OsCAND1 Is Required for Crown Root Emergence in Rice

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ABSTRACT Crown roots are main components of the fibrous root system and important for crops to anchor and absorb water and nutrition. To understand the molecular mechanisms of crown root formation, we isolated a rice mutant defective in crown root emergence designated as *Oscand1* (named after the *Arabidopsis* homologous gene *AtCAND1*). The defect of visible crown root in the *Oscand1* mutant is the result of cessation of the G2/M cell cycle transition in the crown root meristem. Map-based cloning revealed that *OsCAND1* is a homolog of *Arabidopsis CAND1*. During crown root primordium development, the expression of *OsCAND1* is confined to the root cap after the establishment of fundamental organization. The transgenic plants harboring *DR5::GUS* showed that auxin signaling in crown root tip is abnormal in the mutant. Exogenous auxin application can partially rescue the defect of crown root development in *Oscand1*. Taken together, these data show that *OsCAND1* is involved in auxin signaling to maintain the G2/M cell cycle transition in crown root meristem and, consequently, the emergence of crown root. Our findings provide new information about the molecular regulation of the emergence of crown root in rice.

Key words: *Oryza sativa* L.; emergence of crown root; *OsCAND1*; cell cycle.

INTRODUCTION

The crown roots are a major component of the fibrous root architecture of most cereal crops, including rice. The anatomy of different stages of crown root primordium in rice has been characterized in detail (Itoh et al., 2005), and crown root formation can be divided into seven stages. First, the initial cells are formed in a few layers by one or two periclinal divisions of the innermost ground meristem cells (pericycle cells). Then, the epidermis–endodermis, central cylinder, and root cap initial cells are established from the primordia; at this point, the epidermis–endodermis initial cells differentiate into epidermis and endodermis, and endodermal cells form cortical cells, defining the second, third, and fourth stages, respectively. In later developmental stages, the fundamental organization of root is formed with establishment of columella and metaxylem vessel and elongated and vacuolated cells at the fifth and sixth stages. Finally, the crown roots emerge from the stem.

Recently, a few mutants affecting crown root primordia initiation have been identified and characterized in rice, contributing to our understanding of the genetic mechanisms underlying crown root development. The first periclinal division is suppressed in *crl1* (*crown rootless 1*)/*arl1* (*adventitious rootless 1*) (Inukai et al., 2005; Liu et al., 2005). *CRL1*/*ARL1* encodes an AS2/LOB-domain protein. The maize-related gene *RTCS* is also involved in postembryonic shoot-borne root formation (Taramino et al., 2007). In addition, primordia initiation is impaired in *crl4* (*crown rootless 4*)/*Osgnom1* mutants (Kitomi et al., 2008b; Liu et al., 2009). CRL4/OsGNOM1 is highly homologous to the GNOM1 protein in *Arabidopsis thaliana* (*Arabidopsis*). Furthermore, initiation of crown root is delayed in *wox11* (*WUSCHEL-related Homeobox 11*) mutants, which produce fewer crown roots (Zhao et al., 2009). During crown root primordia development, cell elongation and vacuolation of the primordia are suppressed in *crl2* and *crl3* mutants (Inukai et al., 2001; Kitomi et al., 2008a). In spite of these findings, however, knowledge about the molecular mechanisms of crown root primordium emergence remains limited.

A phytohormone critical for plant root development is auxin (De Smet and Jurgens, 2007). The regulatory role of auxin

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signaling in crown root initiation has been emphasized in studies of the *arl1/crl1* mutants, which entirely lacks crown root (Inukai et al., 2005; Liu et al., 2005; Kitomi et al., 2008b; Liu et al., 2009). *ARL1/CRL1* expression is induced by auxin and coincides with auxin distribution in the base of the stem where crown root differentiates. The *CRL1* promoter contains two auxin responsive elements, and one is required for the expression of the gene in the stem base. This element can bind *in vitro* with the auxin response factor (ARF) (Inukai et al., 2005). Further, *ARF* is regulated by microRNA to control crown root initiation in *Arabidopsis* (Gutierrez et al., 2009). Transgene studies have also provided evidence that transgenic rice with overexpression of stabilized *Aux/IAA3* (*AUXIN/INDOLE-3-ACETIC ACID 3*) produces phenotypes with reduced crown roots (Nakamura et al., 2006). Recently, it has been reported that loss-of-function of *CRL4/OsGNOM1*, which encodes a large guanine nucleotide exchange factor for an ADP-ribosylation factor affecting polar auxin transport, yielded a phenotype of no crown root (Kitomi et al., 2008b; Liu et al., 2009). Moreover, RNA interference in a transgenic plant of rice *PIN-formed 1* (*PIN1*) or overexpression of *OsPINOID 1* (*OsPID1*), which alters polar auxin transport, reduces crown roots (Xu et al., 2005; Morita and Kyozuka, 2007). Based on these findings, an important role for auxin signaling and transport in crown root development is apparent. Subsequent patterning of tissues within crown root primordia also requires establishment of an auxin response gradient with a maximum at the tip (Benkova et al., 2003). The role of auxin in maintaining crown root meristem remains to be elucidated.

Previous genetic and molecular studies in *Arabidopsis* have identified the SCF^{TIR1} ubiquitin ligase as a positive regulator of auxin signaling (Gray et al., 2001). Cullin-associated and neddylation-dissociated 1 (CAND1) regulates SCF^{TIR1} assembly and disassembly in both animals and plants (Liu et al., 2002; Zheng et al., 2002; Min et al., 2003; Chuang et al., 2004; Feng et al., 2004; Alonso-Peral et al., 2006). Mutation in *CAND1* results in several phenotypes and reduction of auxin response (Chuang et al., 2004; Feng et al., 2004). CAND1 is required for SCF^{TIR1} activity, which targets members of the auxin/indoleacetic acid (Aux/IAA) family of transcriptional regulators for ubiquitin-mediated proteolysis in response to auxin in *Arabidopsis* (Chuang et al., 2004; Feng et al., 2004). Although it is well understood that *CAND1* is important for auxin signaling transduction, how it regulates root development has not been described.

In this work, we isolated a rice homolog of *AtCAND1* from a mutant defective in crown root emergence (*Oscand1*). Our data indicate that *OsCAND1* is involved in auxin signaling in root tips of crown roots. Loss-of-function of *OsCAND1* results in cessation of the G2/M transition of the cell cycle in the meristem of crown roots and, consequently, failure of the emergence of crown roots. The present results provide insight into the molecular regulation of the emergence of crown roots in plants.

RESULTS

Isolation and Characterization of the *Oscand1* Mutant

One mutant showing no visible crown root was isolated from an ethyl methanesulfonate (EMS)-mutagenized population of rice (cv. Kasalath) (Figure 1A and 1B). The mutant (hereafter referred to as *Oscand1*) plant growth was retarded, and failed to survive after 2 weeks (Supplemental Figure 1). To determine whether the initiation or development of crown root primordia is impaired in the *Oscand1* mutant, cross-sections stained with safranin-fast green (an indicator of cell division; Bryan, 1955) at different developmental stages of crown root primordia were investigated for the wild-type (WT) and the *Oscand1* mutant plants. The results showed the same number of crown root primordia formed in coleoptilar phytomer in the WT and the *Oscand1* mutant, indicating that initiation of crown root primordia is normal in the *Oscand1* mutant (Figure 1C and 1D). The results also indicate that development of crown root primordia progressed to formation of the fundamental organization of crown roots in the *Oscand1* mutant compared with the WT (Figure 1E–1I and 1K–1O). The safranin-fast green staining also showed a rapid differentiation of the formed crown root meristem in the *Oscand1* mutant, as visualized by the faint staining signal of safranin (Figure 1J). However, the actual emergence of crown root failed in the *Oscand1* mutant (Figure 1P and 1Q).

OsCAND1 Is Involved in the G2/M Transition in Crown Root Primordia

To determine whether *OsCAND1* affects the cell cycle in the meristem of matured crown root, the expression of several marker genes for different cell cycling stages was examined in crown root primordia in WT and the mutant. Considering the primary root of *Oscand1* growth stops at 6 d after germination, stem base tissues of 5-day-old seedlings of *Oscand1* and wild-type was used for laser-capture microdissection. The tissues of crown root primordia at the stage before fundamental organization formation and at later stages were sampled using laser-capture microdissection (Figure 2B) for quantitative RT–PCR analysis. The cell cycling marker genes were used, including *OsCDKA;2* (previously referred to as *cdc2Os2*), expressed in the dividing zone of root throughout the cell cycle (Umeda et al., 1999); *OsCDKD;1* (previously referred to as *R2*), expressed preferentially in S-phase (Fabian-Marwedel et al., 2002); *OsCDKB;2*, which is especially abundant from G2 to M phase (Umeda et al., 1999); and *CYCB1;1*, the indicator of mitotic activity from G2 to M (Colon-Carmona et al., 1999). In *Arabidopsis*, *KRP2* negatively affects cell division (De Veylder et al., 2001), and the RB/E2F/DP pathway is considered to control the G1/S transition (Inze and De Veylder, 2006). The homologs of *KRP2* and *RB1* in rice (Guo et al., 2007) were thus also used for the analysis.

Quantitative RT–PCR analysis showed that before establishment of fundamental organization of crown root primordia, cell cycle gene expression did not differ significantly between

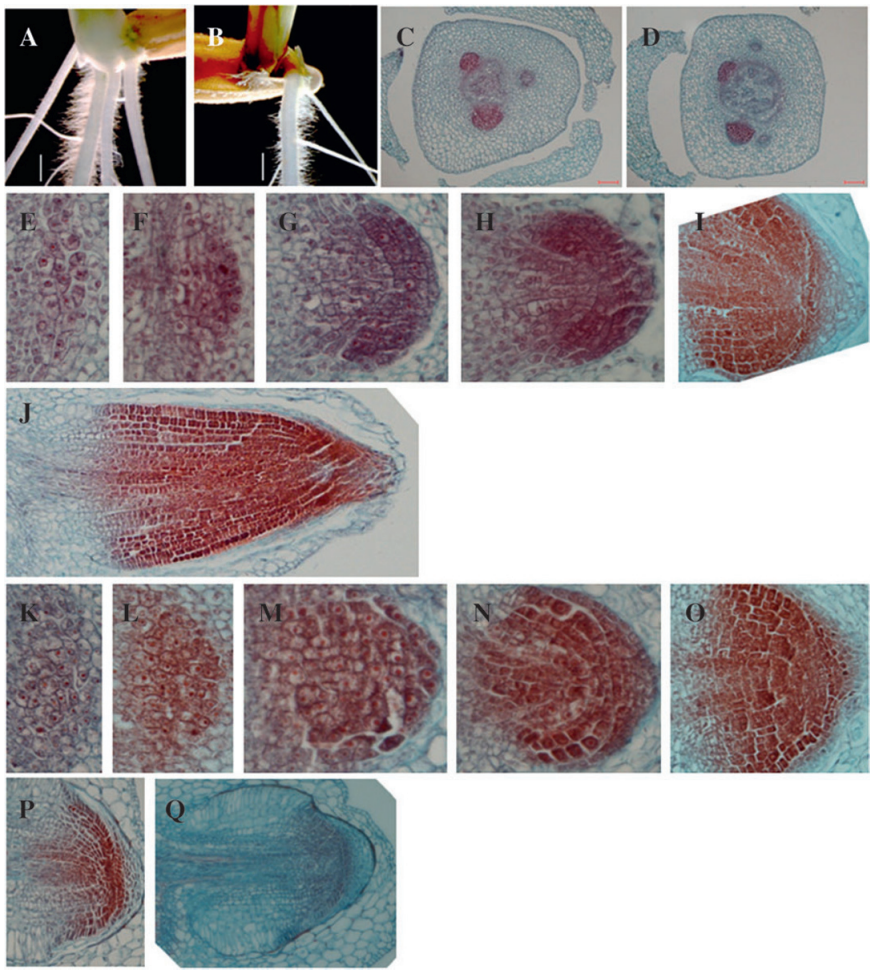

Figure 1. Development of Crown Root Primordia in the Wild-Type (WT) and *Oscand1* Mutant.
(A–B) Crown root growth of 7-day-old seedlings of WT (A) and *Oscand1* mutant (B). Bar = 2 mm.
(C–D) Cross-sections of lower coleoptilar phytomer of 2-day-old seedlings of WT (C) and *Oscand1* mutant (D) with safranin-fast green-stained. Bar = 100 μm.
(E–Q) Developmental processes of crown root primordia in WT (E–J) and *Oscand1* plants (K–Q).

WT and the *Oscand1* mutant. However, after the establishment of fundamental organization, significant repression of *OsCDKB;2* and *OsCYCB1;1*, associated with the G2/M transition, was observed in the mutant compared to WT. In addition, the negative regulator *OsKRP2* was up-regulated in the mutant (Figure 2A). These results indicate that the failure of formation of mature crown root in *Oscand1* is caused by the cessation of the G2/M transition of cell cycle in the meristem of matured crown root primordia.

Cloning and Characterization of *OsCAND1*

Map-based cloning was used to isolate the gene for the mutation. Because the mutant plants could grow only for a couple of weeks, F_2 populations were developed from a cross between heterozygous mutant plants and a japonica rice (Nipponbare). The segregation of WT and *Oscand1* phenotypes at a ratio of 3:1 (52:12, $\chi^2 = 0.01 < \chi^2_{0.05,\ 1} = 3.84$) was found in the F_2 population, indicating a recessive single gene controlling the mutation. Using this F_2 population, we mapped the *OsCAND1* locus on the short arm of chromosome 2 between markers RM1075 and RM12507 in a region of ~26 kb (Figure 3A). This region includes four predicted open reading frames. Sequencing analysis showed a point mutation occurring in a gene coding for a homolog of *CAND1* in *Arabidopsis* (Supplemental Figure 2). This point mutation involves substitution of T to A at 533 bp, which results in premature termination of protein synthesis (Figure 3B).

A complementation test was conducted using a 13.3-kb genomic DNA fragment containing the entire candidate gene (Figure 3C) and subcloned into the plant transformation vector pCAMBIA1300. Because we failed to produce callus from the homozygous *Oscand1* seeds, the gene was transformed into rice callus from heterozygous seeds of the *Oscand1* mutant using *Agrobacterium*-mediated transformation. In the T_0 generation, we identified two independent transgenic lines containing the mutation background (Figure 3F). Sixteen

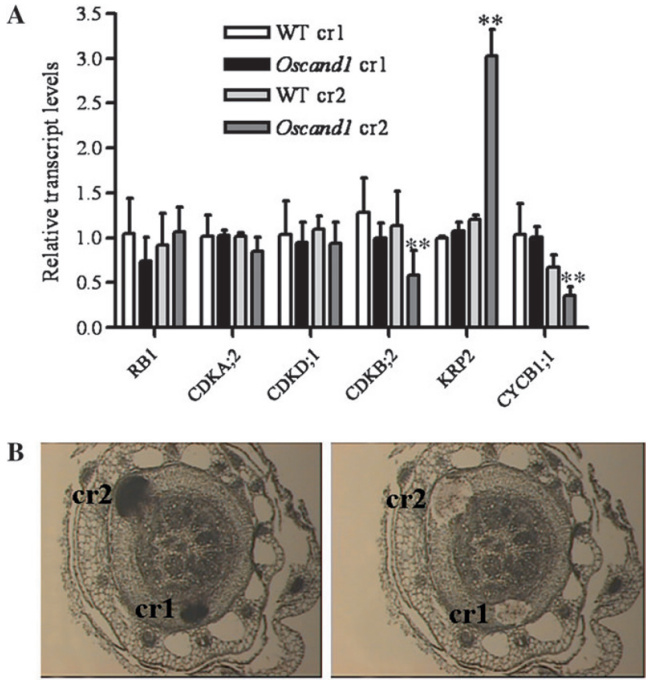

Figure 2. Cell Division Is Reduced in *Oscand1* Mutant.

(A) Relative transcript levels determined by quantitative RT–PCR of six rice cell cycling genes (*OsRB1*, Os*CDKA;2*, *OsCDKD;1*, *OsCDKB;2*, *OsKRP2*, and *OsCYCB1;1*) in two stages of crown root primordia development of *Oscand1* compared with WT. Statistically significant differences are indicated at 1% (**) probability levels (*t*-test).

(B) Schema of LCM sampling of crown root primordia on cross-sections of the stem base. Before captured (right) and after captured (left). cr1, crown root primordia before establishment of fundamental organization; cr2, mature crown root primordia.

and 17 plants exhibited the *Oscand1* mutant phenotype among 321 and 1331 T_1 plants from the two lines (16:1, $P < 0.01$; and 64:1, $P < 0.01$), respectively. The lower segregation of mutants in these T1 populations indicates that introduction of *OsCAND1* into the mutant background can rescue the mutant. The complementation of *OsCAND1* to the mutation was further confirmed by segregation ratio and derived cleavage amplify polymorphism (dCAPs) analysis for the point mutation of *OsCAND1* in self-progenies of the T_1 plants with the WT phenotype (Figure 3D and 3E).

The *OsCAND1* gene encodes a protein of 1218-amino acid (AA) residues with a TIP120 domain (1038–1205 AA) characteristic of CAND1 (Supplemental Figure 2A). The multiple sequence alignments of the full-length CAND1 protein sequences of rice and *Arabidopsis* using the ClustalX program indicated that 82% of the AAs of the TIP120 domain are conserved between OsCAND1 and AtCAND1, and 75% of the AAs outside of the conserved domain are identical between OsCAND1 and AtCAND1 (Supplemental Figure 2A). Phylogenetic analysis between OsCAND1 and its related proteins from several organisms revealed that OsCAND1 is evolutionarily close to AtCAND1 (Supplemental Figure 2B).

Sub-Cellular Localization and Expression Pattern of *OsCAND1*

To examine the sub-cellular localization of OsCAND1, *OsCAND1* was fused in-frame to the N-terminus of synthetic green fluorescent protein (sGFP) and transiently expressed in onion epidermal cells. The OsCAND1–sGFP green fluorescent signal was detected in nuclei (Figure 4A).

Quantitative RT–PCR analysis indicated that *OsCAND1* was expressed in roots, shoots, and the stembase (Supplemental Figure 3). Compared to WT, the expression of *OsCAND1* in the *Oscand1* mutant was reduced in the tested tissues, especially in the stembase, indicating that a positive feedback system may regulate the expression of *OsCAND1*. To examine further the tissue expression pattern of *OsCAND1*, we developed a fused gene of *OsCAND1::GUS* (β-glucuronidase) via promoter of *OsCAND1*. This fused gene can rescue the *Oscand1* mutant phenotype (Figure 4B). The GUS staining analysis showed that during the development of crown root primordia, *OsCAND1* expression is limited to root cap at the stages of fundamental organization formation (Figure 4C). The absence of *OsCAND1* expression in the meristem indicates that *OsCAND1* might regulate crown root meristem cell division in a non-cell autonomous way.

OsCAND1 expression was also detected in the cylinder of vascular bundles in the stembase but not in the peripheral cylinder, which contributes to the initiation of crown root primordia (Figure 4C). In primary and lateral roots, GUS staining was observed above the elongation zone (Figure 4F and 4H), and cross-section showed that *OsCAND1* is expressed in epidermis (Figure 4I). GUS staining was also observed in stamen and leaf vasculature (Figure 4D and 4E).

OsCAND1 Is Required for Proper Auxin Signaling for Maintaining Crown Root Meristem

Auxin signaling is crucial to maintain meristem activity (Benkova and Hejatko, 2009). To check whether auxin signaling was affected in the *Oscand1* mutant, we introduced the *DR5::GUS* construct into heterozygous seeds of the *Oscand1* mutant via *Agrobacterium*-mediated transformation. *DR5* is used as an artificial promoter to monitor auxin responses at the cellular level and *in vivo* auxin distribution (Sabatini et al., 1999; Scarpella et al., 2003). The GUS staining analysis in the transgenic plants showed the same staining pattern between WT and the mutant in the columella of root cap and the central cylinder of the crown root primordia at the stage of fundamental organization formation (Figure 5B and 5C). In contrast, in later crown root primordia stages, the staining in the central cylinder had disappeared, and the gradient staining pattern in the columella had spread throughout the whole root cap in the *Oscand1* mutant, rather than showing the normal staining pattern of the WT (Figure 5D and 5E). It indicates that the auxin signaling has been affected in the later crown root primordia development in the *Oscand1* mutant.

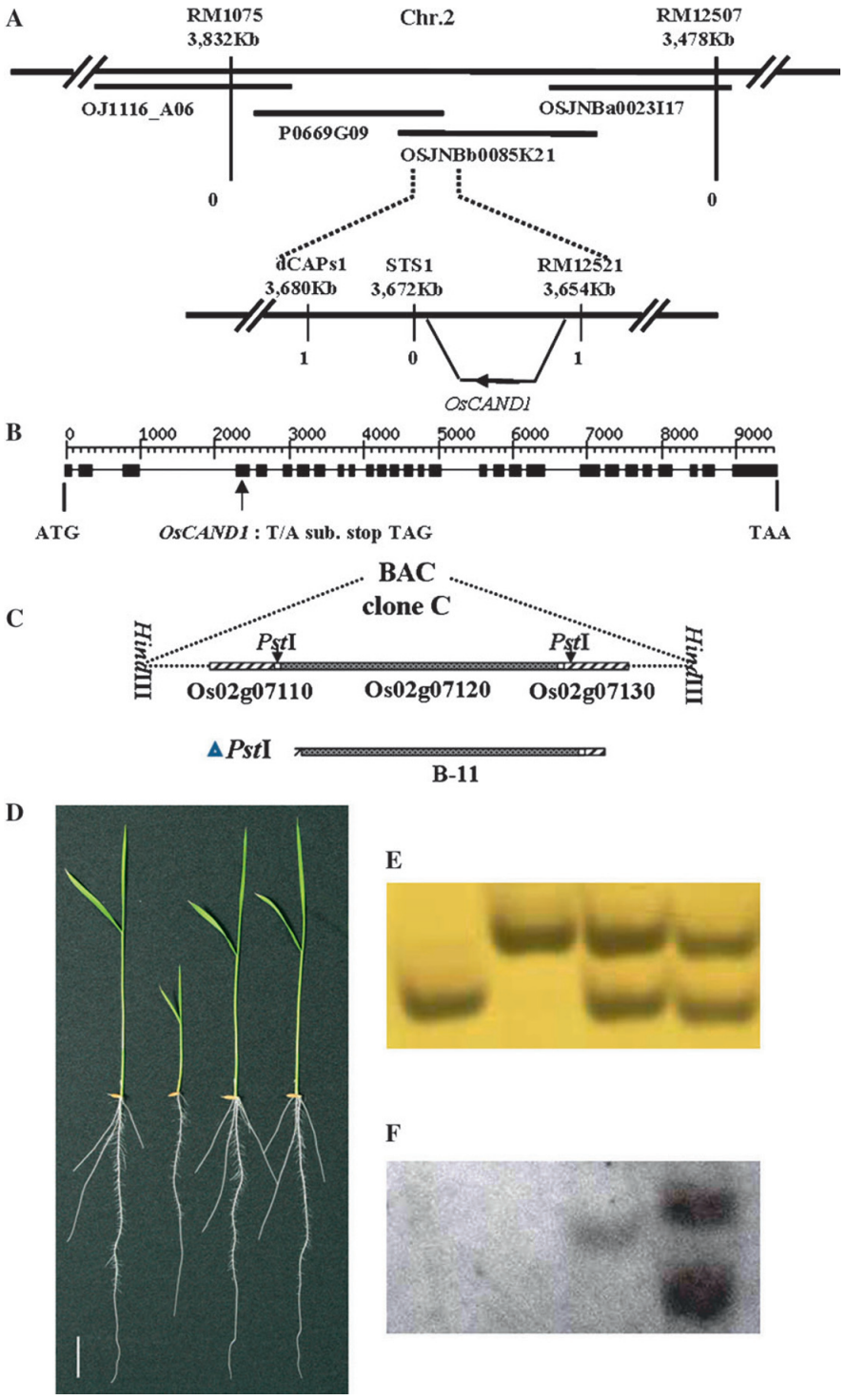

Figure 3. Map-Based Cloning of *OsCAND1* Gene.

(A) Fine mapping of the *OsCAND1* gene between the markers dCAPs1 and RM12521 at the BAC clone OSJNBb0085K21.
(B) Gene structure of the *OsCAND1*. The mutation site of *Oscand1* indicated by the arrow.
(C–F) Complementation analysis of the *Oscand1* mutant. From left to right (C): wild-type, *Oscand1* mutant, two lines of *Oscand1* mutant harboring genomic *OsCAND1*. Bar = 2 cm. Restriction map of the complementing fragment B-11 used in the complementation analysis from BAC cloneC (D). dCAPs analysis (E) and Southern blotting analysis (F) for two lines of transgenic plants.

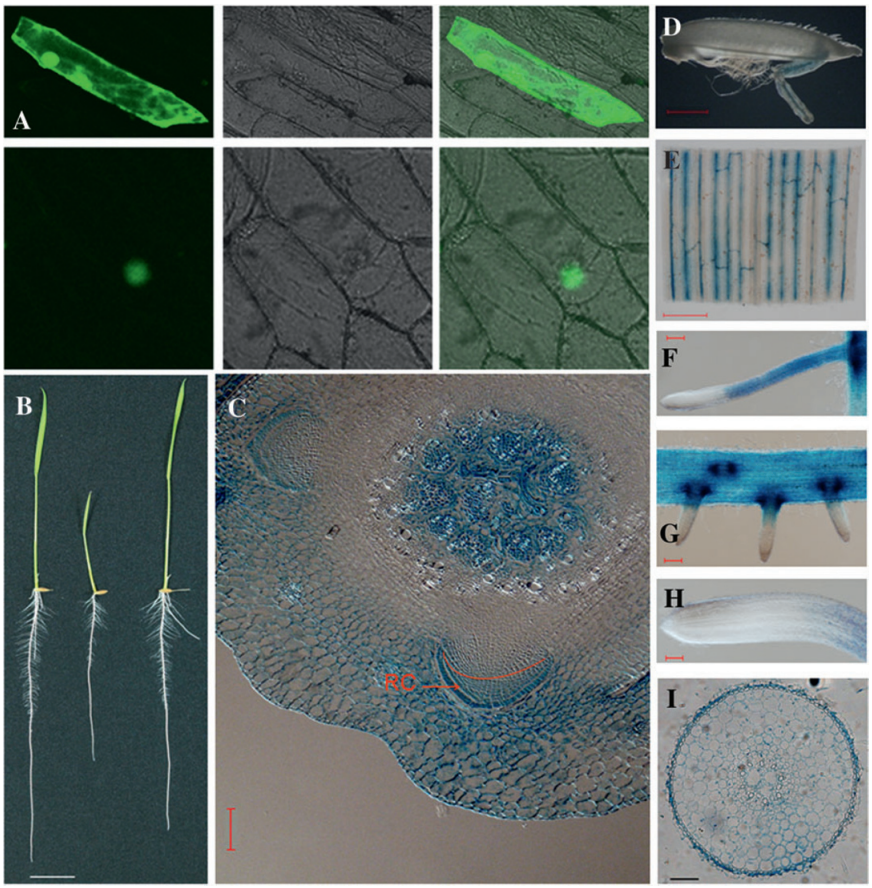

Figure 4. OsCAND1 Protein Localization and Expression Pattern.

(A) OsCAND1 protein sub-cellular localization. Fluorescent microscopy of transiently transformed epidermal onion cells expressing a 35S promoter-driven sGFP fusion protein (upper) and the 35S promoter-driven OsCAND1- sGFP fusion protein (lower). The image of sGFP green fluorescence (left), the same cells under optic microscope (middle) and their merged image (right).

(B) The complementation of *Oscand1* mutant with a construct containing the *OsCAND1* promoter driven expression of OsCAND1–GUS-tagged transgene. This transgene (right) fully complemented defect of crown root development in *Oscand1* mutant. Bar = 2 cm.

(C–I) Expression pattern of *OsCAND1* indicated by GUS stainings in various tissues of vasculature on stembase (C), flower (D), leaf (E), lateral roots (F and G), primary root (H), cross-section of primary root (I). Bar = 100 μm.

To determine whether the crown roots of *Oscand1* mutants can be induced by exogenous auxin, the mutant and WT were exposed to NAA (naphthalene acetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), and IAA in solution cultures. In WT plants, the number of crown roots increased in response to a dose-effect of the exogenous auxins up to 10^{-6} M NAA, 10^{-6} M 2,4-D, and 10^{-5} M IAA (Figure 5A). Similarly, the stimulatory effects of the exogenous auxins were observed in the *Oscand1* mutant. The high concentrations of NAA (10^{-6} M, $P < 0.01$), 2,4-D (10^{-6} M, $P < 0.01$), and IAA (10^{-6} M, $P < 0.01$; 10^{-5} M, $P < 0.05$) significantly induced crown root emergence in the *Oscand1* mutant (Figure 5A).

DISCUSSION

Crown root development has been described in rice (Itoh et al., 2005). Seven stages are defined from the initiation of the crown root primordium to the emergence of crown root from the stem. Our results indicate that a rice homolog of AtCAND1 is involved in the emergence of crown root primordia: crown root primordia cell elongation and vacuolation are suppressed in the *Oscand1* mutant. This phenotype is similar to that of the *crl2* mutant, which has been reported, but the responsible gene is as yet unknown (Inukai et al., 2001). The outgrowth of crown root primordia from the nodes is preceded by death of epidermal cells that cover the root primordia in deepwater rice (Mergemann and Sauter, 2000; Steffens and Sauter, 2009). However, the premise for promoting epidermal cell death is that crown root primordia thereby accomplish fundamental organization and the root apex can reach the stem epidermis (Lorbiecke and Sauter, 1999). By comparison, the *Oscand1* mutant is considered to be in a different stage relative to the emergence event in deepwater rice.

The *CAND1* gene was isolated a decade ago (Yogosawa et al., 1996). Its function in the formation of the SCF complex is conserved in both the animal and the plant kingdoms (Liu

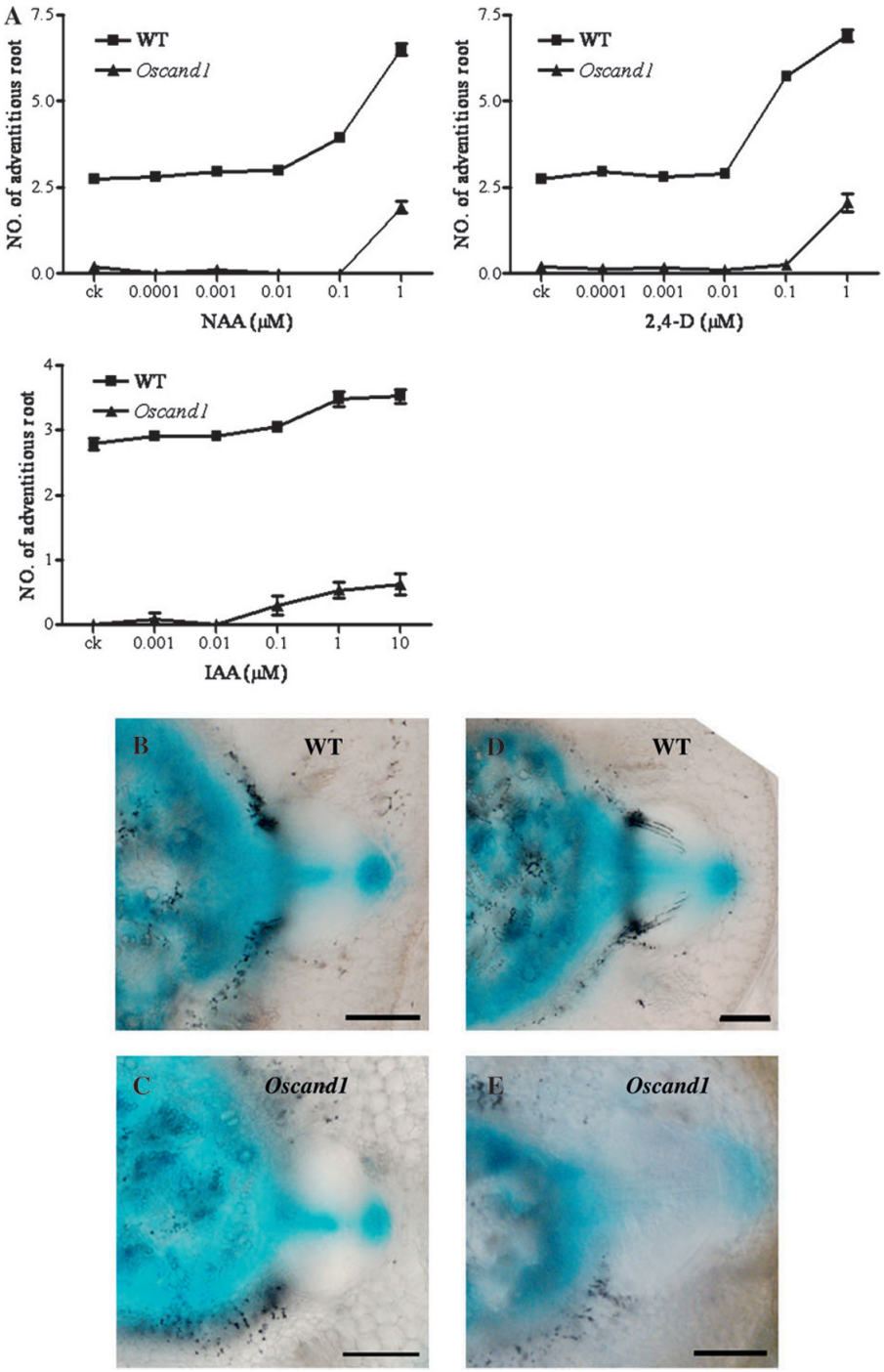

Figure 5. Auxin Signaling Is Changed in the Crown Root Primordia of *Oscand1* Mutant.

(A) Seedlings of wild-type and *Oscand1* mutant grew on culture solution with various concentrations of NAA, 2,4-D, and IAA for 7 d after germination; the numbers of visible crown roots increased in a dose-responsive manner. Data represent the means ± SE of 10 plants in each line.

(B, C) Safranin-fast green-stained cross-section of lower coleoptilar phytomer of 3-day-old seedlings of *Oscand1* without NAA treatment (C) and with NAA treatment (D). Bar = 100 μm.

(D–G) *DR5::GUS* staining in the crown root primordia of WT (D, F) and *Oscand1* (E, G). Bar = 100 μm.

et al., 2002; Zheng et al., 2002; Chuang et al., 2004; Feng et al., 2004). In *Arabidopsis*, CAND1 is considered to be involved in the control of stability of AUX/IAAs in the auxin signaling pathway (Chuang et al., 2004). Loss-of-function of *AtCAND1* results in resistance to auxin and displays short inflorescences and reduced fertility, but in the seedling stage, the mutants grow

normally compared to WT plants (Cheng et al., 2004; Feng et al., 2004). Compared with *Arabidopsis*, auxin resistance can also be found in the *Oscand1* mutant (Supplemental Figure 4), indicating that the function of *CAND1* in auxin signaling may be conserved between *Arabidopsis* and rice. But the *Oscand1* mutant can only survive for 2 weeks; this indicates that *OsCAND1* is an essential gene in rice while *AtCAND1* may be not in *Arabidopsis*. The close evolutionary relationship of AtCAND1 and OsCAND1 supports this idea. In contrast with the dicot model plant *Arabidopsis*, which has a tap root system, the monocot cereal model plant rice has a fibrous root system composed of abundant crown roots. The identification of the function of *OsCAND1* in the emergence of crown root primordia also reveals that during the evolutionary divergence of the tap root and fibrous root systems, the *CAND1* gene may have acquired a novel function in crown root development in crops with a fibrous root system.

The initiation and development of crown root primordia up to the stage of fundamental structure formation in the *Oscand1* mutant are similar to that observed in WT (Figure 1E–1I and 1K–1O). Consistent with this, cell cycle gene expression is normal in both WT and *Oscand1* mutant before the establishment of fundamental organization of crown root (Figure 2A). The expression pattern of *OsCAND1* during the developmental stages of crown root primordia is consistent with its function. The transgenic plant harboring the fused gene of *OsCAND1p:: OsCAND1::GUS* can complement the mutant traits (Figure 4B), indicating normal function of the fused protein. The GUS staining was detected only in the root cap of crown root after the establishment of the fundamental organization of root primordium, which overlapped with GUS staining controlled by the *DR5* promoter in root cap. This deduction is supported by the missing GUS staining in the central cyclinder in the *Oscand1* harboring *DR5::GUS*, and caused the cessation of the G2/M cell cycle transition in the meristem of matured crown root primordia in the *Oscand1* mutant, as indicated by marker genes (Figure 2A) and safranin-fast green staining (Figure 1P and 1Q). These are further confirmed by that auxin application can partially bypass the requirement for *OsCAND1* in the emergence of crown root with rescuing the cell division activity in meristem of crown root primordia in *Oscand1* mutants (Figure 5A–5C).

The tissue expression pattern of *OsCAND1* showed that in primary root and lateral roots, *OsCAND1* is expressed in epidermis in the matured region. The higher expression level was observed in the lateral side of the lateral root primordia in primary root (Figure 4G), while the lateral root development was normal in the *Oscand1* mutant, based on comparison to WT. Using a reflux loop model of auxin signaling in *Arabidopsis* root tip, it has been demonstrated that auxin signals in basal meristem primers adjacent to pericycle cells, resulting in founder cells that determine lateral root initiation (Smet et al., 2007). *OsCAND1* is not expressed in root tip or the elongation zone (Figure 4H). Therefore, we infer that the auxin response of pericycle cells in the basal meristem required for determination of founder cell identity is independent of *OsCAND1*. This expression pattern is different from the expression pattern of AtCAND1 (Chuang et al., 2004). In *Arabidopsis*, *AtCAND1–GUS* was most strongly expressed in the root tips in the older seedlings. But in rice, *OsCAND1–GUS* had no expression in the end of the root tips. This is consistent with all the transgenes made from different transformation. Furthermore, *OsCAND1p:: OsCAND1::GUS* construction has rescued the *Oscand1* mutant. The detailed different expression pattern between *OsCAND* and *AtCAND1* needs to be further investigated.

In this study, we provide evidence that *OsCAND1*, a homolog of *AtCAND1*, has a previously undiscovered function in crown root development. Our data indicate that OsCAND1 is a controller involved in auxin signaling regulating the G2/M transition for meristem cells after the formation of the fundamental organization of the crown root primordia. The regulating mechanism underlying the spatial and temporal distribution of OsCAND1 in the emergence of crown root requires further investigation.

METHODS

Growth Conditions and Hormone Treatments

Hydroponic experiments were conducted using normal rice (*Oryza sativa*) culture solution (Yoshida et al., 1976). The pH of the culture solution was adjusted to 5.0 using 1 M NaOH. In all the hydroponic experiments, seeds were directly grown in each kind of solution culture (3 L) after being germinated in water under a photosynthetic photon flux density of approximately 200 μmol photons m^{-2} s^{-1} with a 12-h light (30°C)/12-h dark (26°C) photoperiod. Humidity was controlled at approximately 70%. To investigate the effects of auxin on crown growth and development, seeds of the wild-type and *Oscand1* mutant were sown on culture solution containing various concentrations of NAA, 2,4-D, and IAA (Sigma-Aldrich, St Louis, MO).

Molecular Cloning, Sequence Alignment, and Phylogenetic Tree Construction

To map *OsCAND1*, linkage analysis was performed using an F2 population of 700 plants derived from the cross between a heterozygous *Oscand1* line (indica variety) and Nipponbare (japonica variety). A BLAST search was performed in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment of OsCAND1 and AtCAND1 were conducted using the CLUSTAL X 1.81 program (Thompson et al., 1997) with default multiple alignment parameters and viewed by GeneDoc 3.2 with default BLOSUM score. The phylogenetic analysis was carried out by the neighbor-joining method. The phylogenetic tree was constructed using ClustalX clustered and MEGA 2.1 (Kumar et al., 2001). Bootstrap analysis was performed using 1000 trials.

Histological Observation

Stem base sections from mutant and wild-type plants were stained with safranin-fast green. The procedures of fixation, dehydration, clearing, infiltration, and embedding were performed according to Liu et al. (2005). The microtome sections (thickness, 8 μm) were mounted on glass slides for imaging.

Histochemical Analysis and GUS Assay

Histochemical GUS analysis was performed as described Liu et al. (2005). Transgenic plant samples were incubated with X-gluc overnight at 37°C. After being stained, the tissues (exclude stem base) were de-colored by ethanol and placed on glass slides for imaging. The stem base was rinsed and embedded though the histogical observation methods without safranin-fast green and then sectioned. Sections were mounted on slides and photographed. For *DR5::GUS* staining, we performed sections (thickness, 0.5–1 mm) in the stem base by hand and then incubated with X-gluc at 37°C for 15 min.

Plasmid Constructs and Plant Transformation

For complementation of the *Oscand1* mutation, the wild-type genomic sequence from –1815 to +11 258 (taking the translation initiation site as +1) was incompletely digested with restriction enzyme *Pst*I from BAC clone which selected from Kasalath BAC library and cloned into pCAMBIA1300. For the *OsCAND1p:: OsCAND1::GUS* construct, the wild-type (Nipponbare) genomic sequence from –2990 to –1 and the cDNA sequence from +1 to +3554 was amplified by PCR and orderly introduced in front of the GUS reporter gene of pCAMBIA1300–GUS (the 35S promoter of *Cauliflower mosaic virus* was substituted by Nopaline synthase promoter) to produce a fusion with the GUS reporter gene. The DR5 promoter–GUS construct was generated as reported previously (Scarpella et al., 2003). All the constructs were transformed into mature embryos developed from seeds of herozygous for *Oscand1* mutation via *Agrobacterium tumefaciens* EHA105 as previously described (Hiei et al., 1994).

Sub-Cellular Localization of OsCAND1

The CaMV35S–OsCAND1–sGFP was subcloned into the binary vector pCAMBIA 1300. The CaMV35S::sGFP empty vector (pCAMBIA1300) was used as control. The resulting construct was sequenced to verify in-frame fusion and used for transient transformation of onion epidermis using a gene gun (Bio-Rad, Hercules, CA, USA). Transformed onion cells were observed under a confocal microscope (Zeiss LSM 510).

Southern Blot Analysis of Transgenic Plants

Genomic DNA was isolated from leaves of wild-type and T_0 transgenic plants using the SDS method (Murray and Thompson, 1980) and was digested with restriction enzyme *Hind*III. Five micrograms of digested DNA were separated on 0.8% agarose gel. After electrophoresis, the digested DNA was transferred to Hybond-N+ Nylon membrane (Amersham Pharmacia, USA) and hybridized with DIG-labeled hygromycin resistant gene probe (DIG Labeling kitII, Roche). The blot was according to product instructions.

Laser-Capture Microdissection

Stem base tissues of 5-day-old seedlings of *Oscand1* and wild-type were fixed in Carnoy's Fluid overnight at 4°C, then dehydrated in graded ethanol series and embedded in low-melting paraffin (Paraplast; Sigma-Aldrich, St Louis, MO). Preparation of sections (thickness, 8 μm) for LCM using the Pix-Cell II LCM system (Arcturus, Mountain View, CA) was performed as described (Liu et al., 2005). RNA was extracted and isolated from captured tissues using the Picopure RNA isolation kit (Aruturus) according to the manufacturer's protocol.

Quantitative RT–PCR Analysis

The first-strand cDNA was synthesized from DNase I treated total RNA by SuperScript VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Quantitative RT–PCR was performed using the gene-specific primers listed in Supplemental Table 1. Quantitative RT–PCR was performed by using the LightCycler 480 SYBR Green I Master and on the LightCycler480 machine (Roche), following the manufacturer's instructions. The amplification program for SYBR Green I was performed at 95°C for 10 s, 58°C for 10 s, 72°C for 20 s. Triplicate quantitative assays were performed on each cDNA sample. The relative quantification of each sample was determined by normalization to the amount of *Actin* cDNA detected in the same sample. Relative expression level was calculated by the formula $2^{-\Delta(\Delta Cp)}$. The gene-specific primers for cell cycle genes were listed in Supplemental Table 1.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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