the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2009) **57**, 264–278 doi: 10.1111/j.1365-313X.2008.03685.x

AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response

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Received 8 May 2008; revised 23 August 2008; accepted 3 September 2008; published online 27 October 2008.
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Summary

Nitrate, the major nitrogen source for most plants, is not only a nutrient but also a signaling molecule. For almost two decades, it has been known that nitrate can rapidly induce transcriptional expression of several nitrate-related genes, a process that is referred to as the primary nitrate response. However, little is known about how plants actually sense nitrate and how the signal is transmitted in this pathway. In this study, a calcineurin B-like (CBL) -interacting protein kinase (CIPK) gene, *CIPK8*, was found to be involved in early nitrate signaling. *CIPK8* expression was rapidly induced by nitrate. Analysis of two independent knockout mutants and a complemented line showed that CIPK8 positively regulates the nitrate-induced expression of primary nitrate response genes, including nitrate transporter genes and genes required for assimilation. Kinetic analysis of nitrate induction levels of these genes in wild-type plants indicated that there are two response phases: a high-affinity phase with a K_m of approximately 30 μM and a low-affinity phase with a K_m of approximately 0.9 mM. As *cipk8* mutants were defective mainly in the low-affinity response, the high-affinity and low-affinity nitrate signaling systems are proposed to be genetically distinct, with CIPK8 involved in the low-affinity system. In addition, CIPK8 was found to be involved in long-term nitrate-modulated primary root growth and nitrate-modulated expression of a vacuolar malate transporter. Taken together, our results indicate that CBL–CIPK networks are responsible not only for stress responses and potassium shortage, but also for nitrate sensing.

Keywords: nitrate signal, primary nitrate response, CIPK, CHL1, nitrate sensing.

Introduction

The growth of plants is governed by their environment. In contrast to animals, plants cannot escape from harsh living conditions and have to adapt to their surroundings. Limited or excessive ion or nutrient availability are environmental stresses frequently encountered by plants, as the concentration of ions or nutrients in various soils can vary by several orders of magnitude (Jackson and Caldwell, 1993). Thus, a rapid nutrient detection mechanism allowing the plant to adapt to dynamic environmental properties is imperative for survival.

Most land plants use nitrate as the major nitrogen source in aerobic soils. Nitrate is taken up into the root by several transporters, such as CHL1 (NRT1.1) (Tsay *et al.*, 1993), AtNRT1.2 (Huang *et al.*, 1999), AtNRT2.1 (Little *et al.*, 2005) and AtNRT2.2 (Li *et al.*, 2007), and assimilated into organic nitrogen by a series of enzymatic reactions catalyzed by nitrate reductase in the cytosol and by nitrite reductase and glutamate synthase in plastids and chloroplasts (Crawford, 1995). Several lines of evidence suggest that, in higher plants, nitrate serves not only as a nutritional source, but also as a signaling molecule. For example, nitrate, but not other nitrogen sources added to the soil or growth medium, induces the expression of several genes involved in nitrate transport and assimilation (Stitt, 1999), induces leaf expansion (Walch-Liu *et al.*, 2000), stimulates lateral root growth (Zhang and Forde, 1998), regulates abscisic acid (ABA)-independent stomatal opening (Guo *et al.*, 2003), and relieves seed dormancy (Alboresi *et al.*, 2005).

Although these nitrate-induced responses could be regulated by downstream metabolites of nitrate, several studies have indicated that nitrate itself regulates gene expression. Genes regulated by nitrate include nitrate transporters

(Lejay *et al.*, 1999), nitrate assimilation enzymes (Gowri *et al.*, 1992), and carbon assimilation enzymes involved in nitrogen/carbon balance (Scheible *et al.*, 1997). These genes are expressed at low levels in the absence of nitrate and are rapidly induced in its presence. The rapid transcriptional induction in response to nitrate is referred to as the primary nitrate response (Redinbaugh and Campbell, 1991). The primary response of these nitrate-regulated genes occurs in the absence of nitrate reductase activity (Deng *et al.*, 1989; Pouteau *et al.*, 1989), suggesting that it is the nitrate ion, rather than the downstream products of nitrate metabolism, that is the signaling molecule responsible for induction. In addition, transcriptional induction occurs in the presence of a protein synthesis inhibitor (Gowri *et al.*, 1992; Redinbaugh and Campbell, 1993), showing that *de novo* protein synthesis is not required for nitrate-induced gene expression in the primary response. Taken together, these data show that nitrate itself serves as the signaling molecule regulating the primary transcriptional response. The underlying mechanism of nitrate sensing, which allows the plant to modulate downstream metabolism in response to the external nitrate concentration, has not yet been identified.

In contrast, several nitrate signaling pathways have been identified in micro-organisms. In *Escherichia coli*, two membrane-spanning histidine kinases, NarX and NarQ, bind nitrate and nitrite via a 17 amino acid periplasmic region called the P-box, then phosphorylate their respective response regulators, NarL and NarP, which then regulate the expression of downstream genes (Cavicchioli *et al.*, 1996; Chiang *et al.*, 1997). In higher plants, similar His-to-Asp two-component systems have been shown to be involved in cytokinin (Inoue *et al.*, 2001) and ethylene (Chang *et al.*, 1993) signaling, but have not been implicated in nitrate signaling. In a second nitrate-sensing system in the bacterium *Klebsiella oxytoca*, nitrate and nitrite induction of assimilation genes is mediated by a transcriptional antitermination mechanism involving an RNA-binding protein, NasR (Chai and Stewart, 1998). In addition, in fungi, the transcription factors *NIT-4* from *Neurospora* (Fu *et al.*, 1989) and *NIRA* from *Aspergillus* (Burger *et al.*, 1991) have been shown to be involved in transcriptional induction of nitrate assimilation genes, and the nuclear localization of NIRA is regulated by nitrate (Bernreiter *et al.*, 2007). While many nitrate signaling components have been identified in bacteria and fungi, there is no evidence that similar homologs function in higher plants, and precisely how higher plants monitor the concentration of nitrate and convert this signal into physiological and developmental responses has puzzled plant biologists for a long time.

In higher plants, the only well-characterized component of the nitrate signaling pathway, ANR1, a transcription factor of the MADS box family, was identified in a mutant defective in nitrate-stimulated proliferation of lateral root elongation (Zhang and Forde, 1998). Several studies have suggested that the dual-affinity nitrate transporter CHL1 (Liu *et al.*, 1999) and the high-affinity nitrate transporter AtNRT2.1 (Little *et al.*, 2005) may be involved in nitrate signaling. For example, mutants of both *chl1* (Guo *et al.*, 2001; Walch-Liu and Forde, 2008) and *atnrt2.1* (Little *et al.*, 2005) exhibit an altered root architecture even in the absence of added nitrate, suggesting that CHL1 and AtNRT2.1 may serve as nitrate sensors or signal transducers. In addition, nitrate-stimulated ANR1-regulated lateral root growth (Remans *et al.*, 2006) and *AtNRT2.1* repression by a high concentration of nitrate (Krouk *et al.*, 2006; Munos *et al.*, 2004) are defective in *chl1* mutants. More detailed analyses are required to understand whether nitrate transporters participate directly or indirectly in the nitrate signaling pathway.

In this study, a calcineurin B-like (CBL)-interacting protein kinase (CIPK) gene, *CIPK8*, was identified by microarray analysis of the *chl1-5* mutant and found to be involved in the primary nitrate response.

Results

Microarray analysis shows that the CBL-interacting protein kinase gene, CIPK8, is down-regulated in the chl1-5 mutant

As CHL1 is one of the major transporters responsible for nitrate uptake, and some studies suggest that it may also play a role in regulating the nitrate response, its influence was analyzed in a global transcriptome study. When microarray analyses of root tissues from wild-type and *chl1-5* mutant plants were performed to explore differences in gene expression between the *chl1-5* mutant and wild-type after 30 min of nitrate induction, the expression of 42 genes in the *chl1-5* mutant was at least 1.7 times lower than normal (*chl1*/wild-type ratio ≤0.59) (Table S1; see Appendix S1 for details of microarray analysis). Interestingly, most of the 42 genes (39/42) were nitrate-inducible genes, indicating that the effects of CHL1 on gene expression correlate well with the nitrate response.

These changes in gene expression could be due to either a reduction in root nitrate content or another additional effect in the *chl1* mutant. To determine which, we first measured nitrate levels in the roots of wild-type *Arabidopsis thaliana* and the *chl1-5* mutant when exposed to various nitrate concentrations. After 30 min exposure, the nitrate content in wild-type roots in 2.5 mM external NO_3^- was similar to that in the roots of the *chl1-5* mutant in 25 mM NO_3^- (indicated by dotted line in Figure 1a). Although the cytosolic nitrate content in the sensing cells is not be faithfully reflected by the total tissue nitrate content, total nitrate content was used as an indicator to facilitate screening. If nitrate content plays a major role in regulating gene expression, as for the MYB transcription factor At1g13300 shown in Figure 1(b), similar expression levels of At1g13300 would be found in the *chl1-5* mutant exposed to 25 mM NO_3^- and in the wild-type

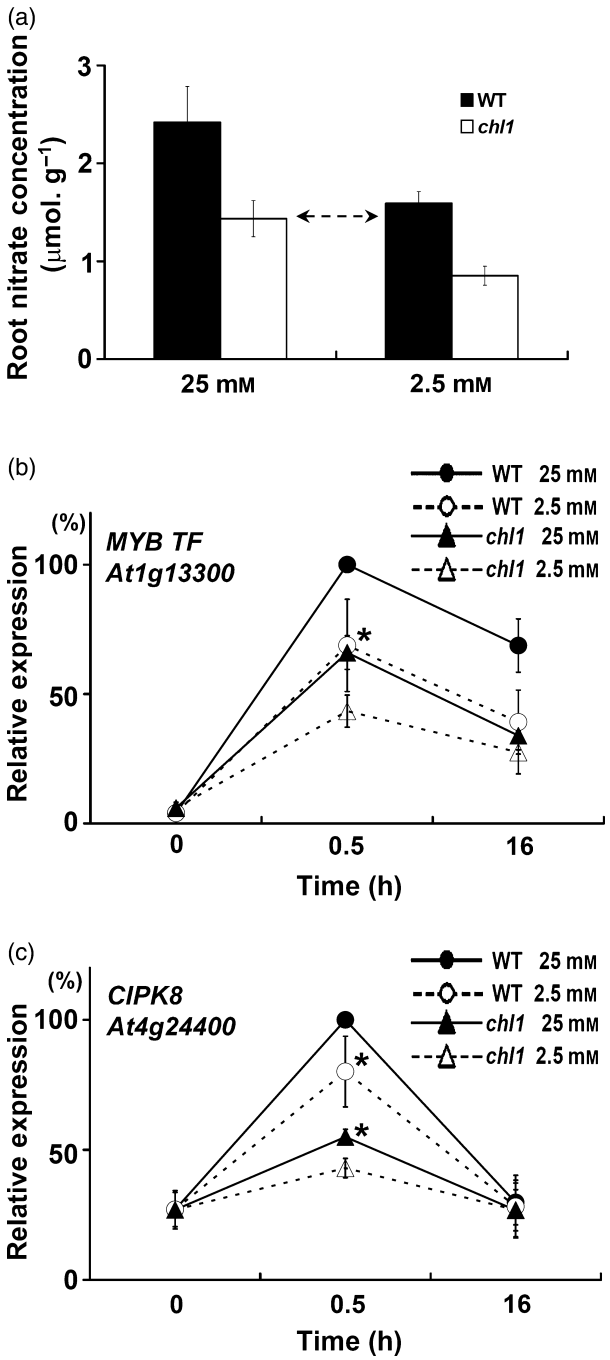

Figure 1. Strategy for classifying 'genes responding to both root nitrate concentration and some additional effects of CHL1' and 'genes responding mainly to alterations of root nitrate concentration'.
(a) Root nitrate content of wild-type and the *chl1-5* mutant. Wild-type and *chl1-5* were grown in NO_3^--free medium (NH_4^+ as the sole nitrogen source) for 10 days, then shifted to 25 mM or 2.5 mM nitrate for 30 min. The root nitrate contents shown are the mean ± SD for four vessels of plants of each type tested in a single experiment.
(b) Quantitative PCR analysis of a putative MYB transcription factor gene (At1g13300), which showed a 'gene responding mainly to alterations of root nitrate concentration' pattern of expression.
(c) Quantitative PCR analysis of *CIPK8*, which showed a 'gene responding to both root nitrate concentration and some additional effects of CHL1' pattern of expression.
In (b) and (c), 10-day-old ammonium-grown wild-type and *chl1-5* plants were exposed to 25 mM or 2.5 mM nitrate for 0.5 and 16 h, then gene expression in the root was measured and normalized to that of the wild-type exposed to 25 mM KNO_3 for 30 min.

exposed to 2.5 mM NO_3^- (labeled by an asterisk in Figure 1b). Genes with this type of expression pattern were classified as 'genes responding mainly to alterations of root nitrate concentration'. On the other hand, genes such as *CIPK8* were classified as 'genes responding to both root nitrate concentration and some additional effects of CHL1', because the expression levels in the *chl1-5* mutant exposed to 25 mM NO_3^- were quite different from the expression levels in wild-type plants exposed to 2.5 mM NO_3^- (labeled with an asterisk in Figure 1c), indicating that, in addition to nitrate, CHL1 plays some role in regulating their expression .

Twenty-two of the 42 genes with significantly decreased expression in the *chl1-5* mutant (Table S1; mainly known transporters, transcription factors, signaling components, or proteins involved in nitrogen assimilation-related pathways, and a few unknown genes with high expression) were selected for further analysis, and their expression in wild-type and *chl1-5* plants exposed to 2.5 or 25 mM NO_3^- for 30 min was measured by RT-PCR or quantitative PCR. Of the 22, 18 were classified as 'genes responding mainly to the alterations of root nitrate concentration' while the remaining four were 'genes responding to both root nitrate concentration and some additional effects of CHL1' (Table S1 and Figure S1). The classification was based on a *t*-test of the expression levels in wild-type plants exposed to 2.5 mM NO_3^- and *chl1-5* mutants exposed to 25 mM for 30 min. CIPK8, one of the 'genes responding to both root nitrate concentration and some additional effects of CHL1', was induced in both the wild-type and the *chl1-5* mutant by 30 min of NO_3^- treatment, and the induction levels were down-regulated in the *chl1-5* mutant (Figure 1c). These data suggest that a novel CBL–CIPK network may be involved in the nitrate response.

CIPK8 is rapidly induced by nitrate

CBLs, a family of Ca^{2+} sensors, are only found in plants. On binding to Ca^{2+}, they activate the serine/threonine CIPKs to trigger a downstream signaling transduction cascade (Albrecht *et al.*, 2001). CBL–CIPK complexes are known to regulate several stress responses, such as salt, cold, drought, abscisic acid signaling and K^+ shortage (Batistic and Kudla, 2004; Gong *et al.*, 2004; Li *et al.*, 2006a; Xu *et al.*, 2006).

Time-course analysis of *CIPK8* expression in wild-type plants by quantitative PCR showed that *CIPK8* was induced very rapidly by 25 mM NO_3^- (Figure 2a). CIPK8 mRNA levels increased at 15 min after nitrate induction, reached

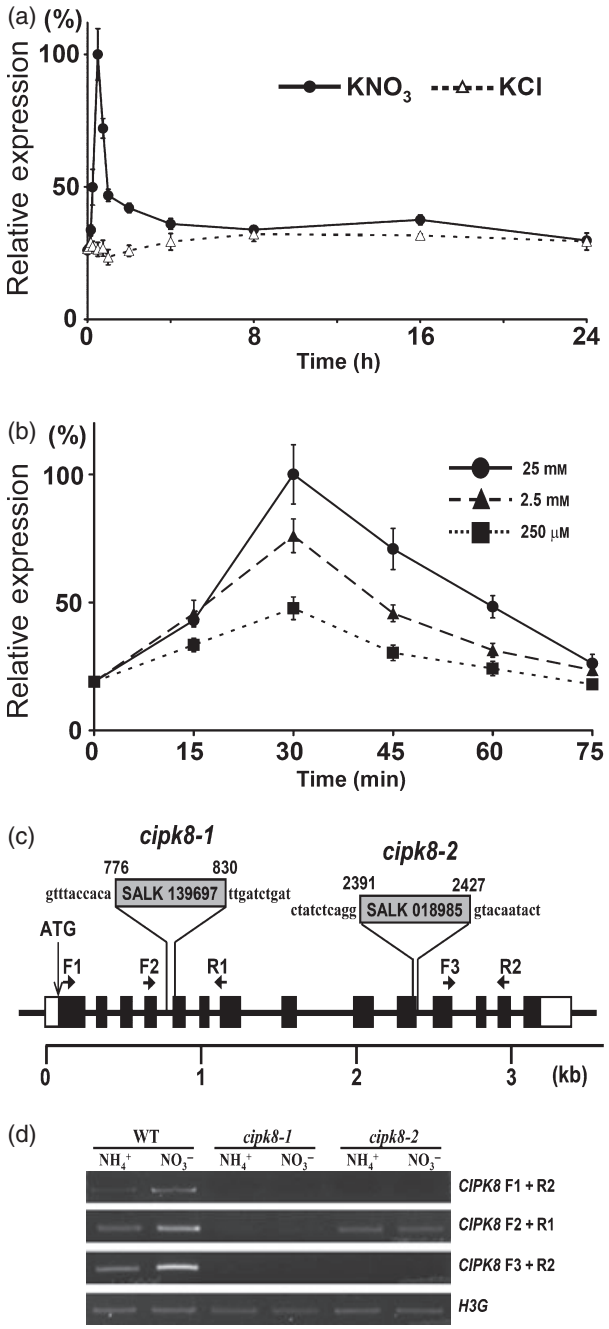

Figure 2. Nitrate induces rapid expression of *CIPK8* in a concentration-dependent manner.
(a) Quantitative PCR analysis of the nitrate induction pattern of *CIPK8* expression in wild-type plants. Plants were exposed to 25 mM KNO_3 or 25 mM KCl as a control for the duration indicated.
(b) Quantitative PCR analysis of *CIPK8* in wild-type plants exposed to the indicated concentration of nitrate. The relative level is the expression normalized to that for *CIPK8* in wild-type plants exposed to 25 mM KNO_3 for 30 min.
(c) Intron–exon organization and T-DNA disruption of the *Arabidopsis CIPK8* gene. The filled and empty boxes indicate exons and untranslated regions, respectively.
(d) RT-PCR analysis of *CIPK8* expression in wild-type and *cipk8* mutants. RNA was isolated from the roots of plants exposed to 25 mM KNO_3 or 12.5 mM $(NH_4)_2$succinate for 30 min.
The primers used here are indicated in (c). H3G (At4g40040) is shown as a control.
The growth conditions of wild-type and *cipk8* plants in (a), (b) and (d) were the same as those described in Figure 1.

maximum levels in 30 min, and fell to basal levels after 2 h. No detectable change was seen when plants were exposed to potassium chloride as a control, indicating that *CIPK8* induction was specific for nitrate and was not due to addition of potassium or removal of ammonium. When wild-type plants were exposed to different concentrations of NO_3^- (250 μM, 2.5 mM and 25 mM), the time courses of CIPK8 expression were similar, with maximum expression at 30 min, but the maximum levels were concentration-dependent (Figure 2b). These results show that *CIPK8* is rapidly induced by nitrate and that the level of induction is concentration-dependent.

CIPK8 is a positive regulator of the primary nitrate response

To study the function of *CIPK8*, two independent T-DNA insertion lines, *cipk8-1* and *cipk8-2*, were isolated. Sequencing analysis of *cipk8-1* and *cipk8-2* revealed that the T-DNA insertions were located in intron 4 after 776 bp and in exon 10 after 2391 bp from the start codon of the *CIPK8* gene, respectively (Figure 2c). RT-PCR analysis using three pairs of primers indicated that, although a truncated form of *CIPK8* transcript was expressed in *cipk8-2*, no full-length transcript was detected in either mutant (Figure 2d).

As CIPK8 is rapidly induced by nitrate and several CIPKs have been implicated in the regulation of gene expression, the expression of several nitrate-induced genes was analyzed in the *cipk8* mutants to determine whether CIPK8 is involved in the primary nitrate response. The genes analyzed included two nitrate-induced nitrate uptake transporter genes, *CHL1* and *NRT2.1*, and three nitrate-assimilation genes, *nitrate reductase 1* (*NIA1*), *nitrate reductase 2* (*NIA2*) and *nitrite reductase* (*NiR*) (Figure 3a–e). When wild-type plants were exposed to 25 mM nitrate, all five genes were highly induced, with expression reaching maximum levels at 30–45 min. In the *cipk8* mutants, these genes were still induced by nitrate with a similar time course, but the induction levels were significantly lower than in the wild-type. Similarly, two other known primary nitrate response genes, *glutamine synthetase 2* (*GLN2*) (Redinbaugh and Campbell, 1993) and *glucose-6-phosphate dehydrogenase 3* (*G6PDH3*) (Redinbaugh and Campbell, 1998) also showed reduced expression in *cipk8* mutants (data not shown). The non-nitrate-regulated histone subunit gene *H3G*, used as a control, had similar expression patterns in wild-type and mutant plants (Figure 3f and Figure S2b).

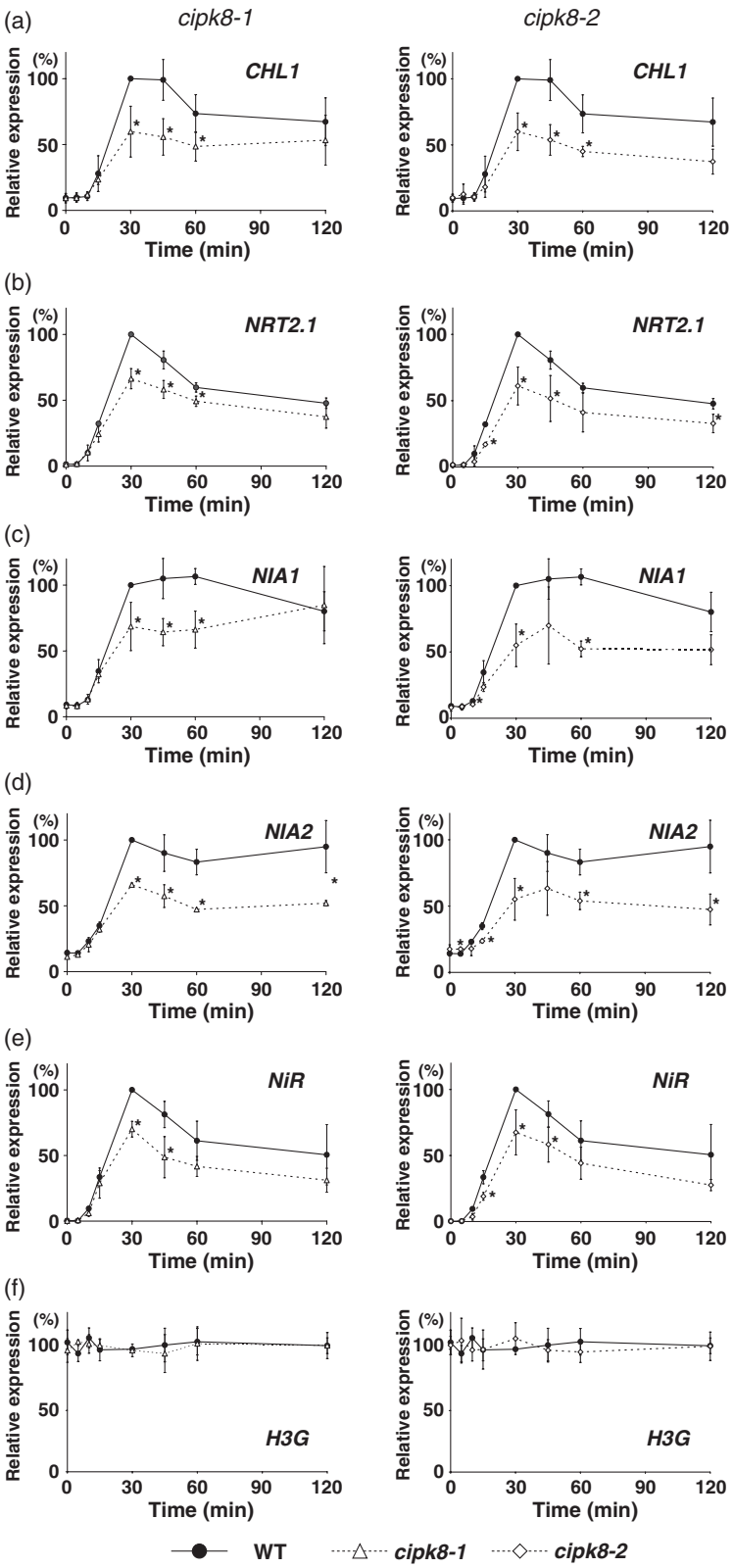

Figure 3. The induction levels of nitrate-regulated genes are reduced in *cipk8* mutants.
Quantitative PCR analysis of the expression of two nitrate uptake transporter genes, *CHL1* (a) and *NRT2.1* (b), three nitrate assimilation genes, *NIA1* (c), *NIA2* (d) and *NiR* (e), and a non-nitrate-regulated gene, *H3G* (f). Wild-type or *cipk8* plants were grown in NO_3^--free medium using $(NH_4)_2$succinate for 10 days, then exposed to 25 mM KNO_3 for the durations indicated. The relative level in the root is the expression normalized to that in wild-type plants exposed to 25 mM KNO_3 for 30 min.

Similar defects in the primary nitrate response were found in both mutants, suggesting that the truncated form of *CIPK8* encoded by the *cipk8-2* mutant is non-functional in this signaling. To further confirm that the defects observed were due to the mutation in *CIPK8*, a complemented line was generated by introducing a full-length cDNA fragment under

the control of the 35S promoter into the *cipk8-1* mutant (see Appendix S1). Quantitative PCR analysis indicated that expression of *CIPK8* transcripts was restored in the complemented line (Figure S2c). When the complemented line was exposed to 25 mM nitrate, the expression of primary nitrate response genes was restored to wild-type levels (Figure 4a and Figure S2a), indicating that the observed phenotypes were associated with the mutations of *CIPK8*. Together, these data suggest that CIPK8 participates in the transcriptional regulation of nitrate response genes.

Using the protein inhibitor cycloheximide, it has been shown that *de novo* protein synthesis is not required for the primary nitrate response (Gowri *et al.*, 1992; Redinbaugh and Campbell, 1993, 1998; Sakakibara *et al.*, 1997). To confirm that CIPK8 is involved in the primary nitrate response, wild-type and *cipk8-1* plants were treated with cycloheximide, and then exposed to nitrate. Consistent with previous studies, in wild-type plants, cycloheximide treatment did not affect nitrate-induced expression of *CHL1* (Figure 4b). With or without cycloheximide treatment, the induction levels in the *cipk8-1* mutants were reduced to 50–60% of the wild-type levels in response to 25 mM nitrate, indicating that the contribution of CIPK8 to transcriptional regulation of the nitrate response genes is not affected by cycloheximide treatment. As a control, the nitrate-induced expression of *LEA5* (Scheible *et al.*, 2004; Wang *et al.*, 2000, 2003), a gene whose expression is known to be cycloheximide-sensitive (Price *et al.*, 2004), was clearly inhibited by cycloheximide treatment, indicating that the dosage of cycloheximide in our study was sufficient to inhibit *de novo* protein synthesis (Figure S3). In addition, nitrate-induction patterns of *LEA5* were not altered in the *cipk8* mutant, indicating that CIPK8 is involved in cycloheximide-insensitive but not cycloheximide-sensitive signaling pathways of nitrate. Interestingly, when *cipk8* mutants were exposed to 250 μM nitrate, the induction levels were almost identical to those in the wild-type, even in the presence of cycloheximide. Similar results were also seen for *NRT2.1* expression (Figure S4). Taken together, these results suggest that the role of CIPK8 in nitrate signaling is nitrate concentration-dependent but cycloheximide-insensitive.

Nitrate-regulated genes are known to be affected by hormones and environmental stimuli, and several CIPKs are known to be involved in hormone, stress and environmental stimulus signaling pathways. To determine whether the effects of CIPK8 observed here were nitrate-specific,

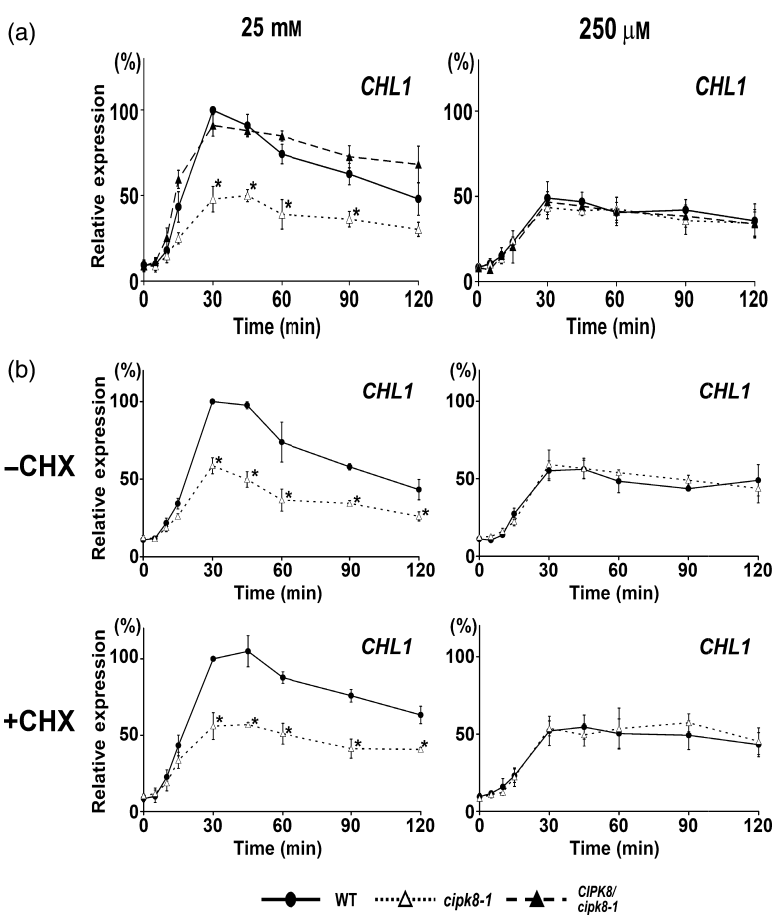

Figure 4. The defect of *cipk8-1* mutant with respect to *CHL1* expression is nitrate concentration-dependent and cycloheximide-independent, and can be rescued in a complemented line.
(a) Quantitative PCR analysis of expression of the *CHL1* gene in response to 25 mM KNO_3 or 250 μM KNO_3 in wild-type, *cipk8-1* and the complemented line.
(b) Quantitative PCR analysis of expression of the *CHL1* gene in response to 25 mM KNO_3 or 250 μM KNO_3 in the absence or presence of cycloheximide (CHX).
Wild-type, *cipk8-1* or the complemented plants were grown in NO_3^--free medium using NH_4^+ as the sole nitrogen source for 10 days, then exposed to 25 mM KNO_3 or 250 μM KNO_3 with or without 100 μM cycloheximide for the duration indicated. For cycloheximide treatment, plants were exposed to 100 μM cycloheximide for 1 h before nitrate induction, and up to the induction duration indicated. The relative level is the expression normalized to that in wild-type plants exposed to 25 mM KNO_3 for 30 min.

several marker genes for stress/ABA (*RD29A*, *RD22*, *KIN1* and *ABI2*) (Cheng *et al.*, 2002; Cheong *et al.*, 2003; Leonhardt *et al.*, 2004; Pandey *et al.*, 2004) and glucose (*HXK1* and *HXK2*) (Li *et al.*, 2006b; Price *et al.*, 2004) were analyzed. The expression of these marker genes was not significantly altered in the *cipk8-1* mutant when plants were exposed to 25 mM nitrate for 30 min (Figure S5). These results suggest that the role of CIPK8 in the primary nitrate response is not mediated by other stress or hormone signals.

In conclusion, neither *de novo* protein synthesis nor other signals are required for the CIPK8-mediated primary nitrate response at higher concentrations (25 mM). However, the defect of *cipk8* is nitrate concentration-dependent.

The cipk8 mutant is defective in the low-affinity phase of the primary nitrate response

To further characterize the roles of CIPK8 in nitrate signaling, the kinetics of *CHL1* and *NRT2.1* expression were analyzed. The temporal patterns of *CHL1* and *NRT2.1* expression, with a peak at 30 min, were not altered when wild-type and *cipk8* plants were exposed to 250 μM or 25 mM potassium nitrate (Figure 4). Therefore, 30 min of nitrate exposure was chosen for further kinetic analysis. Ten-day-old wild-type and *cipk8-1* plants were exposed to various concentrations of nitrate (in the micro- to millimolar range) for 30 min, and the mRNA levels of *CHL1* and *NRT2.1* were determined by quantitative PCR. Expression levels were normalized to the level induced by 25 mM nitrate in wild-type plants. Interestingly, in wild-type plants, the expression of *CHL1* and *NRT2.1* displayed bi-phasic patterns, with a Michaelis–Menten K_m value of approximately 30 μM for the high-affinity phase and approximately 0.9 mM for the low-affinity phase (Table 1 and Figure S6c). The enlarged scale from 0 to 1 mM in Figure 5 illustrates saturation of gene expression in the high-affinity phase, with V_{max} approaching 43–63% of the expression induced by 25 mM nitrate. To further illustrate whether this bi-phasic expression pattern is an attribute of the primary nitrate response caused by nitrate itself not by downstream metabolites, the nitrate reductase-deficient mutant *G4′-3* (*nia1 nia2*) was subjected to kinetic analysis of *CHL1* expression (Figure S6a,c). In *G4′-3* plants, the expression of *CHL1* showed similar bi-phasic expression patterns and K_m values, indicating that nitrate itself is responsible for this bi-phasic transcriptional response. These data suggest that plants can sense different concentrations of nitrate, exhibiting two phases of the transcriptional response.

In the *cipk8-1* mutant, the expression of *CHL1* and *NRT2.1* in the high-affinity phase was comparable to levels in the wild-type, but significant reductions were found in the low-affinity phase (Figure 5). The induction of *CHL1* and *NRT2.1* was nitrate-specific and was not due to the absence of ammonium or to osmotic changes caused by the presence of potassium, because no induction was detected using 250 μM or 25 mM potassium chloride (Figure 5). In three independent experiments, the V_{max}^{HA} of *CHL1* and *NRT2.1* in the *cipk8-1* mutant was reduced by 0–8%, but the V_{max}^{LA} of *CHL1* and *NRT2.1* was reduced by 44–90%, with an mean of 64% for *CHL1* and 59% for *NRT2.1* (Table 1). Therefore, CIPK8 regulates *CHL1* and *NRT2.1* in a concentration-dependent manner with a profound effect in the low-affinity range, suggesting that CIPK8 is involved in a low-affinity nitrate-sensing system.

To determine whether the reduced expression of nitrate-regulated genes in the *cipk8* mutants was due to defective nitrate uptake and/or a reduced nitrate content in the root, plants were grown under the same conditions used for expression studies in nitrate-free medium for 10 days, then shifted to medium containing 25 mM or 2.5 mM nitrate for 30 min. The cumulative nitrate uptake activity (Figure 6a) and root nitrate content (Figure 6b) of the *cipk8-1* mutant (and of the *cipk8-2* mutant, data not shown) were either identical, or similar, to those of the wild-type plant. Taken together, these data suggest that the reduced primary nitrate response in the *cipk8* mutants is more likely due to a defect in signal processing.

Table 1 Kinetic parameters for nitrate-induced expression in wild-type and *cipk8-1* plants

		High-affinity phase[a]			Low-affinity phase[a]			
Gene	Plant	K_m^{HA} (μM)	V_{max}^{HA}	Reduction[b]	K_m^{LA} (mM)	V_{max}^{total}	V_{max}^{LA}[c]	Reduction[b]
CHL1	Wild-type	34 ± 12	55 ± 12	6%	0.9 ± 0.4	91 ± 3	35	64%
	cipk8-1	26 ± 10	52 ± 10		0.2 ± 0.2	65 ± 6	13	
NRT2.1	Wild-type	32 ± 24	49 ± 14	2%	0.8 ± 0.2	92 ± 2	44	59%
	cipk8-1	24 ± 18	47 ± 11		0.4 ± 0.2	65 ± 3	18	

[a]The K_m and V_{max} values shown here are the mean of three independent experiments calculated by the Michaelis–Menten equation, using the double-reciprocal method shown in Figure 5.
[b]The values shown here are reductions in V_{max}^{HA} or V_{max}^{LA} levels in *cipk8* compared to wild-type.
[c]The values shown here are $V_{max}^{total} - V_{max}^{HA}$.

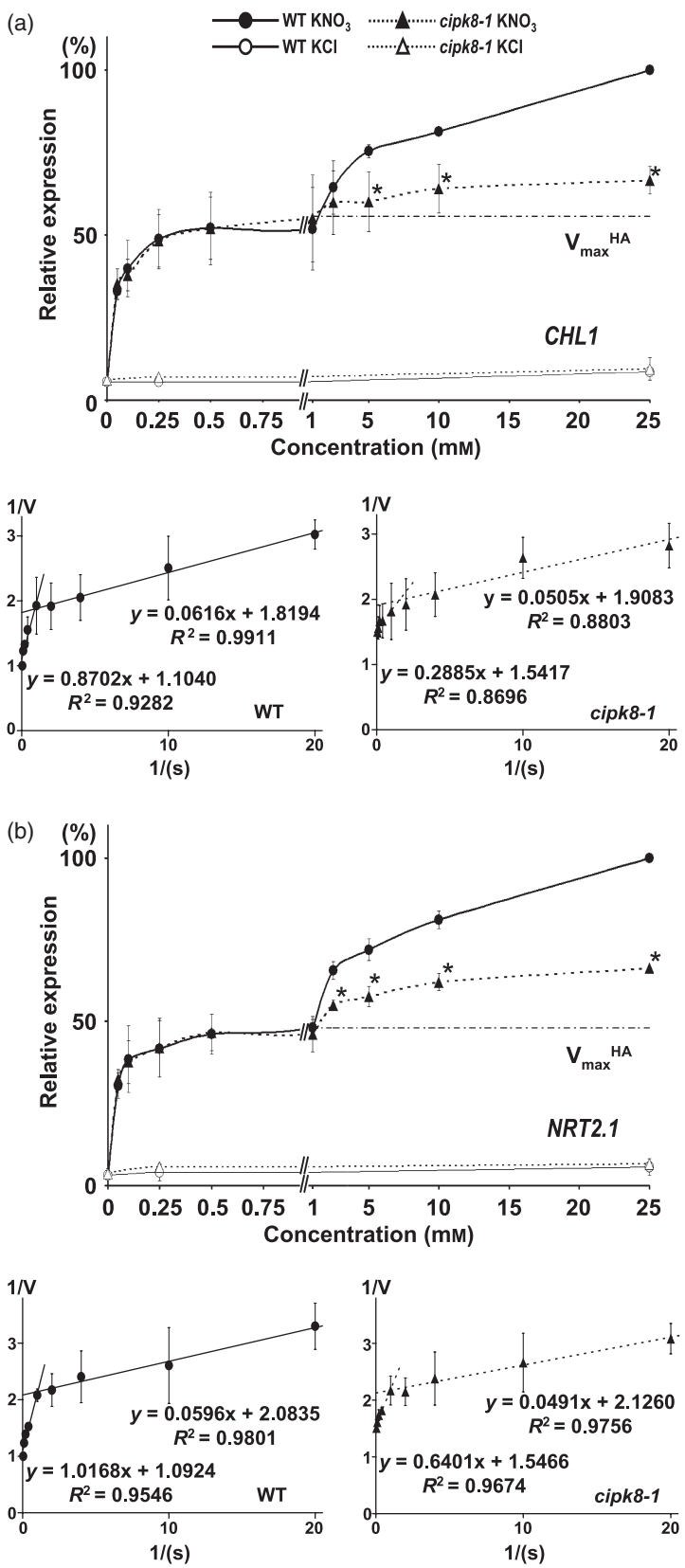

Figure 5. Kinetic analysis of the gene expression levels of *CHL1* and *NRT2.1* in wild-type and *cipk8-1* plants.

Quantitative PCR analysis of *CHL1* (a) and *NRT2.1* (b) expression after 30 min nitrate induction using various concentrations as indicated. Wild-type or *cipk8-1* plants were grown in NO_3^--free medium using NH_4^+ as the sole nitrogen source for 10 days, then exposed for 30 min to various concentrations of KNO_3 or KCl as indicated. The region from 0 to 1 mM is enlarged. The relative level is the expression normalized to that for *CHL1* or *NRT2.1* in wild-type plants exposed to 25 mM KNO_3 for 30 min. The two small panels are double-reciprocal plots of wild-type on the left and *cipk8-1* on the right. The equation and the R^2 value obtained using a linear fitting method in Microsoft Excel (http://www.microsoft.com) are shown in each panel, and were used to determine the kinetic parameters (y intercept = $1/V_{max}$; x intercept = $-1/K_m$) shown in Table 1. V_{max}^{HA}, the saturating level of the high-affinity phase in wild-type plants.

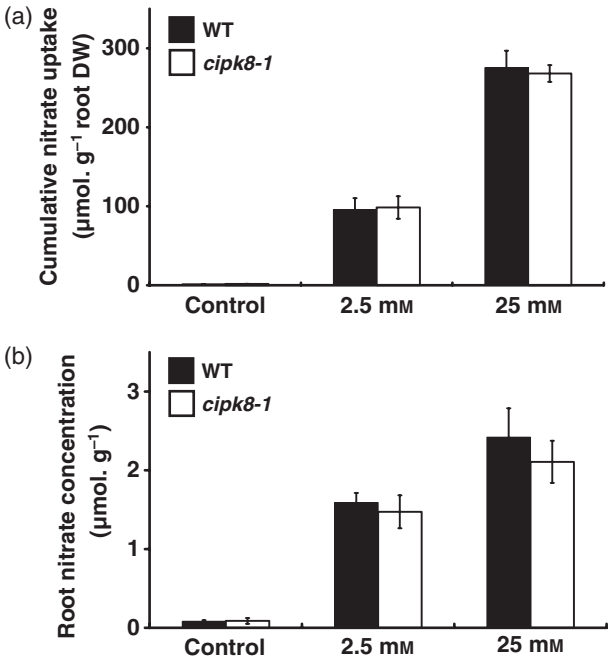

Figure 6. Cumulative nitrate uptake activity and root nitrate content of wild-type and *cipk8-1* plants.

(a) Cumulative nitrate uptake activity of wild-type and *cipk8-1* mutants measured using $^{15}NO_3^-$. Wild-type and *cipk8-1* plants were grown in NO_3^--free medium (NH_4^+ as the sole nitrogen source) for 10 days, then cumulative nitrate uptake was measured by shifting to 25 mM or 2.5 mM $K^{15}NO_3$ for 30 min. The results shown are the mean ± SD for six vessels of each type of plant tested in a single experiment. DW, dry weight.

(b) Root nitrate content of wild-type and *cipk8-1* mutants. Wild-type and *cipk8-1* plants were grown in NO_3^--free medium (NH_4^+ as the sole nitrogen source) for 10 days, then shifted to 25 mM or 2.5 mM NO_3^- for 30 min, after which the roots were harvested and their NO_3^- content determined by HPLC. The results shown are the mean ± SD for four vessels of each type of plant tested in a single experiment.

There were no significant differences between groups grown under the same conditions in (a) and (b). Control, plants before nitrate induction.

CIPK8 is also involved in nitrate-modulated primary root growth

As several *cipk* mutants have been found to be defective in root growth under certain conditions (Cheng *et al.*, 2004; Gong *et al.*, 2002b; Guo *et al.*, 2002), and nitrate has been reported to modulate primary root growth (Tian *et al.*, 2008; Walch-Liu and Forde, 2008), we analyzed the root length of *cipk8* mutants grown under various nitrogen conditions to determine whether CIPK8 was also involved in nitrate-modulated root growth. When the wild-type, *cipk8* and complemented plants were grown on $(NH_4)_2$ succinate plates for 3 days, the germination rates and growth phenotypes of the seedlings were almost identical (data not shown). When the 3-day-old seedlings were transferred to medium containing 2.5 mM $(NH_4)_2$ succinate for an additional 3–5 days, the primary root length of the *cipk8* mutants was similar to that of the wild-type (Figure 7c). In contrast, when transferred to the medium containing 5 mM or 100 μM KNO_3, the primary root length of the *cipk8* mutants was significantly longer than that of the wild-type (Figure 7a,b). This root growth defect was found in both *cipk8-1* and *cipk8-2* mutants, and was restored in the complemented line, indicating that the defect is caused by the mutation of *CIPK8*.

Increased primary root length in the *cipk8* mutants was also observed when seedlings were transferred to medium containing 2.5 mM NH_4NO_3 (data not shown), indicating that the root growth phenotype of the *cipk8* mutant is nitrate-dependent, not due to the absence of ammonium. The concentration dependence of this effect was examined by measuring the root length of plants transferred to media with various concentrations of nitrate. In wild-type plants, high concentrations of external nitrate inhibited primary root growth (Figure S7). The inhibitory effects of high concentration were not nitrate-specific, because similar inhibitory effects were found when KNO_3 was replaced with KCl or $(NH_4)_2$ succinate (data not shown). However, the longer primary root growth in the *cipk8* mutants was nitrate-specific, and was only found in the nitrate-containing media. At all nitrate concentrations tested (1–25 mM), the primary root length of *cipk8* mutants was longer than that of wild-type (Figure S7), indicating that the root growth phenotype of the *cipk8* mutants is concentration-independent. These data suggest that CIPK8 is not only involved in the rapid nitrate-regulated transcriptional control, but also in long-term nitrate-regulated root growth.

CIPK8 regulates expression of the malate and boron transporters

To examine which genes, other than nitrate transporter and nitrate assimilation genes, were regulated by CIPK8, wild-type and *cipk8-1* plants exposed to nitrate for a short period of time were analyzed by microarray. As the defect of *cipk8* mutants in the primary nitrate response is specific to the low-affinity phase and the high-affinity phase is not affected in the mutants, the expression levels of primary nitrate response genes decrease by only approximately 40%, a reduction level that cannot be unequivocally determined by microarray analysis. However, the microarray data did show that a malate/fumarate transporter gene, *AttDT*, involved in pH homeostasis (Emmerlich *et al.*, 2003; Hurth *et al.*, 2005), and a boron transporter gene, *BOR1*, responsible for xylem loading (Noguchi *et al.*, 1997; Takano *et al.*, 2002), were down-regulated in the *cipk8* mutant. The changes in expression of these two genes were confirmed by quantitative PCR analysis, with both *AttDT* (Figure 8a) and *BOR1* (Figure 8b) up-regulated by nitrate in the wild-type after 30 min induction, and reduced induction levels in the *cipk8-1* mutant. When ammonium was the sole nitrogen source, both *AttDT* and *BOR1* were expressed at low levels, and no difference in expression was seen between the wild-type

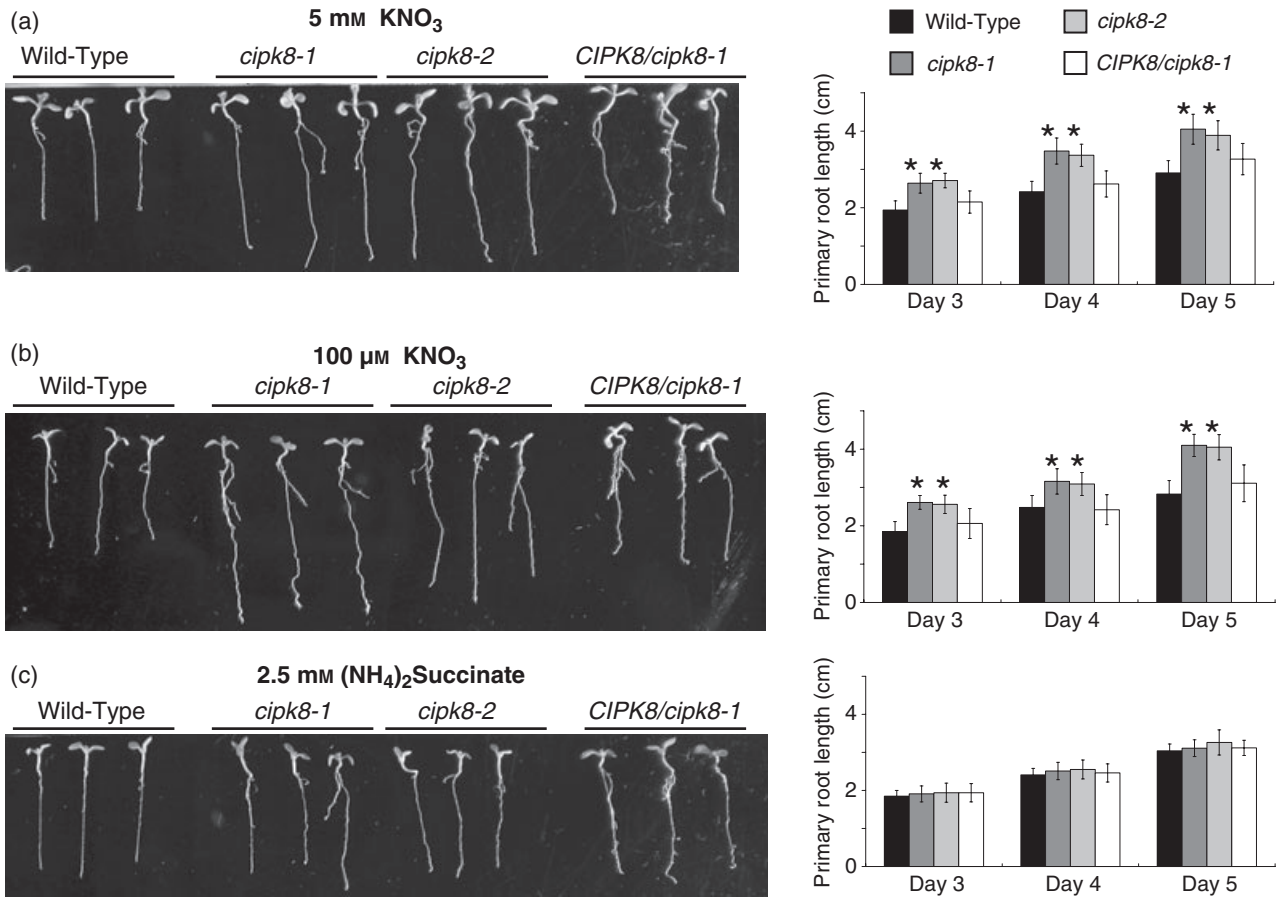

Figure 7. CIPK8 regulates primary root growth in nitrate-containing medium.
Photographs and quantification of primary root growth of plants grown on media containing (a) 5 mM KNO_3, (b) 100 μM KNO_3 or (c) 2.5 mM $(NH_4)_2$succinate. Three-day-old seedlings grown on 2.5 mM $(NH_4)_2$succinate plates were transferred to the medium indicated for 3–5 days (4 days for the photographs). The results shown are the mean ± SD of three independent plate replicates (10 seedlings per plate). The results marked with an asterisk differ significantly ($P < 0.05$ in a *t*-test) from those of wild-type plants receiving the same treatment.

and *cipk8* mutants, indicating that the reduction in the *cipk8* mutants was nitrate-specific. These data suggest that CIPK8 may also participate in regulating the homeostasis between nitrate and other anions, such as malate/fumarate or borate.

Discussion

CIPK8 is involved in regulating the primary nitrate response

The earliest nitrate response detected in higher plants is the induced expression of nitrate assimilation genes and nitrate transporter genes, as well as some other related genes. Although the properties of this primary nitrate response have been intensively studied for nearly two decades (Deng *et al.*, 1989; Pouteau *et al.*, 1989; Redinbaugh and Campbell, 1991), nothing is known about the signaling components involved. In this study, a CBL-interacting protein kinase, CIPK8, was shown to be involved. CIPK8 was identified as a candidate gene involved in the CHL1-regulated nitrate response by transcriptome analysis of the nitrate uptake mutant *chl1-5*. In *cipk8* mutants, the rapid nitrate-induced expression of the seven known primary nitrate response genes, *CHL1*, *NRT2.1*, *NIA1*, *NIA2*, *NiR*, *GLN2* and *G6PDH3*, was reduced to 40–65% of wild-type levels (Figure 3 and data not shown). These results indicated that a kinase cascade and potentially Ca^{2+} signaling are involved in nitrate sensing. Indeed, the involvement of Ca^{2+} and protein phosphorylation in the primary nitrate response has been suggested by Sakakibara *et al.* (1996, 1997), who used EGTA and La^{3+} Ca^{2+} inhibitors as well as protein kinase and phosphatase inhibitors. Using a microarray approach, our data and previous studies (Scheible *et al.*, 2004; Wang *et al.*, 2000, 2003, 2004) show that *CIPK1* and *CIPK3* are also regulated with similar patterns to *CIPK8* in response to nitrate, suggesting that CIPK1 and CIPK3 may also have some function in nitrate signaling. However, analysis of the primary nitrate response in *cipk1* and *cipk3* mutants showed no defects (data not shown). Further studies are required to

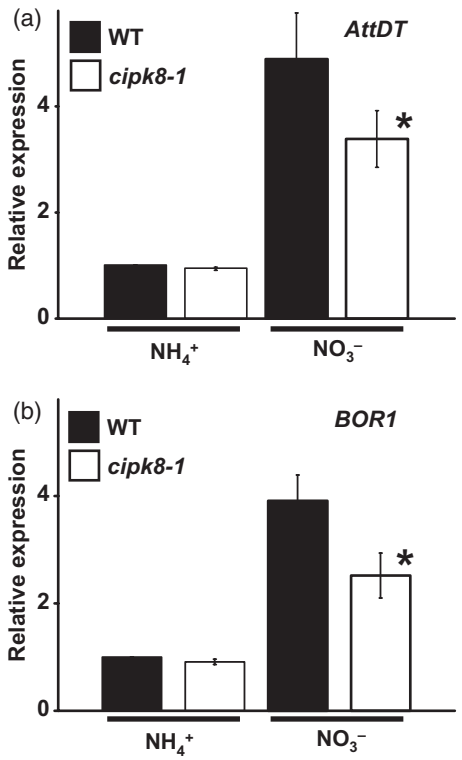

Figure 8. CIPK8 regulates the expression of two nitrate-regulated transporter genes.
Quantitative PCR analysis of relative gene expression of (a) *AttDT* (vacuolar malate/fumalate transporter, At5g47560) and (b) *BOR1* (xylem boron transporter, At2g47160). Ten-day-old wild-type or *cipk8-1* plants were exposed to 25 mM NH_4^+ or NO_3^- for 30 min. The relative level is the expression normalized to that for *AttDT* or *BOR1* in wild-type plants exposed to NH_4^+ for 30 min.

determine whether they are involved in other nitrate-regulated responses.

Roles of CIPK8 in nitrate sensing

Nitrate concentrations in the soil can vary over four orders of magnitude from 1 micromolar to 10 millimolar. To utilize nitrate efficiently under such fluctuating conditions, plants have evolved two nitrate uptake systems, one high-affinity with a K_m in the micromolar range, and one low-affinity with a K_m in the millimolar range. Interestingly, our study shows that nitrate induction levels of *CHL1* and *NRT2.1* in wild-type plants display bi-phasic patterns with a K_m of approximately 30 μM for the high-affinity phase and a K_m of approximately 0.9 mM for the low-affinity phase. Similar results were also seen in the nitrate reductase-deficient mutant *G4′-3*, indicating that nitrate itself rather than downstream metabolites is responsible for this bi-phasic response. The bi-phasic pattern is a novel property of the primary nitrate response, which suggests that, as with nitrate uptake, there are two nitrate-sensing systems for the primary nitrate response, with comparable K_m values to the uptake systems.

Our data further indicate that CIPK8 is a positive regulator in the low-affinity signaling system, as the nitrate-induced expression defects of *cipk8* mutants were found mainly in the low-affinity phase, with a normal or slightly reduced high-affinity response. The normal high-affinity response of *cipk8* mutants suggests that the two nitrate signaling systems are in some way genetically distinct. As the K_m values of the nitrate-sensing systems for the primary response are similar to the K_m values of nitrate uptake systems, it is possible that nitrate sensing and nitrate uptake share some common components. Indeed, some studies have suggested that the dual-affinity transporter CHL1 and the high-affinity transporter NRT2.1 are involved in nitrate signaling (Guo *et al.*, 2001; Krouk *et al.*, 2006; Little *et al.*, 2005; Munos *et al.*, 2004; Remans *et al.*, 2006; Walch-Liu and Forde, 2008). Further studies are required to determine whether nitrate transporters or other kinds of receptors function as nitrate sensors in higher plants. Identification of the first signaling component, CIPK8, of the primary nitrate response should facilitate elucidation of the nitrate-sensing networks in higher plants.

CIPK8 participates in nitrate-mediated regulation of primary root growth

According to microarray analysis by Wang *et al.* (2004) of both the wild-type and a nitrate reductase-deficient mutant, expression of *CIPK8* is induced by nitrate, indicating that *CIPK8* is regulated directly and rapidly by nitrate itself. In addition, similar to the expression of *CHL1* and *NRT2.1*, expression of *CIPK8* in response to nitrate also displayed a bi-phasic pattern, with similar K_m values in both the low- and high-affinity phases (Figure S6b,c). These data indicate that *CIPK8* is a primary nitrate response gene, and that it may be auto-regulated. As *de novo* protein synthesis is not required for the primary nitrate response, the induction of *CIPK8* may play a role in subsequent nitrate-related signaling distinct from the rapid transcriptional regulation of the primary nitrate response. Indeed, after long-term nitrate exposure, from 8 h up to 2 days, the expression levels of two nitrate transporter genes (*CHL1* and *NRT2.1*) were also reduced in *cipk8* mutants (Figure S8b, and data not shown), indicating that CIPK8 is involved in long-term nitrate regulation.

In addition to transcriptional regulation, our study shows that CIPK8 is also involved in the nitrate regulation of root growth. When ammonium was the sole nitrogen source as [$(NH_4)_2$succinate], there was no difference in primary root growth between the wild-type and *cipk8* mutants; however, when nitrate was either the sole nitrogen source (as KNO_3) or was applied together with ammonium (as NH_4NO_3), the primary root was longer in the *cipk8* mutants. As the root nitrate contents of wild-type and the *cipk8* mutant were similar in plants exposed to

KNO_3 for 1 or 2 days (Figure S8a), this suggests that the primary root growth phenotypes of *cipk8* are caused by a defect in nitrate signal processing, rather than being due to changes in root nitrate content. In addition, in contrast to the primary nitrate response, the effects of CIPK8 in primary root growth did not show obvious concentration dependence (Figure S7). The primary nitrate response defect of the *cipk8* mutants was specific to the low-affinity phase, but the root growth phenotype was found at all concentrations tested (from 100 μM to 25 mM), suggesting that different downstream mechanisms are involved in these two processes.

CIPK8 may be involved in both glucose sensing and nitrate sensing

A previous study demonstrated that CIPK8 is involved in glucose sensing (Gong *et al.*, 2002a). In transgenic plants over-expressing a constitutively active form of CIPK8 (PKS11T161D), primary root growth was not inhibited by a high glucose concentration, in contrast to the controls. Our results show that CIPK8 is involved in nitrate sensing. The nitrogen/carbon balance is one of the key factors regulating plant growth and metabolism. For example, several nitrogen-metabolizing genes are up-regulated by glucose (Price *et al.*, 2004), and carbon-metabolizing genes are up-regulated by nitrate (Scheible *et al.*, 2004; Wang *et al.*, 2003). It will be interesting to determine whether CIPK8 could be one of the regulatory links between nitrogen and carbon status.

CIPK8 regulates expression of the vacuolar malate transporter and the boron transporter in response to nitrate

Our study also showed that a vacuolar malate/fumarate transporter gene *AttDT* (Emmerlich *et al.*, 2003) was induced by nitrate, and that CIPK8 was important in this nitrate-induced expression. Synthesis of amino acids from nitrate results in the stoichiometric production of OH^- ions, and this causes a rapid increase in the cytosolic pH (van Beusichem *et al.*, 1985; Touraine *et al.*, 1988). In this situation, OH^- is incorporated into malate, and the increased malate synthesis helps to maintain a neutral pH (Davies, 1986). AttDT, which transports malate into the vacuole, is known to be critical in pH homeostasis (Hurth *et al.*, 2005). The induced expression of *AttDT* by nitrate could be important in reducing the alkaline stress associated with nitrate assimilation. Thus, CIPK8 may be involved in the malate-mediated regulation of pH homeostasis when nitrate is taken up.

Similar to *AttDT*, the boron transporter gene *BOR1*, responsible for transporting borate into the root xylem (Takano *et al.*, 2002), is induced by nitrate and regulated by CIPK8. A recent study showed that boron deficiency affects several aspects of nitrate assimilation leading to decreased nitrate uptake rate, NIA transcript level and nitrate content (Camacho-Cristobal and Gonzalez-Fontes, 2007). The effect of nitrate and CIPK8 on the expression of *BOR1* may provide a regulatory link between nitrate and boron.

CBL–CIPK networks in nutrient sensing

Many studies have shown that CBL–CIPK networks play critical roles in stress adaptation in plants (Batistic and Kudla, 2004; Gong *et al.*, 2004). Recently, CIPK23 has been reported to be involved in sensing potassium limitation; under low-potassium conditions, CIPK23 is activated by binding to CBL1 and CBL9 and enhances potassium uptake by phosphorylating the high-affinity potassium transporter AKT1 (Li *et al.*, 2006a; Xu *et al.*, 2006). In this study, we established a novel role for CIPK in nitrate signaling pathways. Thus, there is growing evidence that, in addition to stress adaptation, CBL–CIPK networks play important roles in sensing nutrient status.

Experimental procedures

Isolation of T-DNA plants disrupted in the cipk8 gene and generation of complemented lines

The *cipk8* T-DNA insertion lines, *cipk8-1* (SALK_139697) and *cipk8-2* (SALK_018985), were obtained from the *Arabidopsis* Biological Resource Center (http://www.arabidopsis.org/abrc/). The insertion in *CIPK8* gene was identified using a T-DNA left border primer and *CIPK8* gene-specific primers (see Appendix S1). To generate the complemented lines, the 35 S::*CIPK8*-c-myc-His fragment was constructed and transformed into *cipk8-1* plants (as described in Appendix S1).

Plant material and treatments

For the nitrate content assay, the nitrate uptake activity assay and quantitative PCR analysis, approximately 100 sterilized seeds of *Arabidopsis thaliana* wild-type, the *chl1-5* mutant or *cipk8* mutants were grown in Magenta vessels (Sigma, http://www.sigmaaldrich.com/) in 60 ml of NO_3^--free liquid growth medium as described previously (Chiu *et al.*, 2004) with 12.5 mM $(NH_4)_2$ succinate as the sole nitrogen source, pH 6.5, and 3–6 vessels (approximately 100 seedlings each) of samples were pooled for each experiment. Ten-day-old plants were shifted to 60 ml of 12.5 mM $(NH_4)_2$ succinate medium in which the pH had been changed to 5.5 by addition of HCl. After 3 h treatment, the plants were washed twice with 10 mM K_2HPO_4/KH_2PO_4, pH 5.5, then shifted for the indicated duration to 60 ml of pH 5.5 growth medium, but with the 12.5 mM $(NH_4)_2$ succinate replaced by KNO_3, KCl or the same concentrations of $(NH_4)_2$ succinate.

For measurement of primary root length, approximately 30 sterilized seeds of the *cipk8* mutants and wild-type were grown on the same 1450 mm^2 plate (Greiner Bio-one, Cellstar 1450 mm tissue culture, http://www.gbo.com) with the NO_3^--free medium described above containing 2.5 mM $(NH_4)_2$ succinate and 0.4% agarose, pH 5.8. Three-day-old seedlings were shifted to a test plate containing the same pH 5.8 medium, but with the 2.5 mM $(NH_4)_2$ succinate replaced by 5 mM KNO_3, 2.5 mM NH_4NO_3 or 2.5 mM $(NH_4)_2$ succinate as indicated in the figures. After 3 days, the primary root length was

measured. Quantification was performed using Adobe Photoshop 9.0 (http://www.adobe.com), and TINA 2.09 image analysis software (Raytest Isotopenmeßgeräte GmbH, http://www.raytest.com/).

Real-time quantitative PCR analysis

Template cDNA samples were prepared using 4 μg of total RNA and the Improm-II™ reverse transcription system (Promega, http://www.promega.com/). Real-time quantitative PCR was performed using a LightCycler System (Roche Diagnostics, http://www.roche-applied-science.com). A LightCycler-FastStart DNA Master SYBR Green I kit (Roche) was used for the PCR reactions. Each PCR reaction contained 10–50 ng of cDNA and 0.5 μM of each of the primer pairs. The sequences of the primers used in this study are listed in Appendix S1. The initial denaturing step of 10 min was followed by 40–55 PCR cycles of 94°C for 0 sec, 60°C for 0 sec and 72°C for 1 sec per 25 bp of the expected product. After the PCR cycles, the melting temperature was tested. Quantification was performed using LightCycler Relative Quantification software version 1.0. Quantitative PCR data presented in the figures are the mean ± SD for three separate experiments, each on a pool of approximately 300 seedlings of each type. Results marked with an asterisk were significantly different ($P < 0.05$, *t*-test) from wild-type plants receiving the same treatment.

$^{15}NO_3^-$ uptake activity assay of Arabidopsis *plant roots*

Root $^{15}NO_3^-$ influxes were assayed using a procedure modified from that described by Munos *et al.* (2004). Briefly, 10-day-old wild-type and *cipk8* plants grown in pH 6.5 NO_3^--free growth medium were transferred for 3 h to pH 5.5 NO_3^--free medium, and were then washed and shifted for 30 min to pH 5.5 nitrate medium containing $K^{15}NO_3$ (approximately 50% atom excess ^{15}N) at the concentrations indicated in the figures, and finally washed three times in 0.1 mM $CaSO_4$. The roots were then collected and dried in an 80°C oven for 4 days. The samples were analyzed for total nitrogen and atom percentage ^{15}N using a continuous-flow isotope ratio mass spectrometer coupled to a C/N elemental analyzer (model ANCA-MS; PDZ Europa, http://www.europa-uk.com) as described by Clarkson *et al.* (1996).

Measurement of the nitrate content of Arabidopsis *roots*

Ten-day-old wild-type and *chl1-5* or *cipk8* plants grown in pH 6.5 NO_3^--free growth medium were transferred to pH 5.5 NO_3^--free medium for 3 h, then shifted to pH 5.5 KNO_3 medium for 30 min, and finally washed three times in Milli-Q water (Millipore, http://www.millipore.com). The pooled roots (0.02–0.06 g) were homogenized in a steel ball mill, then 800 μl of Milli-Q water was added and the mixture was boiled for 20 min and then frozen at –70°C overnight before further processing. The material was then centrifuged at 20 800 ***g*** for 10 min at room temperature, and the supernatant taken up in a 1 ml syringe, passed through a 0.22 μm filter, and its nitrate content determined by HPLC (Thayer and Huffaker, 1980) using a PARTISIL 10 SAX (strong anion exchanger) column (Whatman, http://www.whatman.com).

Acknowledgements

We thank the Salk Institute Genomic Analysis Laboratory for providing the Arabidopsis T-DNA insertion mutants. We thank Shan-Hua Lin in our laboratory for technical support with ^{15}N-labeled GC-MS analysis, the Affymetrix Gene Expression Service Lab of Academia Sinica, Taiwan, for the Affymetrix ATH1 chip service, and the Microarray Core Facility of the Institute of Molecular Biology, Academia Sinica, Taiwan, for assistance with microarray data analysis. This work was supported by grants from the Institute of Molecular Biology, Academia Sinica, Taiwan, and the National Science Council (NSC 95-2321-B-001-001) to Y.-F.T.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Statistical analyses of the expression levels of 'genes responding to both root nitrate concentration and some additional effects of CHL1' and 'genes responding mainly to alterations of root nitrate concentration'.

Figure S2. The defect of *cipk8-1* mutant with respect to *NRT2.1* expression is nitrate concentration-dependent and can be rescued in a complemented line.

Figure S3. Nitrate-induced expression of *LEA5* is cycloheximide-dependent but CIPK8-independent.

Figure S4. The defect of *cipk8-1* mutant with respect to *NRT2.1* expression is cycloheximide-independent.

Figure S5. The expression of several marker genes is not altered in the *cipk8-1* mutant when exposed to 25 mM nitrate.

Figure S6. Kinetic analysis of the gene expression levels of *CHL1* and *CIPK8* in wild-type and *nia1 nia2* plants.

Figure S7. The primary root length of wild-type and *cipk8* mutants grown in various concentrations of nitrate.

Figure S8. After exposure to nitrate, long-term root nitrate contents were not significantly altered but expression of the nitrate-related gene *CHL1* was reduced in *cipk8* mutants.

Table S1. Differential expression of genes in *chl1-5* versus wild-type plants exposed to 25 mM nitrate for 30 min.

Appendix S1. Supplementary experimental procedures.

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References

Alboresi, A., Gestin, C., Leydecker, M.T., Bedu, M., Meyer, C. and Truong, H.N. (2005) Nitrate, a signal relieving seed dormancy in *Arabidopsis*. *Plant Cell Environ*, **28**, 500–512.

Albrecht, V., Ritz, O., Linder, S., Harter, K. and Kudla, J. (2001) The NAF domain defines a novel protein–protein interaction module conserved in Ca^{2+}-regulated kinases. *EMBO J.* **20**, 1051–1063.

Batistic, O. and Kudla, J. (2004) Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. *Planta*, **219**, 915–924.

Bernreiter, A., Ramon, A., Fernandez-Martinez, J. *et al.* (2007) Nuclear export of the transcription factor NirA is a regulatory checkpoint for nitrate induction in *Aspergillus nidulans*. *Mol. Cell. Biol.* **27**, 791–802.

van Beusichem, M.L., Baas, R., Kirkby, E.A. and Nelemans, J.A. (1985) Intracellular pH regulation during NO_3^- assimilation in shoot and roots of *Ricinus communis*. *Plant Physiol.* **78**, 768–773.

Burger, G., Tilburn, J. and Scazzocchio, C. (1991) Molecular cloning and functional characterization of the pathway-specific regulatory gene nirA, which controls nitrate assimilation in *Aspergillus nidulans*. *Mol. Cell. Biol.* **11**, 795–802.

Camacho-Cristobal, J.J. and Gonzalez-Fontes, A. (2007) Boron deficiency decreases plasmalemma H^+-ATPase expression and nitrate uptake, and promotes ammonium assimilation into asparagine in tobacco roots. *Planta*, **226**, 443–451.

Cavicchioli, R., Chiang, R.C., Kalman, L.V. and Gunsalus, R.P. (1996) Role of the periplasmic domain of the *Escherichia coli* NarX sensor-transmitter protein in nitrate-dependent signal transduction and gene regulation. *Mol. Microbiol.* **21**, 901–911.

Chai, W. and Stewart, V. (1998) NasR, a novel RNA-binding protein, mediates nitrate-responsive transcription antitermination of the *Klebsiella oxytoca* M5al nasF operon leader in vitro. *J. Mol. Biol.* **283**, 339–351.

Chang, C., Kwok, S.F., Bleecker, A.B. and Meyerowitz, E.M. (1993) *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. *Science*, **262**, 539–544.

Cheng, W.H., Endo, A., Zhou, L. *et al.* (2002) A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. *Plant Cell*, **14**, 2723–2743.

Cheng, N.H., Pittman, J.K., Zhu, J.K. and Hirschi, K.D. (2004) The protein kinase SOS2 activates the *Arabidopsis* H^+/Ca^{2+} antiporter CAX1 to integrate calcium transport and salt tolerance. *J. Biol. Chem.* **279**, 2922–2926.

Cheong, Y.H., Kim, K.N., Pandey, G.K., Gupta, R., Grant, J.J. and Luan, S. (2003) CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*. *Plant Cell*, **15**, 1833–1845.

Chiang, R.C., Cavicchioli, R. and Gunsalus, R.P. (1997) 'Locked-on' and 'locked-off' signal transduction mutations in the periplasmic domain of the *Escherichia coli* NarQ and NarX sensors affect nitrate- and nitrite-dependent regulation by NarL and NarP. *Mol. Microbiol.* **24**, 1049–1060.

Chiu, C.C., Lin, C.S., Hsia, A.P., Su, R.C., Lin, H.L. and Tsay, Y.F. (2004) Mutation of a nitrate transporter, AtNRT1:4, results in a reduced petiole nitrate content and altered leaf development. *Plant Cell Physiol.* **45**, 1139–1148.

Clarkson, D.T., Gojon, A., Saker, L.R., Wiersema, P.K., Purves, J.V., Tillard, P., Arnold, G.M., Paans, A.J.M., Vaalburg, W. and Stulen, I. (1996) Nitrate and ammonium influxes in soybean (*Glycine max*) roots: direct comparison of ^{13}N and ^{15}N tracing. *Plant Cell Environ*, **19**, 859–868.

Crawford, N.M. (1995) Nitrate: nutrient and signal for plant growth. *Plant Cell*, **7**, 859–868.

Davies, D.D. (1986) The fine control of cytosolic pH. *Physiol. Plant.* **67**, 702–706.

Deng, M., Moureaux, T. and Caboche, M. (1989) Tungstate, a molybdate analog inactivating nitrate reductase, deregulates the expression of the nitrate reductase structural gene. *Plant Physiol.* **91**, 304–309.

Emmerlich, V., Linka, N., Reinhold, T., Hurth, M.A., Traub, M., Martinoia, E. and Neuhaus, H.E. (2003) The plant homolog to the human sodium/dicarboxylic cotransporter is the vacuolar malate carrier. *Proc. Natl. Acad. Sci. USA*. **100**, 11122–11126.

Fu, Y.H., Kneesi, J.Y. and Marzluf, G.A. (1989) Isolation of nit-4, the minor nitrogen regulatory gene which mediates nitrate induction in *Neurospora crassa*. *J. Bacteriol.* **171**, 4067–4070.

Gong, D., Gong, Z., Guo, Y., Chen, X. and Zhu, J.K. (2002a) Biochemical and functional characterization of PKS11, a novel *Arabidopsis* protein kinase. *J. Biol. Chem.* **277**, 28340–28350.

Gong, D., Zhang, C., Chen, X., Gong, Z. and Zhu, J.K. (2002b) Constitutive activation and transgenic evaluation of the function of an *Arabidopsis* PKS protein kinase. *J. Biol. Chem.* **277**, 42088–42096.

Gong, D., Guo, Y., Schumaker, K.S. and Zhu, J.K. (2004) The SOS3 family of calcium sensors and SOS2 family of protein kinases in *Arabidopsis*. *Plant Physiol.* **134**, 919–926.

Gowri, G., Kenis, J.D., Ingemarsson, B., Redinbaugh, M.G. and Campbell, W.H. (1992) Nitrate reductase transcript is expressed in the primary response of maize to environmental nitrate. *Plant Mol. Biol.* **18**, 55–64.

Guo, F.Q., Wang, R., Chen, M. and Crawford, N.M. (2001) The *Arabidopsis* dual-affinity nitrate transporter gene AtNRT1.1 (CHL1) is activated and functions in nascent organ development during vegetative and reproductive growth. *Plant Cell*, **13**, 1761–1777.

Guo, Y., Xiong, L., Song, C.P., Gong, D., Halfter, U. and Zhu, J.K. (2002) A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signaling in *Arabidopsis*. *Dev. Cell*. **3**, 233–244.

Guo, F.Q., Young, J. and Crawford, N.M. (2003) The nitrate transporter AtNRT1.1 (CHL1) functions in stomatal opening and contributes to drought susceptibility in *Arabidopsis*. *Plant Cell*, **15**, 107–117.

Huang, N.C., Liu, K.H., Lo, H.J. and Tsay, Y.F. (1999) Cloning and functional characterization of an *Arabidopsis* nitrate transporter gene that encodes a constitutive component of low-affinity uptake. *Plant Cell*, **11**, 1381–1392.

Hurth, M.A., Suh, S.J., Kretzschmar, T., Geis, T., Bregante, M., Gambale, F., Martinoia, E. and Neuhaus, H.E. (2005) Impaired pH homeostasis in *Arabidopsis* lacking the vacuolar dicarboxylate transporter and analysis of carboxylic acid transport across the tonoplast. *Plant Physiol.* **137**, 901–910.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K. and Kakimoto, T. (2001) Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature*, **409**, 1060–1063.

Jackson, R.B. and Caldwell, M.M. (1993) The scale of nutrient heterogeneity around individual plants and its quantification with geostatistics. *Ecology*, **74**, 612–614.

Krouk, G., Tillard, P. and Gojon, A. (2006) Regulation of the high-affinity NO_3^- uptake system by NRT1.1-mediated NO_3^- demand signaling in *Arabidopsis*. *Plant Physiol.* **142**, 1075–1086.

Lejay, L., Tillard, P., Lepetit, M., Olive, F., Filleur, S., Daniel-Vedele, F. and Gojon, A. (1999) Molecular and functional regulation of two NO_3^- uptake systems by N- and C-status of *Arabidopsis* plants. *Plant J.* **18**, 509–519.

Leonhardt, N., Kwak, J.M., Robert, N., Waner, D., Leonhardt, G. and Schroeder, J.I. (2004) Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *Plant Cell*, **16**, 596–615.

Li, L., Kim, B.G., Cheong, Y.H., Pandey, G.K. and Luan, S. (2006a) A Ca^{2+} signaling pathway regulates a K^+ channel for low-K response in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*. **103**, 12625–12630.

Li, Y., Lee, K.K., Walsh, S., Smith, C., Hadingham, S., Sorefan, K., Cawley, G. and Bevan, M.W. (2006b) Establishing glucose- and ABA-regulated transcription networks in *Arabidopsis* by microarray analysis and promoter classification using a relevance vector machine. *Genome Res.* **16**, 414–427.

Li, W., Wang, Y., Okamoto, M., Crawford, N.M., Siddiqi, M.Y. and Glass, A.D. (2007) Dissection of the AtNRT2.1:AtNRT2.2 inducible high-affinity nitrate transporter gene cluster. *Plant Physiol.* **143**, 425–433.

Little, D.Y., Rao, H., Oliva, S., Daniel-Vedele, F., Krapp, A. and Malamy, J.E. (2005) The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. *Proc. Natl. Acad. Sci. USA*. **102**, 13693–13698.

Liu, K.H., Huang, C.Y. and Tsay, Y.F. (1999) CHL1 is a dual-affinity nitrate transporter of *Arabidopsis* involved in multiple phases of nitrate uptake. *Plant Cell*, **11**, 865–874.

Munos, S., Cazettes, C., Fizames, C., Gaymard, F., Tillard, P., Lepetit, M., Lejay, L. and Gojon, A. (2004) Transcript profiling in the *chl1-5* mutant of *Arabidopsis* reveals a role of the nitrate transporter NRT1.1 in the regulation of another nitrate transporter, NRT2.1. *Plant Cell*, **16**, 2433–2447.

Noguchi, K., Yasumori, M., Imai, T., Naito, S., Matsunaga, T., Oda, H., Hayashi, H., Chino, M. and Fujiwara, T. (1997) *bor1-1*, an *Arabidopsis thaliana* mutant that requires a high level of boron. *Plant Physiol.* **115**, 901–906.

Pandey, G.K., Cheong, Y.H., Kim, K.N., Grant, J.J., Li, L., Hung, W., D'Angelo, C., Weinl, S., Kudla, J. and Luan, S. (2004) The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and biosynthesis in *Arabidopsis*. *Plant Cell*, **16**, 1912–1924.

Pouteau, S., Cherel, I., Vaucheret, H. and Caboche, M. (1989) Nitrate reductase mRNA regulation in *Nicotiana plumbaginifolia* nitrate reductase-deficient mutants. *Plant Cell*, **1**, 1111–1120.

Price, J., Laxmi, A., St Martin, S.K. and Jang, J.C. (2004) Global transcription profiling reveals multiple sugar signal transduction mechanisms in *Arabidopsis*. *Plant Cell*, **16**, 2128–2150.

Redinbaugh, M.G. and Campbell, W.H. (1991) Higher plant responses to environmental nitrate. *Physiol. Plant.* **82**, 640–650.

Redinbaugh, M.G. and Campbell, W.H. (1993) Glutamine synthetase and ferredoxin-dependent glutamate synthase expression in the maize (*Zea mays*) root primary response to nitrate (evidence for an organ-specific response). *Plant Physiol.* **101**, 1249–1255.

Redinbaugh, M.G. and Campbell, W.H. (1998) Nitrate regulation of the oxidative pentose phosphate pathway in maize (*Zea mays* L.) root plastids: induction of 6-phosphogluconate dehydrogenase activity, protein and transcript levels. *Plant Sci.* **134**, 129–140.

Remans, T., Nacry, P., Pervent, M., Filleur, S., Diatloff, E., Mounier, E., Tillard, P., Forde, B.G. and Gojon, A. (2006) The *Arabidopsis* NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. *Proc. Natl. Acad. Sci. USA.* **103**, 19206–19211.

Sakakibara, H., Takei, K. and Sugiyama, T. (1996) Isolation and characterization of a cDNA that encodes maize uroporphyrinogen III methyltransferase, an enzyme involved in the synthesis of siroheme, which is a prosthetic group of nitrite reductase. *Plant J.* **10**, 883–892.

Sakakibara, H., Kobayashi, K., Deji, A. and Sugiyama, T. (1997) Partial characterization of the signaling pathway for the nitrate-dependent expression of genes for nitrogen-assimilatory enzymes using detached maize leaves. *Plant Cell Physiol.* **38**, 837–843.

Scheible, W.R., Gonzalez-Fontes, A., Lauerer, M., Muller-Rober, B., Caboche, M. and Stitt, M. (1997) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *Plant Cell*, **9**, 783–798.

Scheible, W.R., Morcuende, R., Czechowski, T., Fritz, C., Osuna, D., Palacios-Rojas, N., Schindelasch, D., Thimm, O., Udvardi, M.K. and Stitt, M. (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. *Plant Physiol.* **136**, 2483–2499.

Stitt, M. (1999) Nitrate regulation of metabolism and growth. *Curr. Opin. Plant Biol.* **2**, 178–186.

Takano, J., Noguchi, K., Yasumori, M., Kobayashi, M., Gajdos, Z., Miwa, K., Hayashi, H., Yoneyama, T. and Fujiwara, T. (2002) *Arabidopsis* boron transporter for xylem loading. *Nature*, **420**, 337–340.

Thayer, J.R. and Huffaker, R.C. (1980) Determination of nitrate and nitrite by high-pressure liquid chromatography: comparison with other methods for nitrate determination. *Anal. Biochem.* **102**, 110–119.

Tian, Q., Chen, F., Liu, J., Zhang, F. and Mi, G. (2008) Inhibition of maize root growth by high nitrate supply is correlated with reduced IAA levels in roots. *Plant Physiol.* **165**, 942–951.

Touraine, B., Grignon, N. and Grignon, C. (1988) Charge balance in NO_3^- fed soybean: estimation of K and carboxylate recirculation. *Plant Physiol.* **88**, 605–612.

Tsay, Y.F., Schroeder, J.I., Feldmann, K.A. and Crawford, N.M. (1993) The herbicide sensitivity gene *CHL1* of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. *Cell*, **72**, 705–713.

Walch-Liu, P. and Forde, B.G. (2008) Nitrate signalling mediated by the NRT1.1 nitrate transporter antagonises L-glutamate-induced changes in root architecture. *Plant J.* **54**, 820–828.

Walch-Liu, P., Neumann, G., Bangerth, F. and Engels, C. (2000) Rapid effects of nitrogen form on leaf morphogenesis in tobacco. *J. Exp. Bot.* **51**, 227–237.

Wang, R., Guegler, K., LaBrie, S.T. and Crawford, N.M. (2000) Genomic analysis of a nutrient response in *Arabidopsis* reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. *Plant Cell*, **12**, 1491–1510.

Wang, R., Okamoto, M., Xing, X. and Crawford, N.M. (2003) Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. *Plant Physiol.* **132**, 556–567.

Wang, R., Tischner, R., Gutierrez, R.A., Hoffman, M., Xing, X., Chen, M., Coruzzi, G. and Crawford, N.M. (2004) Genomic analysis of the nitrate response using a nitrate reductase-null mutant of *Arabidopsis*. *Plant Physiol.* **136**, 2512–2522.

Xu, J., Li, H.D., Chen, L.Q., Wang, Y., Liu, L.L., He, L. and Wu, W.H. (2006) A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporter AKT1 in *Arabidopsis*. *Cell*, **125**, 1347–1360.

Zhang, H. and Forde, B.G. (1998) An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. *Science*, **279**, 407–409.

