

Short Communication

# *OsDOF11* Promotes Crown Root Formation via Cytokinin in *Oryza Sativa*

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## Abstract

**Background:** Crown root is the main part of root system, which performs an important role in rice growth and development, especially in nutrition and water assimilation. Previously, we reported negative feedback regulation loop between *Oryza sativa* DNA BINDING WITH ONE FINGER 11 (*OsDOF11*) and cytokinin by *Oryza sativa* CYTOKININ OXIDASE/DEHYDROGENASE 4 (*OsCKX4*) in rice development. **Methods:** Reverse transcription quantitative RT-PCR analyses was used to analyze the related gene transcript level. Nitrogen and hormone were measured by CHN-Nitrogen analyser and Liquid chromatography mass spectrometer, respectively. Exogenous application of cytokinin and [<sup>13</sup>C] sucrose labeled stable isotope uptake experiments help us to explain the relationship between *OsDOF11* and cytokinin. **Results:** We demonstrate the role of *OsDOF11* in root development. We note that the loss function of *OsDOF11* displays the reduced crown roots number, low activity of nitrogen assimilation and low content of cytokinin and auxin. The expression level of *WUSCHEL*-related homeobox (*OsWOX11*), *A*-type response regulator 2 (*OsRR2*), *OsRR3*, and *OsCKX4* were decreased in *osdof11-1*, as well as in *OsDOF11* RNA interference 9 mutants (*RNAi-9* lines). Through Exogenous application of multiple concentrations of cytokinin as treatment to *osdof11-1* mutant, *RNAi-9* lines, and wild type (WT). We found that the crown roots number of *osdof11-1* plants were rescued as the cytokinin concentration increased gradually from 1 μM to 10 μM, but the effect was weaker in *RNAi-9* line. And cytokinin inhibited sucrose uptake activity from Murashige-Skoog medium with 3.0% sucrose (MS30) by *OsDOF11* in rice root. **Conclusions:** *OsDOF11* promotes crown root formation via cytokinin in *oryza sativa*. These results provide a physiological basis for further analysis of the *OsDOF11* function of in rice root development.

**Keywords:** *OsDOF11*; cytokinin; nitrogen; crown root; rice

## 1. Introduction

Plant architecture is an important factor in biomass, such as of the root system, tiller number, panicle structure [1,2]. Several development processes or reactions directly or indirectly affect the plant architecture, such as: nutrition transport activity and distribution efficiency of assimilation, plant hormones signaling and mechanism, and so on. Nutrients are assimilated by root system, which provides the fundamental elements for plant development [3,4]. Crown root (also known as adventitious roots or shoot-borne roots) acts as one type of the root component of plant root system, which displays much more contributions to nutrition and water uptake in crops, including rice [5].

The endogenous/exogenous phytohormones are essential for crown roots development, such as auxin, and cytokinin [6,7]. Auxin may induce *Oryza sativa* CROWN ROOTLESS 1 (*OsCRL1*) by two auxin responsive elements at promoter region, which is essential for crown root formation in rice [8]. Also, overexpression of *OsYUCCA1* or RNA interference of rice *PIN-formed 1* (*PIN1*) transgenic

plants displayed reduced crown roots by altering auxin signal transfer or transport, respectively [9,10]. *Oryza sativa* CYTOKININ OXIDASE/DEHYDROGENASE 4 (*OsCKX4*) functions in cytokinin degradation. *OsCKX4* activation line *ren-1* demonstrate more crown roots with lower level cytokinin. Both *RNAi-32* and *RNAi-42*, *OsCKX4* RNA interference lines, have reduced number of crown roots. Two different type response elements of auxin and cytokinin existed in *OsCKX4* promoter. Auxin response factor of *OsARF25*, and *B*-type response regulator of *ORR2* and *ORR3* could bind *OsCKX4* promoter by Yeast-one experiments [11]. In addition, *OsCRL5* and *WUSCHEL*-related homeobox (*OsWOX11*) are also mediated in the cross talk of auxin and cytokinin signaling pathways in regulating crown root formation [12,13]. Over expression of Receptor-like Protein Kinase (*OsRPK1*), which encodes a leucine-rich repeat-receptor-like kinase, reduces crown roots number by auxin transport inhibition. This gene is also regulated by abscisic acid and salt stress [14].



Multiple extrinsic nutrition factors could affect crown root emerging from the stem. *PHOSPHORUS UPTAKE 1 (PUP1)/PHORUS-STARVATION TOLERANCE 1 (PSTOL1)*, expresses in the zone of crown roots initiation of the stem base, might affect crown roots establishment and elongation by contributing to nutrient uptake [15]. *Oryza sativa* *DNA BINDING WITH ONE FINGER 11 (OsDOF11)* is a transcription factor that mediates sucrose transport by binding to the promoter of sucrose transport genes, *Sucrose Transporter (SUT)* and *Sugars Will Eventually be Exported Transporters (SWEET)* genes. Recently, we reported that the *osdof11* mutant and WT germinated on Murashige-Skoog medium without sucrose (MS0) for 7 days. We found that the primary root length and crown roots number were reduced in *osdof11* mutants [16], which were similar with published data of *OsCKX4* RNAi transgenic plant root phenotype [11]. Here, we demonstrated that *OsDOF11* modulates crown roots initially by inducing cytokinin signaling.

## 2. Materials and Methods

### 2.1 Plant Materials and Growth Conditions

Japanica rice (*Oryza sativa* ‘Dongjin’) plants were grown in controlled environment rooms. Seeds were germinated either on an MS medium without sucrose, as previously reported. Progeny of homozygous *osdof11-1* plants and segregating WT plants were selected, as well as *RNAi-9* [17]. Seedlings were cultivated for 7 days at 28 °C under continuous light before being transferred to soil in the greenhouse.

### 2.2 Nitrogen Content

WT, *osdof11-1* and *RNAi-9* seeds were germinated in Yoshida medium with or without nitrogen for 7 days. The seedling plant was harvested, ground in liquid nitrogen, and then filtered through a 100- $\mu$ m sieve. The total nitrogen content was measured using a CHN-Nitrogen analyser (Vario EL cube, Elementar Analysensysteme GmbH, Langenselbold, Germany). The protein contents in the seedling plant and effective leaf blade were calculated on a dry basis.

### 2.3 RT-PCR Analyses

Total RNA was isolated from seedling roots with stem at 7 DAG using RNAiso Plus (TaKaRa, Shiga, Japan; <http://www.takarabio.com>). The cDNAs were synthesized and quantitative real-time RT-PCR was performed as previously described [17]. Expression levels were normalized with rice *UBQ5 (LOC\_Os01g22490)*. All experiments were conducted at least three times, with three or more samples taken at each point. To ensure primer specificity, we performed the experiments when the melting curve showed a single sharp peak. All primers are listed in **Supplementary Table 1**.

### 2.4 Extraction, Purification, and Quantification of Hormones

WT, *osdof11-1* and *RNAi-9* seeds germinated and grew in water for 10 days at growth chamber. The various hormones studied here were extracted, purified and measured as previously reported [18].

### 2.5 Cytokinin Treatment Arrays

WT, *osdof11-1* and *RNAi-9* were germinated in MS medium without sucrose, which contained multiple concentration of kinetin (1  $\mu$ M, 4  $\mu$ M, 10  $\mu$ M, and 50  $\mu$ M).

### 2.6 Statistical Analysis

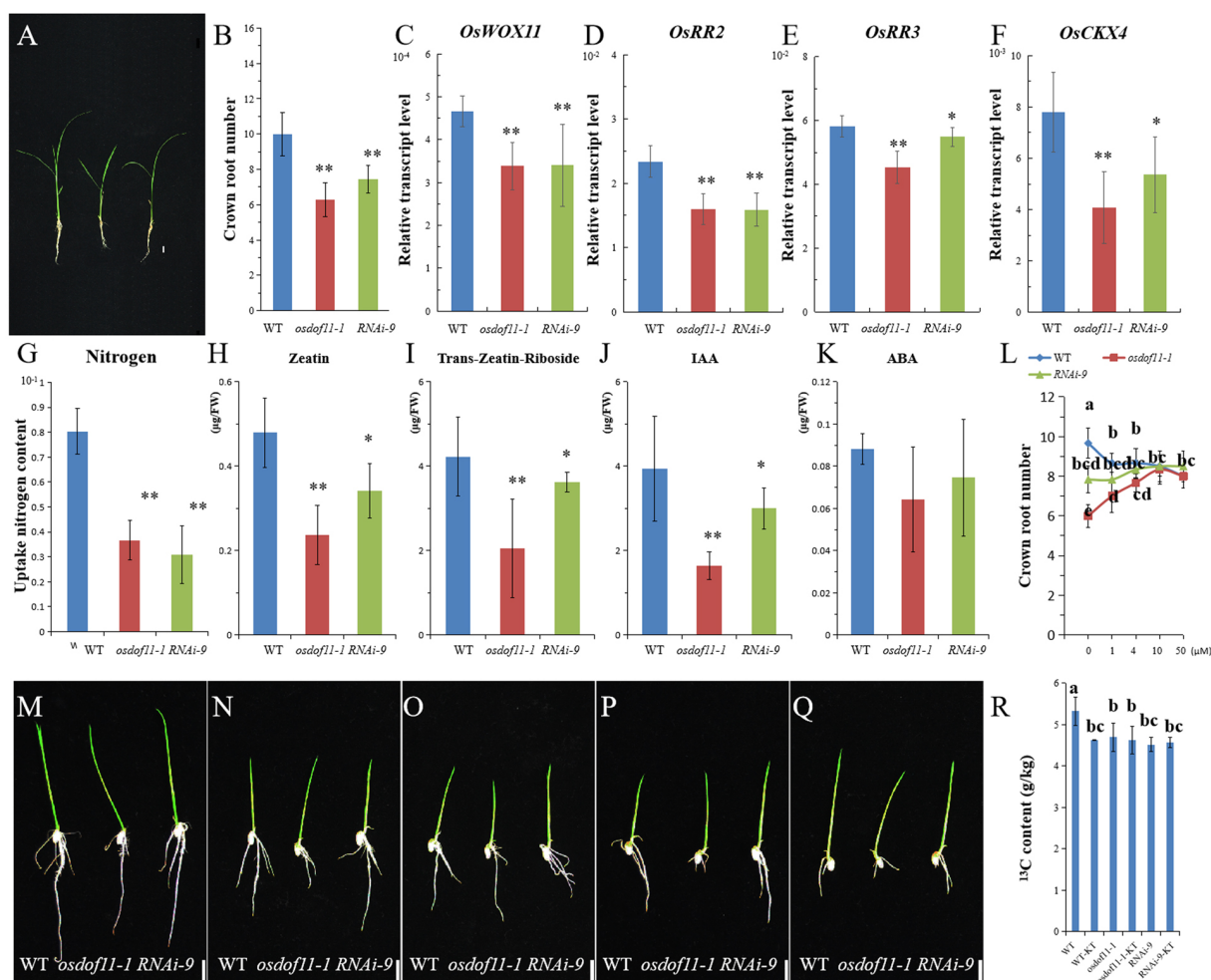
Student's *t*-test by Excel 2010 (Microsoft, Washington, USA) was performed to determine any statistically significant differences among values measured from WT, *osdof11-1*, and *RNAi-9* samples in each experiment. Each data point represents the mean from at least four different plants. Student's *t*-test was used to compare means at a significance level of  $p < 0.05$  or  $p < 0.01$ .

## 3. Result and Discussion

*Oryza sativa* *CYTOKININ OXIDASE/DEHYDROGENASE 4 (OsCKX4)* is a cytokinin inhibitor, which plays a positive role in crown roots formation [11]. Previously, we reported that *Oryza sativa* *DNA BINDING WITH ONE FINGER 11 (OsDOF11)* mediated sucrose transport by sucrose transporter genes and cytokinin degradation by *OsCKX4* [16,19]. To further analyze the function of *OsDOF11* at seedling stage, we chose T-DNA insertion line *osdof11-1* and *OsDOF11* RNA interference line 9 (*RNAi-9*) for analysis, as well as background line of cultivar Dongjin (WT). We found that the crown roots number was reduced in *OsDOF11* mutant (Fig. 1A,B) which was similar with published data of *OsCKX4* RNAi transgenic plant [11]. Reverse transcription quantitative PCR (RT-qPCR) analyses demonstrated that the expression level of *WUSCHEL-related homeobox (OsWOX11)*, *A-type response regulator 2 (OsRR2)*, *OsRR3* and *OsCKX4* was reduced in the *osdof11* mutant root, as well as *RNAi-9* (Fig. 1C–F).

Sucrose not only acts as a type of carbohydrate for providing energy, but is also utilized to synthesize other organic materials. We described that *OsDOF11* participated in sugar distribution and nitrogen assimilation [17,19]. Furthermore cytokinin metabolism and signal are closely associated with nitrogen availability [20], which plays key roles in root development [21,22]. We germinated *OsDOF11* mutant and *RNAi-9* in Yoshida medium for 10 days. Compared with WT, nitrogen assimilated contents were reduced to 45.62% and 38.34% in the *OsDOF11* mutant and *RNAi-9* lines, respectively (Fig. 1G).

We further measured cytokinin, IAA, and ABA contents of the total seedling plant at 10 DAG. These results showed that zeatin contents in *osdof11-1* and *RNAi-9* were



**Fig. 1. OsDOF11 promotes crown roots formation by cytokinin.** (A,B) Crown root number of WT, *osdof11-1*, and *RNAi-9*. (A) Plants at 14 DAG; (B) Crown root number of *OsDOF11* mutant and WT plants at 7 DAG. Bar: A = 2 cm. (C–F) Expression levels of CK-related genes relative to *OsUBQ5*, evaluated in 7 DAG total seedling plant from WT and *osdof11* mutants. (C) *OsWOX11*; (D) *OsRR2*; (E) *OsRR3*; (F) *OsCKX4*. (G) The increased nitrogen content of WT, *osdof11-1*, and *RNAi-9* seedlings grown on MS0 media with or without Nitrogen for 7 days. (H–K) CK, IAA, and ABA content in WT and *osdof11* mutant at 7 DAG. (H) Zeatin; (I) Trans-zeatin-riboside; (J) IAA; (K) ABA. (L–Q) Crown roots number by the treatment of multiple concentration of Kinetin for 7 days. (L) Crown roots number; (M) 0  $\mu$ M; (N) 1  $\mu$ M; (O) 4  $\mu$ M; (P) 10  $\mu$ M; (Q) 50  $\mu$ M. (R)  $^{13}$ C-sucrose content.  $^{13}$ C-sucrose was added into MS medium with 3.0% sucrose and content of the stable isotope labeled sucrose was detected after 3 d. Error bars represent STDEV of at least 3 samples. (B–K): Student's *t*-test was used for statistical analysis. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . (L, R): Values in the same column with different letters are significantly different ( $p < 0.05$ ).

reduced to approximately 49.41% and 71.70%, respectively (Fig. 1H). In addition trans-zeatin-riboside levels were also reduced to about 48.48% and 85.78% (Fig. 1I). This implies that less cytokinin in *OsDOF11* mutant plants might be due to lower activity in synthesis or transport. And the IAA contents in *osdof11-1* and *RNAi-9* were reduced to 41.3% and 75.92%, respectively (Fig. 1J). But the ABA contents was not changed (Fig. 1K). To further analyze the role of cytokinin in *osdof11-1* plants, we applied multiple concentrations of cytokinin (0  $\mu$ M, 1  $\mu$ M, 4  $\mu$ M, 10  $\mu$ M and 50  $\mu$ M) for treatment. We found that crown roots number of WT was inhibited, but that of the *osdof11-1* plants was rescued as the cytokinin concentration increased gradually from 1

$\mu$ M to 10  $\mu$ M (Fig. 1L–Q). These results indicated that *OsDOF11* promoted crown roots number by cytokinin.

*OsDOF11* mediates sucrose transport activity. We germinated WT and *osdof11* mutant seeds on MS medium without sugar for 7 days, and then move to MS liquid medium with 3.0% sucrose (labeled stable isotope [ $^{13}$ C]) for 3 days. Compared with WT, the  $^{13}$ C content in *osdof11-1* and *RNAi-9* seedlings was decreased to 88.28% and 84.88% (Fig. 1R). To analyze the role of cytokinin in sucrose transport, we added 10  $\mu$ M cytokinin in this sucrose uptake experiment. We found that the  $^{13}$ C labeled sucrose content in WT seedlings was reduced by 13.18% under the cytokinin treatment, but *osdof11-1* and *RNAi-9* seedlings

were not affected (Fig. 1R). These results demonstrated that cytokinin inhibited sucrose uptake by *OsDOF11*.

Cytokinin acts as an important factor in rice root development. The concentrations of cytokinin ribosides were reduced in *OsCKX4* activation-tagging mutant [11]. *OsDOF11* appeared to mediate expression of *OsCKX4* directly through binding to the target promoters, and further induced the content of cytokinin in leaf [16]. Defect in sugar transport may cause reduced content of cytokinin and IAA in *OsDOF11* at 10 DAG. The set genes of *OsIPT3*, *OsIPT7*, and *OsIPT8* expressed in leaf blade, which may synthesize the cytokinin [23]. We do not rule out a possibility that cytokinin transport and synthesis are also the reason why cytokinin was abnormal in *OsDOF11* mutant.

## 4. Conclusions

We considered that defect in crown roots number was caused by reduced sugar transport activity and hormone level, especially in cytokinin. Meanwhile, *OsDOF11* controlled sucrose transport and nitrogen metabolism. Cytokinin induced *OsDOF11* transcript levels. However, sucrose uptake activity was inhibited by the 10  $\mu$ M cytokinin treatment. It also indicated that high level of cytokinin decreased root activity. There is the possibility of a relationship between *OsDOF11* and auxin due to the binding of *OsCKX4* to *OsARF25*. Further studies are required to clarify the relationship between hormone and sucrose transport or uptake at the molecular level.

## Author Contributions

XD, WH, XH and YZ performed experiments and analyzed data. YW and FX designed the experiment. YW, GC and FX supervised the experiment. YW, and MZ wrote and edited the manuscript.

## Ethics Approval and Consent to Participate

Not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2708248>.

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