CYTOKININ OXIDASE/DEHYDROGENASE4 Integrates Cytokinin and Auxin Signaling to Control Rice Crown Root Formation

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Crown roots constitute the majority of the rice (\textit{Oryza sativa}) root system and play an important role in rice growth and development. However, the molecular mechanism of crown root formation in rice is not well understood. Here, we characterized a rice dominant mutant, \textit{root enhancer1} (\textit{ren1-D}), which was observed to exhibit a more robust root system, increased crown root number, and reduced plant height. Molecular and genetic analyses revealed that these phenotypes are caused by the activation of a cytokinin oxidase/dehydrogenase (\textit{CKX}) family gene, \textit{OsCKX4}. Subcellular localization demonstrated that \textit{OsCKX4} is a cytosolic isoform of \textit{CKX}. \textit{OsCKX4} is predominantly expressed in leaf blades and roots. It is the dominant \textit{CKX}, preferentially expressed in the shoot base where crown root primordia are produced, underlining its role in root initiation. \textit{OsCKX4} is induced by exogenous auxin and cytokinin in the roots. Furthermore, one-hybrid assays revealed that \textit{OsCKX4} is a direct binding target of both the auxin response factor \textit{OsARF25} and the cytokinin response regulators \textit{OsRR2} and \textit{OsRR3}. Overexpression and RNA interference of \textit{OsCKX4} confirmed that \textit{OsCKX4} plays a positive role in crown root formation. Moreover, expression analysis revealed a significant alteration in the expression of auxin-related genes in the \textit{ren1-D} mutants, indicating that the \textit{OsCKX4} mediates crown root development by integrating the interaction between cytokinin and auxin. Transgenic plants harboring \textit{OsCKX4} under the control of the root-specific promoter \textit{RCc3} displayed enhanced root development without affecting their shoot parts, suggesting that this strategy could be a powerful tool in rice root engineering.

Roots are very important to plants for various functions, including water and nutrient uptake, anchoring, mechanical support, and storage (Hochholdinger et al., 2004; De Smet et al., 2012). Roots also serve as the major interface between plants and various biotic and abiotic factors in the soil environment by both sensing and responding to environmental cues, and they enable plants to overcome the challenges posed by their sessile status (McCully, 1995, 1999; Raven and Edwards, 2001; Malamy, 2005). Rice (\textit{Oryza sativa}) has a dense fibrous root system that includes embryonic roots, crown roots (also known as adventitious roots or shoot-borne roots), and lateral roots. More in-depth research regarding the rice root system should yield more insights regarding the mechanisms that control the differentiation of root primordia from the shoot base and the processes for how the root apical meristems generate structurally differentiated types of roots (Coudert et al., 2010). A comprehensive understanding of root development should also have important implications for the ability to manipulate root architecture, which contributes to both improving crop yield and optimizing agricultural land use (de Dorlodot et al., 2007).

The architecture of the root system is regulated by endogenous factors, such as phytohormones. These factors integrate environmental stimuli (e.g., the availability of water and nutrients) to determine root development (Osmond et al., 2007; Hochholdinger and Zimmermann, 2008; Péret et al., 2009; Petricka et al., 2012). While several phytohormones are involved in regulating root development, auxin and cytokinin are known to be two of the major players. Consequently, coordinating the overlapping activities of these two phytohormones is essential for effective root development (Růžička et al., 2009). Several rice auxin-related mutants show abnormalities in crown root formation. For example, \textit{CROWNLESS ROOT1} (\textit{CRL1})/\textit{ADVENTITIOS

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ROOTLESS1 (ARL1) encodes an ASYMMETRIC LEAVES2 (AS2)/LATERAL ORGAN BOUNDARIES (LOB) transcriptional factor and is known to act as a positive regulator for crown root formation downstream of the indole-3-acetic acid (IAA)- and auxin response factor (ARF)-mediated auxin signaling pathway. The crl1/ar1 mutant has been shown to exhibit few crown roots (Inukai et al., 2006; Liu et al., 2005). Additionally, the CRL4/OsGNOM1 protein is responsible for coordinating the polar localization of the auxin efflux carrier PINFORMED1 (PIN1; Kitomi et al., 2008; Liu et al., 2009). Crown root primordia initiation is strongly inhibited in the crl4/gnom1 mutants. Furthermore, OsCAND1, a homolog of Arabidopsis (Arabidopsis thaliana) CANDI, is involved in auxin signaling to maintain the G2/M cell cycle transition in crown root meristem and, consequently, the emergence of crown roots (Wang et al., 2011b). Moreover, overexpression of the auxin biosynthesis gene OsYUCCA1 promotes crown root formation (Yamamoto et al., 2007). Recently, it was shown that increased expression of rice miR393a or miR393b led to severe defects in auxin response, primary root growth, and crown root initiation (Bian et al., 2012). In Arabidopsis, cytokinins also influence root formation by disrupting lateral root initiation and patterning (Laplaze et al., 2007). The connection between auxin signaling and cytokinin-responsive type A response regulators has also been documented in rice roots, where cytokinin-responsive type A response regulators are involved in the control of cytokinin homeostasis in individual tissues, the maintenance of cytokinin homeostasis in individual tissues, cells, and organelles is crucial. Isopentenyltransferase and cytokinin nucleoside 5′-monophosphate phosphoribohydrolase are two major genes responsible for cytokinin biosynthesis, while CYTOKININ OXIDASE/DEHYDROGENASE (CKX) is responsible for cytokinin degradation (Frébort et al., 2011; Hwang et al., 2012; Tsai et al., 2012). The uncovering of individual genes involved in cytokinin metabolism is a prerequisite for a better understanding of the mechanism of cytokinin action in root development (Werner et al., 2006; Frébort et al., 2011). CKXs are the only known enzymes shown to catalyze the irreversible degradation of cytokinins (Werner et al., 2003). There are seven CKX members identified in Arabidopsis, designated as AtCKX1 to AtCKX7 (Schmülling et al., 2003; Werner et al., 2003). CKX enzymes consist of domains for FAD and substrate binding. Although the FAD-binding and substrate-binding domains are conserved across the various CKX enzymes, the sequences outside these regions display a strong divergence (Galuszka et al., 2007). AtCKX enzymes also differ in their biochemical properties and subcellular localization (Kowalska et al., 2010). Based on database searches, 11 putative CKX homologs were found in the rice genome, but only OsCKX2 has been characterized (Ashikari et al., 2005; Tsai et al., 2012). It was shown that the knockdown or loss of function of OsCKX2 causes cytokinin accumulation in rice inflorescence meristems, leading to enhanced numbers of reproductive organs, finally resulting in an increased grain yield (Ashikari et al., 2005). However, the relationship between CKX enzymes and crown root formation is unknown.

Activation tagging is quite a powerful tool for functional genomics studies and has been successfully used to identify a number of gain-of-function mutants in rice (Lo et al., 2008; Zhang et al., 2009; Wu et al., 2011; Zhao et al., 2013). For example, a dominant mutant, il1-D, with increased lamina joint inclination and hypersensitivity to brassinosteroid, was identified by a screening of rice transfer DNA (T-DNA) activation-tagging lines (Zhang et al., 2009). The mutant phenotype was caused by overexpression of an atypical basic helix-loop-helix rice protein. Moreover, curly flag leaf1 (cfl1), a dominant mutant with curly leaves, was caused by the overexpression of CFI1, which encodes a WW domain protein involved in cuticle development (Wu et al., 2011). However, to date, there have not been any reports in which this methodology has been applied to the study of crown root development. To investigate the molecular mechanism for the regulation of crown root development and identify genes for improving this important trait, we carried out an extensive screening of our T-DNA activation-tagging mutant population (Ma et al., 2009). Among them, a unique rice mutant, root enhancer1 (ren1-D), was isolated and characterized. ren1-D has a more robust root system with more crown roots. Molecular and genetic analyses revealed that the mutant phenotype was caused by the activation of a putative cytokinin oxidase/dehydrogenase gene, OsCKX4. In this article, we have made a detailed analysis of OsCKX4 and its role in modulating rice crown root development.

RESULTS

Phenotypes of ren1-D Are Caused by Activation Tagging

To identify a new component for tuning rice root architecture, an extensive screening of our T-DNA activation-tagging rice mutants was carried out. One of the mutants, ren1-D, with enhanced crown root growth, was identified (Fig. 1A). Examination of a number of cross sections of the coleoptilar node of the wild type and ren1-D mutants revealed that the crown root primordia were present in ren1-D mutants at 2 d after germination (DAG) but not in the wild type (Fig. 1B). Furthermore, the rate of crown root initiation in the ren1-D mutants was faster than that observed in the wild type (Fig. 1C; Supplemental Fig. S1).
Likewise, the mutants were observed to exhibit more crown roots and a longer average root length than the wild type at 7 DAG (Fig. 1, E and F). Consequently, the dry weight of the ren1-D mutant roots was also higher than that of the wild type (Fig. 1G). In contrast to the increased root system, the ren1-D mutants had shorter shoots than the wild type at 7 DAG and significantly reduced plant height at the mature stage (Fig. 1, D and H; Table I).

Segregation analysis of the phenotype and T-DNA in the progeny of the heterozygous population revealed that ren1-D is a dominant mutant with a 3:1 ratio of ren1-D mutant to the wild type, and this phenotype was cosegregated with T-DNA. To isolate the mutated gene responsible for the ren1-D phenotype, SiteFinding-PCR (Wang et al., 2011a) was used to identify the T-DNA flanking sequence. The result showed that the ren1-D plant carried a T-DNA insertion between Os01g0940000 and Os01g0940100 on chromosome 1 (Supplemental Fig. S2A). To confirm whether the T-DNA insertion was responsible for the ren1-D phenotype, we used three PCR primers (P1, P2, and P3) to identify the genotypes of the plants and found that the T-DNA insertion was cosegregated with the observed phenotype in the progeny of the heterozygous population (Supplemental Fig. S2B).

The T-DNA insertion carries four copies of the cauliflower mosaic virus (CaMV) 35S enhancer adjacent to its right border. Consequently, we next examined the expression levels of the genes located within the region 30 kb upstream and downstream of the T-DNA insertion site. Quantitative real-time (qRT)-PCR analysis showed that the transcriptional level of Os01g0940000 was strongly elevated in the ren1-D mutants, whereas the expression of Os01g0940100 was unaffected (Supplemental Fig. S2C). We next transformed wild-type rice with a construct harboring Os01g0940000 complementary DNA (cDNA) under the control of the maize (Zea mays) Ubi-quitin (Ubi) promoter. Although a degree of phenotypic variation was observed among the different lines, eight out of 15 lines recapitulated the ren1-D-like phenotype (Supplemental Fig. S2D). qRT-PCR analysis also confirmed that Os01g0940000 was highly expressed in these lines (Supplemental Fig. S2E). Overall, these results confirmed that the activated expression of Os01g0940000 caused the ren1-D mutant phenotype. Hence, REN1 was Os01g0940000.

![Figure 1. Phenotypes of the ren1-D mutant. A, Root systems of wild-type (WT) and ren1-D seedlings at 7 DAG. Bar = 4 cm. B, Cross sections of the lower coleoptilar nodes of wild-type and ren1-D seedlings at 2 DAG. Red arrows indicate crown root primordia. Bars = 100 μm. C, Phenotypes of wild-type and ren1-D seedlings at 4 DAG. Bar = 1 cm. D, Phenotypes of wild-type and ren1-D plants at the heading stage. Bar = 10 cm. E, Number of crown roots of wild-type and ren1-D seedlings at 7 DAG. Data are means ± SD (n > 15 seedlings; **P < 0.01). F, Root lengths of wild-type and ren1-D seedlings at 7 DAG. Data are means ± SD (n > 15 seedlings; *P < 0.05). G, Root dry weight of wild-type and ren1-D seedlings at 7 DAG. Data are means ± SD (n > 15 seedlings; *P < 0.05). H, Shoot lengths of wild-type and ren1-D seedlings at 7 DAG. Data are means ± SD (n > 15 seedlings; **P < 0.01).](image)

| Table I. Agronomic traits of wild-type and ren1-D mutant plants Average values ± SD are shown. |
|---------------------------------|-----------------|-----------------|
| Agronomic Trait                | Wild Type       | ren1-D          |
| Plant height (cm)              | 104.5 ± 4.2     | 48.6 ± 4.3      |
| Tiller number                  | 10.9 ± 1.4      | 5.6 ± 1.2       |
| Days to flowering              | 107.5 ± 10.5    | 115.3 ± 10.2    |
| Primary branches per panicle   | 13.1 ± 1.1      | 9.9 ± 2.1       |
| Secondary branches per panicle | 38.3 ± 5.2      | 0 ± 0           |
| Grain number per panicle       | 220.2 ± 34      | 38.4 ± 4.5      |
| Grain length (mm)              | 7.4 ± 0.2       | 6.9 ± 0.2       |
| Grain width (mm)               | 3.4 ± 0.1       | 3.2 ± 0.1       |
| 1,000-grain weight (g)         | 28.0 ± 0.2      | 23.7 ± 0.4      |
Os01g0940000, which has been annotated as OsCKX4, belongs to a defined family of cytokinin oxidase/dehydrogenases in plants that is known to catalyze the irreversible inactivation of cytokinins. The OsCKX4 gene consists of five exons comprising 1,590 nucleotides, encoding a 529-amino acid polypeptide with a predicted molecular mass of 58.4 kD. A BLAST search indicated that OsCKX4 contains a FAD-binding domain located at its N terminus and a cytokinin-binding site located at its C terminus. Phylogenetic analysis shows that OsCKX4 is more closely related to Arabidopsis CKX proteins, indicating that separation of the present CKX genes occurred before the separation of monocot and dicot plants (Schmülling et al., 2003; Gu et al., 2010).

Cytokinin Levels Are Reduced in ren1-D

It has previously been reported that cytokinin plays opposite roles in the regulation of shoot and root growth in Arabidopsis (Werner et al., 2003). As described above, the ren1-D mutant exhibited cytokinin-deficient-like phenotypes. To determine whether the ren1-D mutant phenotypes were caused by cytokinin deficiency, we first analyzed the cytokinin levels (free bases, ribosides, and glucosides) in the roots of ren1-D mutants (Fig. 2A). The amount of the cytokinin free base dihydrozeatin was significantly lower than that in the wild type, but other cytokinin free bases were not markedly affected. The concentrations of cytokinin ribosides were reduced in ren1-D mutants; the most affected was isopentenyl adenosine. Additionally, the cytokinin glucosides isopentenyladenine-9-glucoside and trans-zeatin-9-glucoside were also reduced. These findings suggest that OsCKX4 is functional in cytokinin metabolism.

The response of the ren1-D mutants to exogenous cytokinin, the wild-type plants were observed to exhibit higher kinetin concentrations and more severely inhibited shoot elongation and crown root formation than the ren1-D mutants (Fig. 2C). These results, in turn, indicated that the ren1-D mutants were less sensitive to cytokinin than the wild type.

We next examined the expression of type A OsRRs in order to determine whether cytokinin signaling was altered in ren1-D mutants. The result of this analysis demonstrated that the expression of five type A OsRRs (OsRR1, OsRR2, OsRR3, OsRR6, and OsRR7) was poorly altered in the ren1-D mutants. OsRR4, OsRR9, and OsRR10, however, were up-regulated in ren1-D mutants (Fig. 3D), suggesting that different OsRR proteins might functionally differentiate in mediating diverse functions of cytokinin.

Expression Pattern of OsCKX4 and Its Subcellular Localization

To gain more insight into the biological function of OsCKX4, the expression profiling of OsCKX4 in different tissues, including young roots, mature stems, panicles at heading stage, mature leaf blades, mature leaf sheaths, and seeds, was examined by qRT-PCR. Our results revealed that OsCKX4 was predominantly expressed in leaf blades and roots but hardly detected in stems and panicles (Fig. 3A).

To confirm and further clarify OsCKX4 expression in vivo, a 2.3-kb OsCKX4 upstream fragment from the translation start site was fused to the GUS reporter gene and transgenic plants were generated (Fig. 3B). Independent lines of T2 and T3 homozygous progeny were used to detect GUS activities. Histochemical staining of 1-week-old transgenic lines harboring the OsCKX4 promoter:GUS cassette showed that GUS was detected in the primary roots, lateral roots, and crown

Figure 2. Cytokinin content and response in ren1-D mutants. A, Quantification of endogenous cytokinin content in 2-week-old roots. IP, isopentenyladenine; tZ, trans-zeatin; DHZ, dihydrozeatin; iP, isopentenyladenine riboside; iPR, trans-zeatin riboside; DHZR, dihydrozeatin riboside; iP9G, isopentenyladenine-9-glucoside; tZ9G, trans-zeatin-9-glucoside. Data are means ± s.d. (n = 3). B, Kinetin-treated wild-type (WT) and ren1-D seedlings at 6 DAG cultured in one-half-strength MS medium containing 0, 0.1, 1, or 10 μM kinetin (from left to right). Bar = 3 cm. C, Statistics of kinetin treatment on shoot growth in wild-type and ren1-D seedlings. D, Relative expression levels of rice type A response regulator genes in ren1-D mutants. Ubq2 was used as an internal control. Data are means ± s.d. (n = 3).
roots (Fig. 3B, 1–3). Interestingly, GUS was observed to be strongest at the base of shoots where crown root primordia were formed (Fig. 3B, 4). Apart from the roots, GUS activity was detected at a relatively high level in mature leaf sheaths (Fig. 3B, 6) and mature leaf blades (Fig. 3B, 7), suggesting that OsCKX4 might also play a role in regulating other aspects of plant development.

There are 11 putative CKX genes in the rice genome. Data from the Rice Expression Profile Database (http://ricexpro.dna.afric.go.jp) show that different CKX genes have different expression patterns, indicating their different biological functions. To further investigate whether the OsCKXs are involved in crown root initiation, total RNAs were prepared from the specific parts of shoot bases where crown root primordia were formed with 2-week-old wild-type plants, and all OsCKX transcripts were analyzed by qRT-PCR (Fig. 3C). Except for OsCKX4, all other OsCKX genes are expressed relatively lowly in this region. The transcripts of OsCKX6, OsCKX7, and OsCKX10 were under the detection limit. The expression pattern of OsCKX4 is consistent with its specific role in crown root initiation.

To assess OsCKX4 subcellular localization, we performed a transient expression experiment of OsCKX4 in rice protoplasts. The C terminus of OsCKX4 was fused with GFP under the control of the CaMV 35S promoter, and the construct was introduced into rice protoplasts. In cells expressing GFP alone, the signal was detected in the cytoplasm and nucleus (Fig. 3D, top row). In contrast, the fluorescence of the OsCKX4-GFP fusion was observed exclusively in the cytosol (Fig. 3D, bottom row), indicating that OsCKX4 is a cytosolic cytokinin degradation enzyme.

OsCKX4 Is Regulated by Auxin and Cytokinin via ORRs and OsARF-Mediated Signaling Pathways

Analysis of the 5' upstream fragment of OsCKX4 revealed that there are seven cytokinin response elements (core sequence 5'-AGATT-3') and one auxin response element (AuxRE; core sequence 5'-TGTCTC-3') within the 2-kb region upstream of the ATG translation initiation codon (Fig. 4A). To this end, the expression of OsCKX4 was checked after treating rice seedlings in 5 μM 6-benzylaminopurine (6-BA) or 10 μM IAA. qRT-PCR analysis revealed that OsCKX4 is induced within 30 min and maintained throughout 2 h of 6-BA treatment in roots (Fig. 4B). This result suggests that OsCKX4 is a member of the early cytokinin response family. OsCKX4 expression in the roots was also observed to be strongly induced 30 min after IAA treatment (Fig. 4C). Moreover, incubation with 6-BA or IAA for 2 h also rendered a stronger GUS activity in the roots of OsCKX4 promoter::GUS transgenic rice (Fig. 4D). The expression of OsCKX4 was rapidly up-regulated by both auxin and cytokinin, indicating that the auxin and cytokinin cross talk might be realized through transcriptional regulation in mediating root development.

The B-type response regulators (ORRs) have a GARP DNA-binding domain in the middle and are known to be positive regulators of cytokinin signaling (Ito and Kurata, 2006). To investigate if any closely related ORRs are involved in regulating OsCKX4 expression, we also tested whether four transcription factors, ORR1, ORR2, ORR3, and ORR6, bind to the OsCKX4 promoter by yeast (Saccharomyces cerevisiae) one-hybrid analysis. We found that the transcriptional activation domain-fused ORR2 and ORR3 induce β-galactosidase (LacZ) reporter gene expression driven by the OsCKX4 promoter (Fig. 4E). This finding suggests that OsCKX4 is involved in cytokinin signaling.
In addition, ARFs are transcriptional regulators in auxin signaling, binding to AuxREs in the promoters of downstream genes. Furthermore, we identified a potential AuxRE element in the promoter of OsCKX4. To investigate whether ARFs interact with the OsCKX4 promoter region, we performed yeast one-hybrid assays to test whether OsARF1, OsARF12, and OsARF25, three transcription factors known to act in regulating root development (Waller et al., 2002; Inukai et al., 2005; Qi et al., 2012), bind to the OsCKX4 promoter. Our results showed that only OsARF25 directly binds to the OsCKX4 promoter (Fig. 4E), suggesting that OsARF25 might play a role in regulating OsCKX4 expression.

Knockdown of OsCKX4 Displays Phenotypes with Reduced Crown Roots

In order to explore the function of OsCKX4 in crown root development, RNA interference (RNAi) technology was used to suppress OsCKX4 expression in wild-type plants. A gene-specific fragment of OsCKX4 was cloned into a RNAi vector, and transgenic plants were generated via Agrobacterium tumefaciens-mediated transformation. Two independent homozygous lines of the T2 generation (32 and 42) with significantly reduced OsCKX4 transcript were selected for detailed phenotypic analyses (Fig. 5A).

The RNAi plants had significantly fewer crown roots, and primary root growth was also inhibited compared with the wild type at the seedling stages (Fig. 5, B and C; Table II). However, the architecture of RNAi and wild-type plants showed no significant difference at the mature stage, indicating that there is functional redundancy among CKX genes (Supplemental Fig. S3).

The Expression of Auxin-Related Genes and Auxin Content Are Altered in ren1-D

Gravitropic responses are often associated with auxin homeostasis or signal transduction. We examined the root gravitropic response in ren1-D by measuring the curvature after gravistimulation at 90° to the vertical for 24 h (Fig. 6A). Approximately 60% of wild-type roots had root tip angles of 66° to 85°. In contrast, 80% of ren1-D roots were observed to have root tip angles of 66° to 85° (Fig. 6B). The average root tip angle of the wild type was 70.8°, whereas ren1-D roots had average angles of 78.1°. These results suggested that the gravitropic response was enhanced in ren1-D mutants (Supplemental Fig. S4).

We next tested the response of the mutant to N-1-naphthylphthalamic acid (NPA), a drug that inhibits polar auxin transport at the level of auxin efflux. The mutant and wild-type control were treated with different concentrations of exogenous NPA (0, 0.5, or 1 μM). The NPA treatment suppressed crown root and lateral root...
distribution and auxin biosynthesis are regulated by cytokinin, which is consistent with previous studies (Pernisová et al., 2009; Jones et al., 2010).

As crown root initiation was affected in the ren1-D mutants, we measured the expression levels of crown root development marker genes known to be expressed in rice roots, including CRL1, CRL4, and WOX11, by qRT-PCR (Fig. 6G). While CRL1 and CRL4 were found to be up-regulated, no change in the expression of WOX11 was observed in the ren1-D mutants.

Ectopic Expression of OsCKX4 under the Control of the Root-Specific RCC3 Promoter

Given that cytokinin is required for shoot growth, the constitutive activation of OsCKX4 in ren1-D mutants led to severe defects in shoot parts of the mutant plants. Thus, we put the OsCKX4 cDNA under the control of the root-specific promoter RCC3 (Xu et al., 1995) and introduced the construct into wild-type rice (Fig. 7, A and B). Two independent RCC3:OsCKX4 transgenic lines were selected for further analysis. The root-specific over-expression of OsCKX4 was confirmed by qRT-PCR. Specifically, the transgenes were observed to be expressed at high levels in the roots but not in the shoots of the plants (Fig. 7D). Compared with ren1-D mutants, RCC3:OsCKX4 seedlings were observed to exhibit enhanced root system development without suffering from defects in their shoot parts (Fig. 7, C and E).

DISCUSSION

The root system is a vital determinant of plant growth potential. Understanding the regulatory mechanisms underlying root architecture is of great agronomic importance. Screening and characterization of loss-of-function mutants have greatly improved our understanding of the mechanisms driving plant root formation (Inukai et al., 2005; Liu et al., 2005; Liu et al., 2009; Zhao et al., 2009; Kitomi et al., 2011). However, this approach cannot identify genes with functional redundancy. Gain-of-function mutants, on the other hand, may enable researchers to overcome this deficiency. In this study, we utilized activation tagging to identify OsCKX4, a cytokinin metabolism gene in rice. Enhanced expression of OsCKX4 was observed to drive the formation of a robust root system with increased numbers of crown roots. Cytokinins are N6-substituted adenine derivatives that are

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**Table II. Comparison of crown root number and root length between wild-type and OsCKX4-RNAi transgenic plants**

| Time after Germination | Crown Root No. | Root Length |
|------------------------|----------------|-------------|
|                        | Wild Type | RNAi Line 32 | RNAi Line 42 | Wild Type | RNAi Line 12 | RNAi Line 42 |
| 1 week                 |          |              |              |          |              |              |
|                        | 4.3 ± 0.60 | 1.3 ± 0.27** | 3.2 ± 0.31* | 6.0 ± 1.33 | 4.9 ± 0.51* | 5.1 ± 0.54* |
| 2 weeks                | 6.2 ± 1.14 | 5.2 ± 0.66*  | 5.0 ± 0.72* | 13.9 ± 1.64 | 11.1 ± 2.03 | 12.3 ± 2.11 |

Plant Physiol. Vol. 165, 2014 1041
essential to many aspects of plant development. Accordingly, plants have several mechanisms to regulate cytokinin levels, including cytokinin biosynthesis, degradation, and inactivating conjugations. The spatial and temporal distributions of bioactive cytokinin levels are also strictly controlled for plant development events. There exist 11 genes encoding for CKX in the rice genome, with two sets of segmental duplication genes (OsCKX4/OsCKX9 and OsCKX3/OsCKX8) and a pair of tandem duplication genes (OsCKX6/OsCKX7; Ashikari et al., 2005; Gu et al., 2010). Although some characterizations of OsCKX genes have been reported recently (Ashikari et al., 2005; Tsai et al., 2012), only OsCKX2 has been characterized in detail to date. At the protein level, OsCKX2 and OsCKX4 share 45% similarity. However, OsCKX4 is more closely related to Arabidopsis CKX proteins. OsCKX4 contains five exons and four introns, whereas OsCKX2 shows a reduced number of exons from five to four, as a result of the combination of the second and third exons of their ancestors during early evolutionary separation. OsCKX2 was expressed in most tissues, including leaves, culms, inflorescence meristems, and flowers, but was hardly detected in the roots. The knockdown or knockout of OsCKX2 leads to accumulated cytokinin in inflorescence meristems and increased grain numbers, which leads to increased grain yield without affecting the phenology of the rice plant (Ashikari et al., 2005). In contrast, OsCKX4 is predominantly expressed in the roots and leaf blades and has very low levels in inflorescence meristems and flowers, and OsCKX4-RNAi lines did not display enhanced grain number per panicle (Supplemental Table S1). In contrast to OsCKX9 and other OsCKX genes, OsCKX4 expression was observed to be strongest at the point of the shoot base from which crown roots are initiated, which supports our hypothesis that it plays an important role in crown root development. Overall, these findings indicate that different CKXs play distinct roles in the growth and development of rice.

Cytokinin homeostasis in plants is spatially and temporally regulated via a fine balance between biosynthesis and degradation. The cellular and subcellular distribution of biosynthesis and degradation enzymes ensures that endogenous cytokinin levels can be precisely regulated (Frébort et al., 2011). The different isoforms of CKX have been shown to demonstrate a variety of biochemical properties and subcellular localization patterns (Schmülling et al., 2003; Kowalska et al., 2010; Frébort et al., 2011). While the apoplastic CKX enzymes found in Arabidopsis act mainly on cytokinin free bases and ribosides, the cytosolic CKX enzyme prefers cytokinin 9-glucosides. In contrast, the vacuolar isoforms are known to exhibit their highest levels of activity with cytokinin riboside monophosphates, di-phosphates, and triphosphates (Galuszka et al., 2007; Kowalska et al., 2010). While most CKX enzymes appear...
to be localized in the apoplast or vacuoles, there is generally one CKX per plant genome that lacks a translocation signal and, thus, presumably functions in the cytosol (Smehilová et al., 2009). For example, in Arabidopsis, the only nontargeted CKX, AtCKX7, is restricted to the cytosol (Galuszka et al., 2007; Köllmer et al., 2014). Similarly, in maize, ZmCKX10 lacked a translocation signal and also localized in the cytosol (Smehilová et al., 2009). Our results demonstrated that OsCKX4 is also a cytosolic cytokinin degradation enzyme. The cytokinin glucosides isopentenyladenine-9-glucoside and transzeatin-9-glucoside were both observed to be reduced in our ren1-D mutants, confirming the findings of previous research (Galuszka et al., 2007; Kowalska et al., 2010).

The subcellular localization of other rice CKXs, however, is a topic that merits further investigation in the future.

It has been suggested that both auxin and cytokinin play roles in the regulation of root development. The interaction between OsARF25 and the OsCKX4 promoter was confirmed by yeast one-hybrid assay, suggesting that OsCKX4 may participate in auxin signaling. Overexpression of OsCKX4 was observed to reduce auxin biosynthesis, while a knockdown of OsCKX4 was found to enhance auxin biosynthesis (Fig. 6, E and F; Supplemental Fig. S5). Taken together, these results confirm that OsCKX4 has a negative effect on auxin level and suggest that cytokinin is an essential regulator of auxin biosynthesis (Fig. 8). Many genes, including some transcription factors, have been shown to participate in the regulation of crown root development. For example, mutations in CRL1, CRL4, CRL5, and WOX11 are known to cause defects in crown root formation (Inukai et al., 2005; Liu et al., 2005; Kitomi et al., 2008, 2011; Zhao et al., 2009). CRL1 is a direct target of ARF and regulates
crown root initiation in rice (Inukai et al., 2005). CRL4, encoding a protein highly homologous with Arabidopsis GNOM, regulates crown root formation through coordinating auxin transport (Kitomi et al., 2008). In ren1-D mutants, the increased expression level of CRL1 and CRL4 may be responsible for the observed enhanced root phenotype. OsRR2, a type A cytokinin-responsive regulator gene, was reported to be involved in crown root formation (Zhao et al., 2009). In WOX11 overexpression lines, OsRR2 is switched off, resulting in enhanced crown root development (Zhao et al., 2009). In ren1-D mutants, OsRR2 was down-regulated, which may partially account for its root phenotype (Fig. 3D). As described above, these results suggest that OsCKX4 integrates cytokinin and auxin signaling to control crown root development.

Plants with larger root systems have an increased ability to survive under drought and/or nutrient-deficient conditions (Hodge et al., 1999; Liao et al., 2001). Our studies have shown that OsCKX4 is essential to the initiation and development of crown root primordia in rice. Likewise, we have also demonstrated that a change in the OsCKX4 level affects root architecture. ren1-D mutants formed small panicles with a decreased number of branches and spikelets, suggesting that gain-of-function procedures need to be targeted specifically or at particular stages of plant development. Given that cytokinin is required for shoot growth, a systemic reduction of cytokinin status reduces sink strength in the young shoot tissues and, thus, inhibits their growth. Here, we attempted to drive the OsCKX4 gene using a root-specific promoter, Rc3. The Rc3:OsCKX4 transgenic plants were found to exhibit enhanced root phenotypes by producing more and longer crown roots. Moreover, no obvious effects were observed in lateral root development, possibly due to different mechanisms underlying crown and lateral root formation.

In conclusion, we demonstrated that OsCKX4 positively regulates rice crown root initiation and development. This study will facilitate our understanding of the molecular mechanism of root formation in cereal plants and may ultimately be helpful for root engineering in improving cereal crop yield.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The activation-tagging mutant ren1-D of rice (Oryza sativa ssp. japonica ‘Zhonghua 11’) was isolated by screening our T-DNA population for alterations in root development. The cv Zhonghua 11 was used as a wild-type control in this study. Plants were cultivated in the experimental field of the Institute of Genetics and Developmental Biology in Beijing. For root growth assays at the seedling stage, rice seeds were sterilized with 2% (w/v) sodium hypochlorite and then grown on a vertical phytagar plate containing one-half-strength Murashige and Skoog (MS) medium in a growth chamber at 30°C/25°C (day/night) with a 14-h-light/10-h-dark cycle. For our expression profiling analysis, various tissues were collected from plants grown in the experimental field under natural growing conditions.

Identification of the T-DNA-Tagged Locus

The single T-DNA insertion site in the ren1-D mutants was cloned by SiteFinding-PCR (Wang et al., 2011a) using three T-DNA-specific nested primers (T1, 5’-AGGCGATCGATGAGTTTCATG-3’; T2, 5’-CATTGG-CAGCCTGATGCAAGACGTCG-3’; and T3, 5’-GCTTTGCCATTATAAACGACGCGATCG-3’) combined with the arbitrary degenerate primers (S5-1, 5’-CAGCGACACGACACGCTGCTANNNNNNATGCG-3’; SFP1, 5’-CAGCGACACGCTTACTCAACAC-3’; and SP2, 5’-ACTAACAACACCCTCACCAGAAACG-3’) and positively identified by sequencing. Genotyping of the segregating population was performed by PCR using OsCKX4-specific primers (P1, 5’-ATCAACAACACCCTCACCAGAAACG-3’; and P2, 5’-TGGCAGCTTCTT-GATTTCGTA-3’) and a T-DNA left border primer (P5, 5’-TGTATATGGCT-TACCCTCA-3’).

Construction of Transgenic Plants

To generate the OsCKX4 overexpression vector, the coding region of OsD17G040000 was amplified by PCR and cloned into the pEASY-Blunt vector (Transgen) and then subcloned into pXQUbi vector downstream of the maize (Zea mays) Ubi promoter by KpnI and BamHI.

To generate the OsCKX4-RNAi vector, a specific segment of the OsCKX4 (from positions 644 to 1,053 bp) coding sequence was amplified. The resulting PCR product was inserted into the pUCC-RNAi vector (Luo et al., 2006) with both sense and antisense orientations, and then the fragment containing the inserted RNAi sequence of OsCKX4 was transferred into pQXact downstream of the ACTIN promoter for the OsCKX4-RNAi construct.

To generate the OsCKX4 root-specific overexpression vector, a 2,002-bp promoter fragment of Rc3 was amplified by PCR from genomic DNA of cv Zhonghua11. The fragment was first cloned into the pEASY-Blunt vector and further subcloned upstream of the OsCKX4 gene, resulting in the Rc3:OsCKX4 vector.

To generate the OsCKX4 promoter:GUS vector, the promoter region of OsCKX4 (2,308 bp upstream of ATG) was amplified by PCR and inserted into the pCAMBIA2391Z binary vector containing the GUS reporter gene.

The constructs were introduced into Agrobacterium tumefaciens strain AGL1 and transferred into cv Zhonghua 11 by A. tumefaciens-mediated transformation as described previously (Liu et al., 2007). All of the primers used to generate the above-mentioned constructs are listed in Supplemental Table S2, and all of the constructs were confirmed by sequencing. The primers NPTII-F and NPTII-R listed in Supplemental Table S2 were used for genotyping the transgenic plants.

Subcellular Localization of OsCKX4

To examine the subcellular localization of OsCKX4, the coding sequence of OsCKX4 was fused in frame to the N terminus of the enhanced GFP coding sequence under the control of the CaMV 35S promoter. The OsCKX4-GFP fusion construct and empty GFP vector control were transfected into rice protoplasts using a polyethylene glycol-calcium-mediated method followed by an 18-h incubation to allow transient expression. The transformed protoplasts were observed with a confocal laser scanning microscope (Leica TCS SP5) using an excitation wavelength of 488 nm.

Yeast One-Hybrid Assay

To prepare constructs for the yeast (Saccharomyces cerevisiae) one-hybrid assay, the promoter region of OsCKX4 (2,308 bp upstream of ATG) was amplified and cloned into the pLaCZzu vector (Lin et al., 2007) to generate the OsCKX4 promoter:LaZ reporter construct. To generate AD-OsARF1, AD-OsARF12, AD-OsARF25, AD-ORR1, AD-ORR2, AD-ORR3, and AD-ORR6, the full-length coding sequences of OsARF1, OsARF12, OsARF25, ORR1, ORR2, ORR3, and ORR6 were amplified by PCR with the respective pairs of primers and then cloned into the EcoRI-XhoI sites of the pG4-5 vector (Clontech), respectively. All of the primers were designed to generate the above-mentioned constructs are listed in Supplemental Table S2, and all of the constructs were confirmed by sequencing.

Yeast one-hybrid assays were performed according to the previous description (Lin et al., 2007). The plasmids for AD fusions were cotransformed with the OsCKX4 promoter:LaZ reporter construct into the yeast strain EGY48. Transformants were grown on proper dropout plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for blue color development. Yeast transformation was carried out as described in the Yeast Protocols Handbook (Clontech).

RNA Extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). The first-strand cDNA was synthesized from 2 μg of total RNA digested by DNease I.
OsCKX4 Promotes Rice Crown Root Formation

Developmental Biology, Chinese Academy of Sciences) for caring for the plants in both the greenhouse and the field.

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GUS Staining

The GUS activity assay was performed according to the method described previously (Jefferson, 1989). The examined tissues of the OsCKX4 promoter:GUS transgenic lines were incubated in 5-bromo-4-chloro-3-indoly-β-D-glucuronic acid solution for 8 h at 37°C and then dehydrated in an ethanol series (70%, 85%, 95%, and 100%) to remove the chlorophyll. The stained tissues were viewed with a stereomicroscope (Olympus SZX16), and photographs were taken using a digital camera (Canon EOS 600D).

Root Gravitropism Assay

To assess the root gravitropic response, both wild-type and mutant seedlings were grown vertically for 3 d and then immediately gravistimulated with 90° rotation. After 24 h, the root curvature of wild-type and mutant seedlings was quantified and compared. This experiment was performed with a population of more than 30 seedlings per genotype.

Hormone Treatments

For our hormone treatments, 2-week-old uniform rice seedlings were treated with 10 μM IAA or 5 μM 6-BA. Total RNA was extracted after 0, 0.5, 1, 1.5, 2, and 6 h of treatment and analyzed by qRT-PCR. To analyze the effect of kinetin or NPA on the phenotypes, seeds were sterilized in 2% (w/v) sodium hypochlorite, inoculated, and grown aseptically for 5 or 7 d on one-half-strength MS medium containing 3% (w/v) Suc, 1% (w/v) agar (pH 5.8), and kinetin (0, 0.1, 1, or 10 μM) or NPA (0, 0.5, or 1 μM).

Quantification of Free IAA and Cytokinins

Extraction and determination of the IAA content in each sample were performed by using the method described previously (Fu et al., 2012). Cytokinins from the rice roots were extracted and quantified using the polymer monolith microextraction/hydrophilic interaction chromatography/electrospray ionization tandem mass spectrometry method described previously (Liu et al., 2010).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The rate of crown root initiation in wild-type and ren1-D seedlings.

Supplemental Figure S2. Identification of the T-DNA-tagged locus.

Supplemental Figure S3. The phenotype of wild-type and OsCKX4-RNAi transgenic plants at the ripening stage.

Supplemental Figure S4. Comparison of root tip angles between wild-type and ren1-D plants.

Supplemental Figure S5. qRT-PCR analyses revealed the expression of auxin biosynthesis genes in the roots of OsCKX4-RNAi transgenic plants.

Supplemental Table S1. Phenotypes of panicles in ren1-D and ren1-D–RNAi transgenic plants.

Supplemental Table S2. Primers used in this study.

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Microextraction/hydrophilic interaction chromatography/electrospray ionization graphs were taken using a digital camera (Canon EOS 600D). The rice Ubq2 gene was used as an internal reference for all analyses. Three replicates were performed for each analysis, and average and sd values are shown. The primers used for qRT-PCR are listed in Supplemental Table S2.

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