**CIPK23 is involved in iron acquisition of Arabidopsis by affecting
ferric chelate reductase activity**

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Running head: CBL-CIPK and iron nutrient

Abbreviations: CBL, calcineurin B-like; CIPK, CBL-Interacting Protein Kinase;
FCR, ferric chelate reductase; FIT1, FER-like Iron Deficiency Induced Transcription
Factor; FRET, fluorescence resonance energy transfer.

ABSTRACT

Iron deficiency is one of the major limiting factors affecting quality and production of crops in calcareous soils. Numerous signaling molecules and transcription factors have been demonstrated to play a regulatory role in adaptation of plants to iron deficiency. However, the mechanisms underlying the iron deficiency-induced physiological processes remain to be fully dissected. Here, we demonstrated that the protein kinase CIPK23 was involved in iron acquisition. Lesion of *CIPK23* rendered *Arabidopsis* mutants hypersensitive to iron deficiency, as evidenced by stronger chlorosis in young leaves and lower iron concentration than wild-type plants under iron-deficient conditions by down-regulating ferric chelate reductase activity. We found that iron deficiency evoked an increase in cytosolic Ca^{2+} concentration and the elevated Ca^{2+} would bind to CBL1/CBL9, leading to activation of CIPK23. These novel findings highlight the involvement of calcium-dependent CBL-CIPK23 complexes in the regulation of iron acquisition. Moreover, mutation of *CIPK23* led to changes in contents of mineral elements, suggesting that CBL-CIPK23 complexes could be as “nutritional sensors” to sense and regulate the mineral homeostasis in *Arabidopsis*.

Keywords: *Arabidopsis*, iron acquisition, ferric chelate reductase, CIPK, cytosolic Ca^{2+} concentration

Introduction

Iron (Fe) is an essential micronutrient for plant growth and development. Although iron content in soil is high, its bioavailability to plants is often limited, especially in calcareous soils where iron often occurs as insoluble ferric-hydroxides because of the bicarbonate and high soil pH [1, 2]. To cope with iron deficiency, plants have evolved various strategies. These strategies are classified as strategy I in nongraminaceous monocots and dicots, and strategy II in graminaceous monocots [3]. The strategy I mechanism of iron acquisition is reduction-based, including those processes of rhizospheric acidification by up-regulating H^+ -ATPase, reduction of ferric iron to ferrous iron by root ferric chelate reductase (FCR) and up-regulation of high-affinity transporter for ferrous iron [3]. In addition to the physiological responses, strategy I plants can also modify their root system architecture to maximize the acquisition of iron [2, 4]. Iron is transported as iron-citrate complex to shoots thorough the xylem [5, 6]. Transport of iron in the phloem occurs as iron-nicotianamine [7, 8]. Despite extensive studies on iron uptake and translocation in plants [7-11], the mechanisms by which plants sense and respond to iron deficiency remain to be fully elucidated.

The molecular mechanisms underlying the response of strategy I plants to iron deficiency are being unraveled [12-16]. Many genes involved in iron reduction, uptake and translocation have been identified. For instance, among the 12 H^+ -ATPases isoforms in Arabidopsis, AHA2 is the main form responsible for iron deficiency-induced rhizospheric acidification [17]. In Arabidopsis, *FRO2* and *IRT1*

65 encoding plasma membrane FCR and iron transporter have been identified to
66 underpin iron reduction and uptake by plants [18-20]. A FER-like Iron Deficiency
67 Induced Transcription Factor (FIT1) in Arabidopsis belonging to the bHLH
68 transcription factor family is involved in sensing and responding to iron deficiency by
69 regulating expression of *FRO2* and *IRT1* [21]. The bHLH transcription factors
70 function as heterodimers to regulate downstream targets [22]. Up to now, several
71 other genes have been discovered to drive expression of genes involved in plant
72 response to iron-deficiency [23, 24]. Post-transcriptional regulation of *FRO2* and
73 *IRT1* may also be involved in the regulation of iron uptake, because iron deficiency
74 can also induce accumulation of *IRT1* protein and increase FCR activity in transgenic
75 Arabidopsis over-expressing *IRT1* and *FRO2* [25, 26]. Monoubiquitin-dependent
76 endocytosis, trafficking of *IRT1* and the E3 ubiquitin ligase are involved in *IRT1*
77 degradation [15, 27, 28].

78
79 Several plant hormones and messenger molecules have been identified to be
80 associated with response of plants to Fe deficiency. These include ethylene [29, 30],
81 auxin [31], cytokinin [32], nitric oxide (NO) [32, 33], abscisic acid [34] and
82 brassinosteroids [35]. Cytosolic Ca^{2+} is an important signaling molecule to transduce
83 developmental and environmental cues to activate physiological processes in plants
84 [36, 37]. Crosstalk among plant hormones and Ca^{2+} signal has been demonstrated. For
85 instance, both ethylene [38] and nitric oxide (NO) [39] can induce an increase in
86 cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$). Enhanced production of ethylene [29] and NO

[31] has been observed in response to iron deficiency. It is conceivable that changes in $[Ca^{2+}]_c$ may also play a regulatory role in the response to iron deficiency. However, there has been no study to evaluate the role of $[Ca^{2+}]_c$ in plant response to iron deficiency.

An elevation of $[Ca^{2+}]_c$ often acts as a signal to activate downstream events, including diverse array of phosphorylation cascades and expression of genes [36, 37, 40, 41]. Among the $[Ca^{2+}]_c$ -induced downstream components, calcineurin B-like (CBL) proteins are referred to as specific calcium sensors in plants [42, 43]. Ca^{2+} -bound CBLs activate their target proteins, CBL-Interacting Protein Kinase (CIPK), to initiate downstream responses [40, 44-46]. In Arabidopsis, 10 CBL-type calcium sensor proteins that form an interaction network with 26 CIPKs have been reported [47-49]. Recently, a novel mechanism by which activity of CBL-CIPK complexes is regulated has been dissected [50]. CBL-CIPK complexes are involved in mediation of plant response to environmental cues, including drought [51], cold [51, 52], salinity [53, 54] and osmotic stress [55]. In addition to the abiotic stresses, the involvement of CBL-CIPK network in regulation of uptake and translocation of mineral elements is also reported. For instance, a complex formed by SOS3 (CBL4) and SOS2 (CIPK24) mediates Na^+ transport by activating membrane-localized Na^+/H^+ antiporter [56]. CBL1 and CBL9 recruit the kinase CIPK23 to regulate K^+ uptake into roots by phosphorylating the K^+ channel AKT1 [57, 58]. CIPK23 also involves sensing nitrate by phosphorylating nitrate transporter CHL to modulate the affinity of CHL to nitrate

[59]. Moreover, CIPK11 negatively regulates plasma membrane proton ATPase in *Arabidopsis* by phosphorylating the C-terminal regulatory domain of AHA2, preventing its interaction with 14-3-3 proteins [60]. The dependence of H⁺-ATPase on CIPK may affect acquisition of nutrients by plants since changes in activities of H⁺-ATPase can directly alter pH in the rhizosphere, which in turn affects availability of nutrients such as iron, phosphorus and manganese in the rhizosphere. However, there has been no report on the involvement of CBL-CIPK complex in acquisition of these nutrients so far.

Here we report the physiological function of CIPK23 in the mediation of *Arabidopsis thaliana* to iron-deficiency-response. We show that the loss of either *CIPK23* or *CBL1/CBL9* function renders the mutant plants sensitive to iron deficiency. We find that iron deficiency-induced an increase in [Ca²⁺]_c in elongation and root-hair zone of roots, a main region of iron absorption. *lks* mutants that result from mutation of *CIPK23* exhibit lower activity of FCR and are more sensitive to iron deficiency compared to wild-type plants.. We further demonstrate that the regulation of FCR activity by CIPK23 is not achieved by regulating the expression of *FRO2*, *FRO3* and *FRO5* primarily expressed in roots. Taken together, these findings highlight that calcium-dependent CBL-CIPK pathway is involved in response to iron deficiency in *Arabidopsis*.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as wild-type (WT) in the present study. Mutants of *lks* (*lks1-2*, *lks1-3*) and *cbl1cbl9* were kindly provided by Professor Wei-Hua Wu (China Agricultural University, China). These mutants have been well characterized previously [57]. To acquire transgenic materials, the coding region of *CIPK23* was amplified using primers and cloned into the pPLV27 vector [61] for sequence verification. The plasmids of pPLV27-CIPK23 and pSOUP were transferred into *Agrobacterium* (strain C58) and *Arabidopsis* transformation was carried out by the floral dip method (Clough and Bent 1998). The triple mutant of *lks1-3cbl1cbl9* was obtained by crossing *lks1-3* with *cbl1cbl9* and the homozygous lines were identified by RT-PCR. The primers used in these experiments were listed in Table S2.

Seeds were surface-sterilized by incubation for 1 min in 75% ethanol, and rinsed with sterile distilled water, followed by 15 min in 10% (v/v) sodium hypochlorite, and then washed with sterile water. The sterilized seeds were germinated and grown on half-strength Murashige and Skoog (1/2 MS) for 5 d. The germinated seedlings were transferred to agarose medium (pH 5.8) containing varying Fe concentrations (50 μ M (+Fe), 0.5 μ M Fe³⁺ (-Fe) with 0.8% sucrose and 0.7% (w/v) agarose. Concentrations of other mineral nutrients were as followed: 3 mM MgCl₂, 0.25 mM (NH₄)₂SO₄, 1 mM Ca(NO₃)₂, 2 mM KCl, 2.75 mM CaCl₂, 0.18 mM KH₂PO₄, 50 μ M H₃BO₃, 50

153 $\mu\text{M MnSO}_4$, 15 $\mu\text{M ZnSO}_4$, 0.05 $\mu\text{M CuSO}_4$, 0.5 $\mu\text{M Na}_2\text{MoO}_4$, 0.05 $\mu\text{M CoCl}_2$. Iron
154 was supplied as Fe-EDTA. Seedlings were grown in plastic dishes for 4 d, oriented
155 vertically, in a controlled environment with a temperature 22-25°C, 14 h/10 h light
156 cycle and photosynthetic photon flux density of 100-120 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

157 2.2. Ferric chelate reductase enzymatic assays

158 Ferric chelate reductase activity in roots was determined according to protocols
159 described by Waters et al. [30]. Briefly, intact plants treated with iron deficiency were
160 rinsed with water and then transferred to 2 ml Fe(III) reduction assay solution for 1 h.
161 The assay solution consisted of nutrient solution without micronutrients, 100 μM
162 Fe^{3+} -EDTA and 100 μM ferrozine (pH 5.0). The FRO activity was determined by
163 measurement of the absorption at 562 nm of the Fe^{2+} -ferrozine complex formed with
164 a spectrophotometer.

166 2.3. Determination of proton concentrations on root surface

167 Rhizosphere acidification of plants was detected followed the method reported by
168 Santi and Schmidt [17]. Seedlings treated with iron deficiency were watered and
169 exposed to standard nutrient solution without agarose and sucrose containing the pH
170 indicator bromocresol purple (0.005%) either with or without 50 μM Fe-EDTA for 1 h.
171 Proton contents of root surface were accessed by the change of absorption at 590 nm
172 of the pH indicator bromocresol purple.

174 2.4. Measurement of mineral element contents

Plants were desorbed with 5 mM EDTA for 5 min and rinsed for three times with deionized water. Samples were oven-dried at 80°C for 2 d. Plant materials were digested with completely (3–4 h) in 70% (v/v) HNO₃ at 130°C in high pressure tanks. Concentrations of mineral elements including Fe, Mn, Zn, K, Ca, Mg and P were measured with ICP-OES.

2.5. Determination of chlorophyll and root morphology

Chlorophyll (Chl) concentrations were determined as previously described method [35]. Leaves were excised and weighed, then extracted in aqueous acetone (80%, v/v). The extract solutions were measured in 663 nm and 645 nm with a spectrophotometer (Rio-Rad, USA). Total Chl concentration (mg/L) was calculated as $8.02A_{663} + 20.21A_{645}$. The length of primary and lateral roots was measured with a ruler. The number of lateral roots that were greater than approx. ≥ 0.5 mm in length was recorded. Lateral root density is defined as the ratio of secondary root number to primary root length. At least 10 independent replicates were used for each treatment.

2.6. Analysis of gene expression

Real-time PCR was used to study the expression patterns of genes responsible for iron uptake and transport. Roots were ground in liquid nitrogen and total RNA was extracted using RNAiso Plus reagent (TaKaRa). First-strand cDNAs were synthesized from 0.5 µg of total RNA with PrimeScript RT reagent Kit (TaKaRa) which can remove contaminating genomic DNA. Quantitative real-time PCR was performed on

an ABI StepOne Plus instrument. Each reaction contained 5.0 μ l 2 $\times$ UltraSYBR Mixture (With ROX) reagent (Cwbio), 1.5 μ l cDNA samples, and 1.2 μ l of 10 mM gene-specific primers in a final volume of 10 μ l. Thermocycling conditions were 95 °C for 10 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72 °C for 30 s. The primers pairs used for each gene are listed in Supplemental Table 3. Three biological and three technological repeats were performed in RT-PCR. The relative expression level was calculated against that of the internal control gene (*Actin11*) by the comparative C_T method.

2.7. Confocal laser microscopy and measurement of Ca²⁺ concentration in roots

The dynamics of cytosolic calcium Ca²⁺ in roots was measured by fluorescence resonance energy transfer-sensitized emission of the yellow cameleon 3.60 (FRET) described by Rincón-Zachary et al. [63]. Transgenic plants including 35S::YC3.60, 35S::eCFP and 35S::eYFP grown on Fe-sufficient medium were transferred to Fe-deficiency medium for varying periods. Imaging was done with an inverted Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems). Excitation wavelengths were 458 and 514 nm for CFP and YFP, respectively. Single optical sections were taken at a depth of about 30 μ m from the root tip surface. To reduce the effect of the spectra of CFP and YFP overlap, the sensitized emission must be corrected for bleed-through of the emission of the donor (CFP) into the acceptor (YFP) channel and for direct excitation of YFP during the excitation of CFP. An image of the root tip expressing YC3.60 was acquired first. The image was taken after optimizing

settings (pinhole, laser intensity, and photomultiplier gain) for the CFP, FRET, and YFP channels. The images of reference roots (CFP and YFP alone) were obtained using the same settings and used to generate calibration coefficients. FRET efficiency was calculated by the Leica software. At least 15 plants were used for each treatment.

2.8. Western blotting

Root tissues were ground in liquid nitrogen and the protein were extracted by extraction buffer containing 15% glycerol, 6.5% SDS, 0.125 M Tris-HCl, pH 6.8. Samples were centrifuged at 4°C for 15 min at 12 000 g. Protein concentration was determined by the method of Bradford [64] with BSA as a standard. Samples for SDS–PAGE were diluted with 2× sample buffer (125 mM Tris–HCl pH 6.8, 4% SDS, 0.2 M DTT, 20% Glycerol, 0.02% bromophenol blue, 10% β-mercaptoethanol, 0.004%), heated at 95°C for 10 min, and then centrifuged at 12,000g for 5 min. The supernatants were separated by 8% and 12% SDS/polyacrylamide gels and transferred to polyvinylidene fluoride membrane (Immobilon-P; Millipore) by electroblotting, Immunoblot analysis was performed with IRT1 antibodies provided by Professor Kuo-Chen Yeh [65]. Horseradish peroxidase–conjugated anti-mouse secondary antibody (Sangon Biotech) was used to detect the primary antibody, and β-Actin was used as the control protein.

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and the means were

241 compared by Duncan's multiple range test using SAS. Different letters and the "*" on
242 the histograms indicate that the means were statistically different at the $P < 0.05$ level.
243

3. Results

3.1. *lks* mutants are more sensitive to iron deficiency

Previous studies by Xu et al. [57] identified that *LKS* (Low-K-Sensitive) encodes a protein kinase CIPK23. The role of CIPK23 in regulating nutrient uptake and the dependence of plasma membrane H⁺-ATPase on CBL/CIPK complex promoted us to evaluate the role of CIPK23 in iron-nutrition [57-60]. We first compared the phenotypes of WT with the mutants of CIPK23 disfunction (*lks1-2* and *lks1-3*) under iron-sufficient and iron-deficient conditions. Under iron-sufficient conditions, there was no obvious difference between *lks1-2*, *lks1-3* and wild-type (WT) plants (Fig. 1). However, *lks1-2* and *lks1-3* exhibited more chlorosis in young leaves than WT plants when they were grown on iron-deficient medium (Fig. 1). There was a greater reduction in chlorophyll and iron concentrations in *lks1-2* and *lks1-3* than in WT plants by exposure to iron-deficient medium (Fig. 1). These observations that *lks1-2* and *lks1-3* were more sensitive to iron deficiency than WT plants indicate that CIPK23 is involved in iron acquisition.

3.2. *lks* mutants exhibit more lateral roots under iron-deficient conditions

Root morphology plays an important role in adaptation of plants to iron-deficient medium [2, 4]. We first compared the root morphological characteristics of *lks* mutants with those of WT plants. In iron-sufficient medium, there was no significant

difference in primary and lateral root length between WT plants and *lks* mutants (Fig 2). In iron-deficient medium, primary and lateral root length in *lks* mutants were shorter than those in WT plants, while *lks* mutants had greater number of lateral roots than WT plants (Fig. 2). Given that iron uptake mainly occurs in root tips, later root density may be the key factor determining iron uptake. Therefore, these results suggest that the traits of root morphology in *lks* mutants may not be the key factors limiting their iron uptake.

3.3. *lks* mutants display reduced ferric reductase activity

Acquisition of Fe by roots involves acidification of rhizosphere, reduction of Fe^{3+} to Fe^{2+} and transport of Fe^{2+} across plasma membranes of root cells [3]. To explore the mechanisms by which the *lks* mutants accumulate less Fe than in WT plants, we first compared phenotypes of WT with those of *lks* mutants by growing them in low pH medium containing different concentrations of iron. As shown in Figure 3a, a decrease in medium pH did not ameliorate the chlorosis in young leaves of the *lks* mutants. Moreover, proton concentrations on root surface of *lks* mutant were higher than those of WT plants when grown in Fe-deficient medium (Fig 3b). These results indicate that rhizospheric acidification of *lks* does not account for the reduced iron accumulation in the mutants.

The reduction of Fe^{3+} to Fe^{2+} is catalyzed by FCR [66]. To test whether mutation of *lks* affects FCR activity, FCR activities of WT and the two mutants were determined.

A lower FCR activity in roots of the *lks* mutant than in that of WT plants was observed under Fe-sufficient conditions (Fig. 3c). Exposure of WT and mutant plants to Fe-deficient medium led to significant increases in FCR activities in both WT and mutant plants with the increases being less in the mutants than in WT plants (Fig. 3c). These results indicate that CIPK23 may be involved in iron acquisition by regulating FCR activity. Three genes *FRO2*, *FRO3* and *FRO5* primarily expressing in roots are responsible for FCR activity [67]. To further unravel the reasons of the difference in FCR activity between *lks* mutants and WT plants, we compared the expression of the three *FRO* genes in the mutants with that in WT plants by real-time RT-PCR. Our results showed that iron deficiency induced expression of these genes in roots of both *lks1-2*, *lks1-3* and WT plants (Fig. S1). Moreover, a similar iron deficiency-induced up-regulation of *FRO3* and *FRO5* expression in *lks* mutants and WT plants was observed, while *FRO2* expression induced by iron deficiency in *lks1-2* and *lks1-3* mutants was greater than in WT plants (Fig. S1). These results suggest that alteration of FCR activity by CIPK23 may not be achieved by regulating *FRO* gene expression.

IRT1-dependent Fe^{2+} transport is the final step to mediate Fe^{2+} influx into roots in strategy I plants. To test whether the disruption of IRT1-dependent iron transport is responsible for the less accumulation of iron in the *lks* mutants than WT plants, We determined contents of other metal elements such as manganese (Mn) and zinc (Zn) in shoots of WT and the mutants. In addition to transport of iron, IRT1 mediates transport of divalent manganese and zinc into root cells [20]. There was a greater

accumulation of manganese and zinc in *lks* mutants than in WT plants (Table S1), suggesting that IRT1-mediated transport of ferrous iron into root cells is not the rate-limiting step in the uptake of ferrous iron by the *lks* mutants. Moreover, *IRT1* expression and the abundance of IRT1 protein induced by iron deficiency in roots of *lks1-3* were greater than those in roots of WT plants (Fig. S2) also confirms CIPK23 is not involved in mediating Fe²⁺ influx into root cells

3.4. Overexpression and complementation of CIPK23 enhances tolerance to iron deficiency

To further evaluate the role of CIPK23 in iron acquisition, we constructed CIPK23-overexpressing lines and complementation lines, and analyzed the phenotypes of these plants grown on medium supplemented with different levels of iron. The expression of CIPK23 in transgenic lines was markedly up-regulated in transgenic plants (Fig. S3). Overexpression and complementation of CIPK23 conferred transgenic plants tolerance to iron deficiency, as evidenced by less chlorosis, higher concentrations of chlorophyll, iron in shoots, and greater FCR activity in CIPK23-overexpressing and complementation lines than in WT plants under iron-deficient conditions (Fig. 4). These observations corroborate that AtCIPK23 is involved in regulation of iron-deficiency-response.

3.5. CIPK23 upstream CBL proteins are involved in iron nutrient

The CBLs, which are upstream proteins of CIPKs, are involved in plants response to mineral element stress [56-58]. It has been shown that CBL1 and CBL9, directly interact with CIPK23 [57]. To further elucidate the mechanisms underpinning the CIPK23-regulated iron uptake, the phenotypes of *cbl1cbl9* double mutants and WT plants were compared. Similar to *lks1-3*, more evident chlorosis appeared in young leaves of *cbl1cbl9* than those of WT plants when grown on iron-deficient medium (Fig. 5). In addition, *cbl1cbl9* double mutants exhibited reduced chlorophyll and iron concentrations in shoots compared to WT plants (Fig. 5). The triple-mutants *lks1-3cbl1cbl9* did not display any additive sensitivity to iron deficiency compared with *lks1-3* and *cbl1cbl9* (Fig. 5). For instance, the chlorophyll and iron concentrations in shoots of triple-mutants of *lks1-3cbl1cbl9* were consistent with those in *lks1-3* and *cbl1cbl9* under iron-deficient conditions (Fig. 5). These results suggest that the regulation of iron homeostasis by CIPK23 is dependent upon the activity of CBL1/CBL9.

3.6. Iron deficiency evoked an increase in cytosolic calcium concentration in roots

Because CBLs are specific calcium sensors in plants [42] and they activate their target proteins (CIPKs) by cytosolic calcium concentration ($[Ca^{2+}]_c$) [40]. To evaluate the role of the upstream components of CBL1/9 and CIPK23 complex in iron nutrient, we monitored the temporal and spatial changes of cytosolic $[Ca^{2+}]_c$ in primary roots in response to iron deficiency by the method of fluorescence resonance energy transfer

(FRET)-sensitized emission of the yellowameleon 3.60 [63]. An increase in $[Ca^{2+}]_c$ in primary roots was observed after exposure of Arabidopsis plants to iron-deficient medium, and the magnitude of the increase in $[Ca^{2+}]_c$ was positively dependent on the duration of exposure to iron-deficient medium (Fig. 6). Moreover, the iron deficiency-induced increase in $[Ca^{2+}]_c$ occurred predominantly in elongation and root-hair zone (Fig. 6), which is a main region for mobilization and absorption of iron nutrition [20, 26].

4. Discussion

Iron deficiency is one of the important factors limiting crop production in calcareous soils. Plants have evolved a series of strategies, ranging from physiological processes to root morphological changes, to cope with low availability of iron in soil. Numerous signaling molecules and transcription factors have been demonstrated to play a regulatory role in adaptation of plants to iron deficiency [21, 23, 31, 33]. However, the physiological mechanisms underlying the iron deficiency response remain to be fully dissected. CIPK-CBL complexes have been reported to play an important role in regulation of potassium and nitrate transport [57, 59]. In the present study, we demonstrate that iron deficiency evoked an increase in cytosolic Ca^{2+} concentration, which in turn may activate CIPK23 by binding to CBL1 and CBL9. The CBL1/CBL9-CIPK23 complex participates in regulation of iron acquisition by affecting FCR activity in Arabidopsis

There are numerous reports demonstrating protein kinase CIPK23 is involved in regulating response of plants to environmental stresses by interacting with CBLs, such as drought, cold, salinity, osmotic and high pH [51, 55, 60]. In addition, CIPK23 has also been demonstrated to play a regulatory role in acquisition of mineral nutrients of K [57, 58] and nitrate [59]. In the present study, we identify that CBL1/CBL9-CIPK23 was also involved in iron acquisition in plants. We first demonstrated that CBL1/CBL9-dependent CIPK23 was required for iron acquisition by possibly affecting of FCR activity. Several lines of evidence support the

involvement of CBL1/CBL9-CIPK23 complex in iron homeostasis in plants. Firstly, loss of function of CIPK23 led to a decrease in iron deficiency-induced FCR activity (Fig. 3), and thus rendering the mutants less efficient for acquisition of iron under low iron conditions (Fig. 1). Secondly, overexpression and complementation of *CIPK23* conferred greater tolerance of the transgenic plants to low iron supply by up-regulating FCR activity (Fig. 4). Thirdly, in contrast to FCR, mutation of *CIPK23* led to an enhanced rhizospheric acidification under both iron-sufficient and iron-deficient conditions (Fig. 3), which would increase iron availability to *lks* mutants by facilitating mobilization of ferric iron. However, the mutants exhibited less tolerant to iron deficiency than WT plants grown in low pH medium (Fig. 3a). Therefore, it rules out the contribution of proton exudation to iron uptake by *lks* mutants. Moreover, changes in root morphological characteristics can also play a role in adaptation of plants to iron-deficient medium [9]. However, we found that the *lks* mutants exhibited more lateral roots than WT plants when grown on iron-deficient medium (Fig.2). This root phenotype would facilitate iron acquisition by the mutants, thus conferring their tolerant to iron deficiency. These results indicate that the rhizospheric acidification and changes in root morphology of the *lks* mutants are unlikely to account for the phenotypes of *lks* mutants grown on iron-deficient medium. Finally, the less accumulation of iron in the *lks* mutants than WT plants may not result from impairment of IRT1-mediated transport of ferrous iron into root cells because the mutation of *CIPK23* reduced iron concentration in shoots, while concentrations of manganese and zinc in shoots were increased (Table S1). In contrast, mutation of

IRT1 leads to reduction in concentrations of iron, manganese and zinc in shoots [20]. IRT1 is not specifically for transport of ferrous iron, but it also mediates transport of divalent manganese and zinc into root cells [20, 69]. If CIPK23 regulates iron acquisition by targeting IRT1, a comparable reduction in concentration of iron, manganese and zinc in shoots of the *lks* would be expected. However, a reduction in iron concentration in shoots of the *lks* mutants (Fig. 1), while concentrations of manganese and zinc in shoots of the *lks* mutants were enhanced compared to WT plants (Table S1). These results discount the role of IRT1 in the reduced iron concentration in the *lks* mutants.

Cytosolic Ca^{2+} activities have been reported to be involved in regulation of many processes associated with growth and development and responses to abiotic stresses [36, 37, 70]. However, there has been no report on the involvement of the Ca^{2+} in regulation of iron-deficiency-response in the literature so far. In the present study, we discovered that an increase in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) in root tips of WT plants was elicited by iron deficiency (Fig. 6). In addition, spatial information of Ca^{2+} play a role in regulation of physiological processes [40]. Under iron-deficient conditions, the areas of increased $[\text{Ca}^{2+}]_c$ in root tip were similar with those of the expression of iron- uptake genes. These novel findings highlight the involvement of $[\text{Ca}^{2+}]_c$ in iron efficiency response in higher plants. A relatively rapid increase in $[\text{Ca}^{2+}]_c$ is often evoked by metal toxicities (e.g., Al^{3+} , La^{3+} and Gd^{3+}) [63]. In contrast, the increase in $[\text{Ca}^{2+}]_c$ induced by iron deficiency can be detected after 24-h of

deprivation of iron from the incubation medium (Fig. 6). It has been shown that the apoplastic iron is one of the major Fe pools in plants [34]. Deprivation of iron from incubation medium may not impose the plants to iron deficiency immediately. This may explain the delayed response of $[Ca^{2+}]_c$ to iron deficiency. Although the iron deficiency-induced increase in $[Ca^{2+}]_c$ occurred after 24-h of deprivation of iron, the increase in $[Ca^{2+}]_c$ still proceeded the enhancement of FCR activity in response to iron deficiency (data not shown). These results imply that the change in $[Ca^{2+}]_c$ may act as an upstream regulator involved in iron deficiency -response. Along with the time duration of iron deficiency, $[Ca^{2+}]_c$ did not reduce the same level as the control treatment, which was also inconsistent with the changes of $[Ca^{2+}]_c$ induced by metal toxicities [63]. This phenomenon would be due to the effect of other physiological processes induced by iron deficiency, for instance, iron deficiency induces proton-ATPases and this could change the membrane potential to activate calcium channels. It has been reported that external pH changes increase in the $[Ca^{2+}]_c$ [60].

Under iron starvation, strategy I plants are capable of sensing their intrinsic iron status and responding and adapting to iron deficiency [7]. Similar to WT plants, the *lks* mutants also exhibited iron-deficiency response. For instance, iron deficiency induced increases in rhizosphere acidification and FCR activity in the *lks* mutants (Fig. 4). Moreover, expression of many marker genes associated with iron-deficiency-response in the *lks* mutants was also up-regulated by iron deficiency (data not shown), suggesting other signals may also participate in the CIPK23-dependent iron acquisition.

Arabidopsis encodes 10 CBL and 26 CIPK proteins, which form a complex CBL-CIPK network by distinct and selective interactions. In addition to CBL1 and CBL9, CIPK23 can interact with other CBL proteins [57]. Moreover, CBL proteins can interact with K⁺ channel AKT1 independently of CIPK23 [71]. In this work, we found that *cbl1* or *cbl9* single mutant also displayed iron-deficiency symptoms (data not shown), which is inconsistent with the results of K nutrition [57]. Therefore, it is important to further study the pathway of CBL1, CBL9 and CIPK23 or the functions of other CBLs and CIPKs in iron-deficiency response.

CIPK23 directly regulates K and nitrate uptake by targeting K channel (AKT1) [57] and nitrate transporter (CHL1) [59]. It is well known that both K and N deficiency results in chlorosis of plants [2]. Because K and N can be easily translocated within plants, the chlorosis due to their deficiency in the mineral nutrients often first exhibits in old leaves. By contrast, chlorosis usually appears in young leaves of iron-deficient plants [2]. Therefore, although CIPK23 is involved in K and nitrate uptake, we can rule out the possibility that decreases in K and N uptake due to iron deficiency accounts for the sensitivity to iron deficiency in *lks* mutants. Moreover, K contents in shoots of the *lks* mutants grown on Fe-deficient medium were significantly higher than those grown on iron-sufficient medium (Table S1), suggesting that CIPK23-dependent K uptake is enhanced by iron deficiency. In addition to deficiency in mineral nutrients, ion toxicity also results in chlorosis of plants [2]. For instance, Manganese (Mn) toxicity leads to a similar chlorosis in young leaves to that induced

by iron deficiency [72]. Our results that iron deficiency led to an increase in Mn and Zn contents are consistent with those of previous studies [66]. Fe deficiency-induced a similar increase in Mn contents in WT and the mutants (Table S1), discounting that the observed chlorosis in the mutants is unlikely to be accounted for by Mn toxicity. The marked differences in contents of mineral elements including K, Mg, Ca, Fe, Mn, Zn, and P between *lks* mutants and WT plants (Table S1) suggest that CIPK23/CBL complexes could be emerging as “nutritional sensors” to regulate the mineral homeostasis in plants. Therefore, further studies to evaluate the role of CIPK23/CBL complexes in sensing mineral nutrients and regulating mineral homeostasis in plants are warranted.

5. Conclusions

Iron deficiency-induces an increase in $[Ca^{2+}]_c$ in elongation and root-hair zone of roots of Arabidopsis, a main region of iron absorption. Lesion of either *CIPK23* or *CBL1/CBL9* renders plants sensitive to iron deficiency. Enhanced sensitivity of *lks* mutants (mutation of *CIPK23*) to iron deficiency mainly results from their reduced FCR activity. Regulation of iron homeostasis by CIPK23 is dependent upon the activity of CBL1/CBL9. Regulation of FCR activity by CIPK23 may not be achieved by modulation of *FRO2*, *FRO3* and *FRO5* at transcriptional level.

6. Implications

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496 To the best knowledge of the authors, our results show, for the first time, that a
497 member of CIPKs kinase, CIPK23 is involved in acquisition of iron nutrition. We
498 demonstrate that iron deficiency elicited an increase in cytosolic Ca^{2+} , and that the
499 elevated Ca^{2+} would bind to CBL1/CBL9, leading to an activation of CIPK23 protein.
500 The active CBL1/CBL9-CIPK23 complex controls iron status *in planta* by regulating
501 FCR activity. FCR activity in plants not only depends on *FRO* expression, abundance
502 of *FRO* proteins, and but also depends on the levels of heme, ATP and
503 NADPH/NADH [73]. Therefore, it is essential to elucidate the mechanisms by which
504 CBL/CIPK23 complexes regulate FCR activity. In addition to Fe, K and N nutrients,
505 other mineral homeostasis (Ca, Mg, Mn, Zn, P) was also disturbed by mutation of
506 *CIPK23*, suggesting that CIPK23 may serve as a common nutrient sensor to
507 regulate mineral homeostasis in plants.

508

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510

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Figure legends

Fig. 1 *lks* mutants exhibited more sensitivity to low iron availability in growth medium than wild-type (WT) plants.

(a) Comparison of WT and *lks* mutant seedlings response to iron deficiency. (b) Relative chlorophyll contents of WT and mutant seedlings as described in (a). (c) Relative iron contents in shoots of WT plants and *lks* mutants under iron-sufficient and iron-deficient conditions propagated as in (a). Data are means $\pm$ SE (n=5). Different letters in each series indicate a statistically significant difference ($P<0.05$) between genotypes within a given growth condition. “*” indicates a statistically significant difference ($P<0.05$) between iron treatments.

Fig. 2. Root system architecture in WT and *lks* mutants response to iron availability.

(a) Photographs of roots of WT and *lks* mutants grown on iron-sufficient and iron-deficient media. Bar=5 mm. (b) Primary root length, lateral root length and lateral root density of WT and *lks* mutants as propagated as in (a). Data are means $\pm$ SE (n=15). Different letters in each series indicate a statistically significant difference ($P<0.05$) between genotypes within a given growth condition. “*” indicates a statistically significant difference ($P<0.05$) between iron treatments.

Fig. 3 Ferric chelate reductase activity and rhizosphere acidification of WT and *lks* mutants exposed to Fe-deficient. (a) Phenotype of WT and *lks* mutant plants grown in low pH medium. Seedlings were pre-cultured in Fe-sufficient medium and then transferred into low pH (pH=4.8) medium. (b) Rhizosphere acidification of WT and *lks* mutants exposed to Fe-deficient. (c) Relative ferric chelate reductase activity of WT and *lks* mutants treated with iron deficiency. All value are means $\pm$ SE (n=5). Different letters in each series indicate a statistically significant difference ($P<0.05$) between genotypes within a given growth condition. “*” indicates a statistically significant difference ($P<0.05$) between iron treatments.

Fig. 4 *CIPK23* over-expression in WT plants or *CIPK23* complementation in *lks* mutants enhances tolerance to iron deficiency.

(a) Photographs of WT, *lks1-3*, *CIPK23* over-expression lines and *CIPK23* complementation lines grown on iron-sufficient and iron-deficient media. (b) Relative chlorophyll contents of WT, *lks1-3*, *CIPK23* over-expression lines and *CIPK23* complementation lines as shown in (a). (c) Relative ferric chelate reductase activity in root of WT, *lks1-3* and transgenic materials. (d) Relative iron contents of WT and over-expression lines as described (a). Different letters indicate a statistically significant difference ($P<0.05$) between genotypes within a given growth condition. “*” indicates a statistically significant difference ($P<0.05$) between iron treatments.

Fig. 5 Comparison of sensitivity to low iron availability between wild-type Arabidopsis (WT) , *lks1-3*, *cbl1cbl9* and triple-mutant *lks1-3cbl1cbl9* .

(a) Phenotype of WT, *lks1-3*, *cbl1cbl9* and *lks1-3cbl1cbl9* seedlings grown in iron-sufficient and iron-deficient media. (b) Relative chlorophyll contents of WT and mutant seedlings as propagated as in (a). (c) Relative iron contents in shoots of the seedlings propagated as in (a). Data are means $\pm$ SE (n=5). Different letters in each series indicate a statistically significant difference ($P<0.05$) between genotypes within a given growth condition. “*” indicates a statistically significant difference ($P<0.05$) between iron treatments.

Fig. 6 Iron deficiency induced elevations of $[Ca^{2+}]$ in primary roots of Arabidopsis. (a) $[Ca^{2+}]$ -dependent FRET efficiency changes in primary root tip after iron-deficiency for different times. Bars = 200 μ m. (b) Iron-deficiency triggered a $[Ca^{2+}]$ increase in the elongation zone. Wild-type Arabidopsis pre-grown in iron sufficient media were exposed to iron-deficiency media for varying periods. Data are means $\pm$ SE of at least fifteen repeats. Different letters indicate a statistically significant difference ($P<0.05$) between iron treatments at the same treatment period.

Fig. S1. *FRO2*, *FRO3*, *FRO5* expression in roots of WT and *lks* mutants.

Fig. S2. Expression of IRT1 encoding iron transporter in roots of WT and *lks* mutants.

Fig. S3. *CIPK23* expression in *CIPK23* over-expression transgenic lines

Table S1. Relative contents of mineral elements in shoots of WT and *lks* mutant seedlings.

Table S2. A list of primers used in transformation and qRT-PCR.

Figure(s)

Figure(s)

Figure 4

Figure(s)

Figure(s)