.3 promoter and
positively regulated expression of the *NPF7.3* transcript in *Arabidopsis* in response to external K⁺/NO₃⁻ levels. *MYB59* and *NPF7.3* function in same pathway to coordinate root-to-shoot K⁺/NO₃⁻ transport.

**RESULTS**

*Arabidopsis myb59* Mutants Are Sensitive to Low K⁺ Stress.

To identify important components involved in the response to low K⁺, over 400 *Arabidopsis* T-DNA insertion mutants were analyzed for potential low K⁺ phenotypes. Among these mutants, the MYB transcription factor mutant *myb59-1* showed a very sensitive phenotype on low K⁺ (LK, 100 μM K⁺) medium (Figure 1A; Supplemental Figure 1A). When seedlings were grown on LK medium for 10 d, shoots of *myb59-1* became yellow (a typical symptom of K⁺ deficiency), whereas the wild-type shoots remained green (Figure 1A). Under high K⁺ (HK, 5 mM K⁺) conditions, there was no phenotypic difference between wild type and mutant plants (Figure 1A). *MYB59* can produce four distinctively spliced transcripts (*MYB59.1* to *MYB59.4*), and *MYB59.4* appears to have no known function (Li et al., 2006). The other three transcripts are all disrupted in the *myb59-1* mutant (Figure 1B and 1C). A Crispr/Cas9 mutant of *MYB59* (*myb59-2*) was constructed and displayed a similar low K⁺-sensitive phenotype as seen with *myb59-1* (Figure 1A and 1D). Complementation lines of *myb59-1* (COM1 and COM2) generated by transformation with the genomic sequence of *MYB59* rescued the sensitive phenotype of *myb59-1* (Figure 1A and 1C). These data suggested that the transcription factor MYB59 is involved in the low K⁺ response. The transcript levels of *MYB59* in all these plants were analyzed and are shown in Figure 1C and 1E.

*MYB59.3* encodes an R2R3-type transcription factor MYB59.3 that contains two amino acid sequence repeats R2 and R3 in the N terminus (Dubos et al., 2010), while MYB59.1 and MYB59.2 have a truncated N terminus (Figure 1B). All three proteins mediated transcriptional activation activities in yeast (Figure 2A). To test their functions in planta, the coding sequences of each of the three transcripts driven by the native promoter were transformed into *myb59-1*,
respectively. Phenotypic analyses showed that only the longest transcript (MYB59.3) could restore the phenotype of myb59-1 to wild type (Figure 2B). The expression levels of the three transcripts are shown in Figure 2C. In addition, overexpression of MYB59.3 in myb59-1 (myb59-1/ProSuper:MYB59.3-FLAG) also complemented the low K+ sensitive phenotype, but did not lead to an enhanced tolerance phenotype (Supplemental Figure 2). These results suggested that MYB59.3 is the functional form in plants.

**Root-to-Shoot K+ Transport Is Reduced in myb59 Mutants.**

Direct K+ measurements indicated that, under LK conditions, the roots of myb59 mutants accumulated more K+ ions than roots of wild-type plants (Figure 3A). In contrast, the K+ content in mutant shoots was significantly reduced, resulting in the low K+ sensitive phenotype of myb59 mutants (Figure 1A). The increased root/shoot ratios of K+ in myb59 mutants suggested that MYB59 is involved in root-to-shoot K+ transport under LK conditions (Figure 3B). In addition, the K+ concentrations in the xylem exudates of mutant plants were significantly reduced under LK conditions (Figure 3C), further confirming the role of MYB59 in root-to-shoot K+ transport.

We observed that, although the K+ content in myb59 shoots was also slightly reduced on HK medium (Figure 3A), the K+ concentrations in the xylem exudates of myb59 mutants were not significantly changed under HK conditions (Figure 3C). This difference may be due to the different culture conditions used in the two experiments. For K+ content measurement, plants were grown on sealed agar plates for 10 d. In contrast, for the K+ concentration measurement of xylem exudates, plants were grown in vermiculite and irrigated with nutrient solution for 20 d. The differences in substrate, seedling age and transpiration condition may influence K+ translocation rate in xylem. In addition, nutrient solution has more freely diffusible K+ ions compared with agar plates, which prevents the decreased K+ concentration in mutant xylem.

**Transcription of NPF7.3 Is Reduced in myb59 Mutants.**

To identify the target gene of MYB59, we conducted RNA-seq experiments to analyze the
transcriptional changes of K⁺-related genes. Among these genes, the transcriptional levels of HAK5 and NPF7.3 were significantly reduced in myb59 mutant plants under both MS (Murashige & Skoog medium) and LK conditions (Supplemental Table 1; Supplemental Figure 3A). HAK5 functions in high-affinity K⁺ uptake in Arabidopsis root (Gierth et al. 2005). It is considered to be a marker gene in the Arabidopsis response to K⁺ deficiency, because its transcript level is strictly regulated by external K⁺ levels (Gierth et al., 2005; Zhao et al., 2016). Previous studies reported that the transcription factors RAP2.11 and ARF2 directly regulate HAK5 expression (Kim et al., 2012; Zhao et al., 2016). In the present study, the high K⁺ content in myb59 mutant roots may impair or alleviate the K⁺-deficiency signal and thereby could retard the induction of HAK5 transcript in myb59 mutants even under the low K⁺ condition. ⁸⁶Rb⁺ uptake assays also suggested that the high-affinity K⁺ uptake in myb59 mutants was inhibited (Supplemental Figure 3B), and this was consistent with the reduced HAK5 expression in myb59 mutants (Supplemental Figure 3A). Phenotype tests indicated that hak5 mutant plants did not show a similar sensitive phenotype like myb59 under this low K⁺ condition (Supplemental Figure 3C), suggesting that HAK5 may not be a direct target of MYB59. However, the repressed HAK5 transcription in myb59 mutants may further enhance the low K⁺ sensitive phenotype of myb59 mutants.

NPF7.3 is involved in K⁺ and NO₃⁻ translocation from root to shoot (Lin et al., 2008; Li et al., 2017). We found that the NO₃⁻ content in myb59 mutant plants showed a similar pattern to the K⁺ content (Figure 3D to 3F). Therefore, MYB59 is also involved in root-to-shoot NO₃⁻ transport, especially under LK conditions, and this matches the physiological function of NPF7.3.

Both NPF7.3 and MYB59 are mainly expressed in Arabidopsis roots. MYB59 transcripts could be detected in all root tissues and cell types. However, NPF7.3 was specifically expressed in the root stele (Figure 4A). GUS staining and RT-qPCR analyses confirmed that the level of the NPF7.3 transcript was extremely low in roots of myb59 mutants (Figure 4B and 4C). Disruption
of MYB59 also attenuated the level of NPF7.3 in the root stele (Figure 4D). These data suggested that NPF7.3 is a target gene of MYB59.

SKOR is another important K⁺ channel that mediates root-to-shoot K⁺ transport (Gaymard et al., 1998). We also investigated whether SKOR is a target gene of MYB59. Based on the RNA-seq results, under the LK condition, we did not observe differences in SKOR expression between wild type and myb59 mutant plants (Supplemental Table 1). RT-qPCR results further confirmed that the SKOR transcript level was not altered in the myb59 mutants (Supplemental Figure 4A). Moreover, a SKOR loss-of-function mutant (skor) did not display phenotypes that were comparable to myb59 or npf7.3 mutants (Supplemental Figure 4B). Together, these data indicated that SKOR is not a target gene of MYB59 in our experimental conditions.

Root-to-Shoot K⁺/NO₃⁻ Transport Is Impaired in both myb59 and npf7.3 Mutants.

Phenotype tests on LK medium showed that two allelic mutants of NPF7.3 (npf7.3 and lks2) displayed a very sensitive phenotype (yellow shoot) similar to myb59 mutants (Figure 5A; Supplemental Figure 1B). In the lks2 mutant, a single-nucleotide mutation resulted in a G209E substitution in NPF7.3 (Li et al., 2017). With increasing K⁺ concentration in the medium, the yellow shoot phenotype of npf7.3 and myb59 gradually disappeared (Figure 5B). Because NPF7.3 is a key component mediating K⁺ and NO₃⁻ translocation from root to shoot (Lin et al., 2008; Li et al., 2017), the K⁺ and NO₃⁻ contents in npf7.3 and myb59 mutants were determined. The results indicated that these two mutants displayed a very similar pattern regarding ion contents. Both npf7.3 and myb59 mutants had higher K⁺ and NO₃⁻ contents in roots than wild-type plants under LK conditions, whereas the K⁺ and NO₃⁻ contents were significantly reduced in mutant shoots (Figure 5C and 5D). The root/shoot ratios indicated that root-to-shoot transport of both K⁺ and NO₃⁻ is impaired in these mutants (Figure 5E and 5F).

We observed that the root/shoot ratios of NO₃⁻ in npf7.3 and myb59 mutants were more strongly affected than that of K⁺ under LK conditions (Figure 5E and 5F). Besides NPF7.3, the K⁺ channel SKOR and the K⁺ transporter KUP7 also contribute to root-to-shoot K⁺ translocation.
(Gaymard et al., 1998; Han et al., 2016). They are specific for K$^+$ transport, and do not transport NO$_3^-$ Importantly, the transcript levels of both SKOR and KUP7 were not regulated by MYB59 (Supplemental Table 1; Supplemental Figure 4A). In contrast, NPF7.3 has been characterized as the major component for root-to-shoot NO$_3^-$ transport (Lin et al., 2008; Li et al., 2017). Consistent with this, our results show a stronger effect of myb59 and npf7.3 mutants on the NO$_3^-$ distribution than for K$^+$.

To determine whether MYB59 and NPF7.3 function in same pathway, a myb59 npf7.3 double mutant was generated. Phenotype analyses and ion content measurements showed that the double mutant had both a similar sensitive phenotype and similar ion contents as the single mutants (Figure 6A and 6B; Supplemental Figure 1C). In addition, the K$^+$ concentrations in xylem sap in all these single and double mutants were lower than in wild-type plants under LK conditions, and were reduced to the same level (Figure 6C). These results indicated that MYB59 and NPF7.3 function in same regulatory pathway. RT-qPCR results showed that the transcript levels of NPF7.3 were remarkably reduced in both the myb59 single mutant and the myb59 lks2 double mutant, MYB59 transcripts were just slightly increased in npf7.3 and lks2 mutants (Figure 6D). These findings suggested that NPF7.3 acts downstream of MYB59.

**NPF7.3 Represents a Downstream Target Gene of MYB59.**

We performed genetic experiments to further address whether NPF7.3 transcription is regulated by MYB59. A previous study reported that a NPF7.3 coding sequence driven by a PHO1 promoter could complement the phenotype of npf7.3 mutant (Drechsler et al., 2015). PHO1 is involved in phosphate translocation from root to shoot, and its expression pattern in the root stele is similar to that of NPF7.3 (Hamburger et al., 2002). PHO1 transcript levels in myb59 mutants were not significantly different (Supplemental Figure 3A). Therefore, we constructed the transgenic plants npf7.3/ProPHO1::NPF7.3 and myb59-1/ProPHO1::NPF7.3. The NPF7.3 transcripts were no longer reduced in these two transgenic plants (Figure 7A). Phenotype analyses showed that the NPF7.3 genomic sequence driven by PHO1 promoter could
complement the low K+ sensitive phenotype of both npf7.3 and myb59 mutants (Figure 7B; Supplemental Figure 1D and Supplemental Figure 5). The shoot K+ contents in these transgenic plants were significantly increased, even to higher concentrations than that in wild-type plants (Figure 7C). These data clearly demonstrated that the low K+ sensitive phenotype of myb59 is due to reduction of NPF7.3 transcription and identify NPF7.3 as the downstream target gene of MYB59.

**MYB59.3 Binds Directly to the NPF7.3 Promoter.**

As a MYB-type transcription factor, MYB59 contains conserved R2R3 DNA-binding domains at its N terminus that can bind to some cis elements (Mu et al., 2009; Prouse and Campbell, 2012). The NPF7.3 promoter region contains some cis elements that may represent binding sites of MYB59. To determine whether MYB59 binds to the NPF7.3 promoter, ChIP (chromatin immunoprecipitation) and EMSA (electrophoresis mobility shift assay) were performed. Because phenotype analyses indicated that only the transcript MYB59.3 encodes the functional form of MYB59 (Figure 2B), myb59-1/ProSuper::MYB59.3-FLAG transgenic plants were used for ChIP assays. Six DNA fragments (P1 to P6) within the NPF7.3 promoter region (~1.6 kb) were studied (Figure 8A). Two fragments within NPF7.3 exon regions were used as negative controls (Figure 8A). The ChIP results indicated that MYB59.3 could only bind to the P1 and P5 fragments (Figure 8B).

The DNA-binding activity of MYB59 was also confirmed using EMSA. Both MYB59.3 and MYB59.1 (control) were fused with CKS (CTP:CMP-3-deoxy-d-manno-octulosonate cytidylyltransferase) protein and a His tag. The CKS-MYB59-His proteins were expressed in *Escherichia coli* and purified from the soluble fraction using an anti-His antibody. As shown in Figure 8C, MYB59.3 bound to P1 and P5 probes labeled with biotin. The binding signals were gradually reduced by the addition of unlabeled competitive probes. Comparatively, MYB59.1 did not have DNA-binding activity to P1 and P5 fragments (Supplemental Figure 6), suggesting that lack of the R2 domain may abolish the DNA-binding activity of MYB59.1 (Figure 1B). These
data demonstrated that MYB59.3 directly binds to the *NPF7.3* promoter, and imply that this enables to regulate *NPF7.3* transcription.

**Transcription of MYB59 and NPF7.3 Is Responsive to External K⁺ and NO₃⁻ Levels.**

Previous studies have revealed that the transcript level of *NPF7.3* is downregulated under K⁺ and NO₃⁻ deficient conditions (Lin et al., 2008; Li et al., 2017). We asked: as the upstream regulator of *NPF7.3*, how does *MYB59* respond to external K⁺ and NO₃⁻ levels? RT-qPCR results indicated that *MYB59* and *NPF7.3* showed similar expressions pattern in response to external nutrient (K⁺ and NO₃⁻) levels (Figure 9). Their expression was repressed by low K⁺ and low NO₃⁻ treatment, but rapidly resumed when K⁺ and NO₃⁻ were resupplied in the medium (Figure 9). This suggests that both *MYB59* and *NPF7.3* can respond to external K⁺ and NO₃⁻ levels. These data indicated that *MYB59* functions as a positive regulator of *NPF7.3* and that both proteins operate in same pathway and adjust root-to-shoot K⁺/NO₃⁻ transport according to external nutrient levels.

Because MYB59.3 is the functional form regulating *NPF7.3* transcription, we determined the level of MYB59.3 in response to low K⁺ stress using cell-free protein degradation experiments. MYB59.3 protein was expressed in *E. coli* and then the purified protein was incubated with plant total proteins that were extracted from HK- or LK-treated wild-type plants. Our results revealed that MYB59.3 was rapidly degraded upon LK treatment (Figure 10). Moreover, addition of MG132, an inhibitor of protein degradation, totally abolished MYB59.3 degradation (Figure 10). In contrast, the level of MYB59.3 was only slightly reduced when incubated with total proteins extracted from HK-treated plants (Figure 10). These results indicated that low K⁺ signaling posttranscriptionally reduces the protein level of MYB59.3 in order to downregulate *NPF7.3* transcription.
DISCUSSION

MYB59 but not MYB48 Is Involved in the Regulation of Nutrient Transport

Arabidopsis MYB59 belongs to the R2R3-type MYB transcription factor class. A previous report showed that MYB59 is involved in the regulation of cell cycle progression and root growth (Mu et al., 2009). In the present study, we established a function of MYB59 in the regulation of nutrient transport. Previous studies indicated that MYB59 and its closest homolog MYB48 show high similarity in gene structure and amino acid sequence (Li et al., 2006). MYB59 and MYB48 are derived from a relatively recent duplication event, and they share 74.2% nucleotide sequence identity in their coding regions (Chen et al., 2006; Li et al., 2006). In addition, both of them play roles in the JA signaling network (Hickman et al., 2017), suggesting their functional redundancy.

To test whether MYB59 and MYB48 were redundant in the regulation of K^+/NO_3^- transport, the phenotypes of their single and double mutants were tested. Using the Crispr/Cas9 technique, four independent mutant lines of MYB48 were obtained. However, none of them displayed a low K^+ sensitive phenotype like the myb59 mutant (Supplemental Figure 7A). In addition, three myb59 myb48 double mutant lines were also constructed using Crispr/Cas9 in the myb59 background. The double mutant plants showed similar phenotype as the myb59 single mutants (Supplemental Figure 7B). These analyses indicated that only MYB59 rather than MYB48 is involved in Arabidopsis response to low K^+ stress.

MYB59 and MYB48 Genes Undergo Alternative Splicing

Previous reports revealed that both MYB59 and MYB48 undergo similar alternative splicing and have four distinctively spliced transcripts (Li et al., 2006). In the present study, we report that expression of the longest transcript (MYB59.3) is sufficient to bring about the K^+ related function of MYB59 in Arabidopsis (Figure 2B). Moreover, a recent study reported that both Arabidopsis MYB59 and MYB48 showed alternative splicing only in the response to K^+ deficiency, but not to other nutrient deficient conditions (Nishida et al., 2017). After low K^+ treatment, the total
transcript levels of *MYB59* and *MYB48* were reduced, but their longest transcripts *MYB59.3* and *MYB48.3* were significantly increased (Nishida et al., 2017).

We also observed alternative splicing of *MYB59*. Low K\(^+\) treatment repressed the total transcript amount of *MYB59* (Figure 9), but resulted in enhanced accumulation of the *MYB59.3* transcript (Supplemental Figure 8). Resupply of K\(^+\) rapidly restored the normal expression pattern of all three spliced transcripts (Supplemental Figure 8). In contrast, *NPF7.3* forms only one transcript that was downregulated after low K\(^+\) treatment (Figure 9). The total transcript amount of *MYB59* perfectly matched the expression change of *NPF7.3* in *Arabidopsis* response to external K\(^+\) levels (Figure 9). But there is a discrepancy between *MYB59.3* and *NPF7.3* in the accumulation of their transcript levels after low K\(^+\) treatment. According to the results from our phenotype and genetic analyses, *MYB59.3* is a positive regulator of *NPF7.3* and promotes *NPF7.3* expression (Figure 2B; Supplemental Figure 2). Therefore, the increased *MYB59.3* transcript levels are unlikely to be the reason for *NPF7.3* repression under low K\(^+\) conditions. We hypothesized that low K\(^+\) signaling may posttranscriptionally reduce the protein level or transcriptional activity of MYB59.3, which downregulates the *NPF7.3* transcription. MYB59.3 proteins may be degraded, phosphorylated, or modified under K\(^+\) deficient conditions. Such regulation represents a common mechanism for a rapid on-and-off switch of MYB transcription factor activity (Pireyre and Burow, 2015). Indeed, we observed that MYB59.3 protein was degraded after low K\(^+\) stress (Figure 10), resulting in the downregulation of *NPF7.3* transcription. In this regard, the induction of *MYB59.3* transcription after low K\(^+\) stress may be a result of a feedback response.

**Transcriptional Regulation of Transporter Proteins Is Important for K\(^+\) Homeostasis in Plants**

Transcriptional regulation provides an essential strategy in plant response to K\(^+\) deficiency. For example, the transcript levels of K\(^+\) transporter genes are upregulated under K\(^+\) deficient conditions in order to enhance high-affinity K\(^+\) uptake in plant roots (Wang and Wu, 2013, 2017).
At the same time, the root-to-shoot $K^+$ transport must be reduced to keep sufficient $K^+$ in roots, and this represents an important adaptive mechanism to maintain root activity and the $K^+$ balance between root and shoot. Upon $K^+$ depletion in the soil, it is mandatory for the root to sustain a minimal concentration of $K^+$ in order to maintain the required membrane potential and osmotic balance. In contrast, if sufficient $K^+$ were available for growth of the plants, the stem and young leaves represent a major sink that requires large amount of $K^+$. Under this condition, more $K^+$ will be transported toward shoots to support plant growth. Therefore, root-to-shoot $K^+$ transport needs to be fine tuned according to external $K^+$ levels.

In the present study, we show that the modulation of root-to-shoot $K^+$ translocation was controlled by $NPF7.3$ via transcriptional regulation. Furthermore, $NPF7.3$ transcription was regulated by $MYB59$, and $MYB59$ transcription was also modulated by some unknown components in this process. Our data indicate that both $MYB59$ and $NPF7.3$ can respond to external $K^+$ levels (Figure 9). Plants accurately control the transcript levels of $MYB59$ and $NPF7.3$ to achieve the proper $K^+$ distribution between roots and shoots. Under $K^+$ deficient conditions, plants need to represses $MYB59$ and $NPF7.3$ expression to keep enough $K^+$ in roots and maintain proper root activity. However, down regulation of $MYB59$ and $NPF7.3$ expression under low $K^+$ conditions does not mean plants do not need them. In loss-of-function mutants of $MYB59$ or $NPF7.3$, root-to-shoot $K^+$ transport (Figure 6C) was largely abolished, causing $K^+$-deficient symptoms to appear in mutant shoots much earlier than in wild-type plants (Figure 5A). Therefore, the transcript levels of Arabidopsis $MYB59$ and $NPF7.3$ need to be accurately fine-tuned in response to external $K^+$ levels. In addition, we noticed that $MYB59$ expression was upregulated in $npf7.3$ mutant plants (Figure 6D), suggesting that there is feedback regulation of $MYB59$ transcription when $NPF7.3$ function is disrupted.

$MYB59$ and $NPF7.3$ Form a Regulatory Module for Coordination of $K^+$/NO$_3^-$ Transport

Sufficient supplies of potassium and nitrogen are critical for crop yield and quality. However, current fertilization practices in some agricultural regions (e.g. in East Asia) limit the
development of agriculture and result in many problems. For example, overuse of nitrogen fertilizer in conjunction with a lack of potash fertilizer in China not only significantly reduces nitrogen utilization efficiency, but also causes air/water/soil pollution and ecosystem deterioration (Pettigrew, 2008; Guo et al., 2010; Zhang, 2017). The molecular mechanism underlying this phenomenon is the coordination of K/N utilization. Previous studies found that the dual-role transporter NPF7.3 plays essential roles in \( K^+ / NO_3^- \) co-transport in Arabidopsis (Lin et al., 2008; Li et al., 2017). A low \( K^+ \) supply could reduce \( NO_3^- \) transport in \( npf7.3 \) mutants (Figure 5D and 5F), and conversely a low \( NO_3^- \) supply also reduces \( K^+ \) transport towards shoot in \( npf7.3 \) mutant (Li et al., 2017). The transcript level of \( NPF7.3 \) is responsive to external K and N levels (Lin et al., 2008; Li et al., 2017), suggesting that \( NPF7.3 \) is part of an important mechanism for maintaining K/N balance. In the present study, we identified a transcription factor that positively regulates \( NPF7.3 \) expression to balance \( K^+ / NO_3^- \) transport (Figure 11). Under \( K^+ / NO_3^- \) sufficient conditions, MYB59 binds to the \( NPF7.3 \) promoter and facilitates \( NPF7.3 \)-mediated root-to-shoot \( K^+ / NO_3^- \) transport. When plants are subjected to \( K^+ / NO_3^- \) deficient stresses, \( MYB59 \) is downregulated, which subsequently impairs the accumulation of the \( NPF7.3 \) transcript. Therefore, root-to-shoot \( K^+ / NO_3^- \) transport is restricted in order to balance the \( K^+ / NO_3^- \) distribution between root and shoot. In conclusion, the present study establishes a central role for transcriptional regulation as a mechanism of \( K^+ / NO_3^- \) coordination in plants response to fluctuating nutrient environments.

**METHODS**

**Plant Materials**

The Arabidopsis T-DNA insertion lines (Columbia ecotype, Col-0), including \( myb59-1 \) (GABI_627C09), \( npf7.3 \) (SALK_043036), and \( hak5 \) (SALK_005604) were obtained from NASC or ABRC. The \( lks2 \) and \( skor \) mutants were obtained as described previously (Li et al., 2017).

To produce the Crisp/Cas9 construct, the vector pCBC-DT1T2 (Xing et al., 2014) was used as the template and a DT1-BsF/DT1-F0/DT2-R0/DT2-BsR four-primer mixture (as mentioned in
Supplemental Table 2) was used with DT1-F0/DT2-R0 diluted 20 fold with DT1-BsF or DT2-BsR, resulting in DT1T2-PCR. To clone the DT1T2-PCR product into the pHSE401 vector (Xing et al., 2014), the restriction/ligation reaction was performed in a total volume of 15 μL, which contained 2 μL DT1T2-PCR product, 2 μL pHSE401 vector, 1.5 μL 10×NEB T4 Buffer, 1.5 μL 10×BSA, 1 μL BsaI (NEB), 1 μL T4 Ligase (NEB), 6 μL ddH2O. The reaction was performed at 37°C for 5 h, 50°C for 5 min, and 80°C for 10 min, resulting the final Crispr/Cas9 construct. To generate the myb59-2 mutant, the Crispr/Cas9 construct for MYB59 was transformed into wild-type (Col-0) plants. The Crispr/Cas9 construct for MYB48 was transformed into wild-type (Col-0) plants or the myb59-1 mutant respectively, generating myb48 mutants and myb59 myb48 double mutants. Genomic DNA from plants in the T1 generation was sequenced for construct verification.

The MYB59 genomic sequence was cloned into the pCAMBIA1300 vector (Cambia) driven by its native promoter, and this construct was transformed into the myb59-1 mutant to obtain two complementation lines (COM1 and COM2). Sequences encoding each of the three MYB59 transcripts driven by the native promoter were cloned separately into the pCAMBIA1305 vector (Cambia) and then transformed into the myb59-1 mutant to obtain complementation lines corresponding to each transcript (MYB59.1 COM, MYB59.2 COM, MYB59.3 COM). The NPF7.3 genomic sequence was cloned into the pCAMBIA1300 vector driven by PHO1 promoter, and was transformed into myb59-1 mutant and npf7.3 mutant respectively to generate myb59-1/ProPHO1:NPF7.3 and npf7.3/ProPHO1:NPF7.3 plants. MYB59 and NPF7.3 promoter fragments were cloned into the pCAMBIA1381 vector (Cambia) and were transformed into wild-type plants to generate ProMYB59:GUS or ProNPF7.3:GUS plants, respectively. The myb59-1/ProSuper:MYB59.3-FLAG lines were generated by cloning the coding sequence of MYB59.3 into the SUPER1300 vector (Cambia) and transforming into myb59-1 mutant. Homozygous T4 transgenic plants were used.
Phenotypic Analyses and Growth Conditions

The K⁺-sufficient medium and low-K⁺ medium for phenotypic analyses were modified from MS (Murashige & Skoog) medium as follows: 1.5 mM MgSO₄ was unchanged, 1.25 mM KH₂PO₄ and 2.99 mM CaCl₂ were replaced by 1.25 mM NH₄H₂PO₄ and 2.99 mM Ca(NO₃)₂, 20.6 mM NH₄NO₃ and 18.79 mM KNO₃ were removed. The actual and final K⁺ concentration in the K⁺ sufficient medium and low-K⁺ medium were adjusted to 5 mM and 100 µM respectively by adding KCl. For the phenotypic analyses using different concentrations of K⁺, media were also supplemented with KCl.

For NO₃⁻ treatments, media were modified from K⁺-sufficient medium as follows: 1.5 mM MgSO₄ and 1.25 mM NH₄H₂PO₄ were unchanged, 2.99 mM Ca(NO₃)₂ was replaced by 2.99 mM CaCl₂ and then either 5 mM KNO₃ or 5 mM KCl was added respectively, representing NO₃⁻-sufficient medium or NO₃⁻-deficient medium.

All the media used in the study contained 0.9% (w/v) agar and 3% (w/v) sucrose. Seeds were surface sterilized using 6% (v/v) NaClO and germinated on K⁺ sufficient medium or low-K⁺ medium at 22°C under constant illumination (60 µmol m⁻² s⁻¹) for 10 d. For phenotypic assays, four plates were considered four biological replicates in one independent experiment. Each plate contained 7-10 seedlings for each plant material. In one phenotype test, at least three independent experiments were performed. A photograph of one plate is shown in a figure to represent one phenotypic test.

For seed harvest, Arabidopsis plants were grown in a potting soil mixture (rich soil(vermiculite = 2:1, v/v) and kept in a growth chamber (22°C, illumination at 120 µmol m⁻² s⁻¹ for a 16 h daily light period; the relative humidity was approximately 70%).

Measurement of K⁺ and NO₃⁻ Content in Plant Tissues

Seedlings were germinated and grown on K⁺ sufficient medium or low K⁺ medium for 10 d, and then shoots and roots were harvested separately. The plant samples were washed thoroughly three times with double-distilled water.
For K⁺ content analyses, samples were dried at 80°C for 24 h to a constant weight and the dry weight was measured. Samples were treated in a muffle furnace at 300°C for 1 h and at 575°C for 5 h and dissolved in 0.1 N HCl. The K⁺ concentrations were measured using the 4100-MP AES system (Agilent). Three biological replicates were used in one independent experiment. For low K⁺ treatment, about 90-100 individual seedlings from two plates were collected and used as one biological replicate. For K⁺ sufficient treatment, about 40-45 individual seedlings from one plate were collected and used as one biological replicate. Three independent experiments were performed in one measurement analysis.

For NO₃⁻ content analyses, the fresh weight of plant shoots and roots was measured. Plant tissues were boiled in double-distilled water for 20 min and then frozen at -80°C. The NO₃⁻ concentrations of plant samples were measured using HPLC. Three biological replicates were used in one independent experiment. For low K⁺ treatment, about 80-100 individual seedlings from two plates were collected and used as one biological replicate. For K⁺ sufficient treatment, about 20-25 individual seedlings from one plate were collected and used as one biological replicate. Three independent experiments were performed in one measurement analysis.

**Measurement of K⁺ and NO₃⁻ Content in Xylem Sap**

*Arabidopsis* seeds were germinated on K⁺-sufficient medium for 10 d and then transferred into vermiculite. Plants were irrigated with 1/4-strength nutrient solution containing 5 mM K⁺ (K⁺-sufficient) or 100 μM K⁺ (K⁺-deficient). The nutrient solution was renewed every week. After growing in a growth chamber for 20 d, the plant shoots were excised and xylem sap was collected using capillaries within 2 h. Three biological replicates were used in one independent experiment. For each replicate, the xylem sap was collected from 20-24 individual plants. The total volume for each replicate was approximately 40-60 μL. The K⁺ and NO₃⁻ concentrations in xylem sap were measured using the 4100-MP AES system or HPLC.
Transcription Analyses

For both RT-PCR and RT-qPCR analyses, total RNA was extracted from roots of 8-d-old seedlings by using the Trizol reagent (Invitrogen), and then treated with DNase I (RNase Free, Takara) to eliminate genomic DNA contamination. The cDNA was synthesized by SuperScript\textsuperscript{II} RNase reverse transcriptase (Invitrogen). Oligo (dT) primers (Promega) and Random Hexamer primers (Promega) were used for RT-PCR and RT-qPCR analyses, respectively.

For RT-PCR, about 80-100 individual seedlings from two plates were collected and used as one sample. Three independent experiments were performed. The $E F 1 \alpha$ gene was used as an internal standard for normalization of gene expression levels. Two pairs of specifically designed primers were used to amplify $M Y B 5 9$ transcripts of different sizes: $M Y B 5 9$ \_fw2 and $M Y B 5 9$ \_rev2 as described (Li et al., 2006), $M Y B 5 9$ \_fw3 and $M Y B 5 9$ \_rev3 (see Supplemental 2 for all primer sequences). The primers $M Y B 5 9$ \_fw2 and $M Y B 5 9$ \_rev2 were used to amplify $M Y B 5 9 . 1$ (expected size 643 bp). PCR was performed for 30 cycles, each at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s. The primers $M Y B 5 9$ \_fw3 and $M Y B 5 9$ \_rev3 were used to amplify $M Y B 5 9 . 2$ (expected size 336 bp) and $M Y B 5 9 . 3$ (expected size 304 bp). PCR was performed for 35 cycles, each with 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s.

RT-qPCR was conducted using a Power SYBR Green PCR Master Mix (Applied Biosystems, USA) on a 7500 Real Time PCR System machine (Applied Biosystems). The amplification reactions were performed in a total volume of 20 μL, which contained 10 μL SYBR Green premix, 7 μL ddH$_2$O, 2 μL forward and reverse primers (1 μM), and 1 μL cDNA. PCR was conducted as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For NO$_3^-$ treatment experiments, $E I F 4 a$ was used as an internal standard for normalization of gene expression levels. For other experiments, RT-qPCR results were calculated by normalization to Actin2/8. Three biological replicates were used in one independent RT-qPCR experiment. Each replicate contained 80-100 individual seedlings from two plates. Three independent experiments were performed in one RT-qPCR analysis.
Transcriptional Activation Assays in Yeast

cDNAs encoding three of the MYB59 transcripts were separately cloned into the pGBKT7/BD vector (Clontech) containing the GAL4 DNA-binding domain. Either pBD-MYB59.1, pBD-MYB59.2, pBD-MYB59.3, the positive control pBD-GAL4, or the negative control pBD vector was transformed into the yeast strain AH109 (Clontech). The transformed strains were cultured on SD/-TRP plates and confirmed by PCR, and then were spotted on SD/-TRP or SD/-TRP-HIS plates by diluting to different concentrations. Medium lacking Trp (SD/-TRP) was used to indicate the presence of constructs in the pGBKT7/BD vector, and medium without both Trp and His (SD/-TRP-HIS) was to identify whether these clones could activate the expression of the reporter gene HIS3 (Reece-Hoyes and Walhout, 2012). The transcription activation activity of each transcript was evaluated according to the growth status of the corresponding yeast cells. The LacZ marker gene was examined by X-gal assay.

ChIP-qPCR Assays

Seedlings of myb59-1/ProSuper:MYB59.3-FLAG were germinated on K+ sufficient medium for 10 d, and then roots from 2000-2500 individual seedlings (about 0.5-1 g fresh weight) were collected as one replicate for ChIP experiments. The samples were first crosslinked using formaldehyde. Then the crosslinked nuclei were purified and sonicated to shear the chromatin into suitably sized fragments. The anti-FLAG antibody (MBL, Lot number: M185-3L) was used to immunoprecipitate protein/DNA complexes from the chromatin. 5-10 μL anti-FLAG antibody was added to positive sample of 2 mL. The DNA in the complexes was recovered and analyzed by qPCR methods. qPCR was performed for 40 cycles, each at 95°C for 15 s, 50°C for 20 s and 60°C for 45 s. Six primer combinations were used to amplify fragments within the promoter of NPF7.3. Two exon fragments in the NPF7.3 coding sequence were amplified as internal controls. The relative enrichment of each fragment was calculated by comparing samples treated with antibodies to samples treated without antibodies. Three technical replicates were used in one independent experiment. Three independent experiments were performed.
Electrophoretic Mobility Shift Assays (EMSA)

EMSA assays were performed using the LightShift Chemiluminescent EMSA kit (Pierce) following the manufacturer’s protocol. The recombinant proteins CKS-MYB59.3-His, CKS-MYB59.1-His and CKS-His were expressed in and purified from *E. coli*. Two fragments (P1 and P5) in the *NPF7.3* promoter were obtained by PCR, and either left unlabeled or labeled with 5’-biotin. The unlabeled fragments of the same sequences were used as competitors, and the CKS-His protein alone was used as the negative control.

Cell-Free Degradation Assays

Five-day-old wild-type seedlings were transferred to K⁺ sufficient medium (5 mM K⁺), low-K⁺ medium (100 μM K⁺), or low-K⁺ medium containing 10 μM MG132 for 3 d. Roots of 200-250 individual seedlings from two plates were collected and used as one replicate. The roots were harvested and ground into a fine powder in liquid nitrogen. Total proteins were extracted in degradation buffer containing 25 mM Tris-HCl, pH7.5, 10 mM MgCl₂, 10 mM NaCl, 4 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 10 mM ATP. The protein concentration was determined using the Bio-Rad protein assay. To monitor the degradation of recombinant protein CKS-MYB59.3-His, 250 ng purified recombinant protein was incubated in 20 μL of root protein extract (50 μg) at 22°C for the indicated time periods. Recombinant CKS-His was used as a control. The abundance of CKS-MYB59.3-His or CKS-His was analyzed by immunoblotting with an anti-His antibody (EASYBIO, Lot number: BE2019) used at a 1:5000 dilution.

86Rb⁺ Uptake Assay

86Rb⁺ uptake assays were performed as described previously (Zhao et al., 2016). Seven-d-old seedlings that were grown on MS medium were transferred to LK medium for 2 d. Then, the plants were incubated in 1.5 mL uptake solution (5 mM MES and 0.2 mM CaCl₂) that was K⁺-free and supplemented with 100 μM RbCl containing 0.005 μCi/nmol 86Rb⁺ (Amersham
Biosciences). At the end of the uptake period, plants were blotted with tissue paper and immediately washed two times for 30 min in 4°C washing solution (K⁺-free and supplemented with 1.75 mM nonradio-labeled RbCl). The radioactivity in plants was measured with a scintillation counter (Perkin-Elmer 1450 MicroBeta TriLux) after the addition of 1 mL scintillation liquid. Three biological replicates were used in one independent experiment. The fresh weight of 12 individual seedlings was measured as one replicate before the materials were used for ⁸⁶Rb⁺ uptake assays.

**RNA Isolation and RNA-seq**

Five-d-old seedlings were transferred from MS medium to low K⁺ medium for 2 d. Total RNA was extracted from roots using the Trizol reagent (Invitrogen). RNA degradation was monitored on 1% agarose gels, and RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Sequencing libraries were generated using the NEBNext® Ultra TM RNA Library Prep Kit for Illumina® (NEB, USA). The library preparations were sequenced on an Illumina Hiseq 4000 platform and 150-bp paired-end reads were generated. Raw sequence data were cleaned by filtering out reads containing unknown bases (more than 10% of the total bases), adapter sequences, or more than 50% of the total bases with quality values of <5.

**Accession Numbers**

Sequence data for the genes described in this article can be found in the *Arabidopsis* TAIR database ([https://www.arabidopsis.org](https://www.arabidopsis.org)) under the following accession numbers: At5g59780 for *MYB59*, At3g46130 for *MYB48*, At3g23430 for *PHO1*, At1g32450 for *NPF7.3*, At4g13420 for *HAK5*, At3g02850 for *SKOR*. Germplasm used included *myb59-1* (GABI_627C09), *npf7.3* (SALK_043036), *hak5* (SALK_005604), along with the *lks2* and *skor* mutants (Li et al., 2017).
Supplemental Data

Supplemental Figure 1. Dry Weight of Various Plant Materials.

Supplemental Figure 2. Phenotype of the MYB59.3 Overexpressing Line.

Supplemental Figure 3. Phenotype of the hak5 Mutant and 86Rb+ Uptake Test in myb59 and npf7.3 Mutants.

Supplemental Figure 4. Phenotype of the skor Mutant.

Supplemental Figure 5. Phenotypes of the Transgenic Lines Expressing NPF7.3 Driven by the PHO1 Promoter.

Supplemental Figure 6. EMSA Analysis of the Binding Activity of MYB59.1 to the NPF7.3 Promoter.

Supplemental Figure 7. Phenotypes of the myb48 Single Mutant and the myb59 myb48 Double Mutant.

Supplemental Figure 8. RT-PCR Analysis Showing the Changes of Three MYB59 Transcripts in the Arabidopsis Response to Low K+ Stress.

Supplemental Table 1. Expression Analyses of K+-Related Genes in the myb59 Mutant Using RNA-Seq Method.

Supplemental Table 2. Primer Sequences Used in this Study.

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AUTHOR CONTRIBUTIONS

W.H.W., J.L, J.K., and Y.W. designed the research. X.Q.D., F.L.W., H.L., S.J., and M.Y. conducted the experiments. X.Q.D. and Y.W. wrote and revised the article.
REFERENCES


Figure 1. The \textit{myb59} Mutant Is Sensitive to Low K\textsuperscript{+} Stress.

(A) Phenotypic comparison among wild-type plants (Col), two \textit{myb59} mutants and two complementation lines (COM1 and COM2). Seedlings were germinated and grown on K\textsuperscript{+} sufficient (5 mM) medium or low K\textsuperscript{+} (100 \mu M) medium for 10 d.

(B) Schematic representation of four spliced transcripts of \textit{MYB59}. The mutation sites are indicated by white triangles in \textit{MYB59.1}. The red triangles show primer sites for the RT-PCR analysis described below. The two MYB domain repeats (R2 and R3) are indicated with sets of arrows.

(C) RT-PCR analysis of \textit{MYB59} transcripts in various plants using the primer sites shown in (B). \textit{MYB59.1} was amplified using the upper pair of primers, \textit{MYB59.2} and \textit{MYB59.3} were amplified using the lower pair of primers. The \textit{EF1\alpha} gene was used as an internal standard for normalization of gene expression levels.

(D) Partial alignment of the \textit{myb59-2} mutant sequence with the wild-type sequence.

(E) RT-qPCR analysis of \textit{MYB59} expression in various plants. Data are shown as means ± SE (n=3). Student’s $t$ test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance, and “#” represents control.
Figure 2. The MYB59.3 Is Able to Rescue the Low K⁺ Sensitive Phenotype of myb59.

(A) Transactivation resulting from the expression of three transcripts of MYB59 in yeast as described in Methods.

(B) Effect of K⁺ concentration on growth phenotype. Wild-type plants, myb59 mutant plants and the complementation lines with three different MYB59 transcripts (MYB59.1 COM, MYB59.2 COM, and MYB59.3 COM) were grown in either 5 mM or 100 μM K⁺ for 10 d.

(C) RT-PCR analysis of three MYB59 transcripts in various plants.
Figure 3. The *myb59* Mutant Is Defective in Root-to-Shoot Transport of K⁺ and NO₃⁻.

(A) and (D) K⁺ and NO₃⁻ content measurement in various plant lines and organs after being germinated and grown on K⁺ sufficient (5 mM) medium or low K⁺ (100 μM) medium for 10 d.

(B) and (E) The root/shoot ratios of K⁺ and NO₃⁻ per plant in various plant lines and organs.

(C) and (F) K⁺ and NO₃⁻ concentrations in xylem exudates from *myb59* mutants and complementation lines after being cultured in solutions containing different K⁺ concentrations (5 mM and 100 μM) for 20 d. Data in this figure are shown as means ± SE (*n* = 3). Student’s *t* test (*P* < 0.05 and **P** < 0.01) was used to analyze statistical significance, and “#” represents control.
Figure 4. MYB59 Positively Regulates NPF7.3 Expression.

(A) GUS staining of ProMYB59:GUS and ProNPF7.3:GUS transgenic plants. Bars in black = 1 mm, and bars in red = 100 μm.

(B) GUS staining showing expression of transcript NPF7.3 in myb59 mutant and wild-type plants. Bars = 1 mm.

(C) RT-qPCR analyses of MYB59 and NPF7.3 expression in myb59 mutants and complementation lines. Seedlings were germinated and grown on K⁺ sufficient (5 mM) medium or low K⁺ (100 μM) medium for 8 d, and then roots were collected and used for RT-qPCR assays. Data are shown as means ± SE (n=3). Student’s t test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance, and “#” represents control.

(D) GFP fluorescence observation showing the expression of NPF7.3 in myb59 mutant and wild-type plants. Bars = 100 μm.
Figure 5. The myb59 and npf7.3 Show Similar Low K⁺ Sensitive Phenotype.

(A) Effect of K⁺ concentration on growth of myb59, npf7.3, and lks2 mutants. The lks2 is a single-nucleotide mutant of NPF7.3.

(B) Phenotypic analysis of myb59 and npf7.3 mutant plants after growth on medium containing different K⁺ concentrations for 10 d.

(C) and (D) K⁺ and NO₃⁻ content measurement in various plant lines and organs.

(E) and (F) The root/shoot ratios of K⁺ and NO₃⁻ per plant in various plant materials. Data are shown as means ± SE (n = 3). Student’s t test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance, and “#” represents control.
Figure 6. MYB59 and NPF7.3 Function in Same Pathway in Response to Low K⁺ Stress.

(A) Effect of K⁺ concentration on growth of single and double mutants of myb59 and npf7.3.

(B) K⁺ content measurement in various plant materials.

(C) K⁺ concentrations in xylem exudates from various plants after being cultured in solutions containing different K⁺ concentrations (5 mM and 100 µM) for 20 d. Data are shown as means ± SE (n = 3). Student’s t test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance, and “#” represents control.

(D) RT-qPCR verification of MYB59 and NPF7.3 expression in various plants as indicated.
Figure 7. Genetic Complementation of myb59 Mutant by Expressing NPF7.3 under Control of the PHO1 Promoter.

(A) RT-qPCR verification of MYB59 and NPF7.3 expression in various plants as indicated.

(B) Phenotype comparison among wild-type plants, myb59 mutant, myb59/ProPHO1: NPF7.3, and npf7.3/ProPHO1: NPF7.3 transgenic plants.

(C) K⁺ content measurement in various plant materials. Data in (A) and (C) are shown as means ± SE (n = 3). Student’s t test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance, and “#” represents control.
Figure 8. MYB59 Directly Binds to the *NPF7.3* Promoter.

(A) Diagram of the promoter and gene regions of *NPF7.3*. P1 to P6 together with Exon1 and Exon2 are the fragments used in ChIP-qPCR assay. Triangles with different color represent *cis*-elements. Blue triangles represent MRE: AACCaaa, and red triangles indicate ERE: ATTTCAAA.

(B) ChIP-qPCR analysis of MYB59 DNA-binding activity to the *NPF7.3* promoter. Chromatin was isolated from 10-d-old seedlings of *myb59/ProSuper:MYB59.3-FLAG* grown on K⁺-sufficient medium, and immunoprecipitated with FLAG antibody. Then the six fragments were tested using qPCR. Data are shown as means ± SE (*n*=3).

(C) EMSA to analyze the binding activity of MYB59.3 to P1 and P5 fragments in the *NPF7.3* promoter. An excess of unlabeled probe was added to compete with labeled probe.
Figure 9. MYB59 and NPF7.3 Transcription Respond to External K+/NO3– Levels.

RT-qPCR analyses of MYB59 and NPF7.3 expression levels after control or low K+/NO3– treatment. Wild-type (Col) plants germinated on K+/NO3– sufficient (5 mM) medium were transferred to K+/NO3– sufficient (5 mM, +K/+NO3–) medium, or low K+ (100 μM, -K) medium (A), or low NO3– (0 μM, -NO3–) medium (B) for 2 d, respectively. Then the plants were transferred back to K+ or NO3– sufficient (5 mM) medium. Roots were collected at different time points as indicated for RT-qPCR assay. Data are shown as means ± SE (n=3).
Figure 10. MYB59.3 Protein Is Degraded after Low K⁺ Stress.

Cell-free degradation assay for MYB59.3 protein. MYB59.3 protein (CKS-MYB59.3-His) was expressed in *E. coli*. Then the purified protein was incubated with plant total proteins that were extracted from HK (5 mM) or LK (100 μM) treated wild-type plants. The proteasome inhibitor MG132 (10 μM) was added to reduce the degradation of ubiquitin-conjugated proteins. The abundance of MYB59.3 protein was determined by immunoblotting with anti-His antibody. Recombinant CKS-His was used as control.
Figure 11. Working Model of *NPF7.3* Transcriptional Regulation by MYB59 in *Arabidopsis* Response to Low K+/NO₃⁻ Stresses.

Under K+/NO₃⁻ sufficient conditions, MYB59 directly binds to the *NPF7.3* promoter and facilitates *NPF7.3*-mediated root-to-shoot K+/NO₃⁻ transport. When plants are subjected to K+/NO₃⁻ deficient stresses, *MYB59* is down-regulated and subsequently inhibits the transcript level of *NPF7.3*. Therefore, the root-to-shoot K+/NO₃⁻ transport is restricted.
The Transcription Factor MYB59 Regulates K+/NO3- Translocation in the Arabidopsis Response to Low K+ Stress
Xin-Qiao Du, Feng-Liu Wang, Hong Li, Si Jing, Miao Yu, Jigang Li, Wei-Hua Wu, Joerg Kudla and Yi Wang

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