RESEARCH ARTICLE

The calcium sensor OsCBL1 modulates nitrate signaling to regulate seedling growth in rice

Jing Yang1, Xiaolong Deng1, Xiaoxin Wang1, Jingzhang Wang1, Shiyun Du2, Yangsheng Li1*

1 State Key Laboratory of Hybrid Rice, College of Life Sciences, Wuhan University, Wuhan, P. R. China, 2 Institute of Rice Research, Anhui Academy of Agricultural Sciences, Hefei, P. R. China

*lysh2001@whu.edu.cn

Abstract

Nitrate signaling integrates and coordinates gene expression and plant growth; however, the underlying molecular mechanisms involved remain poorly understood. Our previous study revealed that rice calcineurin B-like protein 1 (OsCBL1) modulates lateral root elongation by affecting auxin biosynthesis. Here, we report that OsCBL1 also modulates nitrate signaling to regulate rice seedling growth. Compared with wild-type seedlings, seedlings of OsCBL1-knockdown (OsCBL1-KD) plants showed a suppressed growth phenotype, which included reduced root and shoot fresh weights and shorter radicles, crown roots, and lateral roots, when grown in nitrogen-free conditions. Although the growth defects of OsCBL1-KD plants could be partially rescued by the addition of nitrate to the growth conditions, the nitrate uptake capability of the OsCBL1-KD plants did not differ from that of wild-type plants as assessed via nitrate content and 15NO3− influx experiments. The nitrate-regulated expression of nitrate signal sentinel genes (OsNRT2.1 and OsNRT2.2) was affected in the OsCBL1-KD plants under both long- and short-term nitrate treatments. Overall, our results showed a novel role for OsCBL1 in the regulation of nitrate signaling and nitrate-mediated rice growth.

Introduction

Because they cannot escape from harsh environmental conditions like animals can, plants have evolved a sophisticated system to sense and adapt to changes in their surrounding environment, including nutrient variations. Nitrate (NO3−) is a major nitrogen source for most land plants and is known to be a dual-function molecule. NO3− is not only a nutrient source but also a signaling molecule at the center of communication between plant genetic programs and the environment. The NO3− signaling has both long- and short-term effects. The long-term effects are important for triggering different physiological events involving plant growth affected by NO3−, including seed germination, major root and leaf growth, and the transition to the reproductive stage [1–5]. The short-term effects involve the regulation of gene expression after a short period of exposure to NO3−. At the molecular level, NO3− application can strongly and rapidly affect gene expression, which is
thought to be crucial for the ability of plants to sense nutrient conditions and alter their growth process [6]. These rapid and often transient transcriptional inductions in response to NO$_3^-$ are the short-term effects of NO$_3^-$ signaling and are also referred to as the primary nitrate response (PNR) [7].

The PNR can occur in nitrate reductase (NR)-null mutants, which means the NO$_3^-$ itself triggers the induction rather than its downstream assimilation products [8]. The PNR can also occur in the presence of protein synthesis inhibitors, showing that it does not require de novo protein synthesis [9, 10]. In Arabidopsis, many NO$_3^-$ transport and assimilation genes, such as NRT2.1, CHL1/NRT1.1, NIA1, NIA2, and NiR, serve as sentinels for the PNR [2, 11]. One of the first genes found to affect the PNR was CIPK8, which encodes a calcineurin B-like (CBL)-interacting kinase that is rapidly induced by NO$_3^-$ and differentially regulated in CHL1/NRT1.1 NO$_3^-$ transceptor mutants (chl1–5) (9). Several PNR sentinels, including NRT2.1, CHL1/NRT1.1, NIA1, NIA2 and NiR, reduce the magnitude of induction in cipk8 mutants exposed to high-nitrate conditions, suggesting that CIPK8 is a positive regulator during the low-affinity phase of the PNR [9]. Expression of the CIPK23 gene is also transiently induced by NO$_3^-$ and acts as a negative regulator of the PNR in the both low- and high-affinity phases [11].

The regulatory effect of CBL-interacting protein kinases (CIPKs) on the PNR indicates that a Ca$^{2+}$ signal is involved in the perception and transmission of NO$_3^-$ signaling. Moreover, recent evidence has shown that nitrate treatment increases cytoplasmic Ca$^{2+}$ concentrations and activates Ca$^{2+}$-sensor protein kinases (CPKs), which phosphorylate NLP transcription factors to regulate nitrate-responsive gene expression [2, 12]. As another kind of Ca$^{2+}$ sensor, CBLs contain four EF-hand domains for Ca$^{2+}$ binding and specifically interact and activate CIPKs to transduce calcium signals [13]. CBL7 is involved in the regulation of the low-NO$_3^-$ response in Arabidopsis [14]. Whether and how CBLs play roles in the regulation of NO$_3^-$ signaling remain unclear. In the present work, we provide evidence that OsCBL1 is involved in both long- and short-term NO$_3^-$ signaling regulation, which in turn modulates rice seedling growth.

**Materials and methods**

**Plant materials and growth conditions**

Experiments were performed with wild-type (WT) rice (ShijinB) and transgenic OsCBL1-knockdown (OsCBL1-KD) plants reported in our previous study [15]. Seeds of the WT and knockdown plants were surface sterilized with 5% (v/v) NaClO at room temperature for 30 min and then rinsed with double-distilled water. The seeds were subsequently germinated in water at 30˚C for 2 days prior to placement in 5-L vessels that contained H$_2$O or solutions of different NaNO$_3$ concentrations for an additional 7 days. The plants were grown in a growth chamber at 26/22˚C and under a 16/8-h light/dark photoperiod. To evaluate the PNR, 7-day-old plants growing in H$_2$O were treated with different concentrations of NaNO$_3$ or NaCl for the indicated time.

**Gene expression analysis**

Total RNA was isolated from the root using TRIzol reagent (Invitrogen, Cat no. 15596026). An amount of ~ 2 μg of total RNA was extracted and treated with RNase-free DNase I before it was reverse transcribed to cDNA. Quantitative real-time PCR (qRT-PCR) was performed in a Bio-Rad CFX96™ Real-time System (Bio-Rad, http://www.bio-rad.com) in conjunction with SYBR Green real-time PCR Master Mix. Data analysis was performed with Bio-Rad CFX
Manager 3.0 software. The relative expression of target genes was normalized using the housekeeping gene *Actin* and *EF-1a*. The primers used for qRT-PCR are listed in S1 Table.

**Measurement of NO$_3^-$ content and $^{15}$N influx**

Seven-day-old plants were used to measure the NO$_3^-$ content and $^{15}$N influx. The total amount of NO$_3^-$ was measured as previously described [16]. The shoots and roots of 7-day-old seedlings grown under different NO$_3^-$ concentrations were collected. Approximately 0.1 g of fresh tissue samples was then ground to powder in liquid nitrogen, suspended in 1 mL of water and incubated at 45˚C for 1 hour. The supernatant was collected after centrifuging at 10000 g for 15 min at 4˚C and sequentially reacted with salicylic acid–H$_2$SO$_4$ for 20 min. After adding 2 mL of 2 M NaOH, the solution was measured at a 410-nm wavelength, and then the NO$_3^-$ concentration was calculated according to a standard curve.

A $^{15}$N-influx assay was performed with $^{15}$N-labeled NaNO$_3$ (98% atom $^{15}$N-NaNO$_3$, Sigma-Aldrich). Seedlings were grown in H$_2$O for 7 days and then treated with 0.2 or 2 mM $^{15}$N-NaNO$_3$ for 30 min. The seedlings were then transferred to H$_2$O for 3 min and treated with 0.1 mM CaSO$_4$ for 1 min to remove the $^{15}$N-Na NO$_3^-$ from the root surfaces. The roots were subsequently collected and dried at 75˚C. Finally, the roots were ground, and the $^{15}$N content was determined using a Vario ISOTOPE cube analyzer (Elementar Analysensysteme, https://www.elementar.de/en.html) following the manufacturer’s instructions.

**Phenotypic characterization**

Root images were collected using a Canon600D camera. The lengths of the radicle, crown roots, and lateral roots (near the base of the radicle, 0.5–2 cm from the seed) were measured using ImageJ software (http://imagej.nih.gov.ij/).

**Results and discussion**

The inhibited-growth phenotype of *OsCBL1*-knockdown plants can be partially rescued by NO$_3^-$

Our previous study showed that decreasing the expression of *OsCBL1* (i.e., *OsCBL1*-KD) inhibited the growth of rice roots under 1/2-strength Murashige and Skoog (MS) medium growth conditions [15]. Root growth is inextricably linked to nutrient elements. The *CBL1* gene has been reported to be involved in the uptake of K$^+$ and NH$_4^+$ in *Arabidopsis* [17, 18]; furthermore, OsCBL1 localizes to the plasma membrane, and CBL1 is also involved in the regulation of K$^+$ uptake in rice [19]. To further study how OsCBL1 participates in the regulation of rice growth and development and whether the regulation is related to the uptake of nutrient elements, we compared the growth of WT and *OsCBL1*-KD plants in H$_2$O and in solution with different concentrations of NO$_3^-$.

When the plants were grown in water, the growth of the *OsCBL1*-KD plants was significantly inhibited compared with that of the WT plants; when 0.2–2 mM NO$_3^-$ was supplied, the growth difference between *OsCBL1*-KD and WT was partially reduced (Fig 1 and S1 Fig). Low NO$_3^-$ concentrations (0.2–0.5 mM) significantly promoted the growth of rice seedlings, and the growth was more pronounced for *OsCBL1*-KD than for WT (Fig 1B–1K). High NO$_3^-$ concentrations (1–2 mM) suppressed the growth of WT seedlings, but this effect was weaker for *OsCBL1*-KD than for WT under 1 mM NO$_3^-$ conditions. Therefore, compared with the WT plants, the *OsCBL1*-KD plants were more sensitive to the stimulatory effects of low NO$_3^-$ but were insensitive to the inhibitory effects of high NO$_3^-$.

These results indicate that in rice, OsCBL1 plays an important role in self-development programs and in the regulatory effects of NO$_3^-$ on rice growth.
The growth inhibition of OsCBL1 knockdown plants is not associated with NO$_3^-$ uptake or transport

To investigate how OsCBL1 influences the regulatory effects of NO$_3^-$ on rice growth, we first analyzed the NO$_3^-$ content in 7-day-old WT and OsCBL1-KD plants under different growth conditions. There were no significant differences in the content of NO$_3^-$ in the roots or shoots between the WT and OsCBL1-KD plants (Fig 2A and 2B); the NO$_3^-$ content in seeds also did not differ (Fig 2C). Using $^{15}$N-labeled NO$_3^-$, we then compared the uptake of NO$_3^-$: The WT plants absorbed slightly more $^{15}$NO$_3^-$ than did the OsCBL1-KD plants when supplied with 2 mM $^{15}$NO$_3^-$ for 30 min, but no significant difference was detected when the plants were supplied with 0.2 mM $^{15}$NO$_3^-$ (Fig 2D). Similar to what occurred for the NO$_3^-$ content, there was no significant difference in nitrogen content between the WT and OsCBL1-KD plants after $^{15}$NO$_3^-$ treatment (Fig 2E). These results indicated that the growth difference between the WT and OsCBL1-KD plants was not due to the difference in NO$_3^-$ uptake capability or NO$_3^-$ content.

OsCBL1 affects the expression of NO$_3^-$ transport-related genes under different NO$_3^-$ conditions

In addition to being an essential nutrient, NO$_3^-$ acts as a signaling molecule to regulate gene expression. NO$_3^-$ signaling is at the center of communication between plant genetic programs...
and the environment and regulates plant growth, development and stress responses [20]. Many NO$_3^-$ transport- and assimilation-related genes have also been found to be involved in NO$_3^-$ signaling. To further investigate how NO$_3^-$ affects the growth of WT and OsCBL1-KD plants under different growth conditions, we evaluated the expression of some NO$_3^-$ transport-related genes (OsNRT2.1, OsNRT2.2, OsNAR2.1, and OsNAR2.2) under different growth conditions. The results showed that with the addition of NO$_3^-$, the expression of OsNRT2.1, OsNRT2.2, and OsNAR2.1 decreased in both the WT and OsCBL1-KD plants (Fig 3A–3C), suggesting that the expression of these genes was induced by nitrogen starvation, similar to the results for nitrate transporter genes (AtNRT2.1, AtNRT2.4, AtNRT2.5) in Arabidopsis [14, 21, 22]. Under conditions of no and low NO$_3^-$ content, the expressions of NRTs and NARs was higher in the OsCBL1-KD plants than in the WT plants (Fig 3A–3D), indicating the presence of altered NO$_3^-$ sensing in the OsCBL1-KD mutant. Considering that the NO$_3^-$ content in both the WT and OsCBL1-KD plants increased after NO$_3^-$ addition, and the lack of significant difference between WT and CBL1-KD plants (Fig 2A and 2B), these results indicate that the difference in the expression of these genes did not directly affect NO$_3^-$ uptake or translocation but may have affected the sensing and/or transmission of NO$_3^-$ signal, subsequently regulating rice growth. Compared with WT plants, the OsCBL1-KD plants in the same NO$_3^-$ conditions
were not more NO$_3^-$ starved but seemed to respond more intensely to nitrogen starvation signals. Therefore, OsCBL1 likely plays an important role in signaling pathways involved in intracellular NO$_3^-$ perception.

OsCBL1 regulates the primary nitrate response

As a sentinel for PNR, AtNRT2.1 is induced not only by nitrogen starvation but also by short-term NO$_3^-$ treatment [7]. To further confirm that the NO$_3^-$ signaling changed in OsCBL1-KD plants, the expression of six NO$_3^-$ induced genes was analyzed in OsCBL1-KD plants to determine whether OsCBL1 is involved in the regulation of the PNR. These genes included two NO$_3^-$ uptake transporter genes, OsNRT2.1 and OsNRT2.2, and their partners, OsNAR2.1 and OsNAR2.2, as well as two NO$_3^-$ assimilation genes, OsNR1 and OsNR2. Wild-type and OsCBL1-KD plants were grown in H$_2$O for 7 days and then were exposed to different concentrations of nitrate solution. The expression levels of all six genes were significantly induced by NO$_3^-$ in both the WT and OsCBL1-KD plants. The magnitude of induction of OsNRT2.1, OsNRT2.2, and OsNR2 was significantly reduced in OsCBL1-KD plants compared with WT plants under NO$_3^-$ induction (Fig 4A, 4C and 4E), while the expressions levels of OsNAR2.1.
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A. NRT2.1

B. NAR2.1

C. NRT2.2

D. NAR2.2

E. NR2

F. NR1

G. NRT2.1

H. NRT2.2
OsNAR2.2, and OsNR1 were similar between the WT and OsCBL1-KD plants (Fig 4B, 4D and 4F). A decrease in the NO$_3^-$-induced expression of OsNRT2.1 and OsNRT2.2 occurred under both low and high NO$_3^-$ concentrations, while the expression of OsNR2 was repressed only under high nitrate concentration (Fig 4A, 4C and 4E). These data suggest that the existence of the PNR pathways that either involve or do not involve OsCBL1. We further surveyed the time course of the expression of OsNRT2.1 and OsNRT2.2 under 2 mM NO$_3^-$ concentrations.

Although the expression of the two genes was relatively high in OsCBL1-KD under nitrogen-free conditions, the expression increased more quickly and intensely in the WT plants under NO$_3^-$ treatment (Fig 4G and 4H). The expression of AtNRT2.1 in plants growing under high-N condition is inhibited [7] but is induced when exposed to nitrate for a short period of time regardless of whether plants grow under N-sufficient or N-deficient conditions [23].

These different regulatory activities indicate that there are different regulatory pathways between long-term and short-term nitrate signaling. Many genes have been characterized to regulate the expression of AtNRT2.1, such as NLP6, NLP7, LBD37/38/39, and NIGT1, which are involved in short-term nitrate signaling and NLP7, TGA1/4, and HIN9/IWS1, which are involved in long-term nitrate signaling [24]. Our results indicate that OsCBL1 is involved in both long- and short-term nitrate signaling and plays different roles in the regulation of OsNRT2.1 and OsNRT2.2 expression.

In Arabidopsis, two CBL-interacting protein kinases, CIPK8 and CIPK23, are involved in PNR regulation. The CIPK8 gene is rapidly induced by NO$_3^-$, and CIPK8 acts as a positive regulator in PNR because the induction of several PNR sentinel genes by NO$_3^-$ is reduced in the cipk8 mutant under high NO$_3^-$ concentrations [9]. The CIPK23 gene is also transiently induced by NO$_3^-$, and the induction of NRT2.1 by NO$_3^-$ is higher in the cipk23 mutant than in WT plants at both high and low NO$_3^-$ concentrations [11]. The OsCBL1 gene was not induced by NO$_3^-$ under long- or short-term treatment (S2 Fig), and its product differentially regulated the expression of different PNR marker genes depending on the NO$_3^-$ concentration (Fig 4). These results suggest that OsCBL1 may function as a converter that accepts different Ca$^{2+}$ signals induced by different NO$_3^-$ concentrations and transduces Ca$^{2+}$ signals downstream by activating different OsCIPKs and regulating gene expression.

A recent study revealed the function of Ca$^{2+}$ sensor CPKs to be master regulators that regulate NO$_3^-$-activated signaling [2]. Here, we revealed the role of another type of Ca$^{2+}$ sensor CBL in NO$_3^-$ signaling. Considering that CIPK is also involved in the regulation of NO$_3^-$ signaling [9, 11], the CBL–CIPK pathway should be another NO$_3^-$-coupled Ca$^{2+}$ signaling mechanism that regulates the plant nutrient-growth network. The complex interaction between CBL and CIPK members indicates that the CBL–CIPK module might play an important role in relaying NO$_3^-$ signaling specifically to downstream targets. Future studies are likely to clarify how CBLs sense distinct Ca$^{2+}$ signatures caused by nutrient signaling and identify targets of CIPKs, such as channels, transporters, transcription factors and other regulators involved in all aspects of nutrient-mediated growth regulation in plants.
Supporting information

S1 Fig. The root phenotype of WT and OsCBL1-knockdown plants under different nitrate concentration. Radicle (A) and crown root (B) length of 7-day-old plants were measured grown under different nitrate concentrations. *, p < 0.05, **, p < 0.01 and ***, p < 0.001 compared to the WT (t test).

(TIF)

S2 Fig. The expression pattern of OsCBL1 under different nitrate treatment. (A) The relative expression levels of OsCBL1 in 7-day-old WT plants grew under different NaNO₃ concentrations. (B) The relative expression levels of OsCBL1 in WT plants which grew under non-nutritional condition for 7 days and then were treated by different NaNO₃ or NaCl (control) concentration solution for 2 hours.

(TIF)

S1 Table. Primer sequences used in this study.

(DOCX)

Author Contributions

Conceptualization: Jing Yang, Shiyun Du, Yangsheng Li.
Data curation: Jing Yang, Xiaolong Deng, Xiaoxin Wang, Jingzhang Wang.
Formal analysis: Xiaolong Deng, Xiaoxin Wang, Jingzhang Wang.
Funding acquisition: Shiyun Du, Yangsheng Li.
Investigation: Jing Yang, Xiaolong Deng, Xiaoxin Wang, Jingzhang Wang.
Writing – original draft: Jing Yang, Xiaolong Deng.
Writing – review & editing: Jing Yang, Yangsheng Li.

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