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A novel role of the calcium sensor CBL1 in response to phosphate deficiency in *Arabidopsis thaliana*

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ABSTRACT

Phosphorus acts as an essential macroelement in plant growth and development. A lack of phosphate (Pi) in arable soil and phosphate fertilizer resources is a vital limiting factor in crop yields. Calcineurin B-like proteins (CBLs) act as one of the most important calcium sensors in plants; however, whether CBLs are involved in Pi deficiency signaling pathway remains largely elusive. In this study, we utilized a reverse genetic strategy to screen *Arabidopsis thaliana* T-DNA insertion mutants belonging to the CBL family under Pi deficiency conditions. The *cbl1* mutant exhibited a relatively tolerant phenotype, with longer roots, lower anthocyanin content, and elevated Pi content under Pi deficiency, and a more sensitive phenotype to arsenate treatment compared with wild-type plants. Moreover, *CBL1* was upregulated, and the mutation of *CBL1* caused phosphate starvation-induced *(PSIs)* genes to be significantly induced under Pi deficiency. Histochemical staining demonstrated that the *cbl1* mutant has decreased acid phosphatase activity and hydrogen peroxide concentrations under Pi deficiency. Collectively, our results have revealed a novel role of CBL1 in maintaining Pi homeostasis.

1. Introduction

Phosphorus (P) is an essential macronutrient for plant growth and development that plays vital roles in multiple biochemical processes in plant cells, including energy metabolism, signal transduction, photosynthesis, and respiration. Although P is abundant in soil, most organic and inorganic phosphorus are unavailable to plants. The concentration of phosphate (Pi), the inorganic form of P that is taken up and utilized by plants, is extremely low in soil (less than 10 μM) (Luan, 2009; Lambers et al., 2015). A lack of Pi leads to the accumulation of anthocyanin, with dark green leaves, growth retardation, and reduced yield. Thus, Pi deficiency is becoming one of the most limiting factors for sustainable crop production (Puga et al., 2017). In order to improve agricultural productivity, a large amount of Pi fertilizer is applied to soil, which causes serious environmental problems such as eutrophication of water sources (Conley and Likens, 2009). Hence, exploring mechanisms for achieving sustainable P utilization efficiency has become an urgent objective in plant breeding research (Heuer et al., 2017).

Pi deficiency seriously inhibits primary root growth and induces the formation of lateral roots and root hairs. To cope with Pi deficiency, plants have evolved a range of adaptations that improve Pi absorption and translocation, thus maintaining cellular Pi homeostasis (Rouached et al., 2010). The initial uptake and remobilization of Pi are regulated by Phosphate Transporter 1 (PHT1) proteins. PHT1 proteins play important roles in Pi uptake and allocation from the rhizosphere (Gu et al., 2016; Versaw and Garcia, 2017; Xu, 2018). PHT1;1 and PHT1;4, which are functionally redundant, mainly function in Pi uptake in roots under both low- and high-Pi conditions (Shin et al., 2004). PHO1, a member of the SPX-EXS subfamily, functions in Pi transfer from root epidermal and cortical cells to xylem and facilitates Pi translocation from root to shoot (Hamburger, 2002; Vogiatzaki et al., 2017). PHR1 (Phosphate-starvation Response 1) and PHR1-LIKE1 (PHL1), which have been identified as master transcription factors, play central roles in controlling the phosphate response of multiple targets by interacting with P1BS *cis*-elements, including *PHTs*, *IPS1*, *RNS1*, and *SPX1* (Rubio et al., 2001; Bustos et al., 2010; Thibaud et al., 2010).

Calcium (Ca^{2+}) is a ubiquitous second messenger in eukaryote cells that plays an important role in signal transduction in response to internal and external stimuli. Ca^{2+} signatures are perceived by Ca^{2+} sensors, such as calmodulin (CaMs), calmodulin-like protein (CMLs),

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calcium-dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs) families. The CBL protein harbors four elongation factor hands (EF-hands) which are responsible for Ca^{2+} binding (Luan, 2009; Hashimoto and Kudla, 2011; Kudla et al., 2018).

CBLs can form functional complexes with CBL interacting protein kinases (CIPKs), which not only respond to external adverse stimuli, such as drought, salt, cold, and immune stress responses, but also regulate the dynamic balance of diverse intracellular ions, including K^+, Na^+, Mg^{2+}, NO_3^-, and PO_4^{3-} (Zhu, 2016; Kudla et al., 2018; Zhang et al., 2019; Chai et al., 2020; Lu et al., 2020). For example, the CBL1/9-CIPK23 complex activates AKT1 (*Arabidopsis* K^+ transporter 1) and HAK5 (high-affinity K^+ transporter 5) to enhance K^+ absorption under low-potassium conditions (Xu et al., 2006; Ragel et al., 2015); moreover, the CBL1-CIPK23 complex can phosphorylate and activate CHL1/NRT1.1 (nitrate transporter1.1) to mediate both high-affinity and low-affinity nitrate uptake (Ho et al., 2009; Leran et al., 2015), and the CBL1/CBL9-CIPK23 complex can trigger the phosphorylation of S-type anion channel SLAC1 or SLAH3 to mediate stomatal opening (Tobias et al., 2014). The well-characterized salt overly sensitive (SOS) pathway comprising CBL4-CIPK24-SOS1 (a plasma membrane Na^+/H^+ antiporter) plays a vital role in salt tolerance (Liu et al., 2000; Qiu et al., 2002, 2004). CBL2/CBL3 with CIPK3/9/23/26 are involved in vacuolar Mg^{2+} sequestration to protect plants from Mg^{2+} toxicity (Ren-Jie et al., 2015). Additionally, previous studies have demonstrated that CBL1 mutations can impair plant responses to drought, salt, cold, glucose, and aluminum (Cheong, 2003; D'Angelo et al., 2006; Li et al., 2013; Ligaba-Osena et al., 2017). Although the CBL-CIPK complexes exhibit essential roles in the homeostasis of various ions in cells, whether they are also involved in the regulation of the Pi deficiency signaling pathway remains unknown.

In this study, we utilized a reverse genetic strategy to identify CBL1 as a negative regulator in the Pi deficiency signaling pathway in *Arabidopsis*. The isolated *cbl1* mutant exhibited a relatively tolerant phenotype under Pi deficiency conditions and a more sensitive phenotype under arsenate treatment compared to wild-type plants. The *CBL1* mutation led to increased transcription of multiple phosphate starvation-induced genes, including *PHT1;1*, *PHT1;4*, *PHT1;5*, *IPS1*, and *RNS1*. Moreover, the *cbl1* mutant had decreased acid phosphatase activity and hydrogen peroxide concentrations. This novel discovery expands our understanding of CBL1 functions in Pi homeostasis.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana Ws seedlings (ecotype *Wassilewskji*), except for materials in Fig. S1, were used as the wild type in various experiments. The T-DNA insertion mutant lines including *cbl1* (NASC ID: N9888), *cbl2* (SALK_151426), *cbl3* (SAIL_785_C10), *cbl5* (GK-276F07), *cbl7* (SAIL_100_F5), and *cbl9* (SALK_142774) were obtained from the Nottingham *Arabidopsis* Stock Center (NASC). For phenotypic assays, seeds of *Arabidopsis thaliana* were surface sterilized with 8% NaClO (v/v) and then stratified for three days at 4 °C. Seeds were sown on Petri dishes containing 1/2 Murashige and Skoog Basal medium (MS) for five days, and then seedlings were transferred to Pi-sufficient (1/2 MS) or LP (MS without phosphate, Caisson, USA) medium to contain 50 μM Pi (supplied with KH_2PO_4) or 1/2 MS + As(V) media (As(V) final concentration: 500 μM). LP contained different IAA supplementations (50, 100 nM) as indicated in the figures, with 1% sucrose and 1% agar (Solarbio, China) at pH 5.7 with a 16-h light / 8-h dark cycle at 22 °C.

For the complementation test of the *cbl1* mutant, a DNA fragment harboring the 1991-bp promoter, the gene, and 1.0-kb downstream sequence of *CBL1* (AT4g17615) was amplified and cloned into a binary vector pCAMBIA1381. *35S:CBL1* overexpression lines were generated by cloning the coding sequence of *CBL1* into a pCAMBIA1307 vector. The resultant vectors were transformed into the *cbl1* mutant by the *Agrobacterium*-mediated floral dip method, and the homozygous lines were obtained. The primers used for plasmids constructions are listed in Table S1.

2.2. Physiological measurements

Anthocyanin content was measured as previously described (Lu et al., 2014). Seedlings were homogenized in extraction buffer (propanol/HCl/water [18:1:81]). After centrifugation for 10 min at 12,000 *g*, the supernatant was collected for measuring absorbance at 535 nm and 650 nm. Relative anthocyanin concentration was calculated using the following equation: (A_{535} – [2*A_{650}]) / fresh weight (grams).

Hydrogen peroxide accumulation was determined by staining roots using a DAB Immunohistochemistry Color Development Kit according to the manufacturer's instructions (Sangon Biotech).

In vivo APase activity staining was detected as follows (Tomscha et al., 2004). Five-day-old seedlings grown for an additional four days in MS and Pi deficiency medium were transferred to 0.1 % (w/v) 5-bromo-4-chloro-3-indolyl phosphate solution and incubated at 37 °C for 30 min. After clearance in 70 % (w/v) alcohol for 4 h, roots were captured using the Olympus microscope.

To determine Pi content, seven-day-old seedlings germinated on 1/2 MS (Murashige and Skoog Basal medium) were transferred to the modified Hoagland solution (1 mM KNO_3, 1 mM $CaNO_3$, 0.4 mM $MgSO_4$, 0.2 mM $NH_4H_2PO_4$, 3 μM H_3BO_3, 1 μM $(NH_4)_6Mo_7O_{24}$, 0.4 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 20 μM Fe(III)-EDTA) for two weeks. Seedlings were then transferred to control and LP solutions (final Pi concentration, 5 μM) for seven days, and shoots and roots were harvested for Pi content analysis using ascorbate-molybdate-antimony methods (Liu et al., 2015; Zheng et al., 2019).

GUS analysis

For construction of *proCBL1:GUS*, a 1991-bp promoter region of *CBL1* was amplified and fused to a pCAMBIA1381 vector containing the *uidA* gene encoding β-glucuronidase (GUS) and transformed into wild type by the *Agrobacterium*-mediated floral dip method. For GUS staining, T3 homozygous transgenic lines were used. Seedlings were immersed in staining buffer (O'BioLab, Beijing) and then incubated at 37 °C for 6 h. Stained samples were subjected to gradient ethanol (20 %, 35 %, 50 %, and 70 %) to remove chlorophyll. Samples were observed with a microscope (MZ10 F, Olympus).

2.3. Quantitative real-time PCR analysis

For expression analysis of *CBL1* in response to Pi deficiency, seeds were grown on Petri dishes containing Pi-sufficient (1/2 Murashige and Skoog Basal medium, Sigma) medium for seven days, and then seedlings were transferred to 1/2 MS and Pi-deficient medium for three days. Total RNA was extracted using TRIzol reagent (TIANGEN). The first-strand cDNA was synthesized by 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. *ACTIN2* was used as an internal control. The primers used for qRT-PCR are listed in Table S1.

2.4. Statistical analysis

Statistical significance of differences between mean values was determined using Student's *t* test. Different asterisks against error bars of histograms are used to indicate means that are statistically different at $P < 0.05$.

3. Results

3.1. The cbl1 mutant is tolerant to Pi deficiency and sensitive to arsenate

To determine whether CBL family proteins are involved in Pi deficiency signaling transduction, we implemented a reverse genetic

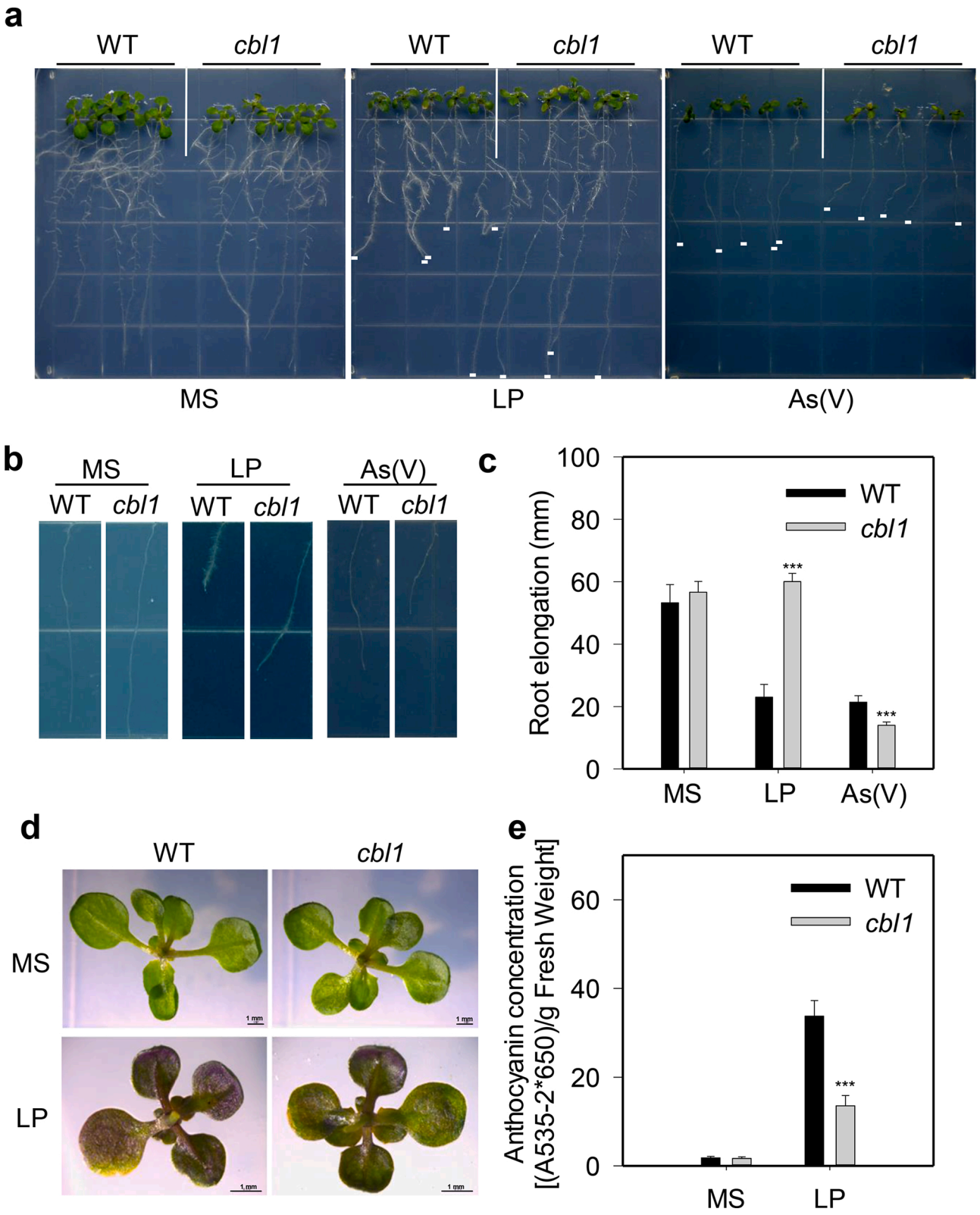

Fig. 1. The *cbl1* mutant is tolerant to Pi deficiency and sensitive to arsenate. **a** Phenotype of WT and *cbl1* mutant under MS, LP and As(V) medium. Seeds were germinated on 1/2 MS agar medium for five days, then seedlings were transferred to 1/2 MS, Pi deficient medium (50 μM), and 1/2 MS + As(V) (500 μM) for another seven days. **b** Root hair development in WT and *cbl1* mutant under Pi deficient and As(V) medium. **c** Statistics on root elongation of seedlings as in a. **d** Leaf color of WT and *cbl1* mutant under MS and Pi deficient medium. Bars: 1 mm. **e** Anthocyanin concentration of WT and *cbl1* mutant under MS and Pi deficient medium (50 μM). The data are representative of three independent experiments. Results are expressed as the means ± SD. Asterisks indicate significant differences between the *cbl1* mutant and WT (Student's *t*-test: ***, $P < 0.001$).

strategy to screen genes encoding CBLs for T-DNA insertion mutants (*cbl1*, *cbl2*, *cbl3*, *cbl5*, *cbl7*, and *cbl9*) under Pi deficiency conditions (Fig. S1). Strikingly, we found that the *cbl1* knockout mutant displayed a significantly tolerant phenotype under Pi deficiency conditions (LP, 50 μM), with much longer roots, fewer and shorter root hairs, and lower anthocyanin content relative to wild-type (WT) plants, while root elongation of the *cbl1* mutant was similar to that of WT plants grown on 1/2 MS medium (Fig. 1). As arsenate As(V) has chemical properties that resemble those of phosphate, it can readily enter plant cells via phosphate transporters (Shin et al., 2004; Catarecha et al., 2007). Thus, we treated *cbl1* mutant with high As(V) concentrations and found that the *cbl1* mutant exhibited a more sensitive phenotype in response to As(V) stress than did WT plants, with 35 % less root elongation (Fig. 1a,c). These results indicated that the *cbl1* mutant was more tolerant to Pi deficiency stress and more sensitive to arsenate treatment than WT plants.

3.2. Total Pi accumulation enhanced by CBL1 mutation

To examine whether the *CBL1* mutation influences Pi accumulation, we grew WT and *cbl1* mutant seedlings in 1/5 modified Hoagland solution for two weeks and then transferred the seedlings to either Pi-sufficient (Control) or Pi-deficient (LP, 5 μM) Hoagland solution for another week. The Pi content of the shoots and roots was measured and analyzed. Pi content accumulation in *cbl1* mutant shoots was higher than that in the WT under the control conditions, and both the shoots and roots Pi content of the *cbl1* mutant were significantly elevated under LP conditions compared with the control conditions (Fig. 2). Thus, the *cbl1* mutant exhibited enhanced Pi uptake.

3.3. CBL1 expression is upregulated by Pi deficiency

To obtain insight into whether CBL1 is involved in Pi deficiency signaling, the levels of *CBL1* expression in the shoots and roots of plants under 1/2 Murashige and Skoog Basal medium (MS) and LP conditions were determined by real-time qRT-PCR. We found that *CBL1* was expressed ubiquitously in shoot and root tissues, and the expression of *CBL1* increased significantly both in shoots and roots under Pi deficiency conditions (Fig. 3a). To determine the tissue-specific expression of *CBL1*, we fused an approximately 2.0-kb *CBL1* promoter to the *uidA* gene encoding GUS and then introduced the construct into WT plants through *Agrobacterium*-mediated transformation. GUS staining of homozygous lines revealed tissue-specific differences in GUS activity between treatments. In the shoots, strong GUS activity was observed in leaves, including within the hypocotyl zone; in the roots, GUS expression was confined to the central vascular tissue in mature zones and root tips under MS medium, with GUS staining enhanced under the Pi deficiency treatment (Fig. 3b). To confirm the induction of *CBL1* expression under Pi deficiency, we also conducted the GUS staining on plants grown under 0, 1, 10, and 100 μM Pi conditions (Fig. S2). Collectively, these results showed that *CBL1* gene expression was regulated by Pi availability.

3.4. Transcription of PSI genes promoted by CBL1 mutation

To cope with Pi deficiency, plants have evolved a series of Pi starvation response processes that regulate Pi transporter activity at the transcriptional and/or post-transcriptional level to maintain Pi homeostasis. Pi deficiency significantly induces the expression of multiple phosphate starvation-induced (*PSI*) genes, including phosphate transporters (*PHTs*), *AtIPS1*, and *AtRNS1* (Bari et al., 2006; Martin et al., 2010; Lin et al., 2013; Sun et al., 2016). We performed qRT-PCR to measure transcription levels of transporter genes in WT and *cbl1* mutant plants. Thus, Pi deficiency stress induced expression of *PHT1s*, especially in the *cbl1* mutant; *PHT1;4* and *PHT1;5* gene expression was considerably elevated in *cbl1* mutant compared to WT plants. The expression levels of the Pi starvation-induced genes *IPS1* and *RNS1* were also markedly increased in the *cbl1* mutant under Pi deficiency (Fig. 3c). These findings may suggest that *CBL1* negatively regulates the Pi deficiency response.

3.5. Phenotype of CBL1 complementation and overexpression lines

To confirm that CBL1 is involved in Pi deficiency signal transduction, we conducted a complementation test on the *cbl1* mutant by introducing a WT *CBL1* gene comprising an approximately 2.0-kb promoter and full genomic sequence of *CBL1* into the *cbl1* mutant. The *CBL1* transcript was not detectable in the *cbl1* mutant, but the transcript was restored to the WT level in the complementation lines *COM#1*, *COM#2*, and *COM#3*, as shown by RT-PCR (Fig. 4a) The tolerant phenotype of the *cbl1* mutant was fully restored in the *cbl1* mutant in the T3 generation of the complementation lines under Pi deficiency conditions (Fig. 4b,c).

Meanwhile, we also generated *CBL1* overexpression lines by introducing the coding sequence of *CBL1* into the *pCAMBIA1307* vector. The T3 overexpression lines were determined by qRT-PCR, and the *CBL1* transcript level in the transgenic lines *OE#3*, *OE#5*, and *OE#6* was at least five-fold higher than WT (Fig. 4d). Phenotypic analysis demonstrated that the root growth was significantly inhibited in *OE#3*, *OE#5*, and *OE#6* compared with WT (Fig. 4e,f). These results indicated that CBL1 shapes the root system architecture under Pi deficiency.

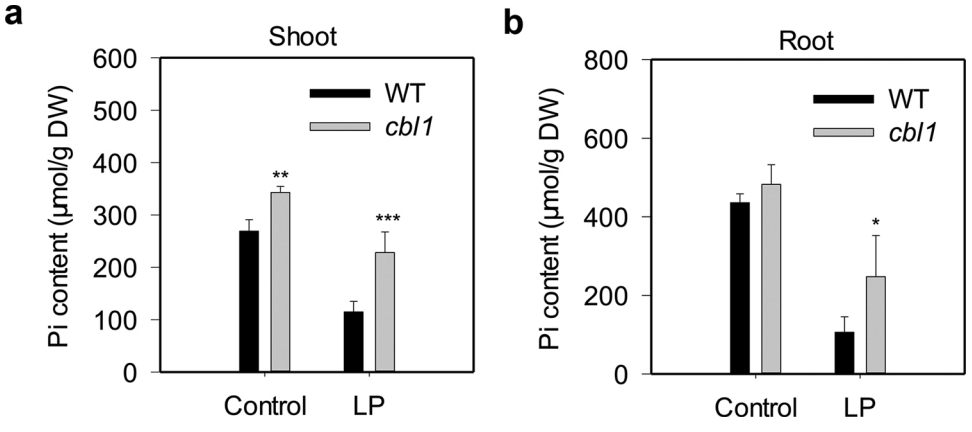

Fig. 2. Mutation of *CBL1* enhances total Pi accumulation. **a** Total Pi content in shoots of WT and *cbl1* mutant under control and Pi deficient conditions. Seedlings were cultured in Hoagland solution (control, Pi sufficient) for two weeks, then seedlings were transferred to control and Pi deficient solution (5 μM) for another seven days, then total Pi content was measured. **b** Total Pi content in roots of WT and *cbl1* mutant under control and Pi deficient conditions. Seedlings were cultured in Hoagland solution (Control, Pi sufficient) for two weeks, then seedlings were transferred to control and Pi deficient solution (5 μM) for another seven days, then total Pi content was measured. The data are representative of three independent experiments. Results are expressed as the means ± SD. Asterisks indicate significant differences between *cbl1* mutant and WT (Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$).

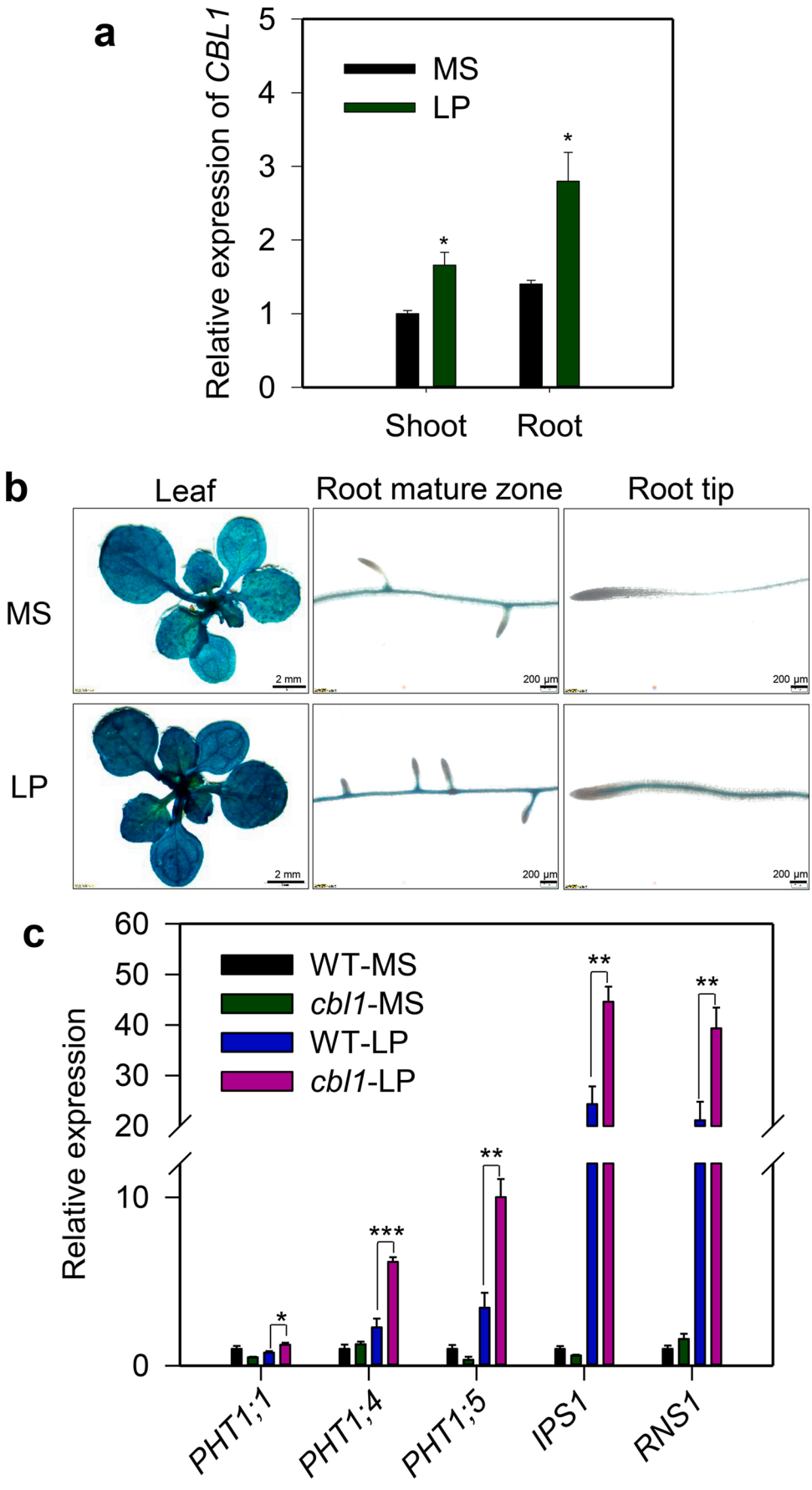

Fig. 3. *CBL1* mutation causes increased transcription of *PSI* genes. **a** Expression of *CBL1* in response to Pi deficiency. qRT-PCR analysis of *CBL1* expression from shoots and roots of wild-type seedlings. Transcript level of *CBL1* was quantified relative to *ACTIN2*. Values represent means ± SD of biological replicates. **b** Tissue-specific expression pattern of *CBL1* in response to Pi deficiency. 1991 bp of *CBL1* promoter was fused with *uidA* gene encoding GUS and transformed into WT plants for GUS analysis. **c** Relative expression of *PHT1;1*, *PHT1;4*, *PHT1;5*, *IPS1*, and *RNS1* gene. Transcript level of different genes was quantified relative to *ACTIN2*. Results are expressed as the means ± SD. Asterisks indicate significant differences (Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$).

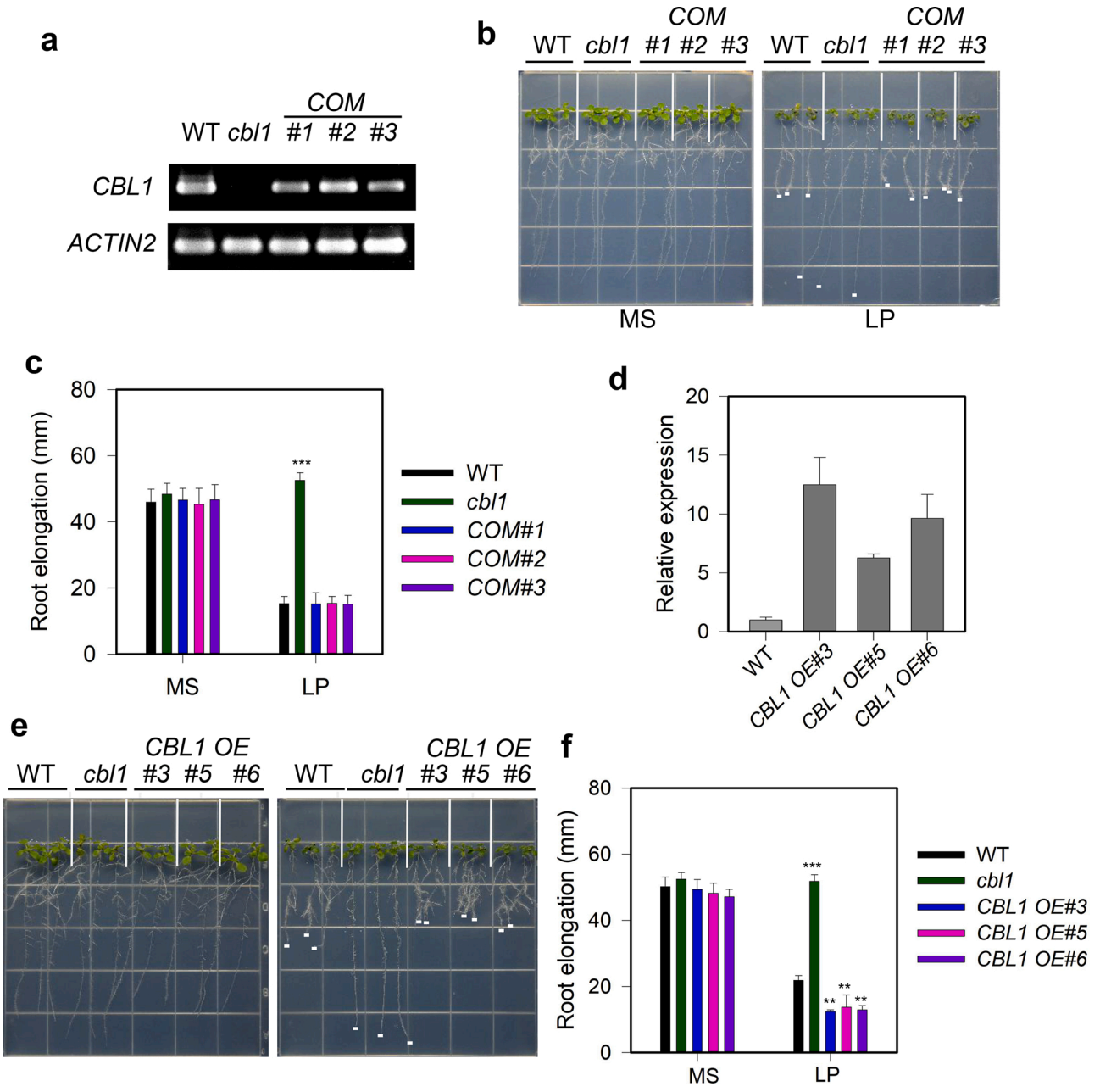

Fig. 4. Phenotypic analysis of *CBL1* complementation and overexpression lines. **a** RT-PCR analysis of *CBL1* and *ACTIN2* mRNA levels in WT, *cbl1* mutant, and three complementation lines transformed with *CBL1* genomic DNA (*COM#1 COM#2* and *COM#3*). **b** Phenotype of WT, *cbl1* mutant, and complementation lines under MS and LP medium. **c** Statistics on root elongation of seedlings as in b. **d** Expression level of *CBL1* in transgenic overexpression lines (*OE#3*, *OE#5* and *OE#6*) by qRT-PCR. **e** Phenotype of WT, *cbl1* mutant, and *CBL1 OE#3*, *OE#5, OE#6* under MS and LP medium. **f** Statistics on root elongation of seedlings as in e. Values represent means ± SD of biological replicates. Asterisks indicate significant differences (Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$).

3.6. Mutation of CBL1decreases acid phosphatase activity and hydrogen peroxide

As Pi deficiency can induce additional physiological responses, including increased acid phosphatase (APase) activity in roots (Del Vecchio et al., 2014), we determined the impact of the *CBL1* mutation on APase activity. On the Pi-deficient medium, roots of WT seedlings exhibited higher levels of staining, indicating that APase activity was stimulated as expected. In contrast, less staining of APase activity was observed in the *cbl1* mutant, while there was stronger staining in the *CBL1 OE#3* line (Fig. 5a,b). Thus, the decreased APase of *cbl1* mutant may be a feedback regulation result, but not the reason of Pi increase.

Reactive oxygen species (ROS) play a key role in many metabolic processes in plants. The ROS content of roots increases rapidly under Pi deficiency, and the ROS distribution changes associated with Fe accumulation may determine the architecture of root systems under Pi-deficient conditions (Chiou and Lin, 2011; Ham et al., 2017; Zheng et al., 2019). To investigate whether increased root elongation in low-Pi medium was induced by altered ROS concentrations, we performed *in situ* DAB staining to measure H_2O_2 accumulation in root tips. There was no difference in DAB staining intensity among the root tips of WT, *cbl1* mutant, and *CBL1 OE#3* plants grown on the 1/2 MS medium; however, under Pi deficiency conditions, the DAB staining signal in the *cbl1* mutant was significantly lower than that in the WT plants, suggesting that H_2O_2 concentrations were reduced in the *cbl1* mutant, but there was no difference between the *CBL1 OE#3* line and WT plants (Fig. 5c, d).

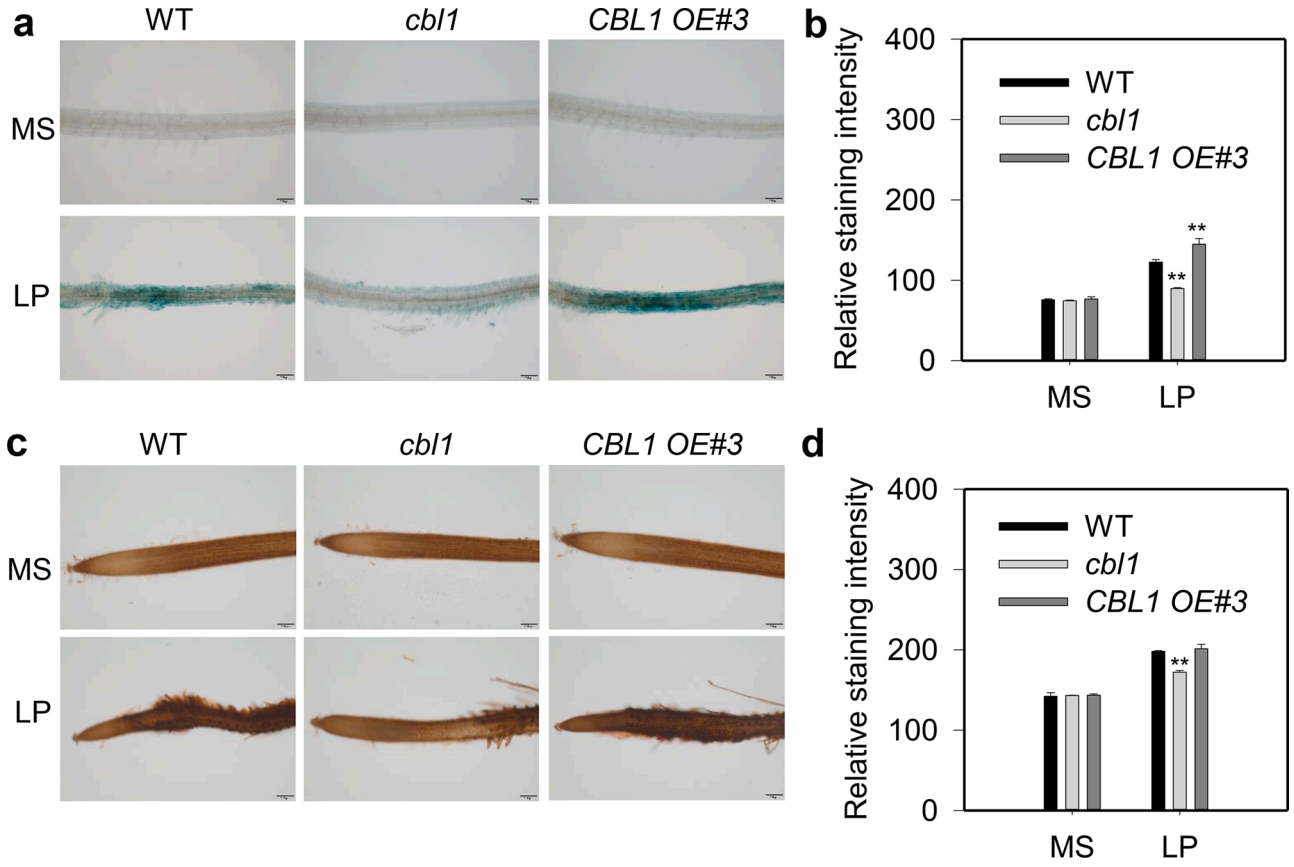

Fig. 5. *CBL1* mutation leads to decreased acid phosphatase activity and hydrogen peroxide accumulation. **a** Acid phosphatase (APase) activity staining in root tips of the WT, *cbl1* mutant and *CBL1 OE#3* line under MS and LP medium. Seeds were germinated on 1/2 MS agar medium for five days, then seedlings were transferred to 1/2 MS and Pi deficient medium for another four days, then APase activity staining was performed. **b** Relative staining intensity of APase activity was determined by Image J software. **c** DAB staining for hydrogen peroxide (H_2O_2) in root tips of the WT, *cbl1* mutant, and *CBL1OE#3* line under MS and LP medium. Seeds were germinated on 1/2 MS agar medium for five days, then seedlings were transferred to 1/2 MS and Pi-deficient medium for another four days, then DAB staining was performed. **d** Relative staining intensity of DAB was determined by Image J software. The data are representative of three independent experiments. Results are expressed as the means ± SD. Asterisks indicate significant differences between *cbl1* mutant and WT (Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$).

Available evidence demonstrated that auxin plays an essential role in mediating root system architecture under phosphate deficiency (Pérez-Torres et al., 2008; Bhosale et al., 2018). The primary root growth of the WT, *cbl1* mutant, and *CBL1 OE* lines in response to IAA supplementation was then characterized. Results demonstrated that the Pi deficiency phenotype among WT, *cbl1* mutant, and *CBL1 OE* transgenic lines was disappeared, indicating that the response of root system architecture of CBL1 in response to Pi deficiency may be involved in the auxin signal pathway (Fig. S3).

4. Discussion

Pi is a macronutrient that is important for plant growth and development. However, the amount of available Pi in soil for plant growth is often limited, and about 70 % of global soil suffers from Pi deficiency, which has become a major factor limiting sustainable crop production (Chiou and Lin, 2011; Xu et al., 2019). Pi deficiency causes changes in plant root system architecture modifications, such as the inhibition of main root elongation and increased density and length of lateral roots and root hairs, thereby increasing the absorption area of Pi in plants. Pi deficiency also induces the secretion of organic acids and acid phosphatases from roots, thus increasing the concentration of Pi in the rhizosphere (Ticconi et al., 2009; Lopez-Arredondo et al., 2014). To cope with Pi deficiency, plants have evolved different strategies to balance Pi availability, including modulating the root system architecture, thereby enhancing the capacity to uptake Pi and mobilize Pi from intracellular stores (Muller et al., 2015; Kanno et al., 2016; Balzergue et al., 2017).

Calcium (Ca^{2+}), as an essential intracellular secondary messenger in plants, plays important roles in the response to environmental stimuli (Poovaiah and Reddy, 1993). Extensive evidence has demonstrated that multiple abiotic stresses, including cold, heat, salt, drought, and light, can trigger the elevation of cytoplasmic Ca^{2+} concentrations (Kudla et al., 2010; Liao et al., 2017). Ca^{2+} signals are in turn decoded by CBLs, CaMs, or CDPKs, which could lead to modulations of downstream transcriptional and phosphorylation events (Poovaiah and Reddy, 1993; Luan, 2009). CBL-CIPK complexes are involved in the homeostasis of various ions, including K^+, Na^+, Mg^{2+}, NH_4^+, and NO_3^- (Zhu, 2016; Kudla et al., 2018), but little is known about the function of the CBL-CIPK complex in the Pi balance of plant cells. In the present study, we have described a novel function of CBL1 in negatively regulating Pi homeostasis under Pi deficiency, and this novel discovery opens a new avenue for exploring molecular mechanisms of Pi homeostasis and tolerance in plants.

Given the extensive evidence that CBL-CIPK complexes act as master regulators in plants responding to perturbations of homeostasis for various ions, we initiated this work by assessing tolerance to Pi deficiency of *cbl*$_s$ mutants (*cbl1*, *cbl2*, *cbl3*, *cbl5*, *cbl7*, and *cbl9*) through a reverse genetic screen. Only the *cbl1* mutant plants were significantly tolerant to Pi deficiency compared with WT plants, with much longer roots, decreased APase activity, and lower anthocyanin concentrations (Fig. 1 and Fig. 5a,b). In general, CBLs interact with their interacting CIPK proteins to form complexes and phosphorylate downstream substrates (Luan, 2009). Unfortunately, in screening candidate CIPKs that may be involved in CBL1-mediated Pi deficiency signaling, no *cipk*$_s$

mutants showed a tolerant phenotype in response to Pi deficiency, indicating the possible functional redundancy of 26 CIPKs in *Arabidopsis* (Li et al., 2009). As(V) is a chemical analog of phosphate (Pi), and it can disrupt Pi-dependent metabolism (Tu and Ma, 2003; Wu et al., 2011). The *cbl1* mutant exhibited a more sensitive phenotype upon As(V) treatment (Fig. 1a,b), which was consistent with the antagonistic relationship between Pi and As(V) for the same transporters in the plasma membrane, implying that CBL1 regulates the transcription or transporter activities of *PHT1* genes through interaction with unknown CIPKs in vivo. To determine whether the tolerant phenotype of the *cbl1* mutant was related to increased Pi uptake, we assayed the Pi content of shoots and roots of WT and *cbl1* mutant plants under control and LP conditions, finding that shoot Pi content was significantly higher in *cbl1* mutant plants even under control conditions. However, under LP conditions, both shoot and root Pi content were remarkably increased in *cbl1* mutant plants, indicating that the *CBL1* mutation could lead to Pi over-accumulation in plants (Fig. 2). The tolerant phenotype of *cbl1* mutant plants was fully rescued in the transgenic complementation lines under the LP treatment, indicating the *CBL1* mutation was responsible for the tolerant phenotype under Pi deficiency (Fig. 4a-c). In contrast, *CBL1* overexpression lines showed inhibited root growth under Pi deficiency (Fig. 4d-f).

To obtain insight into the response of CBL1 to Pi deficiency, we analyzed the *CBL1* transcription level in the WT background, where Pi deficiency significantly induced the *CBL1* expression (Fig. 3a). Tissue-specific GUS expression analysis revealed that CBL1 was highly expressed in leaves and vascular tissues in root mature zones and root tips; GUS activity was obviously higher under Pi deficiency, which was consistent with the mRNA expression results (Fig. 3b, Fig. S2). Considering the increased root elongation of the *cbl1* mutant under Pi deficiency, we suggested that mutation of *CBL1* may influence Pi uptake or translocation in plants. The *cbl1* mutant accumulated more total Pi than did WT plants in Pi-deficient media (Fig. 2). Furthermore, qRT-PCR analysis showed that not only was the expression of the main phosphate transporters (PHT1;1, PHT1;4, PHT1;5) remarkably increased, but also *PSI* genes such as *IPS1* and *RNS1* were elevated in the *cbl1* mutant under Pi-deficient conditions (Fig. 3c). These results demonstrated that CBL1 may function as a negative regulator in regulating Pi deficiency response, and the *CBL1* mutation could induce Pi accumulation in plants. In addition, we found the activity of APase was lower in the *cbl1* mutant than in WT seedlings (Fig. 5a,b); this may be a feedback regulation result of Pi increase.

Reactive oxygen species (ROS) act as essential signaling molecules in regulating root growth and development in response to Pi deficiency (Tsukagoshi, 2016). Thus, we performed DAB staining to observe the H_2O_2 accumulation in primary root tips. There was no difference in the accumulation of DAB between WT and *cbl1* mutant seedlings on 1/2 MS medium, but DAB accumulation was obviously decreased in the *cbl1* mutant grown on Pi-deficient medium (Fig. 5c,d).

In summary, we present evidence that the *CBL1* mutation can greatly alleviate the severe root inhibition defects that occur under Pi deficiency, thus producing a tolerant phenotype, indicating that CBL1 may function as a novel negative regulator of Pi homeostasis in plant cells. However, the underlying molecular mechanism through which CIPKs or target proteins of CBL1 negatively regulate the Pi deficiency signaling pathway remains to be demonstrated. Our future research will focus on the identification of CBL-interacting CIPKs or other target proteins that respond to Pi deficiency, exploring the important role of Ca^{2+} in the phosphate signaling pathway, in an attempt to lay the theoretical foundation for molecular breeding of crops with better Pi utilization efficiency.

5. Conclusion

CBL1 may function as a novel negative regulator of Pi homeostasis in plant cells under Pi deficiency, thus facilitating physiological adaptation of plants to constantly changing soils.

Author contributions

This research was designed by Wang C and Gao H. Gao H, Wang C, Li L, Fu D, Zhang Y, Yang P, and Zhang T performed the experiments. Gao H and Wang C analyzed the data. Gao H and Wang C wrote the manuscript with comments from all authors.

CRediT authorship contribution statement

Huiling Gao: Methodology, Investigation, Validation, Data curation. **Chuanqing Wang:** Conceptualization, Supervision, Writing - review & editing, Methodology, Investigation, Validation, Data curation. **Lili Li:** Methodology, Investigation, Validation, Data curation. **Dali Fu:** Methodology, Investigation, Validation, Data curation. **Yanting Zhang:** Writing - review & editing, Data curation. **Peiyuan Yang:** Writing - review & editing, Data curation. **Tianqi Zhang:** Writing - review & editing, Data curation. **Cun Wang:** Conceptualization, Supervision, Writing - review & editing, Methodology, Investigation, Validation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2020.153266.

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