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CHL1 Functions as a Nitrate Sensor in Plants

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SUMMARY

Ions serve as essential nutrients in higher plants and can also act as signaling molecules. Little is known about how plants sense changes in soil nutrient concentrations. Previous studies showed that T101-phosphorylated CHL1 is a high-affinity nitrate transporter, whereas T101-dephosphorylated CHL1 is a low-affinity transporter. In this study, analysis of an uptake- and sensing-decoupled mutant showed that the nitrate transporter CHL1 functions as a nitrate sensor. Primary nitrate responses in CHL1T101D and CHLT101A transgenic plants showed that phosphorylated and dephosphorylated CHL1 lead to a low- and high-level response, respectively. In vitro and in vivo studies showed that, in response to low nitrate concentrations, protein kinase CIPK23 can phosphorylate T101 of CHL1 to maintain a low-level primary response . Thus, CHL1 uses dual-affinity binding and a phosphorylation switch to sense a wide range of nitrate concentrations in the soil, thereby functioning as an ion sensor in higher plants.

For a video summary of this article, see the PaperFlick file with the Supplemental Data available online.

INTRODUCTION

Plants acquire most of their essential nutrients from the soil. Ions in the soil not only serve as essential nutrients, but also function as signal molecules regulating plant development, gene expression, and metabolism. The ability to sense changes in soil ion concentrations and to respond metabolically to these changes is vital for nonmobile plants to survive in harsh conditions and to sustain maximal growth in nutrient-sufficient conditions. However, the plasma membrane ion sensor that detects these nutrient changes in the soil has not yet been identified in higher plants.

Nitrogen is a key limiting factor for plant growth and crop productivity. For most plants, nitrate is the primary nitrogen source (Crawford, 1995). To be assimilated, it has to be taken up from the soil and converted into ammonium by nitrate reductase and nitrite reductase, and then into amino acid by enzymes such as glutamate synthase (Crawford, 1995). In addition to being an essential nutrient, nitrate also serves as a signaling molecule. For instance, it is known to regulate root architecture, stimulate shoot growth, delay flowering, regulate abscisic acid-independent stomata opening, and relieve seed dormancy (Walch-Liu et al., 2005).

The best known nitrate-induced response is the primary nitrate response (Redinbaugh and Campbell, 1991), in which gene expression of nitrate assimilatory enzymes and nitrate transporters, such as CHL1 and NRT2.1, is rapidly induced (within 0.5 to 1 hr) by nitrate (Wang et al., 2003). A normal primary nitrate response is seen in nitrate reductase mutants (Deng et al., 1989) and in the presence of protein synthesis inhibitors (Redinbaugh and Campbell, 1993), indicating that nitrate itself is responsible for the response and that de novo protein synthesis is not required. The molecular identities of the signaling components in the nitrate response are just beginning to be determined. For example, the transcription factor ANR1 is involved in the nitrate regulation of root architecture (Zhang and Forde, 1998), and recent studies have shown that the transcription factor NLP7 (Castaings et al., 2009) and the calcineurin-like protein (CBL)-interacting protein kinase CIPK8 (Hu et al., 2009) are positive regulators of the primary nitrate response.

In contrast to nitrate signaling, the molecular mechanism of nitrate uptake is well characterized. Three families of nitrate transporters, *AtNRT1* with 53 members, *AtNRT2* with 7, and *AtCLC* with 7, have been identified in *Arabidopsis* (De Angeli et al., 2009; Forde, 2000; Tsay et al., 1993). Two *AtNRT1* genes (*AtNRT1.1* [*CHL1*] and *AtNRT1.2*) and two *AtNRT2* genes (*AtNRT2.1* and *AtNRT2.2*) are involved in nitrate uptake. Nitrate concentrations in the soil can vary by four orders of magnitude from micromolar to millimolar. To cope with this wide range of concentrations, plants have evolved two nitrate uptake systems: a high-affinity system, with a K_M of about 50 μM, and a low-affinity system, with a K_M of about 5 mM. AtNRT2.1 and AtNRT2.2 are involved in high-affinity uptake (Li et al., 2007; Little et al., 2005) and AtNRT1.2 in low-affinity uptake (Huang et al., 1999), whereas AtNRT1.1 (CHL1) functions as a dual-affinity transporter involved in both high- and low-affinity uptake (Liu et al., 1999; Wang et al., 1998). CHL1 functions as a high-affinity nitrate transporter when T101 is phosphorylated and as a low-affinity nitrate transporter when T101 is dephosphorylated (Liu and Tsay, 2003). The phosphorylation of CHL1 at T101, triggered by changes in external

nitrate availability, is responsible for the shift from low- to high-affinity uptake mode, thus adapting transporter function to the levels of available resources in the root environment.

Several lines of evidences suggest that, in addition to uptake, CHL1 is also involved in nitrate signaling. For example, the nitrate signaling responsible for the repression of *AtNRT2.1*, stimulation of ANR1-regulated lateral root growth, inhibition of L-glutamate-induced root architecture modulation, and stimulation of seed germination was found to be changed in a *chl1* mutant (Alboresi et al., 2005; Munos et al., 2004; Remans et al., 2006; Walch-Liu and Forde, 2008). Further evidence was therefore required to determine whether these defects in the *chl1* mutant were due to reduced nitrate uptake, and therefore to reduced accessibility of nitrate to the unknown sensors, or were due to direct involvement of CHL1 in nitrate sensing.

In this study, by characterizing an uptake- and sensing-decoupled mutant *chl1-9*, we provide strong evidence that CHL1 is a nitrate sensor responsible for the primary nitrate response and that uptake activity is not required for sensor function. In addition, we reveal a novel mechanism of ion sensing, in which plants use the dual affinity binding of CHL1 and a phosphorylation switch at residue T101 to sense a wide range of nitrate concentrations in the soil and generate different levels of the primary response. Low, but not high, nitrate concentrations stimulate CHL1T101 phosphorylation, which then reduces the primary nitrate response to a low level. The CBL-interacting protein kinase CIPK23 was found to be responsible for CHL1T101 phosphorylation in response to low nitrate concentrations. The identification of this sensing mechanism raises the possibility that this could be a universal mechanism for plants and other organisms to sense ion changes in the environment.

RESULTS

CHL1 Is Involved in the Primary Nitrate Response

Recent studies have suggested that CHL1 participates in the nitrate sensing signaling pathway (Munos et al., 2004; Remans et al., 2006). To determine whether CHL1 is involved in the primary nitrate response, changes in the expression of the primary nitrate response gene *AtNRT2.1* were examined in wild-type and *chl1-5* mutant plants. Consistent with our previous study (Hu et al., 2009), time course analysis of the wild-type during exposure to nitrate for 2 hr showed that, for all nitrate concentrations tested, expression of this primary nitrate response gene peaked at 30 min. *AtNRT2.1* mRNA levels in the wild-type and *chl1-5* mutant after exposure to various concentrations of nitrate for 30 min were then measured by quantitative PCR (Q-PCR). As shown in Figure 1A, and consistent with our previous studies, increased expression of *AtNRT2.1* was seen in wild-type plants, which displayed a biphasic pattern with a K_M of 24 ± 8 μM for the high-affinity phase and a K_M of 764 ± 175 μM for the low-affinity phase (Table 1 and the double reciprocal plots in Figure S1A, available online). However, in the *chl1-5* mutant, only basal expression of *AtNRT2.1* was seen in both the high-affinity and low-affinity nitrate concentration range, the level of expression being similar to that of *AtNRT2.1* in the wild-type treated with potassium chloride (as a control for nitrate). These data show that CHL1 is required for the primary nitrate response. Furthermore, like nitrate uptake, the primary response also displayed a two-phase pattern, suggesting that the nitrate sensing system can sense not only the presence or absence of nitrate but also the concentration of nitrate.

CHL1 Functions as a Nitrate Sensor (Transceptor), Independent of Its Uptake Activity

The mutant *chl1-5* is a deletion mutant (Tsay et al., 1993) in which no CHL1 protein or transcript is expressed and both nitrate uptake and the primary nitrate response are defective. The primary nitrate response defect in the *chl1-5* mutant could be due to either reduced nitrate uptake into the plant or direct involvement of CHL1 as a nitrate sensor. To determine whether the sensor function of CHL1 could be decoupled from its uptake function, the primary nitrate responses of additional CHL1 mutants were examined. One of these mutants, *chl1-9*, generated by EMS mutagenesis and isolated by chlorate selection (Liu et al., 1999), was found to have normal levels of CHL1 mRNA and protein (Liu et al.,1999) (Figure 1B). Sequence analysis showed that a point mutation in *chl1-9* resulted in Leu replacing Pro492 in the loop region between the 10^{th} and 11^{th} transmembrane domains (Figure 1C). Pro492 is conserved in 50 of the 53 *Arabidopsis* NTR1 (PTR) transporters. A nitrate uptake defect of *chl1-9*, previously demonstrated by HPLC analysis (Liu et al., 1999), was confirmed using a short-term ^{15}N-labeling approach, *chl1-9* being shown to be defective in both high- and low-affinity nitrate uptake (Figure 1D). Nitrate uptake by *chl1-9* was lower than in the wild-type, but similar to that in the deletion mutant *chl1-5* in both the high-affinity and low-affinity range. Interestingly, despite the nitrate uptake defect, *chl1-9* still showed a typical biphasic primary nitrate response and K_M values in both affinity ranges (Figure 1E, Table 1, Figures S1B and S1C, and Figure S2). Consistent with this, when the *chl1-9* genomic fragment was introduced into *chl1-5*, the signaling defect but not the uptake defect was rescued (Figure S3). These data show that transporter activity is not required for the sensing function of CHL1 and that CHL1 functions directly as a nitrate sensor (transceptor).

The two modes of action of CHL1 in nitrate transport are switched by phosphorylation/dephosphorylation of Thr101, which lies between the second and third transmembrane domains (Figure 1C) (Liu and Tsay, 2003). To determine whether this phosphorylation regulatory mechanism is also involved in the primary nitrate response, transgenic plants expressing T101D or T101A, mimicking, respectively, phosphorylated or dephosphorylated CHL1, were generated. *CHL1* genomic DNA containing the T101D or T101A mutation was placed under the control of its own promoter and introduced into the *chl1-5* deletion mutant. In contrast to the primary nitrate response in the wild-type, the response in transgenic *chl1-5* plants expressing T101D (*CHL1 promoter::CHL1T101D*) was monophasic, showing that T101D could recover the high-affinity, but not the low-affinity, primary nitrate response (Figures 2A and S4A). The K_M and V_{max} values for the high-affinity phase of the primary nitrate response in the wild-type plants (24 ± 8 μM and 60 ± 8%) and CHL1T101D plants (30 ± 9 μM and 58 ± 2%) were similar (Table 1). In contrast, expression of *CHL1 promoter::CHL1T101A* in *chl1-5* mutants resulted in an increased

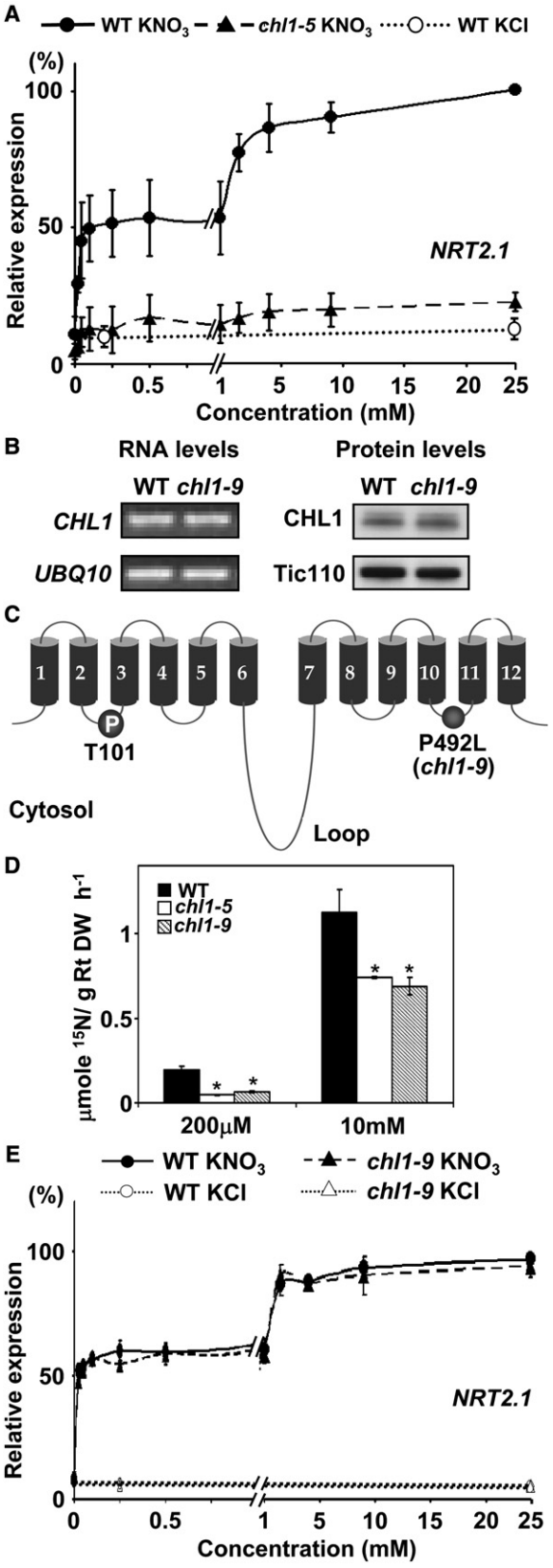

Figure 1. Decoupled Mutant *chl1-9* Is Defective in Nitrate Uptake but Shows a Normal Primary Nitrate Response

(A) The primary nitrate response is defective in *chl1-5* mutant plants. *NRT2.1* expression was analyzed by Q-PCR. The plants were grown for 10 days in NO_3^--free medium using NH_4^+ as the sole N source, treated with fresh medium overnight and again for an additional 3 hr, and then exposed for 30 min to the indicated concentrations of KNO_3 or KCl. The scale between 0 and 1 mM is enlarged. The data are shown as the mean ± standard deviation (SD) of triplicate experiments. The relative expression is the expression normalized to that for *NRT2.1* in wild-type plants exposed to 25 mM KNO_3 for 30 min. The double reciprocal plots for the wild-type and the equation, together with the R^2 value obtained using the linear fitting methods, are shown in Figure S1, and the kinetic parameters (y intercept = $1/V_{max}$; x intercept = $-1/K_M$) determined are shown in Table 1.

(B) CHL1 mRNA and protein levels in the *chl1-9* mutant. The RNA and protein were isolated from wild-type and *chl1-9* plants grown in NH_4^+ as the sole N source. Tic110 (At1g06950), a non-NO_3^--inducible chloroplast membrane protein, or *UBQ10* was used as the loading control for protein and RNA, respectively.

(C) Schematic diagram showing the *chl-9* mutation (492 Pro→Leu), the phosphorylation site T101, and the putative membrane topology of CHL1.

(D) Defective nitrate uptake in *chl1-9* plants. Nitrate uptake of wild-type plants and the *chl1*-5 and *chl1-9* mutants measured using 10 mM or 200 µM $K^{15}NO_3$ for 30 min (as described in Hu et al. [2009]). The data are the mean ± SD for three experiments; $p < 0.01$ compared to the wild-type (t test).

(E) Q-PCR analysis of *AtNRT2.1* expression in the *chl1-9* mutant. The plants were grown as in (A). The data are the mean ± SD for three experiments.

primary nitrate response with a high-affinity K_M of 51 ± 14 µM, similar to that in the wild-type, but with a high-affinity phase V_{max} twice that of the wild-type and similar to the wild-type low-affinity V_{max} (Figure 2B, Table 1, and Figure S4B). When exposed to high concentrations of nitrate, *AtNRT2.1* and *CHL1* expression in the T101A plants was significantly higher than that in the wild-type and also higher than the maximum level seen in their own high-affinity phase. CHL1T101D rescued the high-affinity phase of the primary response, but showed no further increase in the low-affinity phase, suggesting that phosphorylated CHL1 is involved in maintaining the primary nitrate response at a low level. In contrast, CHL1T101A enhanced both the high-affinity and low-affinity phases of the primary nitrate response, suggesting that unphosphorylated CHL1 results in a high primary response. These results show that T101 phosphorylation/dephosphorylation is also involved in regulating the primary nitrate response and that phosphorylation

Table 1. Kinetic Parameters of Nitrate-Induced Gene Expression in Various Plants

	High-Affinity Phase		Low-Affinity Phase		
	K_M^{HA} (µM)	V_{max}^{HA} (%)	K_M^{HA} (µM)	V_{max}^{total} (%)	V_{max}^{LA} (%)
Wild-type	24 ± 8	60 ± 8	764 ± 175	102 ± 4	43
chl1-9	7 ± 3	59 ± 5	684 ± 234	104 ± 11	45
T101D	30 ± 9	58 ± 2	—	—	—
T101A	51 ± 14	103 ± 8	434 ± 129	116 ± 5	13
cipk23-3	30 ± 14	91 ± 6	504 ± 48	123 ± 9	14

Data are shown as the mean ± SD for three independent expressions in *chl1-9*, T101D, T101A, or *cipk23-3* and for the five independent experiments in the wild-type and were obtained by fitting to the Michaelis-Menten equation ($V_{max}^{LA} = V_{max}^{total} - V_{max}^{HA}$).

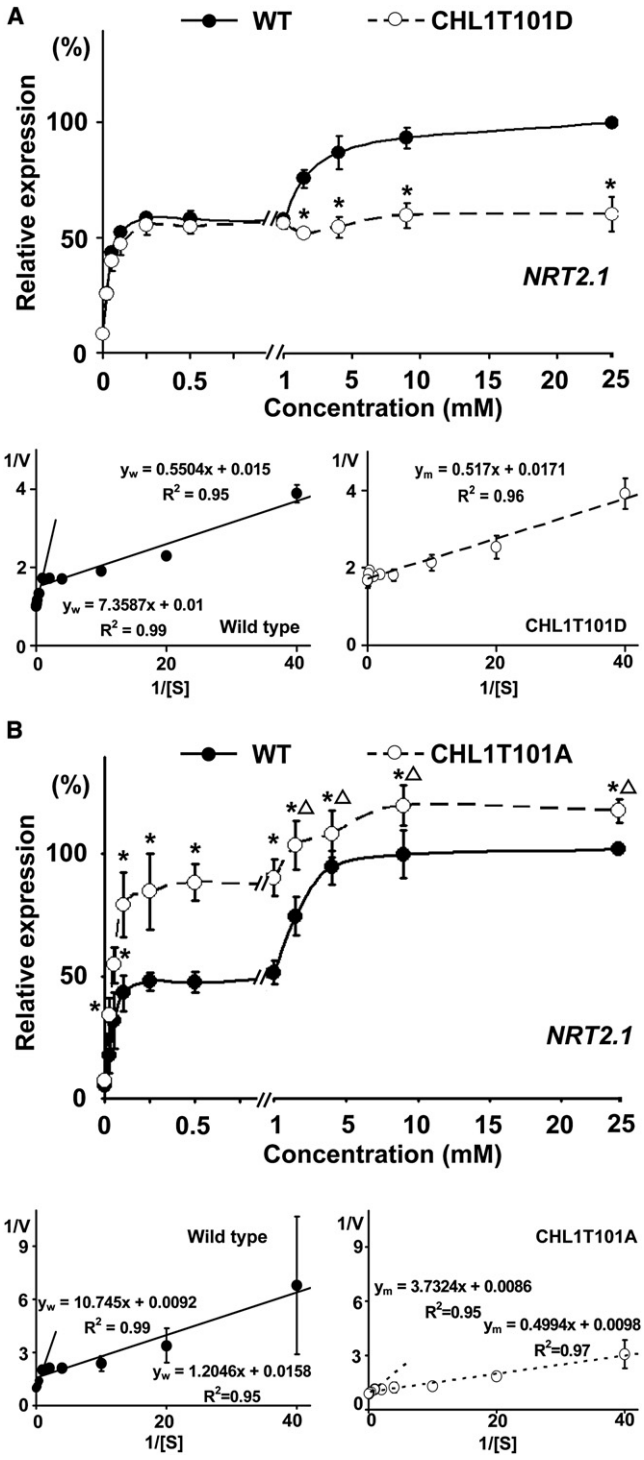

Figure 2. CHL1T101D and T101A Transgenic Plants Show, Respectively, a Low or a High Primary Nitrate Response

Q-PCR analysis of *NRT2.1* expression in CHL1 T101D plants (A) or CHL1 T101A plants (B). See the legend to Figure 1 for the seedling growth and data analysis. The small panels are the double reciprocal plots. The kinetic parameters determined are shown in Table 1. The data are the mean ± SD for three experiments. *p < 0.01 compared to the wild-type (t test). Δ, significant increase in CHL1 T101D plants exposed to a concentration of nitrate higher than 1 mM (p < 0.01) compared to the values for the same plants exposed to 0.5 mM nitrate.

of CHL1 T101 is required to reduce the primary nitrate response to low levels.

CIPK23 Is Involved in the Primary Nitrate Response

To determine how the phosphorylation of T101 is regulated and how it is involved in nitrate sensing, protein kinase genes showing an interesting expression pattern in a microarray analysis of the *chl1-5* mutant (Hu et al., 2009) were identified as candidates for further analysis. In our microarray analysis, a protein kinase, CIPK23 (CBL-interacting protein kinase 23/SOS2-like protein kinase [PSK17]), was found to be downregulated in the *chl1* mutant. In addition, Q-PCR analysis showed that expression of *CIPK23* was transiently induced by nitrate (Figure 3A). The *Arabidopsis* genome contains 25 putative CIPKs (Kolukisaoglu et al., 2004), several of which have been shown to activate plant membrane transport proteins and participate in Na^+, salt, cold, drought, and abscisic acid signaling and the K^+ shortage response (Batistic and Kudla, 2004; Cheng et al., 2004; Gong et al., 2004; Li et al., 2006; Xu et al., 2006). To determine whether CIPK23 is involved in the primary nitrate response, two mutants, *cipk23-3* (referred to as *iks1-3* in Xu et al. [2006]) and *cipk23-4,* with T-DNA inserted in the seventh or tenth intron of the *CIPK23* gene, respectively (Figure 3B), were obtained from the *Arabidopsis* Biological Resource Center.

RT-PCR analyses showed that no full-length *CIPK23* transcript was expressed in either mutant (Figure 3C). Time course analysis showed that the temporal pattern of the primary nitrate response (peak at 30 min) was not changed in the two mutants (data not shown). The expression of the two primary nitrate response genes *AtNRT2.1* and *CHL1* in the mutants exposed for 30 min to various concentrations of nitrate was examined by Q-PCR analysis (Figure 3D and Figure S5) and found to be quite similar to that in T101A transgenic plants, with increased expression at all concentrations tested. Kinetic analysis of the primary nitrate response using double reciprocal plots showed that, in the *cipk23* mutants, the high-affinity K_M (30 ± 14 μM) was similar to that in both wild-type and T101A plants, whereas the V_{max} for the high-affinity phase was similar to that in T101A plants, i.e., twice as high as in the wild-type, and similar to the wild-type low-affinity phase V_{max} (Table 1). Another similarity with T101A plants was that, when the plants were exposed to high concentrations of nitrate, *AtNRT2.1* expression in the *cipk23* mutant was higher than in the wild-type and also higher than the maximal levels seen in its own high-affinity phase (Figure 3D and Figure S5). The increased high-affinity phase of primary nitrate response in the *cipk23* mutants suggests that in low nitrate, CIPK23 is a negative regulator of the primary nitrate response. The observation of similar phenotypes in the *cipk23* mutants and T101A plants suggests that CHL1 is a potential target of CIPK23.

Phosphorylation of Threonine 101 in CHL1 Is Reduced in *cipk23* Mutants

To determine whether CIPK23 could regulate CHL1T101 phosphorylation, 10-day-old wild-type and *cipk23* mutant plants

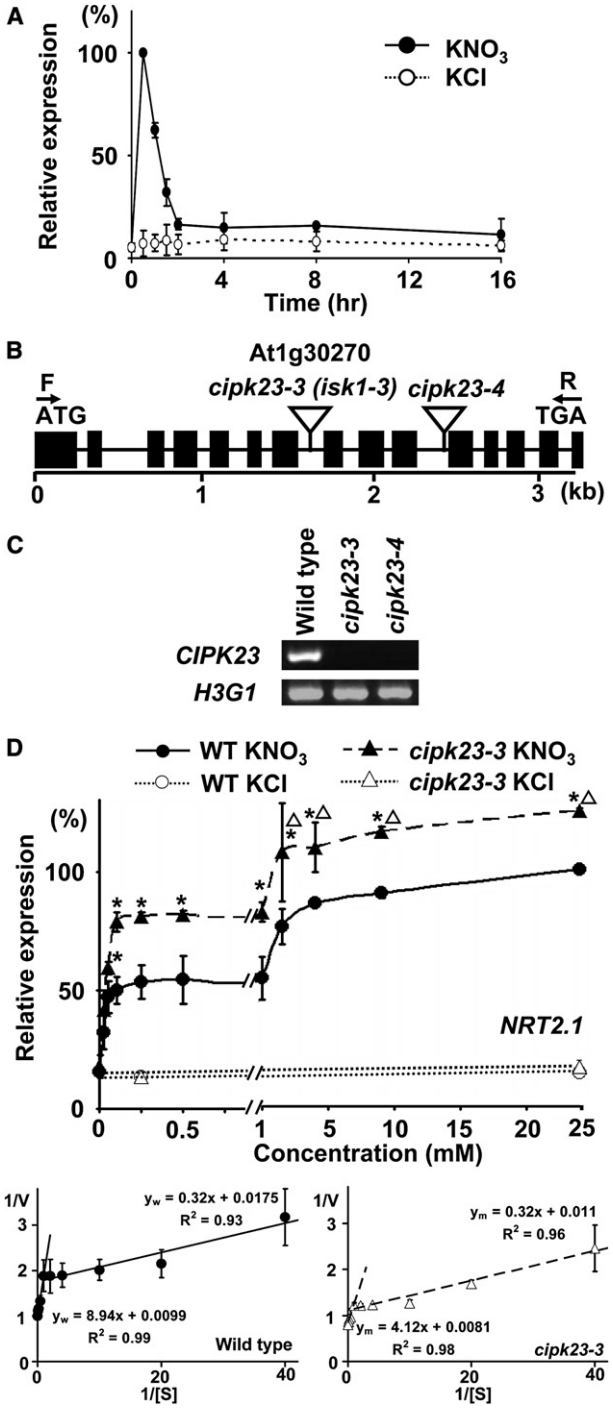

Figure 3. CIPK23 Is a Negative Regulator of the Primary Nitrate Response

(A) Q-PCR analysis of nitrate-induced expression of *CIPK23*. Wild-type plants were exposed to 25 mM KNO_3 or to 25 mM KCl as a control for the indicated time. The relative expression is the expression normalized to that of plants exposed to 25 mM KNO_3 for 30 min. The data are shown as the mean ± SD for three experiments, each in triplicate.

(B) Schematic maps of the *cipk23-3* and *cipk23-4* mutants with T-DNA inserted in the seventh or tenth intron, respectively, of the *CIPK23* gene. The filled box represents exons and the line introns. F, *CIPK23* forward primer; R, *CIPK23* reverse primer.

(C) RT-PCR analysis of *CIPK23* transcript levels in *cipk23-3* and *cipk23-4* mutants using primers F and R. *H3G1* (At4g40040) was used as the loading control.

(D) Primary nitrate response in *cipk23-3* mutant. Plant growth conditions and data analysis were as described in Figure 1. The double reciprocal plots for the wild-type (left) and *cipk23-3* (right), together with the equations and R^2 values, are shown in the bottom panels. The data are the mean ± SD for three experiments. *$p < 0.01$ compared to the wild-type (t test). Δ, significant difference ($p < 0.01$) between the values for the *cipk23* mutant exposed to nitrate concentrations higher than 1 mM or 0.5 mM nitrate.

were shifted from nitrate-free medium to medium containing 200 μM or 25 mM NO_3^- for time course analysis of CHL1 phosphorylation. Membrane proteins were isolated and analyzed by immunoblotting with anti-CHL1 antibodies or anti-P-T101 antibodies, which specifically recognize T101-phosphorylated CHL1 (Liu and Tsay, 2003). As shown in Figures 4 and S6, CHL1 protein levels were quite similar in the wild-type and *cipk23* mutants, and were unchanged after 60 min induction with either high or low concentrations of nitrate. In wild-type plants exposed to a high nitrate concentration (25 mM), phosphorylation of CHL1-T101 remained unchanged for at least 60 min (Figure 4C), whereas exposure to a low nitrate concentration (200 μM) resulted in a transient increase in phosphorylation, peaking at 15 min (Figure 4A). This transient increase in CHL1 T101 phosphorylation in response to low-concentration nitrate was not seen in *cipk23* mutants. These data show that CHL1 T101 phosphorylation is differentially regulated by high and low concentrations of nitrate and that CIPK23 is important for the increase in CHL1 phosphorylation in response to low-concentration nitrate.

CIPK23 Can Interact with CHL1

Because phosphorylation of CHL1T101 was reduced in the *cipk23* mutants, it was possible that CIPK23 might directly interact with CHL1. We used Dualsystem yeast two-hybrid analyses (Stagljar et al., 1998) to test for interaction of CHL1 with CIPK23. As shown in Figure 5A, CIPK23 interacted with the full-length (TM1–12) and the N-terminal half of CHL1 (TM1–6). The control CIPK24 (SOS2), which shows 70.6% sequence similarity with CIPK23, showed no interaction with the CHL1, demonstrating that the interaction between CHL1 and CIPK23 was specific. The specific interaction between the CHL1 and CIPK23 was confirmed using a quantitative β-galactosidase activity assay (Figure 5B).

To confirm the interaction in plant cells, a bimolecular fluorescence complementation (BiFC) assay based on split yellow fluorescence protein (YFP) was performed (Waadt et al., 2008). The different combinations of the N- or C-terminal end of YFP fused to CHL1 or CIPK23 were transiently coexpressed in *Arabidopsis* protoplasts. As shown in Figures 5C and S7, for two combinations (CHL1-YC+CIPK23-YN and YN-CHL1+YC-CIPK23) a fluorescent signal was seen in the plasma membrane, whereas no significant signals were observed in controls lacking CHL1 or CIPK23. The results of the protein interaction demonstrated in yeast cells and by the BiFC assay in plant cells show that CIPK23 interacts with CHL1 in the plasma membrane.

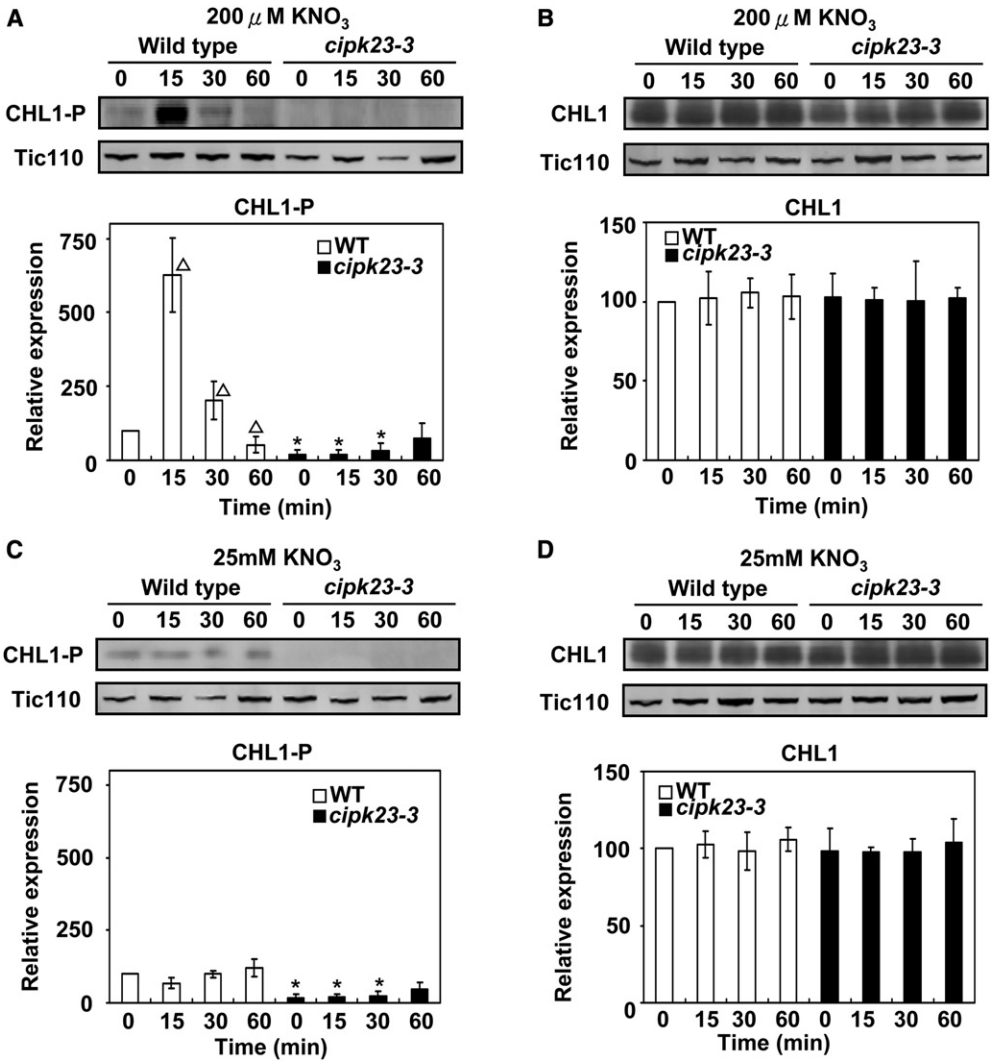

Figure 4. CHL1T101 Phosphorylation Is Reduced in *cipk23* Mutant Plants

CHL1 protein and phosphorylation of CHL1-T101 in the wild-type and *cipk23-3* mutant exposed to 200 μM KNO_3 (A and B) or 25 mM KNO_3 (C and D). Growth conditions were as in Figure 1. After electrophoresis and transfer, membrane proteins on the blots were probed with anti-CHL1-P-T101 antibodies (CHL1-P; left panel) (Liu and Tsay, 2003), anti-CHL1 antibodies (CHL1; right panel), or anti-Tic110 antibodies as the loading control. The band intensities for CHL1-P and CHL1 were first normalized to those for Tic110 and then expressed relative to that in the wild-type plants at time 0. Quantification of CHL1 protein and phosphorylation of CHL1T101 levels is shown in each lower panel. The data are the mean ± SD for three experiments. *$p < 0.01$ compared to the wild-type (t test). Δ, significant change ($p < 0.01$) in the wild-type plant after nitrate induction compared to before induction.

CIPK23 Inhibits the Low-Affinity Transport Activity of CHL1

To determine whether CIPK23 directly regulates phosphorylation of CHL1T101 and influence CHL1 uptake kinetics, nitrate uptake was examined in oocytes coinjected with *CHL1*, *CIPK23*, and *CBL9* cRNAs (Figures 6A, 6B, and S8) using 150 μM and 10 mM nitrate. Oocytes injected with *CIPK23* or *CBL9* alone showed no observable nitrate transport, whereas those injected with *CHL1* alone exhibited both high- and low-affinity nitrate transport activities. Because it is already known that CIPK23 is activated through CBL9 modulation to activate downstream transporters (Cheong et al., 2007; Xu et al., 2006), nitrate uptake was then investigated in oocytes coinjected with *CHL1*, *CIPK23*, and *CBL9* cRNAs. In the triple-injected cells, nitrate uptake in 150 μM and 10 mM nitrate was, respectively, 285% and 64% of that seen in cells injected only with *CHL1*, showing that high-affinity uptake activity was significantly increased and low-affinity uptake activity significantly reduced. Uptake measured in 10 mM nitrate includes contributions from both the low- and high-affinity components; when the contribution of the high-affinity component (activity measured in 150 μM nitrate) was subtracted, the triple-injected oocytes were found to have only 15% of the low-affinity uptake of oocytes injected with *CHL1* alone (Figure 6B). Together, these data show that in oocytes coinjected with *CHL1*, *CIPK23*, and *CBL9*, high-affinity uptake was higher than that in oocytes injected with only

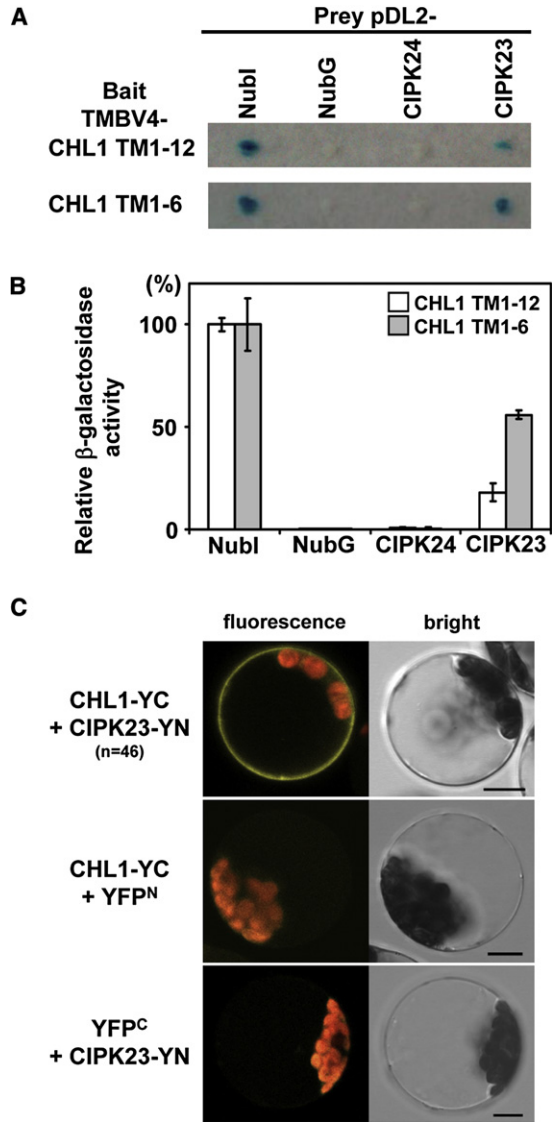

Figure 5. CIPK23 Interacts with CHL1

(A and B) Interaction of CIPK23 and CHL1 in a yeast-based split ubiquitin system. An X-Gal filter assay (A) and quantitative β-galactosidase measurements (B) were performed using CHL1 (TM1-6 and TM1-12)-Cub-PLV as bait and NubG, NubI, NubG-CIPK23, or NubG-CIPK24 as prey. The N-terminal part of wild-type ubiquitin (NubI) or of a mutant that does not interact with the C-terminal part of ubiquitin (NubG) was used as the positive and negative control, respectively. CIPK24 was included as a specificity control. The data in (B) are shown as the mean ± SD for three experiments, each in triplicate.

(C) BiFC analysis of the CIPK23 and CHL1 interaction in plant protoplasts. Protoplasts were transfected with different combinations of expression vectors encoding CIPK23-YFP^N, CHL1-YFP^C, YFP^C, or YFP^N (control). The numbers (n) of the protoplasts showing similar pattern are indicated. Identical results of each combination were observed in two or three batches of protoplasts. Other combinations for interaction are shown in Figure S6.

CHL1, whereas low-affinity uptake was largely reduced. These results are consistent with the finding that CHL1 phosphorylation level is reduced in *cipk23* mutant.

CHL1 Can Be Phosphorylated by CIPK23

Because CIPK23 was able to directly interact with CHL1 and switch its nitrate uptake mode, we examined whether CIPK23 could phosphorylate CHL1 at T101. Two GST fusion proteins, GST-CIPK23 and GST-CBL9, purified from *E. coli*, were used in an in vitro kinase assay. Membrane proteins isolated from the roots of wild-type plants grown in ammonium were incubated with or without the purified CIPK23 and CBL9 proteins, and then subjected to immunoblot analysis using anti-CHL1 or anti-P-T101 antibodies. As shown in Figures 6C and S9, compared to the no-CIPK23 control, phosphorylation of CHL1T101 was greatly increased after coincubation with GST-CIPK23 and GST-CBL9, showing that protein kinase CIPK23 can directly phosphorylate CHL1 at Thr101.

DISCUSSION

CHL1 Functions as a Nitrate Sensor (Transceptor) in the Primary Nitrate Response

Our study provided new insights into the dual-affinity nitrate transporter CHL1 and its role in nitrate signaling. In the CHL1 deletion mutant *chl1-5*, the primary nitrate response was reduced to ~30% of the wild-type level, showing that CHL1 is involved in nitrate sensing (Figure 1A). In addition, the single amino acid substitution mutant *chl1-9* was defective in nitrate uptake, but showed a normal primary nitrate response, indicating that, independent of its uptake activity, CHL1 serves as a sensor for nitrate signaling in the primary nitrate response (Figures 1D and 1E). Reduction of primary response in *chl1* mutants varied from 85% to 50% depending on growth conditions (Figure S10), suggesting that there could be other nitrate sensors, with the contribution of each in the primary response depending on some other internal and/or external environmental factors. In eukaryotic cells, several nontransporting carrier homologs, e.g., glucose sensors Snf3 and Rgt2 in yeast (Ozcan et al., 1996) and SGLT3 in mammalian cells (Diez-Sampedro et al., 2003), as well as active transport carriers, such as the ammonium sensor/carrier Mep2 (Lorenz and Heitman, 1998) and the amino acid sensor/carrier Gap1 in yeast (Donaton et al., 2003), have been shown to function as sensors monitoring nutrient levels in the extracellular environment. However, despite being essential for the survival of nonmobile plants, the nutrient sensors responsible for monitoring levels of extracellular nutrients are poorly understood in higher plants. Our data for the mutant *chl1-9* indicate that plants utilize a transporter responsible for nitrate uptake to monitor changes in nitrate concentrations in soil to activate the expression of nitrate-related genes.

Proline 492 of CHL1 Is Important for Nitrate Uptake, but not Nitrate Sensing

Several reports have suggested that CHL1 is involved directly or indirectly in nitrate signaling (Remans et al., 2006; Walch-Liu and Forde, 2008). In this study, the results for the uptake- and sensing-decoupled mutant *chl1-9* provided strong evidence that CHL1 is indeed a nitrate sensor. In addition, they also revealed that, although the transport and sensing functions of CHL1 share something in common, i.e., dual-affinity behavior, they have different requirements. Pro492, which is mutated in

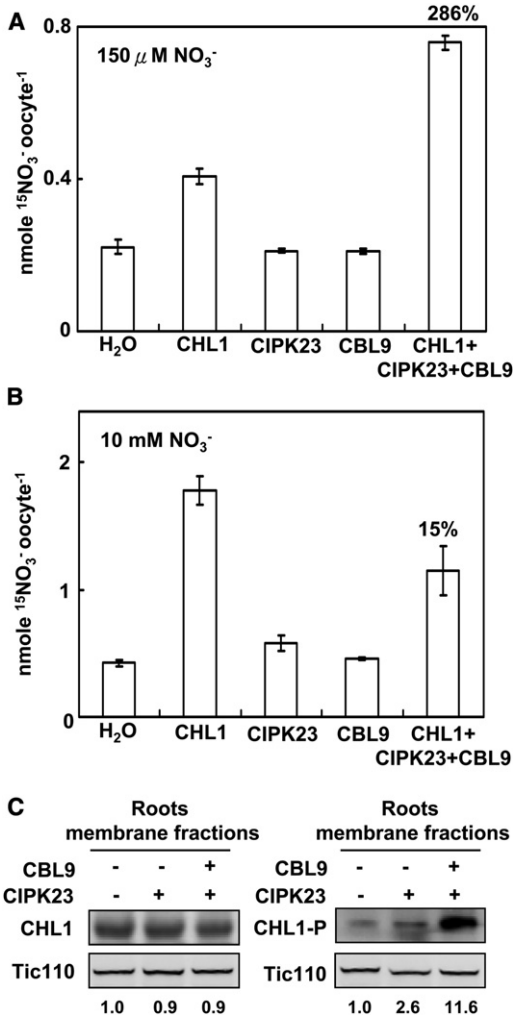

Figure 6. CIPK23 Can Convert CHL1 into a High-Affinity Nitrate Transporter and Phosphorylate It at Residue Threonine 101

(A) Uptake activities of oocytes injected with *CIPK23*, *CBL9*, or *CHL1* alone or coinjected with all three. Uptake activities were determined using 150 μM and 10 mM ^{15}N-labeled nitrate to assess the level of high-affinity (A) and low-affinity (B) uptake. The values are the mean ± SD (n = 6). Similar results were obtained with oocytes isolated from three other frogs. The numbers above the last bar in (A) indicate uptake expressed as a percentage of wild-type CHL1 uptake after subtraction of background uptake (water-injected oocytes), whereas those in (B) show the actual low-affinity nitrate uptake calculated by subtracting the contribution of the high-affinity component from the 10 mM nitrate uptake.

(C) In vitro phosphorylation of CHL1 by CIPK23. In vitro kinase assays were performed on GST-CIPK23 using, as substrate, total membrane protein from the roots of plants grown in NO_3^--free medium using NH_4^+ as the sole N source for 10 days. Levels of CHL1T101 phosphorylation were determined by western blot analyses using anti-CHL1 antibodies (CHL1; left panel) or anti-CHL1-P-T101 antibodies (CHL1-P; right panel) (Liu and Tsay, 2003), with Tic110 as the loading control (stained with anti-Tic110 antibodies). Full film for the CHL1 phosphorylation is shown in Figure S8. Similar results were obtained in three independent experiments. The band intensities for CHL1-P and CHL1 were first normalized to that for Tic110 and then expressed relative to CHL1-P or CHL1 protein levels without incubation with CIPK23.

the *chl1-9* mutant, was required for nitrate transport, but not for nitrate sensing. The mutant *chl1-9* displayed a normal biphasic primary nitrate response, showing that Pro492 was not required for nitrate binding. In the case of Ca^{2+}-ATPase, the calcium binding site in the middle of the transmembrane region faces the cytosol in the E1 stage (Toyoshima and Mizutani, 2004) and binds calcium in the cytosol. To expel calcium to the exterior (or to the sarcoplasmic reticulum lumen), a conformational change is required to convert the E1 stage into the E2 stage to move the binding site to face the exterior. Pro492 in CHL1 might be required for a similar conformational change to transport nitrate across the membrane. However, the conformational changes triggered by nitrate binding to initiate nitrate signaling and CHL1T101 phosphorylation were not defective in the substitution mutant P492L (Figure S11). Only 3 of the 53 *Arabidopsis* NTR1 transporters do not contain a Pro in the corresponding region. It would be interesting to know whether these three NRT1s are nontransporting sensors.

Similar to CHL1-mediated nitrate uptake, the primary nitrate response is pH dependent (Figure S12A). As shown in Figures S3C and S13, no nitrate uptake and no nitrate-induced current change were detected in oocytes injected with *chl1-9*, suggesting that membrane potential changes are not required for the nitrate response. Compared to nitrate, chlorate is less competent at triggering the primary nitrate response (Figure S12B). Interestingly, a 10 s nitrate pulse could trigger a statistically significant response; it was, however, much lower than the 30 min response, suggesting that nitrate levels are sensed rapidly and that continuous exposure can have a cumulative effect. These studies suggested that the primary nitrate response could be initiated by conformational change of CHL1 induced by nitrate binding to activate or recruit downstream signaling components.

Role of CHL1 T101 Phosphorylation in Nitrate Signaling

We previously showed that phosphorylation of CHL1T101 is important for the functional switch between its high-affinity and low-affinity transport modes (Liu and Tsay, 2003). The evidence presented here indicates that CHL1T101 phosphorylation is also involved in the regulation of the primary nitrate response. Compared to the two-phase pattern of the primary nitrate response in the wild-type, CHL1T101D rescued the high-affinity phase of the *chl1* mutant, but did not cause any further increase in the low-affinity phase, whereas CHL1T101A enhanced both phases (Figure 2). These results show that the phosphorylated form of CHL1 is responsible for reducing the primary nitrate response to a low level, whereas the dephosphorylated form results in a high-level (or full) primary nitrate response. The primary nitrate response and CHL1T101 phosphorylation levels in the wild-type were also consistent with this. When wild-type plants were exposed to a low concentration of nitrate, CHL1T101 phosphorylation increased transiently (Figure 4A) and gene expression in the primary nitrate response in the high-affinity phase was low, whereas, when they were exposed to high concentrations of nitrate, no increase in CHL1T101 phosphorylation was seen (Figure 4C) and gene expression in the low-affinity phase was high (Figure 1). These results support the model that in response to a low concentration of nitrate, the phosphorylated form of CHL1 leads to a low level primary

response. Because of the phosphorylation switch, CHL1 is able to sense a wide range of nitrate concentrations and trigger different levels of the nitrate response.

The roles of CHL1T101 phosphorylation are somewhat different in nitrate uptake and nitrate signaling. Transgenic plants expressing T101A, which mimics dephosphorylated CHL1, exhibited only low-affinity nitrate uptake, but could sense nitrate in the high-affinity range and their high-affinity nitrate response was double that in the wild-type. These properties suggest that, in wild-type CHL1 and the T101A mutant, there could be two nitrate binding sites, one high affinity and one low affinity, and that, in the T101A mutant, only nitrate bound to the low-affinity site can be transported across the membrane. Different from uptake, nitrate binding to both sites of T101A mutant could trigger the nitrate response. This could be why the CHL1T101A mutant still exhibited a biphasic primary response (Figure 2B). Transgenic plants expressing T101D, which mimics phosphorylated CHL1, exhibited only high-affinity nitrate uptake activity and similarly triggered only a high-affinity primary nitrate response, suggesting that T101D can only bind nitrate with high affinity. Thus, it is possible that the low-affinity binding site might be blocked by T101 phosphorylation. The two nitrate binding sites described here might reflect two conformations of a single binding site. Taken together, these data suggest that, for sensing, phosphorylation of T101 keeps the primary nitrate response at a low level, whereas, for uptake and substrate binding, T101 phosphorylation may block low-affinity nitrate binding and is required for high-affinity transport. Crystal structure analyses are required to understand the details of the conformational changes in CHL1 and how these changes are regulated by T101 phosphorylation.

CIPK23 Phosphorylates CHL1T101 in Response to Low Nitrate Concentrations

The primary nitrate response of the *cipk23* mutants was similar to that of transgenic T101A plants. The increased high-affinity primary response in the *cipk23* mutants suggested that CIPK23 is a negative regulator of the high-affinity nitrate response. An in vitro kinase assay and oocyte coinjection experiments showed that, once activated by CBL9, CIPK23 can phosphorylate CHL1 at T101 and convert CHL1 into a high-affinity nitrate transporter. In contrast to the wild-type, no transient increase in T101 phosphorylation was seen in response to low concentrations (high-affinity range) of nitrate in the *cipk23* mutant. Consistent with CBL9 being a calcium binding protein that activates CIPK23, as in the *cipk23* mutant, primary nitrate responses were enhanced in calcium-depleted media (Figure S12A). These in vivo and in vitro findings show that, when exposed to low-concentration nitrate, CIPK23 is responsible for the phosphorylation of CHL1T101, which then reduces the primary nitrate response to a low level.

Our previous study (Hu et al., 2009) showed that another CBL-interacting protein kinase, CIPK8, is also involved in the primary nitrate response. However, CIPK8 and CIPK23 play different roles in nitrate signaling: CIPK8 is a positive regulator of the low-affinity response, whereas CIPK23 is a negative regulator of the high-affinity response. In addition, our preliminary data indicate that CHL1 T101 is not the direct target of CIPK8.

Two CIPKs can have different effects in the same signal pathway and a single CIPK can be involved in multiple signaling pathways (Kolukisaoglu et al., 2004). Previous studies showed that *Arabidopsis* CIPK23 can activate the potassium channel AKT1 to enhance potassium acquisition in response to low-potassium conditions (Li et al., 2006; Xu et al., 2006). In this study, we demonstrated a novel function of CIPK23 in nitrate signaling. Because KCl did not induce expression of nitrate-related genes, with only basal expression in both the wild-type and *cipk23* mutant plants (Figure 3), the increased primary nitrate response seen in the *cipk23* mutant was nitrate specific. Moreover, different potassium concentrations (10 mM or 0.2 mM) in the induction medium will not affect the primary nitrate response level (Figure S12C), indicating a lack of crosstalk between nitrate and potassium. Therefore, CIPK23 is involved independently in both potassium sensing and nitrate sensing. Because CIPK23 was able to bind directly to CHL1, CHL1 may also serve as a scaffold protein to recruit the signaling components to trigger a nitrate-specific response. It will be interesting to determine whether AKT1 also functions as a potassium sensor. More generally, the sensing mechanism described here might be a common means of ion sensing in plants and other organisms.

A Model for the Sensing Mechanism of CHL1

We propose a working model to illustrate how CHL1 might sense a wide range of nitrate concentrations in the external environment and thus generate different levels of the primary nitrate response (Figure 7). Nitrate binding is required to trigger signals: in CHL1, this could occur at two separate sites with different affinities or at a single binding site, the affinity being modified by conformational changes. For the purpose of illustration, two binding sites are shown in Figure 7. Nitrate binding to CHL1 is required to trigger the primary nitrate response, and phosphorylation of threonine 101 by CIPK23 could modulate the levels of primary response. When exposed to low concentrations of nitrate, nitrate binds to the high-affinity site. This high-affinity binding then activates or recruits CIPK23 to phosphorylate CHL1 at T101, and phosphorylated CHL1 prevents higher primary nitrate response at low-nitrate concentrations. In contrast, at high concentrations, nitrate can bind to the low-affinity site, which inhibits the phosphorylation of CHL1 T101, and the unphosphorylated CHL1 then generates a high primary nitrate response. In the transgenic CHL1T101D plants, CHL1 was fixed in the phosphorylated form, whereas, in CHL1T101A plants, it was fixed in the unphosphorylated form, leading to low or high monophasic primary responses, respectively. In the *cipk23* mutants, CHL1 could not be phosphorylated in response to low concentrations of nitrate and, as with the transgenic CHL1 T101A plants, there was a monophasic, high-level primary response. Thus, using dual-affinity binding and T101 phosphorylation, CHL1 can sense a wide range of nitrate concentrations in the soil and trigger different levels of response.

EXPERIMENTAL PROCEDURES

Plant Material and Treatments

The plant growth conditions for the Q-PCR analysis, nitrate uptake assay, and protein blot assay were described previously (Hu et al., 2009; Liu and Tsay,

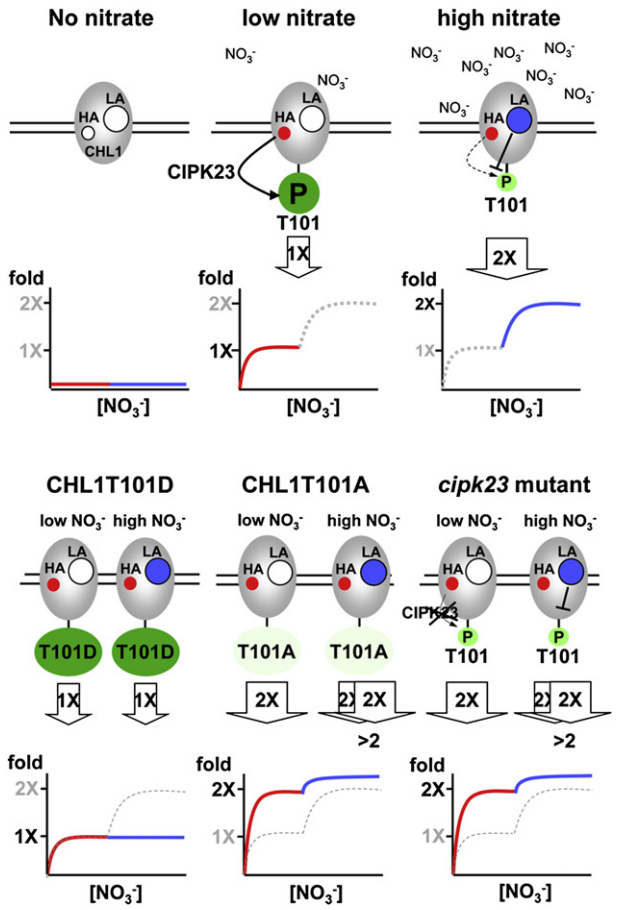

Figure 7. Schematic Model for CIPK23- and CHL1-Mediated Nitrate Sensing in the Primary Nitrate Response

The top panel shows the nitrate sensing mechanism, while the bottom panel shows gene expression during the primary nitrate response. The gray ovals represent CHL1 in the plasma membrane. The small and large empty circles represent the high- and low-affinity nitrate binding sites, respectively. The red and blue circles indicate nitrate binding to the high- and low-affinity binding site, respectively. P denotes phosphorylated CHL1T101 and the green color gradient represents the level of CHL1T101 phosphorylation. The panels below the cartoons represent the level of gene expression in the primary nitrate response, with red for the high-affinity phase and blue for the low-affinity phase.

2003). Three vessels (~100 seedlings each) of samples were pooled for each independent experiment. Unless indicated, plants were grown in 12.5 mM $(NH_4)_2$ succinate medium at pH 6.5 for 10 days, transferred to a fresh 12.5 mM $(NH_4)_2$ succinate medium at pH 5.5 overnight/16 hr, and then transferred to another fresh 12.5 mM $(NH_4)_2$ succinate medium at pH 5.5 for an additional 3 hr. After 3 hr treatment, the plants were shifted for the indicated time to 60 ml of the same pH 5.5 growth medium, but with the $(NH_4)_2$ succinate replaced with KNO_3 or KCl.

cipk23 Gene T-DNA Plant Isolation and the CHLT101D and CHL1T101A Transgenic Plants Generation

The *cipk23* T-DNA insertion lines *cipk23-3* (SALK_036154) and *cipk23-4* (SALK_112091) were obtained from the *Arabidopsis* Biological Resource Center (http://www.arabidopsis.org/abrc/). The insertions were identified using a T-DNA left border primer and CIPK23-specific primers (listed in Supplemental Experimental Procedures). To generate the transgenic plants, *CHL1 promoter::CHL1T101D/A* genomic DNA fragments were constructed and transformed into *chl1-5* plants (details in Supplemental Experimental Procedures). All plasmid constructions are given in the Supplemental Experimental Procedures.

Real-Time Q-PCR Analysis

The primers for the PCR reactions are listed in the Supplemental Experimental Procedures. Real-time Q-PCR was performed as described previously (Hu et al., 2009) using a LightCycler System (Roche Diagnostics). Q-PCR presented are the means ± SD of triplicate, each on ~200 seedlings grown in three boxes. Statistic analyses were performed using t test in Excel program. Identical results were obtained in another experiment or two other experiments.

Yeast Two-Hybrid Assays

For split-ubiquitin analysis (Stagljar et al., 1998), the full-length cDNAs of interest were cloned in frame with either the C-terminal (Cub) or N-terminal (NubG; wild-type I-13 replaced by G) subdomain of ubiquitin, and then introduced into yeast strain DSY2 and DSY1 by the lithium acetate method (Schiestl and Gietz, 1989). For β-galactosidase activity assay see the Supplemental Experimental Procedures.

BiFC Assays

As described previously (Waadt et al., 2008), mesophyll protoplasts were isolated from 2-week-old wild-type plants (ecotype Columbia) and transformed with different YFP fusion protein combinations (http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html), and then, after incubation at 23°C for 12–18 hr, YFP fluorescence in the transformed protoplasts was imaged using a confocal laser scanning microscope (LSM510; Carl Zeiss).

In Vitro Phosphorylation Assay and Western Blots

Fifteen micrograms of GST fusion proteins expressed in *E. coli* strain BL21 and purified using glutathione Sepharose 4B (Pharmacia) and 20 μg of membrane fractions from *Arabidopsis* root were subjected to in vitro phosphorylation assays at 25°C for 30 min in 20 mM Tris-HCl (pH 7.2), 2.5 mM $MnCl_2$, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM DTT, and 1 μM ATP in a final volume of 20 ml. The reaction was stopped by addition of 6 μl of 4× loading buffer and the proteins were resolved by SDS-PAGE and detected by western blotting using anti-P-CHL1T101 antibody. The protocols and antibodies used have been described previously (Liu and Tsay, 2003).

$^{15}NO_3^-$ Uptake Assay in *Xenopus* Oocytes

The oocytes were injected with distilled water (50 nl as control), CHL1 cRNA (50 ng in 50 nl), *chl1-9* cRNA (50 ng in 50 nl), CIPK23 cRNA (100 ng in 50 nl), or CBL9 cRNA (50 ng in 50 nl) or a CHL1, CBL9, and CIPK23 cRNA mixture (50:100:50 ng in 50 nl). As described previously (Almagro et al., 2008), after 2 days the oocytes were incubated for 90 min in $^{15}NO_3^-$ medium and the amount of nitrate retained in the oocytes was determined.

SUPPLEMENTAL DATA

Supplemental Data contain Supplemental Experimental Procedures, 13 figures, and a video summary can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00842-3.

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