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https://escholarship.org/uc/item/9q9519b3

Wang, Chao
Zhang, Wenjing
Li, Zehui
et al.

2018

10.3389/fpls.2018.00593

Peer reviewed
FIP1 Plays an Important Role in Nitrate Signaling and Regulates CIPK8 and CIPK23 Expression in Arabidopsis

Chao Wang1,2, Wenjing Zhang1, Zehui Li1, Zhen Li1, Yingjun Bi1, Nigel M. Crawford3 and Yong Wang1*

1 National Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Tai’an, China,
2 Section of General Biology, Department of Life Science and Engineering, Jining University, Jining, China, 3 Section of Cell and Developmental Biology, Division of Biological Science, University of California at San Diego, La Jolla, CA, United States

Unraveling the molecular mechanisms of nitrate regulation and deciphering the underlying genetic network is vital for elucidating nitrate uptake and utilization in plants. Such knowledge could lead to the improvement of nitrogen-use efficiency in agriculture. Here, we report that the FIP1 gene (factor interacting with poly(A) polymerase 1) plays an important role in nitrate signaling in Arabidopsis thaliana. FIP1 encodes a putative core component of the polyadenylation factor complex. We found that FIP1 interacts with the cleavage and polyadenylation specificity factor 30-L (CPSF30-L), which is also an essential player in nitrate signaling. The induction of nitrate-responsive genes following nitrate treatment was inhibited in the fip1 mutant. The nitrate content was also reduced in fip1 seedlings due to their decreased nitrate uptake activity. Furthermore, the nitrate content was higher in the roots but lower in the roots of fip1, which may result from the downregulation of NRT1.8 and the upregulation of the nitrate assimilation genes. In addition, qPCR analyses revealed that FIP1 negatively regulated the expression of CIPK8 and CIPK23, two protein kinases involved in nitrate signaling. In the fip1 mutant, the increased expression of CIPK23 may affect nitrate uptake, resulting in its lower nitrate content. Genetic and molecular evidence suggests that FIP1 and CPSF30-L function in the same nitrate-signaling pathway, with FIP1 mediating signaling through its interaction with CPSF30-L and its regulation of CIPK8 and CIPK23. Analysis of the 3′-UTR of NRT1.1 showed that the pattern of polyadenylation sites was altered in the fip1 mutant. These findings add a novel component to the nitrate regulation network and enhance our understanding of the underlying mechanisms for nitrate signaling.

Keywords: Arabidopsis, FIP1, CPSF30-L, nitrate signaling, nitrate uptake and assimilation, CIPK8, CIPK23

Abbreviations: APA, alternative polyadenylation; CPSF, cleavage and polyadenylation specificity factor; GUS, β-glucuronidase; N, nitrogen; NiR, nitrite reductase; NR, nitrate reductase; NUE, nitrogen use efficiency; PNR, primary nitrate response; YFP, yellow fluorescent protein.
INTRODUCTION

Nitrogen is an essential macronutrient, and its availability in soil is a major limiting factor for plant growth and development. N fertilizers are routinely used to increase agricultural productivity; however, the low NUE of many crops means that a large portion of the N application cannot be absorbed by plants and is lost to the environment, leading to various environmental and ecological problems, such as eutrophication and soil acidification, as well as increased economic costs for farmers (Canfield et al., 2009; Konishi and Yanagisawa, 2013; Marchive et al., 2013). 

The CIPK23-CBL9 protein triggers nitrate responses, regulating the expression of only nitrate sensor to have been identified thus far, NRT1.1 (Guo et al., 2014, 2017; Xu et al., 2016; Li et al., 2017). The NIA2 and NRT1.1 play crucial roles in regulating nitrate-responsive genes such as ANRI, NPR1, NRT1.1 (NITRATE TRANSPORTER 1.1), also called NPF6.3 and CHL1), AFB3-miR393, NLP7, TCP20, HRS1, and HHO1 (Zhang and Forde, 1998; Castaings et al., 2009; Ho et al., 2009; Wang et al., 2009; Vidal et al., 2010, 2013; Guan et al., 2014, 2017; Medici et al., 2015). Oligopeptide signals have also been shown to mediate N-dependent root architecture via the CLE-CLVATA1 module (Araya et al., 2014) and via leucine-rich repeat receptor kinases (LRR-RKs) (Tabata et al., 2014).

In the short term, nitrate induces the PNR in roots and shoots, during which the expression of more than 1000 genes can be rapidly altered (Wang et al., 2003, 2007; Scheible et al., 2004; Krouk et al., 2010a; Alvarez et al., 2012). Important nitrate regulatory genes have been identified over the last decade that play crucial roles in regulating nitrate-responsive genes such as NRT1.1, NRT2.1, NRT2.2, NITRATE REDUCTASE 1 (NIA1), NIA2, and NR (Castaings et al., 2009; Alvarez et al., 2014; Guan et al., 2014, 2017; Xu et al., 2016; Li et al., 2017). The only nitrate sensor to have been identified thus far, NRT1.1, triggers nitrate responses, regulating the expression of CIPK8 as a positive factor and CIPK23 as a negative factor during PNR (Ho et al., 2009; Hu et al., 2009). The CIPK23-CBL9 protein complex has been implicated in the switch between the dual affinities of NRT1.1, through the phosphorylation of a threonine residue (Thr101) (Ho et al., 2009). NLP6 and NLP7 act as key activators of nitrate assimilatory genes (Castaings et al., 2009; Konishi and Yanagisawa, 2013; Marchive et al., 2013). 

Furthermore, TGA1 can interact with CIPK23, suggesting that phosphorylation may be important for TGA1 activation (Yazaki et al., 2016). Recently, a 65-kDa subunit of the CPSF CPSF30-L was found to function upstream of NRT1.1 in nitrate signaling, where it affects nitrate uptake and assimilation (Li et al., 2017); however, the structure of the regulatory network modules and the underlying molecular mechanisms remain uncharacterized. CPSF30 has two spliced forms, a larger one (CPSF30-L) and a smaller one (CPSF30-S) (Delaney et al., 2006). CPSF30-S interacts with FIP1 (factor interacting with poly(A) polymerase 1), an important regulator of the nuclease activity of CPSF30 (Addepalli and Hunt, 2007); however, whether CPSF30-L can interact with FIP1 has yet to be reported. Here, we demonstrate that FIP1 interacts with CPSF30-L and plays an important role in nitrate signaling. Our results show that FIP1 also modulates the nitrate content in plants by regulating their nitrate transport, allocation, and assimilation. Moreover, FIP1 negatively regulates the expression of CIPK8 and CIPK23. Molecular and genetic analyses revealed that FIP1 and CPSF30-L function in the same nitrate signaling pathway.

MATERIALS AND METHODS

Plant Materials

Arabidopsis thaliana (Columbia-0 ecotype) and homozygous transgenic seeds containing the NRP-YFP construct (SS204-9) (Wang et al., 2009) were used as the wild types (WTs). The mutant lines chl1-13 (original name: Mut21; containing a NRT-YFP construct) (Wang et al., 2009), cipk8-1 (Hu et al., 2009), cipk23-3 (Ho et al., 2009), nrg2-3 and nlp7-4 (Xu et al., 2016), and cpsf30 (original name: Mut65; containing a NRT-YFP construct) (Li et al., 2017) were described previously. The fip1 mutant (Salk_087117), containing a T-DNA insertion in the sixth exon of FIP1, was obtained from ABRC and used for further analysis (Alonso et al., 2003). The construct p35S:FIP1 was used as a destination vector pMDC43 (Thermo Fisher Scientific) was transformed into the fip1 mutant using the Agrobacterium-mediated floral dip method (Xu et al., 2016). Homozygous transgenic lines were isolated as complementation line (FIP1/fip1) for further investigation.
Growth and Treatment Conditions
Seeds were germinated on nylon mesh floating in a 2.5-mM ammonium succinate [(NH₄)₂Suc] solution for 7 days. To detect the expression of the nitrate-responsive genes, the roots were treated with 10 mM KNO₃ or KCl for 2 h and then harvested. For fluorescence microscopy, seedlings were grown on the KNO₃ medium for 4 days before being observed using a Nikon Eclipse Ti-S microscope (Nikon, Tokyo, Japan). The fluorescence intensity was quantified using Image J (Schneider et al., 2012).

To determine expression profiles, various tissues were harvested either from plants grown for 7 days in 1/2 MS medium (pH = 5.7, 10 mM KNO₃, and 10 mM NH₄NO₃) or for 6 weeks in soil. Roots and shoots were harvested from seedlings grown in 1/2 MS solution for 7 days and used to determine their nitrate concentration, NR activity, and amino acid content. The expression levels of the genes involved in nitrate transportation and assimilation were determined in the roots and shoots of these seedlings, whereas the expression of the nitrate regulatory genes was measured in seedlings grown in 2.5 mM (NH₄)₂Suc, 10 mM KNO₃, and 10 mM NH₄NO₃ for 7 days.

qPCR Analysis
Total RNA was isolated from Arabidopsis roots and shoots using a Total RNA Miniprep Kit (CWBio, Beijing, China). cDNA synthesis was carried out using the RevertAid first-stand Synthesis System Kit (Thermo Fisher Scientific, Waltham, MA, United States). An UltraSYBR Green Mixture qPCR Kit (CWBio) was used for the qPCR reaction, following the manufacturer’s protocol. Gene expression was determined by real-time PCR using an ABI7500 Fast Real-Time PCR System (Thermo Fisher Scientific). TUB2 (At5g62690) was used as the internal reference gene.

Expression Profile Analysis
A GUS assay was performed according to Xu et al. (2016). A 381-bp genomic sequence located upstream of the FIP1 start codon was cloned into the pMDC163 destination vector (Thermo Fisher Scientific). Transgenic plants containing the Pro_FIP1::GUS construct were grown on 1/2 MS for 7 days or in soil for 6 weeks before their GUS activity was assessed.

Nitrate, NR Activity, and Amino Acid Content Assays
Plant nitrate content was determined using the salicylic acid method, as described previously (Zhao and Wang, 2017). The amino acid content and NR activity of the seedlings were tested using a Micro Amino Acid Content Assay Kit and a Micro Nitrate Reductase (NR) Assay Kit (Solarbio, Beijing, China), respectively.

Yeast Two-Hybrid Assays
Full-length cDNA fragments of CPSF30-L or CPSF30-L containing a point mutation in nucleotide 376 (G to A; mCPSF30-L) were introduced into the pGBK7T vector (Clontech Laboratories, Mountain View, CA, United States), while a full-length cDNA fragment of FIP1 a 1335-bp sequence encoding the N-terminal of FIP1 (FIP1-S1), a 2196-bp sequence encoding the C-terminal of FIP1 (FIP1-S2), were ligated into the pGADT7 vector (Clontech Laboratories). The two-hybrid interaction was performed following the instructions provided by the manufacturer (Clontech Laboratories).

GST Pull-Down Assays
Full-length cDNA of CPSF30-L or mCPSF30-L was cloned into pGEX4T-1 (GE) to produce a GST-CPSF30-L or GST-mCPSF30-L product as bait protein, respectively. FIP1 or FIP1-S1 was cloned into pET28a (Novagen) to produce His-FIP1 or His-FIP1-S1 product as prey protein, respectively. The constructs were introduced into Escherichia coli strain BL21. The preparation and immobilization of the bait protein, the preparation and capture of the prey protein, and bait-prey elution were performed using a GST protein interaction pull-down kit (Thermo). The prepared eluent was loaded into wells and electrophoresis was run in the stacking and separating gel, respectively. Following SDS-PAGE, the proteins were transferred onto blotting membrane and blocked, then adding anti-HIS mAb (Zoonbio) as primary antibody and HRP-conjugated secondary antibody (Zoonbio). The Chemiluminescent (ECL) was used to visualize protein bands as recommended by the manufacturer (Thermo).

BiFC Analysis
Transient bimolecular fluorescence complementation (BiFC) assays in Arabidopsis mesophyll protoplasts were performed as described (Xu et al., 2016). Full-length cDNA of CPSF30-L and mCPSF30-L were cloned into the Gateway compatible binary vectors pSITE-NEYFP vectors containing the N-terminal fragments of YFP (YFPN). Full-length cDNA of FIP1 and FIP1-S1 were cloned into pSITE-CEYFP containing the C-terminal fragments of YFP (YFPC), respectively. These vectors CPSF30-L-YFPN and FIP1-YFPC, CPSF30-L-YFPN and FIP1-S1-YFPC, and mCPSF30-L-YFPN and FIP1-YFPC were cotransfected into protoplast and the empty vectors YFPN and YFPC were used as negative controls. The fluorescence of transfected protoplast was observed using confocal microscope (Leica TCS SP5II).

Analysis of Polyadenylation in NRT1.1 3′-UTR
For analysis of NRT1.1 3′-UTR, the nested PCR technique was used as described (Li et al., 2017). The seedlings of WT, fip1, cpsf30, and fis1cpsf30 were grown on 10 mM KNO₃ for 7 days and total RNA were extracted, and then reverse transcription polymerase chain reaction (RT-PCR) was performed. The 3′-UTR of NRT1.1 after two rounds of PCR was analyzed by polyacrylamide gel electrophoresis (PAGE).

RESULTS
The Interaction Between FIP1 and CPSF30-L
We first tested if FIP1 can interact with CPSF30-L using a yeast two-hybrid assay. Full length FIP1 and FIP1 segments were used for the assays: segment 1 comprised the N-terminal
445 amino acid residues while segment 2 contained the C-terminal 731 amino acid residues (Figure 1A). The CPSF30-L protein and a CPSF30-L variant containing a point mutation resulting in a conversion of Gly to Arg at the 126th amino acid in the third zinc finger, mCPSF30 (Li et al., 2017), were used as bait proteins. We found that CPSF30-L and FIP1 interacted with each other; however, mCPSF30 did not interact with FIP1 (Figure 1B). Specifically, CPSF30-L

![FIGURE 1](image_url)

**FIGURE 1** | FIP1 interacts with CPSF30-L. (A) Secondary structure of FIP1 defined using SMART software. FIP1-S1, N-terminal of FIP1, comprising 445 amino acids with a Fip1 conserved domain (indicated by the gray box); FIP1-S2, C-terminal of FIP1, comprising 731 amino acids. The seven fuchsia boxes indicate regions of low complexity. (B) Yeast two-hybrid (Y2H) assay of the FIP1 and CPSF30-L interaction. CPSF30-L, a BD vector containing cDNA encoding CPSF30-L (a 65-kDa transcript of At1g30460.2); mCPSF30-L, a BD vector containing CPSF30-L cDNA with a point mutation at nucleotide 376 (G to A); FIP1, an AD vector containing FIP1 cDNA (a transcript of At5g58040); FIP1-S1, an AD vector containing cDNA comprising a 1335-bp sequence encoding the N-terminal of FIP1; FIP1-S2, an AD vector containing cDNA comprising a 2196-bp sequence encoding the C-terminal of FIP1; DDO (LT), SD medium lacking leucine and tryptophan used to verify the co-transformation of AD and BD plasmids; QDO (LTHA), SD medium lacking leucine, tryptophan, histidine, and adenine used to test for autoactivation or interaction. Positive controls (BD-pGBKT7-53 and AD-pGADT7-T) and negative controls (BD-pGBKT7-Lam and AD-pGADT7-T) are also shown. (C) GST pull-down assay to detect the in vitro interaction of FIP1 and CPSF30-L. The tagged protein GST-CPSF30-L, GST-mCPSF30-L, His-FIP1, and His-FIP1-S1 were synthesized in *Escherichia coli*. His-FIP1 (a) or His-FIP1-S1 (b) was pulled down using glutathione agarose beads with GST-CPSF30-L. (c) GST pull-down with GST-mCPSF30-L, as detected using an anti-His antibody. (D) BiFC analysis for interaction between FIP1 and CPSF30-L. CPSF30-L and mCPSF30-L were fused to N-terminal fragments of YFP, FIP1 and FIP1-S1 were fused to C-terminal fragments of YFP, respectively. Different combinations of expression vectors and negative control (indicated on the left of the panel) were transfected into *Arabidopsis* mesophyll protoplast. Presence of YFP signal indicates reconstitution of YFP through protein interaction of the tested pairs. Yellow arrows indicate the nucleus.
interacted with the N-terminus but not the C-terminus of FIP1.

To confirm the interaction between FIP1 and CPSF30-L, in vitro GST pull-down assay and in vivo test with BiFC assays in Arabidopsis mesophyll protoplast were performed. In pull-down assay, the interaction of GST tagged CPSF30-L and His-tagged FIP1 was inspected. His-FIP1 or His-FIP1-S1 was readily pulled down by glutathione agarose beads with GST-CPSF30-L, as detected using an anti-His antibody (Figure 1Ca,b). When mCPSF30-L was used, His-FIP1 could not be pulled down (Figure 1Cc). In BiFC assay, a direct interaction was observed between FIP1 and CPSF30-L as well as FIP1-S1 and CPSF30-L in the nucleus of the protoplast when CPSF30-L-YFP was coexpressed with FIP1-YFP and FIP1-S1-YFP. But

FIGURE 2 | FIP1 regulates the expression of nitrate-responsive genes. (A) Schematic map of the T-DNA insertion site in the fip1 mutant. Exons and introns are represented by black boxes and lines, respectively. The location of the T-DNA insertion in FIP1 is indicated by a triangle. (B) RT-PCR analysis of FIP1 mRNA levels in the wild type (WT) and fip1. Total RNA was isolated from 7-days-old seedlings grown on 1/2 MS. TUB2 serves as the internal control. (C) The expression of nitrate-responsive genes in the roots of the fip1 mutant and complementation line (FIP1/fip1). Seedlings were grown on medium with 2.5 mM ammonium succinate as the sole nitrogen source for 7 days, and then treated with 10 mM KNO3 or KCl as a control for 2 h. The transcripts of the nitrate-responsive genes were quantified using qPCR. Error bars represent the SD of the biological replicates (n = 4). Asterisks indicate significant differences (p < 0.05, U-test). (D) Nitrate responsiveness in 4-days-old fip1-SS seedlings grown on a medium containing KNO3, revealed using a fluorescent reporter system (NRP-YFP). (E) Quantification of root fluorescence in the WT and the fip1-SS mutant described in (D). Error bars represent the SD of the biological replicates (n = 60), asterisks indicate significant differences to WT (p < 0.05, U-test).
no interaction was found when mCPSF30-L-YFP\textsuperscript{N} and FIP1-YFP\textsuperscript{C} were coexpressed (Figure 1D). These results suggest that CPSF30-L interacts with the N-terminus of FIP1, and this interaction depends on a Gly at the 126th amino acid of FIP1.

**The *fip1* Mutant Is Defective in the PNR**

To explore the function of FIP1, we obtained a *fip1* mutant (Salk_087117), containing a T-DNA insertion in the sixth exon (Figure 2A), from ABRC. FIP1 expression was undetectable in this mutant (Figure 2B). To determine whether FIP1 is involved in the PNR, we determined the expression levels of three known nitrate-responsive genes, *NRT2.1* (encoding a high-affinity nitrate transporter), *NIA1*, and *NiR*, using qPCR. As shown in Figure 2C, the induction of these three genes by nitrate treatment was significantly decreased in the *fip1* mutant, but was restored to WT levels in the FIP1/*fip1* complementation line, indicating that FIP1 plays an important role in nitrate signaling. In addition, we previously constructed a transgenic Arabidopsis line containing a nitrate-responsive reporter (SS204-9) that exhibited strong YFP fluorescence in the roots of Arabidopsis plants in the presence of nitrate (Wang et al., 2009). We introduced the nitrate-responsive reporter into the *fip1* mutant by crossing it with SS204-9 to generate *fip-SS*. The *fip1-YFP* line had a significantly reduced fluorescence in the presence of nitrate in comparison with the WT (Figures 2D,E). These results show that FIP1 functions as an important regulatory gene in the PNR.

To test whether FIP1 expression can be induced by nitrate, the seedlings were grown for 7 days on medium containing 2.5 mM ammonium succinate as the sole N source, and then treated with 10 mM KNO\textsubscript{3}. We found that the expression of FIP1 was not significantly altered in the roots following nitrate treatment (Supplementary Figure 1), indicating that FIP1 expression is not induced by nitrate.

**FIP1 Is Mainly Expressed in the Vascular Tissues of the Leaves and Roots**

To further explore the regulation of the nitrate response by FIP1, its expression pattern was investigated using qPCR. FIP1 was found to be expressed in all tissues investigated, but was particularly highly expressed in the roots and leaves (Figure 3A). Histochemical analyses using transgenic lines harboring the GUS gene driven by the FIP1 promoter revealed that FIP1 was predominantly expressed in the vascular tissues of the roots and leaves (Figure 3B). GUS staining was also observed in the trichomes and stomata on the leaves, as well as in the flowers (Figure 3B). The expression profile of FIP1 suggests that it may function in the nitrate signaling pathway of several tissues.

**FIP1 Regulates the Uptake and Allocation of Nitrate Between the Shoot and Root**

Some nitrate regulators affect the accumulation of nitrate within the plant (Castaings et al., 2009; Wang et al., 2009; Xu et al., 2016; Li et al., 2017). To test the physiological effects of FIP1, we measured the nitrate content of plants grown on 1/2 MS medium. Nitrate accumulation was significantly decreased in the *fip1* seedlings compared with the WT (Figure 4A). This decreased nitrate content may result from defects in nitrate uptake and/or increased nitrate assimilation; therefore, we determined the remaining nitrate concentration in the solution after 7 days of seedling growth. The remaining nitrate concentration in the solution was significantly higher for *fip1* than for WT (Figure 4B). We tested the nitrate uptake of seedlings grown in the presence of 2.5 mM ammonium succinate for 7 days and then treated with 5 mM KNO\textsubscript{3} for different durations, and found that the nitrate content was reduced significantly in the *fip1* mutant at the time points tested (Figure 4C). Taken together, these results indicate that FIP1 affects nitrate uptake. To further investigate the distribution of nitrate in the plants, we quantified the accumulation of nitrate in both the shoots and roots. The nitrate content was higher in the roots but lower in the shoots of *fip1*, and this phenotype was recovered in the FIP1/*fip1* complementation line (Figures 4D,E), suggesting that the allocation of nitrate within the plant is altered in the *fip1* mutant.
mutant. Taken together, these findings show that FIP1 regulates nitrate uptake and allocation.

The decreased nitrate content in fip1 led us to investigate the expression of genes known to be involved in nitrate transport and assimilation. For this assay, steady state levels of mRNA after a week of growth on nitrate were determined by growing seedlings on 1/2 MS for 7 days then harvesting roots and shoots separately for RNA extraction. Our qPCR analysis showed that NRT1.5 expression was increased in the roots of fip1 compared with the WT, while the expression of NRT1.5 and NRT1.8 was decreased in the fip1 shoots in comparison with the WT (Figures 5A,B). These phenotypes were recovered in the FIP1/fip1 complementation line. No significant differences in expression were found for the other genes tested (Supplementary Table 1).

NRT1.8 functions in nitrate unloading from the xylem to the shoots (Li et al., 2010). Therefore, the decreased expression of NRT1.8 in the shoots may result in the lower nitrate content in the shoots but higher in the roots of fip1.

We also determined the expression levels of the nitrate assimilation-related genes. The expression levels of NIA1 and NiR were much higher in fip1 than in the WT (Figure 5C), while in the roots only the expression of NiR was higher in fip1 (Figure 5D and Supplementary Table 2). Furthermore, we detected the NR activity and amino acid content of the plants. The NR activity in the shoots of fip1 was higher than that of the WT (Figure 5E), while the amino acid content was lower in the roots and higher in the shoots of fip1 compared with the WT (Figure 5F). These results demonstrate that FIP1 also affects the expression of the genes involved in nitrate assimilation, which
may be another reason for the lower nitrate content in the shoots of *fip1*.

**FIP1 Regulates the Expression of CIPK8 and CIPK23 in the Presence of Nitrate and Functions in the Same Nitrate-Signaling Pathway as CPSF30**

To further explore the relationship between FIP1 and the previously characterized nitrate regulatory genes, we grew nitrate regulation mutant plants, lacking *CPSF30, NRT1.1, NLP7, CIPK8, CIPK23,* or *NRG2,* for 7 days under three N sources (2.5 mM ammonium succinate, 10 mM KNO$_3$, or 10 mM NH$_4$NO$_3$), and then quantified their expression of FIP1. There was no significant difference in the expression of FIP1 between the WT and each mutant under the different N sources (Supplementary Figure 2), indicating that the above nitrate regulatory genes do not regulate its expression. We also detected the expression of these genes in *fip1*, revealing that the expression of CIPK8 and CIPK23 in *fip1* was significantly increased in comparison with the WT in the presence of nitrate, and that these changes were recovered in the FIP1/*fip1* complementation line (Figures 6A,B). No significant differences were found for the other genes tested (Supplementary Table 3). CIPK8 and CIPK23 are important regulatory genes involved in nitrate signaling (Ho et al., 2009; Hu et al., 2009); therefore, our results suggest that FIP1 may play an essential role in the PNR by regulating the expression of the nitrate regulatory genes CIPK8 and CIPK23.

The expression of CIPK8 and CIPK23 is also known to be controlled by NRT1.1 (Ho et al., 2009; Hu et al., 2009). As
FIGURE 6 | FIP1 regulates the expression of CIPK8 and CIPK23 and affects seedling nitrate content in the presence of nitrate. RNA was extracted from seedlings grown for 7 days with various sources of nitrogen [(NH₄)₂ Suc, KNO₃, or NH₄NO₃ media]. The expression levels of CIPK8 (A) and CIPK23 (B) in the fip1 mutant, determined using qPCR. The expression levels of CIPK8 (C) and CIPK23 (D) in the cpsf30 mutant, determined using qPCR. Error bars represent the SD of the biological replicates (n = 4). (E) Nitrate content in the CIPK23 overexpression line (CIPK23-OE) grown in 1/2 MS medium for 7 days. Error bars represent the SD of the biological replicates (n = 5). Asterisks and different letters indicate significant differences to the WT (p < 0.05, U-test).

CPSF30-L regulates the expression of NRT1.1, we investigated whether CPSF30-L affected the expression of CIPK8 and CIPK23. We found that CIPK8 and CIPK23 expression was significantly higher in the cpsf30 mutant than in the WT in the presence of nitrate, a phenotype that was recovered in the CPSF30-L/cpsf30 complementation line (Figures 6C,D). These results demonstrate that, like FIP1, CPSF30-L can modulate the expression of CIPK8 and CIPK23.

CIPK23 was previously reported to phosphorylate Thr101 of NRT1.1, thereby facilitating a shift to its high-affinity status (Ho et al., 2009). In theory, the increased expression of CIPK23 may strengthen the phosphorylation of NRT1.1, enhancing its high-affinity nitrate transport activity and reducing nitrate uptake in the presence of sufficient nitrate. We therefore quantified the nitrate content of the CIPK23 overexpression line (CIPK23-OE). The nitrate content of this line was reduced, whereas no
A significant difference was observed between the nitrate contents of *cipk23* and the WT (Figure 6E). These results suggest that the increased expression of *CIPK23* in *fip1* may enhance the phosphorylation of NRT1.1, resulting in the lower nitrate accumulation of this mutant.

To further investigate the relationship between *FIP1* and *CPSF30-L*, we constructed a double mutant (*fip1cpsf30*) by crossing *fip1-SS* and *cpsf30*. RT-PCR results showed that the expression of *FIP1* was undetectable and sequencing results exhibited that the point mutation of the *cpsf30* mutant was present in the double mutant (Supplementary Figure 3). The fluorescence intensity, indicating nitrate responsiveness, was significantly lower in *fip1* than in the WT but higher than in *cpsf30*, while no significant difference was found between *fip1* and the *fip1cpsf30* double mutant (Figures 7A,B), suggesting that *FIP1* and *CPSF30-L* may regulate the nitrate response via the same pathway. The expression levels of the nitrate-responsive genes *NIA1*, *NiR*, and *NRT2.1* were found to be higher in the *fip1cpsf30* double mutant than in *cpsf30*, while no significant difference in expression was observed between the *fip1cpsf30* double mutant and the *fip1* single mutant (Figure 7C). This further confirms that *FIP1* and *CPSF30-L* function in the same nitrate-signaling pathway. The similar phenotype of *fip1* mutant and the double mutant may be explained by the possibility that...
FIP1 and CPSF30-L may be part of a larger complex. Loss of FIP1 can result in a change in the complex that reduces the complex's activity by a certain amount. Loss of CPSF30-L may result in a larger change that reduces activity more than loss of FIP1. Loss of both genes results in a change that mimics loss of FIP1 alone. This would happen if CPSF30-L binding requires FIP1 binding. Loss of FIP1 prevents CPSF30-L binding so that the phenotype of double mutant looks like that of the fip1 single mutant.

Previous study has shown that CPSF30-L can affect APA of 3′-UTR in NRT1.1 mRNA (Li et al., 2017). Since FIP1 and CPSF30 function in the same nitrate signaling pathway, it's possible that FIP1 also affects the APA of the NRT1.1. Therefore, 3′-UTR of NRT1.1 was amplified by nested PCR from the WT, fip1, cpsf30, and fip1cpsf30. The seedlings were grown on 10 mM KNO3 for 7 days and total RNA was extracted for RT-PCR.

![FIGURE 8](https://example.com/figure8.png)

**FIGURE 8** | FIP1 can affect the APA in 3′-UTR of NRT1.1 mRNA. Polyacrylamide gel electrophoresis (PAGE) was run to separate the amplified bands of different 3′-UTR polyadenylated forms of NRT1.1 in WT, fip1, cpsf30, and fip1cpsf30. The seedlings were grown on 10 mM KNO3 for 7 days and total RNA was extracted for RT-PCR.

**DISCUSSION**

In Arabidopsis, CPSF30 has two spliced forms, a smaller one (CPSF30-S) and a larger one (CPSF30-L) (Delaney et al., 2006). CPSF30-S plays a role in a number of distinct developmental processes and physiological responses, partly resulting from a global shift in the poly(A) site choice of numerous responding genes (Zhang et al., 2008; Bruggeman et al., 2014; Liu et al., 2014); however, the function of CPSF30-L was largely unknown (Delaney et al., 2006). Recently, we reported that a nitrate regulatory mutant, Mut65, lacked CPSF30 function (Li et al., 2017). Further investigation demonstrated that CPSF30-L and not CPSF30-S was responsible for the nitrate signaling (Li et al., 2017). It is known that CPSF30-S can interact with FIP1, a putative core component of a pre-mRNA processing complex (Addepalli and Hunt, 2007) and that this interaction regulates the nuclease activity of CPSF30. However, whether CPSF30-L could interact with FIP1 and whether FIP1 was involved in nitrate signaling were not known. The data we present here indicate that the N-terminus of FIP1 does indeed interact with CPSF30-L (Figure 1) and plays an important role in nitrate signaling.

To determine whether FIP1 participates in nitrate signaling, we quantified the expression of the nitrate-responsive genes in fip1 and found that this mutant was defective in the PNR (Figure 2). Our histochemical assay and qPCR analysis showed that FIP1 was mainly expressed in the stelar region of the primary root, basal lateral root, vascular tissue of the leaf, and stomata, and was not induced by nitrate (Figure 3 and Supplementary Figure 1). The expression profile of FIP1 was coincident with that of CPSF30 (Li et al., 2017). The common expression sites of both genes further support the suggestion that FIP1 interacts with CPSF30-L.

CPSF30-L is known to regulate nitrate uptake and the expression of the nitrate transporter gene, NRT1.1 (Li et al., 2017). Here, we demonstrated that the nitrate content was lower in the fip1 mutant seedlings because of its defective nitrate uptake (Figures 4A–C); however, the expression levels of the known genes involved in nitrate uptake, such as NRT1.1 and NRT1.2, were not affected in fip1 (Supplementary Table 1). Furthermore, the expression of CIPK23 was increased in fip1 (Figure 6B). As CIPK23 can phosphorylate NRT1.1 to switch its activity to have a high affinity for nitrate, the increased expression of CIPK23 may upregulate the phosphorylation and nitrate transport activity of NRT1.1, reducing nitrate uptake under conditions with sufficient nitrate. Indeed, the nitrate concentration in CIPK23-OE was decreased (Figure 6E), suggesting that the increased expression of CIPK23 might result in the lower nitrate content observed in the fip1 mutant.

In addition, FIP1 affects the distribution of nitrate between the roots and shoots. The fip1 mutant accumulated more nitrate than WT in the roots, but less in the shoots (Figures 4D,E), in contrast to the cpsf30 mutant, which accumulates less nitrate than the WT in both its roots and shoots (Li et al., 2017). The lower nitrate content in the shoots and higher in the roots of the fip1 mutant may be due to the following possibilities: (1) the lower expression of NRT1.8 in the shoots may decrease nitrate...
unloading from the xylem to the shoot tissues (Figure 5B); (2) the increased expression of the nitrate assimilation genes may cause more nitrate to be reduced in the shoots (Figure 5D). The NR activity and amino acid contents in the fip1 mutant were indeed higher than in the WT (Figures 5E,F). In the cpsf30 mutant, the decreased expression of NRT1.8 and the increased expression of the nitrate assimilation genes in the shoots also led to a lower nitrate content in the shoots (Li et al., 2017). These results indicate that FIP1 plays an important role in regulating nitrate uptake, transport, and assimilation.

We investigated the relationships between FIP1 and the nitrate regulatory genes, revealing that FIP1 functions as a negative regulator to modulate the expression of CIPK8 and CIPK23 in the PNR, similar to the role of CPSF30-L (Figure 6). It has been reported that both CIPK8 and CIPK23 are involved in regulating the nitrate response (Ho et al., 2009; Hu et al., 2009); thus, our results suggest that FIP1 may play an essential role in the PNR by regulating the expression of CIPK8 and CIPK23. Moreover, our genetic and molecular findings suggest that FIP1 and CPSF30-L work in the same nitrate-signaling pathway (Figure 7), FIP1 also alter the 3′-UTR of NRT1.1, similar with CPSF30-L (Figure 8); however, the fip1 and cpsf30 mutants have some phenotypic differences, such as their nitrate distributions and their regulation of NRT1.1 expression, reflecting the complexity of nitrate regulation.

FIP1 is a core component of a pre-mRNA processing complex that is conserved between plants, yeast, and humans (Preker et al., 1995; Kaufmann et al., 2004; Forbes et al., 2006). In plants, FIP1 is an RNA-binding protein and its N-terminus (containing 137 amino acids) can interact with poly(A) polymerase and stimulate its activity. In addition, the N-terminus of FIP1 interacts with other protein machinery involved in the 3′-end processing of pre-mRNAs such as CstF77, CFlm-25, PabN1, and CPSF30 (Forbes et al., 2006). Furthermore, it has been reported that three distinct hubs centered around FIP1, CPSF100, and CLPS are involved in poly(A) processing in Arabidopsis (Hunt et al., 2008). The biological function of FIP1 is unknown; however, the findings presented here indicate that FIP1 plays an important role in nitrate signaling and the regulation of nitrate uptake, transport, and assimilation.

In Arabidopsis, the 28-kDa CPSF30-S subunit contains three zinc finger motifs and functions in polyadenylation, while the 65-kDa CPSF30-L subunit contains a YTH (YT521 homology) RNA-binding domain in addition to three zinc fingers (Delaney et al., 2006). In both splicing forms, the first zinc finger binds RNA and the third zinc finger has nuclease activity, but the function of the second zinc finger domain is unknown. FIP1 was previously shown to interact with the third zinc finger of CPSF30-S, thereby regulating its nuclease activity (Addepalli and Hunt, 2007). Furthermore, a disulfide bond can be formed between the side chains of two cysteine residues in the third zinc finger domain, which, when reduced, results in the loss of endonuclease activity in CPSF30-S (Addepalli et al., 2010). CPSF30-S plays a variety of roles in a number of distinct developmental and physiological responses, partly resulting from a global shift in the poly(A) site choice of the corresponding genes (Zhang et al., 2008; Bruggeman et al., 2014; Liu et al., 2014); however, whether CPSF30-L functions in the same way to influence APA remains unknown. Interestingly, a mutation in the third zinc finger motif of CPSF30-L affects the poly(A) processing of NRT1.1 mRNA, resulting in the altered expression of NRT1.1 (Li et al., 2017). These findings suggest that CPSF30-L mediates APA function in a similar manner to CPSF30-S; therefore, the function of CPSF30-L in regulating nitrate signaling may depend on its endonuclease activity. Here, we found that FIP1 could interact with CPSF30-L and also alter the APA in the 3′-UTR of NRT1.1. FIP1 and CPSF30-L function in the same pathway to regulate nitrate signaling. There results suggest that FIP1 and CPSF30-L may modulate the expression of nitrate-related genes through affecting their APA process.

AUTHOR CONTRIBUTIONS
YW and NC designed the project. CW, WZ, ZhL, and YB performed the experiments. YW, CW, and ZeL analyzed the data. YW, NC, and CW wrote the manuscript.

FUNDING
This work was supported by the National Key R&D Program of China (Grant No. 2016YFD0100701), the NSFC (Grant No. 31670247), and funding from the Funds of Shandong “Double Tops” Program (Grant No. SYL2017YSTD01) awarded to YW.

ACKNOWLEDGMENTS
We thank Prof. Weihua Wu for providing the CIPK23-OE line and Dr. Fei Ni for the useful discussion.

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00593/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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