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RESEARCH PAPER

The Ca^{2+}-regulated protein kinase CIPK1 integrates plant responses to phosphate deficiency in Arabidopsis thaliana

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ABSTRACT

- Phosphate (Pi) deficiency severely restricts plant growth and development, as Pi is an essential macronutrient. Calcium (Ca^{2+}) is a ubiquitous second messenger in plants; calcineurin B-like proteins (CBL) and CBL-interacting protein kinases (CIPK) are signalling pathways that act as an important Ca^{2+} signalling network which integrates plants to fine tune the response to stress; however, whether CIPK are involved in Pi deficiency stress remains largely unknown.
- In this study, we carried out a reverse genetic strategy to screen T-DNA insertion mutants of CIPK isoforms under Pi deficiency in *Arabidopsis thaliana*. Then Pi content, transcription of phosphate starvation-induced (*PSI*) genes, acid phosphatase activity and hydrogen peroxide were determined in the wild-type (WT) and *cipk1* mutant, respectively. The phenotype of *CIPK1* complementation lines was analysed.
- The *cipk1* mutant had a more sensitive phenotype, with lower root elongation and root length, and decreased Pi content compared with the WT under Pi deficiency. Moreover, *CIPK1* mutation caused phosphate starvation-induced (*PSI*) genes to be significantly induced under Pi deficiency. Histological staining demonstrated that the *cipk1* mutant had increased acid phosphatase activity and hydrogen peroxide concentration under Pi deficiency. By using the yeast two-hybrid system, we further demonstrated the interaction between CIPK1 and the WRKY transcription factors, WRKY6 and WRKY42.
- Overall, we demonstrate that CIPK1 is involved in the Pi deficiency signalling pathway in *A. thaliana*, revealing the important role of Ca^{2+} in the Pi nutrition signalling pathway, and potentially providing a theoretical foundation for molecular breeding of crops with better Pi utilization efficiency.

INTRODUCTION

Phosphorus (P), a plant macronutrient, plays essential roles in energy metabolism, signal transduction, photosynthesis and respiration. Plant growth and development depend on the availability of inorganic phosphate (Pi) in soil. A deficiency of Pi in soil (<10 μM) has become one of the most limiting factors for agricultural production (Luan, 2009; Lambers *et al.,* 2015). To cope with Pi deficiency, farmers generally use a large amount of phosphate fertilizer to improve crop productivity, which can cause serious environmental problems, such as water eutrophication (Conley & Likens, 2009). Therefore, exploring the molecular mechanism of plant Pi uptake and distribution to enhance Pi utilization efficiency has become urgent and imperative for sustainable crop production (Heuer *et al.,* 2017).

To ameliorate the shortfall in availability of Pi in soil, plants have evolved a range of mechanisms to improve Pi assimilation and translocation, thus maintaining cellular Pi homeostasis (Rouached *et al.,* 2010). The Phosphate Transporter 1 (PHT1) protein family functions in the initial uptake and remobilization of Pi into plant cells from the rhizosphere (Gu *et al.,* 2016; Versaw & Garcia, 2017; Xu, 2018). The family members PHT1;1 and PHT1;4, with functional redundancy, are mainly involved in Pi uptake from both low and high Pi conditions (Shin *et al.,* 2004). The family member PHO1 functions in Pi transfer from root epidermal and cortical cells to the xylem, and facilitates Pi translocation from the root to the shoot (Hamburger, 2002; Vogiatzaki *et al.,* 2017). Recently, vacuolar Pi efflux transporters (OsVPE1 and OsVPE2) and a nitrate sensor (NRT1.1B) that interacts with the Pi signalling repressor SPX4 have been reported to integrate the nitrogen (N) and Pi signalling networks in rice (Hu *et al.,* 2019; Xu *et al.,* 2019). Four *Arabidopsis* WRKY proteins (WRKY6, WRKY42, WRKY45 and WRKY75) have been characterized as functioning in the Pi deficiency signalling pathway. Both WRKY45 and WRKY75 have been reported to act as positive regulators of PHT1.1 (Devaiah *et al.,* 2007; Wang *et al.,* 2014). Members WRKY6 and WRKY42 can repress *PHO1* expression by binding to the W-box motifs within the *PHO1* promoter, and thus the repression of *PHO1* by WRKY can be released under low Pi conditions (Chen *et al.,* 2009; Su *et al.,* 2015). Moreover, Pi deficiency was found to significantly induce Pi starvation-induced (*PSI*) genes, including the phosphate transporters genes (*PHT*), *IPS1*, *RNS1*, *microRNA399* and *microRNA827* (Bari *et al.,* 2006; Martin *et al.,* 2010; Lin *et al.,* 2013; Sun *et al.,* 2016).

Calcium (Ca^{2+}) is a ubiquitous second messenger in all eukaryotes and plays an essential role in signal transduction in response to external stimuli. The Ca^{2+} signatures are decoded by Ca^{2+} sensors, such as calmodulin (CaM), calmodulin-like protein (CML), calcium-dependent protein kinase (CDPK) and calcineurin B-like protein (CBL) families, and these Ca^{2+} sensors can bind Ca^{2+} and change their structural characteristics, thus triggering an interaction with their downstream target proteins (Luan, 2009; Hashimoto & Kudla, 2011; Kudla *et al.,* 2018). The calcineurin B-like protein (CBL)-interacting protein kinase (CIPK) network, which has been largely elucidated in recent years, not only responds to multiple external adverse stimuli, such as salt, drought, cold and immune stress responses, but more particularly also regulates the intracellular ion homeostasis, *e.g.* K^+, Na^+, Mg^{2+} and NO_3^- (Zhu, 2016; Kudla *et al.,* 2018). In response to K^+ deficiency, the Ca^{2+} sensors CBL1 and CBL9 positively regulate CIPK23 and activate AKT1 (*Arabidopsis* K^+ transporter 1) and HAK5 (high-affinity K^+ transporter 5) for optimal K^+ absorption (Xu *et al.,* 2006; Ragel *et al.,* 2015); moreover, the CBL1/9–CIPK23 complex also phosphorylates CHL1/NRT1.1 (nitrate transporter1.1) to mediate nitrate (NO_3^-) uptake (Ho *et al.,* 2009; Leran *et al.,* 2015). The well documented salt overly sensitive (SOS) pathway plays an important role in salt tolerance, *e.g.* the CBL4-CIPK24-SOS1 (a plasma membrane Na^+/H^+ antiporter) (Liu *et al.,* 2000; Qiu *et al.,* 2002; Qiu *et al.,* 2004). Furthermore, CBL2/CBL3 with CIPK3/9/23/26 have been reported to function in vacuolar Mg^{2+} sequestration, therefore protecting plants from Mg^{2+} toxicity (Ren-Jie *et al.,* 2015). In addition, previous studies found that a *CBL1* mutation impairs plant response to drought, salt, cold, glucose and aluminium stresses; loss of *CIPK1* function also renders plants hypersensitive to ABA (Cheong, 2003; D'Angelo *et al.,* 2006; Li *et al.,* 2013; Ligaba-Osena *et al.,* 2017). Although the CBL-CIPK modules are indispensable in many ion homeostasis processes in plant cells, whether they are involved in the Pi deficiency signalling pathway remains unknown.

Here, we show that CIPK1 positively regulates the Pi deficiency signalling pathway using a reverse genetic strategy in *Arabidopsis*. The *cipk1* mutant had a more sensitive phenotype under Pi deficiency than the wild type (WT). A mutation in *CIPK1* leads to increased transcription of multiple Pi starvation-induced genes, such as *PHT1;1*, *PHT1;5* and *IPS1*. Using a yeast two-hybrid system, we further demonstrate the interaction between CIPK1 and the WRKY transcription factors WRKY6 and WRKY42. Taken together, our findings show a novel role for CIPK genes in the Pi deficiency signalling pathway and provide a theoretical foundation that could be useful for future crop engineering.

MATERIAL AND METHODS

Plant material and growth conditions

Arabidopsis thaliana Ws seedlings (ecotype *Wassilewskji*) were used as the WT in this study. The *cipk1* T-DNA insertion mutant line (N9889) was obtained from the Nottingham *Arabidopsis* Stock Center (NASC, UK). For phenotypic assays, seeds were surface-sterilized with 8% NaClO (v/v) and then stratified for 3 days at 4 °C. Seeds were germinated on 1/2 MS medium for 5 days and then transferred to Pi-sufficient (1/2 MS) or LP (MS without Pi, Caisson) medium containing 50 μM Pi (supplied as $NH_4H_2PO_4$) for another 7 days, containing 1% sucrose and 1% agar (Solarbio, Cat#A8190, China), pH 5.7, with a 16-h light/8-h dark cycle at 22 °C.

For the complementation assay of the *cipk1* mutant, a DNA fragment containing the 2.0 kb promoter, the *CIPK1* gene and 1.0 kb downstream sequence of *CIPK1* was amplified and cloned into the binary vector pCAMBIA1381. The resultant vector was transformed into the *cipk1* mutant using the *Agrobacterium*-mediated floral dip method. Homozygous individuals were screened using PCR with the primers listed in Table S1.

Physiological measurements

To determine the Pi content, 7-day-old seedlings germinated on 1/2 MS medium were transferred to the 1/2 MS or LP medium for 7 days, and then shoots and roots were harvested for Pi content analysis using the ascorbate–molybdate–antimony method (Liu *et al.,* 2015; Zheng *et al.,* 2019).

Staining for hydrogen peroxide was assessed by staining roots using a DAB Immunohistochemistry Color Development Kit according to the manufacturer's instructions (Sangon Biotech, Beijing, China). *In vivo* APase activity staining was detected as previously described (Devaiah *et al.,* 2007).

Quantitative real-time PCR analysis

For expression analysis of *CIPK1* in response to Pi deficiency, seeds were grown in Petri dishes containing Pi-sufficient (1/2 MS; Sigma, USA) medium for 7 days and then transferred to 1/2 MS or Pi-deficient medium for 3 days. Total RNA was extracted using TRIzol reagent (TIANGEN, China); the first-strand cDNA was synthesized using the FastKing RT Kit with gDNAase (TIANGEN). Quantitative real-time PCR (qRT-PCR) was performed using ChamQ SYBR qPCR Master Mix (Vazyme) on a Roche Applied Science LightCycler 480 system (Roche, Switzerland). The *ACTIN2* gene was used as an internal control. The primers used for qRT-PCR are listed in Table S1.

Yeast-two-hybrid experiment

For the interaction of CIPK1 with WRKY transcription factors, the full-length open-reading frames (ORF) of WRKY and *CIPK1* were amplified and subcloned into the pGAD GH and pGBT9 vectors, respectively. The vectors were transformed into the yeast strain Y2HGold. Interactions were visualized on SD/-LT and SD/-LTH medium. Sequences of all primers used for vector construction are provided in Table S1.

Statistical analysis

Statistical significance of differences between mean values was determined using Student's *t*-test. Asterisks above error bars in the figures indicate mean value that are statistically different at $P \leq 0.05$.

RESULTS

The *cipk1* mutant exhibits a sensitive phenotype in response to Pi deficiency

To investigate whether the CIPK family is involved in Pi deficiency stress, we carried out a reverse genetic strategy to

screen *CIPK* T-DNA insertion mutants under Pi deficiency (Figure S1), *i.e. cipk1*, *cipk4*, *cipk5*, *cipk7*, *cipk8*, *cipk11*, *cipk14*, *cipk17*, *cipk21*, *cipk23* and *cipk1cipk8.* Strikingly, we found that the *cipk1* knockout mutant displayed a significantly sensitive phenotype under Pi deficiency, with reduced root elongation compared with the WT, while root elongation of the *cipk1* mutant was similar to that of the WT on 1/2 MS medium (Fig. 1A and B; Figure S2). To further confirm phenotypic differences of the *cipk1* mutant under Pi deficiency, we then conducted a germination experiment. Similarly, the sensitive phenotype of the *cipk1* mutant was also assessed with a germination experiment, showing a 50% decrease in root length of the WT under Pi deficiency (Fig. 1C and D). These results indicate that CIPK1 is involved in the Pi deficiency signalling pathway and plays a positive role.

Mutation of *CIPK1* decreases Pi accumulation

Previous studies have shown that CIPK1 is localized in the plasma membrane, cytosol and nucleus, and that physical interaction of CIPK1 with CBL1 and CBL9 targets the CIPK1 kinase to localization in the plasma membrane (D'Angelo *et al.,* 2006). Considering the differences in the Pi deficiency stress phenotype between the *cipk1* mutant and the WT, we further examined whether the *CIPK1* mutation influences Pi accumulation in plants. Seedlings were grown on 1/2 MS medium for 7 days, then both the WT and *cipk1* mutant were transferred to 1/2 MS or LP medium for another 7 days. The results showed that the Pi content of whole seedlings significantly decreased in the *cipk1* mutant under Pi deficiency (Fig. 2A). Furthermore, we also found that the Pi content obviously declined both in shoot and root tissues in the *cipk1* mutant compared with the WT under Pi deficiency, whereas there were no significant differences in Pi content in 1/2 MS medium (Fig. 2B and C). These results demonstrate that *CIPK1* mutation can influence Pi homeostasis in plant cells.

Phenotype of *CIPK1* complementation lines

To confirm that the Pi deficiency-sensitive phenotype of the *cipk1* mutant is attributable to loss of the *CIPK1* gene, we generated CIPK1 complementation lines by introducing a WT CIPK1 with the 2.0 kb promoter and full genomic sequence of this gene into the *cipk1* mutant. Root elongation of the *cipk1* mutant was obviously much less than in the WT under Pi deficiency, and roots of transgenic lines #2 and #4 were similar to those of the WT, while there was no significant difference in root elongation in 1/2 MS medium (Fig. 3). These results indicate that the Pi deficiency-sensitive phenotype observed in the *cipk1* mutant was caused by loss of the *CIPK1* gene.

The *CIPK1* mutation promotes transcription of *PSI* genes

To further determine whether CIPK1 is involved in Pi deficiency signalling, the expression level of *CIPK1* was analysed using real-time qRT-PCR in the shoot and root of seedlings under 1/2 MS or Pi deficiency. The results showed that *CIPK1* was expressed ubiquitously in shoot and root tissues, but expression of *CIPK1* was not induced upon Pi deficiency (Fig. 4A).

Plants have evolved a series of Pi starvation adaptations to regulate phosphate transporter activity at the transcriptional or post-transcriptional level to maintain Pi homeostasis in cells. Deficiency of Pi regulates the expression of multiple phosphate starvation-induced (*PSI*) genes, and there have been several investigations into whether CIPK1 is involved in Pi deficiency activation of these genes, including the phosphate transporters *PHT1*, *AtIPS1* and *AtRNS1* (Bari *et al.,* 2006; Martin *et al.,* 2010; Lin *et al.,* 2013; Sun *et al.,* 2016). Total RNA was extracted from 7-day WT and *cipk1* seedlings that were subjected to Pi deficiency stress for 1 day, then qRT-PCR was performed. The results showed that levels of *PSI* genes were significantly higher in the *cipk1* mutant under Pi deficiency, especially *PHT1;1*, *PHT1;5*, *IPS1* and *RNS1* genes, indicating a severe Pi deficiency response in the *cipk1* mutant. However, the

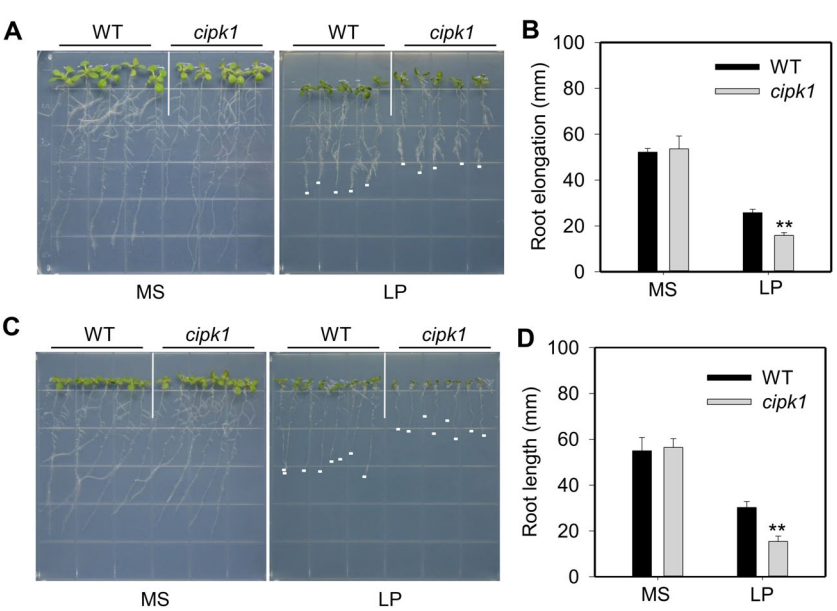

Fig. 1. The *cipk1* mutant is sensitive to Pi deficiency. A: Phenotypic analysis of the *cipk1* mutant on LP medium. Seeds were germinated on 1/2 MS medium for 5 days, then transferred to 1/2 MS or LP medium (50 μM) for another 7 days. B: Root elongation of seedlings as in (A). C: Phenotypic analysis of the *cipk1* mutant germinated on LP medium. Seeds were germinated on 1/2 MS and LP medium for 10 days. D: Root elongation of seedlings as in (C). Data represent three independent experiments. Results are expressed as mean ± SD. Asterisks indicate significant differences between the *cipk1* mutant and WT (Student's *t*-test: $*P < 0.05$ and $**P < 0.01$).

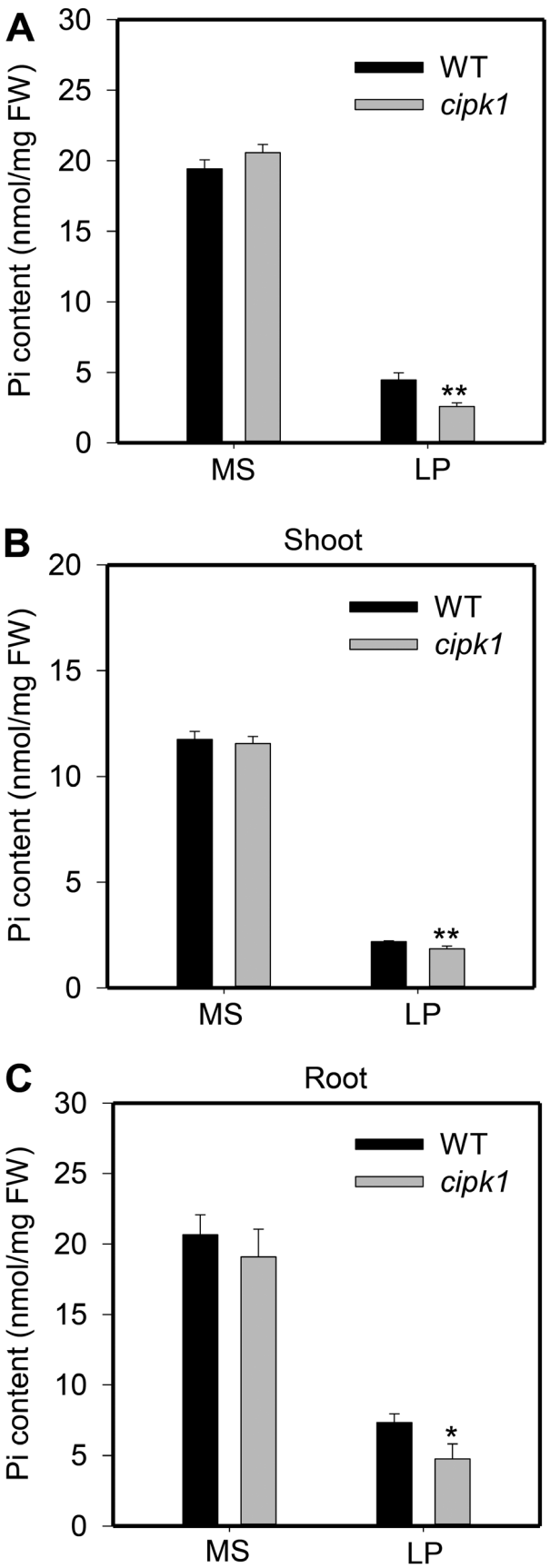

Fig. 2. Mutation of *CIPK1* decreases Pi accumulation. A: Pi content in whole seedlings of WT and *cipk1* mutant on MS or LP medium. Seeds were germinated on 1/2 MS medium for 7 days, then transferred to 1/2 MS or LP medium (50 μM) for another 7 days, before Pi content of whole seedlings was assessed. B and C: Shoot and root Pi content in WT and *cipk1* mutant under MS and Pi deficiency. Seeds were treated as described above, then Pi content of shoot and root tissues assessed. Data represent three independent experiments. Results are expressed as mean ± SD. Asterisks indicate significant differences between *cipk1* mutant and WT (Student's *t*-test: $*P < 0.05$ and $**P < 0.01$).

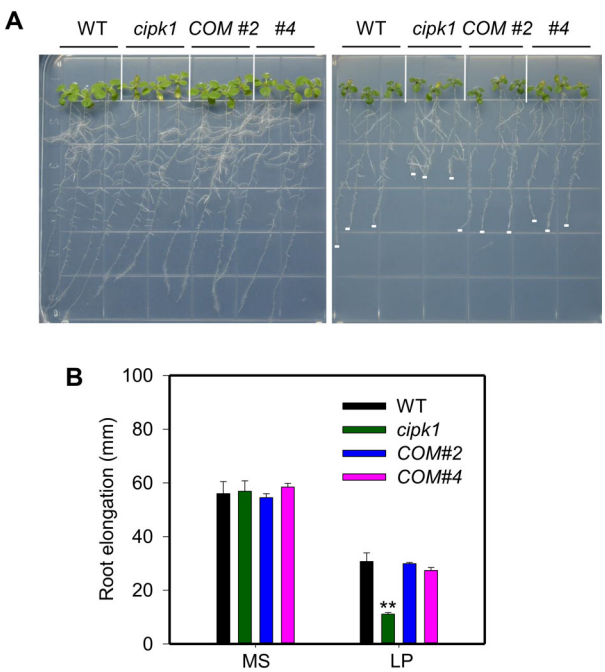

Fig. 3. Phenotypic analysis of *CIPK1* complementation lines. A: Phenotype of WT, the *cipk1* mutant and *COM#2* and *COM#4* on MS or LP medium. B: Root elongation of seedlings in (A). Values represent mean ± SD of biological replicates. Asterisks indicate significant differences (Student's *t*-test: $**P < 0.01$).

PHO1 gene significantly decreased in the *cipk1* mutant compared with the WT under Pi deficiency. There was obvious up-regulation of *WRKY6* and *WRKY42* in the *cipk1* mutant, which has been reported to negatively affect Pi translocation by repressing expression of *PHO1* in response to Pi deficiency (Chen *et al.*, 2009; Su *et al.*, 2015) (Fig. 4B). These findings suggest that CIPK1 is required in the Pi deficiency signalling pathway.

The *cipk1* mutant has increased acid phosphatase activity and hydrogen peroxide concentration

Deficiency of Pi can stimulate additional physiological responses, including elevation of acid phosphatase (APase) activity in roots, thus releasing Pi from organic or inorganic sources in the soil (Del Vecchio *et al.*, 2014). We therefore determined the impact of *CIPK1* mutation on APase activity. There was no significant difference in APase activity to that in the WT, *cipk1* or complementation lines (*COM#2* and

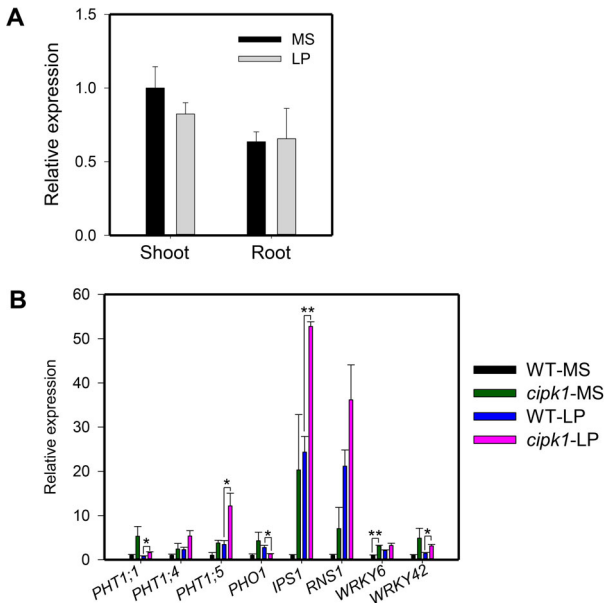

Fig. 4. *CIPK1* mutation causes increased transcription of *PSI* genes. A: Expression of *CIPK1* in response to Pi deficiency. qRT-PCR analysis of *CIPK1* expression from shoots and roots of WT seedlings. Transcript level of *CIPK1* was quantified relative to *ACTIN2*. Values represent mean ± SD of biological replicates. B: Relative expression of *PHT1;1*, *PHT1;4*, *PHT1;5*, *PHO1*, *IPS1*, *RNS1*, *WRKY6* and *WRKY42* genes. Transcript level of different genes was quantified relative to *ACTIN2*. Results are expressed as mean ± SD. Asterisks indicate significant differences (Student's *t*-test: $*P < 0.05$ and $**P < 0.01$).

COM#4) on 1/2 MS medium. In contrast, there was much stronger staining of APase activity in the *cipk1* mutant, but APase activity of the complementation lines (*COM#2* and *COM#4*) was fully restored to the WT level (Fig. 5A and B).

Reactive oxygen species (ROS) play a crucial role in multiple metabolic pathways in plants. The ROS content increases rapidly following Pi deficiency, and variation in ROS distribution associated with Fe^{3+} accumulation may determine the root system architecture under Pi deficiency (Chiou & Lin, 2011; Ham *et al.*, 2017; Zheng *et al.*, 2019). To investigate whether the Pi deficiency-dependent sensitive phenotype in the *cipk1* mutant was induced by an alteration in ROS concentration, we performed *in situ* DAB staining to detect H_2O_2 accumulation in roots. There was no significant difference in DAB staining intensity of root tips between the WT, *cipk1* mutant or complementation lines (*COM#2* and *COM#4*) on 1/2 MS medium; however, under Pi deficiency, the DAB staining signal in the *cipk1* mutant was significantly stronger than in the WT, suggesting an increase in H_2O_2 concentration in the *cipk1* mutant, while the DAB staining signal of the complementation lines (*COM#2* and *COM#4*) was fully restored to the WT level (Fig. 5C and D). These results further indicate that CIPK1 is a positive regulator in the Pi deficiency signalling pathway through altered ROS accumulation, which may be associated with iron accumulation in the roots.

The CIPK1 interacts with WRKY6 and WRKY42 transcription factors

Since members of the CIPK family function as protein kinases to phosphorylate target proteins in response to different stimuli, we performed a yeast two-hybrid screen to search for CIPK1-interacting proteins involved in the Pi signalling pathway. Interestingly, we found that yeast cells transformed with WRKY28-AD and CIPK1-BD or WRKY75-AD and CIPK1-BD or with the empty vector AD and CIPK1-BD could not grow on plates without added tryptophan/leucine/histidine (–THL). However, yeast cells transformed with WRKY6-AD and CIPK1-BD or WRKY42-AD and CIPK1-BD grew well on the –THL medium (Fig. 6); these results suggest that CIPK1 interacts with WRKY6 and WRKY42 in yeast.

DISCUSSION

Plant growth and development as well as crop production are dependent on the availability of inorganic Pi in the soil. Although the total amount of soil P is abundant, inorganic Pi, the form which can be utilized by plants, is severely limited in many agricultural ecosystems. A deficiency in Pi has become a factor of extreme importance in achieving sustainable crop production (Chiou & Lin, 2011; Xu *et al.*, 2019). The typical features of modifications to plant root system architecture are inhibition of root elongation, increased density of lateral roots and root hairs; moreover, Pi deficiency can also trigger the secretion of organic acids and acid phosphatases from roots, which promote Pi uptake from the rhizosphere (Ticconi *et al.*, 2009; Lopez-Arredondo *et al.*, 2014). To cope with Pi deficiency, plants have evolved multiple strategies to enhance Pi uptake and distribution (Muller *et al.*, 2015; Kanno *et al.*, 2016; Balzergue *et al.*, 2017).

Calcium (Ca^{2+}), as a vital intracellular second messenger in plants, plays a vital role in responses to biotic and abiotic stress (Poovaiah & Reddy, 1993). Previous studies have demonstrated that abiotic stresses, *e.g.* salt, drought, cold, heat and light, can lead to elevation of the Ca^{2+} concentration in the cytoplasm (Kudla *et al.*, 2010; Liao *et al.*, 2017). The Ca^{2+} signals are decoded by Ca^{2+} sensors, CBL, CaM or CDPK, which trigger the downstream transcriptional or phosphorylation events (Poovaiah & Reddy, 1993; Luan, 2009). Extensive evidence shows that the CBL–CIPK network is involved in ion homeostasis in plant cells, *e.g.* Na^+, K^+, Mg^{2+}, NH_4^+ and NO_3^- (Zhu, 2016; Kudla *et al.*, 2018), but whether the CBL–CIPK complex functions in the Pi deficiency signalling pathway is poorly understood. In this study, we identified a novel role of CIPK1 in modulating Pi deficiency stress, and our data provide new information that might be useful for future crop engineering.

To better understand whether the CBL–CIPK complex is involved in Pi homeostasis in plant cells, we initiated a study to assess the sensitivity of *cipk* (*cipk1*, *cipk4*, *cipk5*, *cipk7*, *cipk8*, *cipk11*, *cipk14*, *cipk17*, *cipk21*, *cipk23*, *cipk1cipk8*) mutants to Pi deficiency through a reverse genetic screen (Figure S1). The results showed that only the *cipk1* mutant is significantly sensitive to Pi deficiency compared to the WT, with shorter root elongation, increased APase activity and H_2O_2 concentration (Figs 1 and 5).

To determine whether the sensitive phenotype of the *cipk1* mutant was due to decreased Pi uptake, we first analysed the Pi content of whole seedlings in the WT and *cipk1* mutant under MS or Pi deficiency, and found that Pi content was obviously decreased in the *cipk1* mutant under Pi deficiency (Fig. 2A); the Pi content also declined in both shoot and root tissues, implying that *CIPK1* mutation could lead to reduced Pi

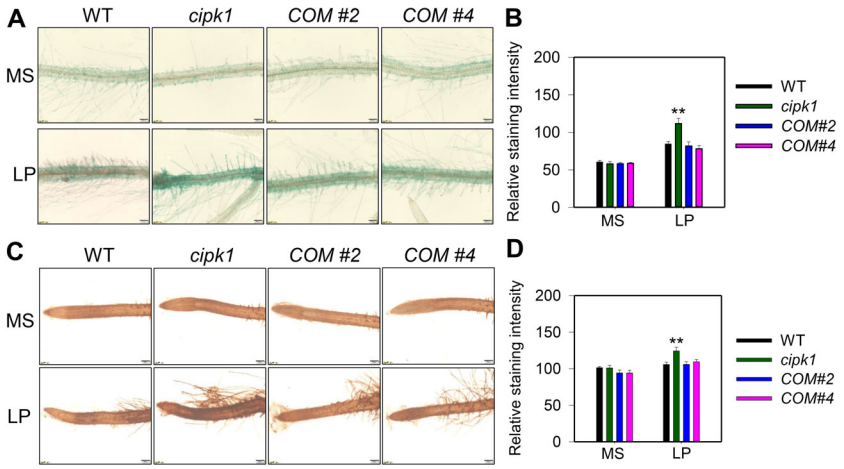

Fig. 5. *CIPK1* mutation leads to increased acid phosphatase activity and H_2O_2 accumulation. A: Acid phosphatase (APase) activity staining in root tips of WT and *cipk1* mutant on MS or LP medium. Seeds were germinated on 1/2 MS agar medium for 5 days, transferred to 1/2 MS or LP medium for another 4 days, then APase activity staining was performed. B: Relative staining intensity of APase activity determined with Image J software. C: DAB staining for H_2O_2 in root tips of the WT and *cipk1* mutant on MS or LP medium. Seeds were treated as described above then transferred and DAB staining performed. D: Relative staining intensity of DAB determined with Image J software. Data represent three independent experiments. Results are expressed as mean ± SD. Asterisks indicate significant differences between the *cipk1* mutant and WT (Student's *t*-test: $*P < 0.05$; $**P < 0.01$).

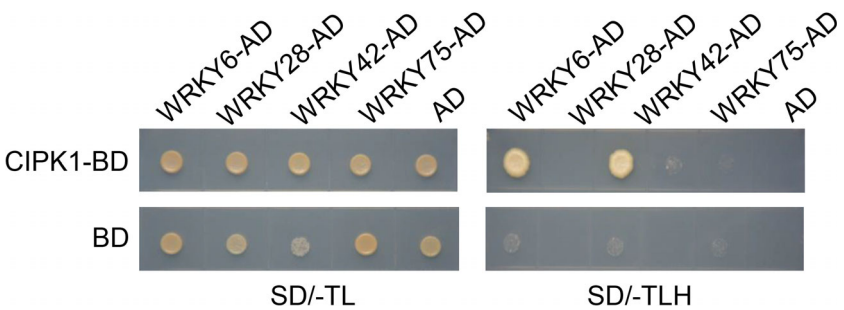

Fig. 6. CIPK1 interacts with WRKY6 and WRKY42. Yeast two-hybrid analysis showing the interaction of CIPK1 with WRKY6, and CIPK1 with WRKY42. Yeast cells were grown on synthetic dropout medium without Trp and Leu (SD/-TL, left) or on SD medium without Trp/Leu/His (right).

accumulation in plants (Fig. 2B and C). The sensitive phenotype of the *cipk1* mutant was fully rescued in the transgenic complementation lines under LP treatment, indicating the *CIPK1* mutation was responsible for the sensitive phenotype under Pi deficiency (Fig. 3).

To gain insight into CIPK1 in response to Pi deficiency, we analysed the *CIPK1* transcription level of the shoot and root in the WT background and found that *CIPK1* was constitutively expressed in both shoot and root tissues, and transcription could not be induced under Pi deficiency (Fig. 4A). Considering the sensitive phenotype of the *cipk1* mutant under Pi deficiency, we suggest that the *CIPK1* mutation may disturb Pi homeostasis in plants. Results of qRT-PCR showed that not only the expression of major phosphate transporters (PHT1;1 and PHT1;5) was remarkably increased, but also *PSI* (phosphate starvation-induced) genes, such as *IPS1* and *RNS1*, was also elevated. Moreover, activity of the *PHO1* gene significantly decreased in the *cipk1* mutant under Pi deficiency; it was previously demonstrated that this gene has essential roles in Pi translocation from root to shoot (Poirier *et al.*, 1991). Both *WRKY6* and *WRKY42* were also up-regulated in the *cipk1* mutant (Fig. 4B). These results demonstrate that the severe sensitive phenotype of the *cipk1* mutant can lead to elevation of *PSI* gene transcription under Pi deficiency, which is a stress response, but is not the underlying cause. Previous studies demonstrated that overexpression of both WRKY6 and WRKY42 negatively affects Pi accumulation in plants subjected to Pi deficiency by repressing the expression of PHO1 (Chen *et al.*, 2009; Su *et al.*, 2015). The *CIPK1* mutation could significantly increase expression of *WRKY6* and *WRKY42*, which may negatively repress the *PHO1* gene, thus decreasing Pi translocation in plants. If CIPK1 is instead a positive regulator of Pi accumulation, we might hypothesize that the CIPK1–WRKY interaction could lead to decreased activity of these two transcription factors. To investigate the mechanism through which CIPK1 functions, we performed a yeast two-hybrid screen to search for CIPK1-interacting proteins involved in the Pi signalling pathway. The results showed that CIPK1 interacts with WRKY6 and WRKY42 transcription factors (Fig. 6), indicating that it may function in the Pi deficiency signalling pathway by interacting with WRKY6 and WRKY42, although the molecular mechanism remains to be explored.

In summary, we present evidence that a *CIPK1* mutation can greatly aggravate the severe root inhibition defects generated under Pi deficiency, thus producing a sensitive phenotype. This indicates that CIPK1 may function as a novel regulator in regulating Pi homeostasis in plant cells. However, the molecular mechanism remains to be elucidated of how CIPK1 is involved in the WRKY6- and WRKY42-mediated Pi deficiency signalling pathway. Our future research will focus on the molecular mechanism of CIPK1 in response to Pi deficiency, to reveal the important role of Ca^{2+} in the Pi nutrition signalling pathway, and provide a theoretical foundation for molecular breeding of crops with more efficient Pi utilization.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Phenotypic analysis of *cipk*$_s$ mutants.

Figure S2. Root elongation of *cipk1* mutant with different exogenous Pi supply.

Table S1. Primers used for PCR.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

There were no potential conflicts of interest.

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