Original research article

CRL4 regulates crown root formation through auxin transport in rice

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Abstract: Adventitious (crown) roots account for the majority of the root system of monocots. It is reported that auxin plays an important role in the formation of crown roots, but the underlying molecular mechanisms are still unknown. We characterized a rice (*Oryza sativa* L.) mutant *crown rootless4* (*crl4*) that was found to have defective crown root formation. Besides reduced crown root number, the *crl4* mutant showed auxin-related abnormal phenotypical characteristics such as reduced lateral root number and impaired root gravitropism. *CRL4* encodes a protein highly homologous with *Arabidopsis* GNOM, which mediates auxin-dependent plant growth by coordinating the polar localization of auxin efflux carrier PIN1. In the *crl4* mutant, auxin transport was impaired in shoots and roots. Besides, the GUS staining controlled by *DR5* promoter in the node of *crl4* mutant was fainter and spread wider than that of wild-type. These results indicate that maintaining an appropriate auxin accumulation and gradient through *CRL4* in the basal portion of shoots is essential for crown root formation in rice.

Keywords: auxin transport, crown root, mutant, rice (*Oryza sativa* L.)

Introduction

The root system of most dicot plants usually develops from the radicle, while monocot plants have a fibrous root system, characterized by numerous adventitious (crown) roots (Klepper 1992). Crown and lateral roots develop postembryonically from differentiated cells, while a radicle develops during embryogenesis. The phytohormone auxin is essential for root development. Exogenous treatment with auxin induces ectopic formation of lateral and adventitious roots (Schiefelbein 2003). The transmission of auxin signaling is controlled by the interaction between AUX/IAA and ARF proteins (Liscum and Reed 2002). An auxin signal captured by TIR1, an auxin receptor (Tan et al. 2007), promotes the ubiquitination of IAA protein, a repressor of the auxin responsive transcription, through the SCF^{TIR1} complex (Gray et al. 2001). The ubiquitinated IAA proteins are then degraded by the 26S proteasome, which allows the auxin responsive transcription to be regulated by ARF proteins; these proteins then act as transcriptional activators or repressors (Gray et al. 2001).

Some mutants in *Arabidopsis* showed the morphological abnormalities in lateral roots, and they were particularly resulted from stabilized IAA protein. The number of lateral roots was reduced in *iaa1*/*axr5*, *iaa3*/*shy2*, *iaa12*/*bdl*, *iaa14*/*slr*, *iaa19*/*msg2*, and *iaa28*, and increased in *iaa7*/*axr2* and *iaa17*/*axr3* (Leyser et al. 1996, Rouse et al. 1998, Tian and Reed 1999, Nagpal et al. 2000, Rogg et al. 2001, Fukaki et al. 2002, Hamann et al. 2002, Tatematsu et al. 2004, Yang et al. 2004). Recently, it was also reported that auxin-related mutants show abnormalities in root formation in rice, e.g., *crl1*/*arl1* has few crown roots, and that *CRL1*/*ARL1* encodes plant-specific AS2/LOB transcriptional factors, which act downstream of IAA and ARF-mediated auxin signaling pathways (Inukai et al. 2005, Liu et al. 2005). Similarly, lateral root initiation was strongly inhibited in the *arf7 arf19* double mutant (Okushima et al. 2005).

On the other hand, endogenous auxin, indole-3-acetic acid (IAA) is synthesized at the shoot apical meristem and in young leaves, and then transported to the basal portion of shoots and tip of roots. Molecular genetic studies using *Arabidopsis*

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mutants have revealed that a balance between influx and efflux in the auxin transport system is essential for lateral root initiation, and subsequently for primordial development (Fukaki et al. 2007). However, crown root formation has not been studied in detail due to the shortage of monocot mutants.

Here, we report on a newly isolated rice *crown rootless4* (*crl4*) mutant that was found to have defective crown root formation. The *crl4* mutant showed severe defects not only in crown root but also in lateral root formation. We suspect that *CRL4* is involved in auxin-related root formation. This study revealed that *CRL4* encodes a protein highly homologous with *Arabidopsis* GNOM responsible for coordinating the polar localization of auxin efflux carrier PIN1 (Steinmann et al. 1999). We also showed that polar auxin transport was impaired and that auxin accumulation and distribution in the basal portion of shoots was distorted in accordance with the reduction of auxin transport ability in the *crl4* mutant. These results indicate that maintaining an appropriate auxin accumulation and gradient through *CRL4* in the basal portion of shoots is essential for crown root formation in rice.

Materials and Methods

Plant growth conditions

The *crl4* mutant was derived from the somaclonal variation associated with the tissue culture. Seedlings of wild-type rice (*Oryza sativa* cv. Nipponbare), *crl4* mutant and F_2 plants derived from the cross between *crl4* mutant (*japonica* variety) and Kasalath (*indica* variety) were grown in a growth chamber at 29.5°C under continuous light. These plants were grown in water or 0.8% agar medium without nutrients. Transgenic plants were grown in Murashige and Skoog medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose and 0.3% Gelrite.

Histological analysis

Plant tissues were fixed in FAA (formaldehyde:acetic acid : 50% ethanol at 1 : 1 : 18) for 24 hours at 4°C and then dehydrated in a graded ethanol series. Dehydrated samples were embedded in Paraplast plus (Oxford Labware, St. Louis, MO), sectioned into 8-μm-thick sections by using a rotary microtome, and then stained with toluidine blue.

Map-based cloning, sequence alignment and phylogenetic tree construction

To map the *CRL4* gene, linkage analysis was performed using F_2 plants derived from the cross between *crl4* mutant (*japonica* variety) and Kasalath (*indica* variety). BLAST search was performed and the predicted protein sequences were clustered using ClustalW, then Tree View was used to generate the phylogenical output.

Plasmid constructs and plant transformation

For complementation of the *crl4* mutation, the wild-type genomic sequence from −4359 to +1314 (taking the translation initiation site as +1) was cut off from bacterial artificial chromosome (BAC) clone OSJNBa0056E06 and cloned into pCAMBIA1300. *pDR5::GUS* construct were generated as reported previously (Scarpella et al. 2003). The resulting fusion construct was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation. *Agrobacterium*-mediated transformation of rice was performed as described previously (Hiei et al. 1994). Transgenic plants were selected on media containing 50 mg L^{-1} hygromycin.

Expression analysis

cDNA synthesis for semiquantitative RT-PCR was carried out by using an Omniscript RT kit (Qiagen, Valencia, CA), and RNA was extracted by using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR was performed essentially as described by the manufacture. The primer sequences were 5'-CTGTGGAGCTTGATGAATACAC-3' and 5'-CAAGCTTCTCAGGCAACAAATG-3' for *CRL4*, 5'-AGACCATGCAGGAGGTTATCCG-3' and 5'-ACAACACCAAATCCACCTCCCA-3' for *CRL4-like* and 5'-GACTACATACAACTCCATCATG-3' and 5'-AGCATTTCCTGTGCACAATGG-3' for *ACT1*.

In situ hybridization

In situ hybridization was performed as previously described (Kouchi and Hata 1993). For the *CRL4* probe, a 695-nt fragment cut off by *Sac* I from the complementation construct was subcloned into pBluescript® II KS (–) (Stratagene, La Jolla, CA). Hybridization conditions were at 55°C overnight and the probe was mounted 0.2 μl per slide.

Auxin transport and accumulation assays

Auxin transport and accumulation were measured as described by Chhun et al. (2007) with some modifications. To measure the auxin transport ability in shoot, the shoots of 7-day-old seedlings were decapitated and the cut stem of 1.2 cm in length was applied the vaseline paste containing 1 μM 3-[5(n)-^{3}H]-

Indolylacetic acid ([^{3}H]-IAA, Amarsham, USA). After 4 hours incubation at 28°C by water culture, the 3 mm segments were cut from 3 mm upper of the basal node. The 1.2 cm seminal root tips were obtained from 7-day-old seedlings then each was placed to keep the contact of the apical or the basal end of root tip with the vaseline paste containing 1 µM [^{3}H]-IAA on a slide glass, for measuring the transport ability in root. After 4 hours incubation at 28°C in humid condition, the 3 mm segments were cut from the basal or apical end of the root tip. Each sample was placed into 5 ml of scintillation fluid and radioactivity was counted with the scintillation counter (LSC-5100, Alaka). To measure the auxin accumulation, the shoots of 5-day-old seedlings were decapitated and the cut stem of 1 cm in length was applied the vaseline paste containing 1 µM [^{3}H]-IAA. After 4 hours incubation at 28°C, the 3 mm segments of the basal end of shoot and whole seminal root were cut and placed into 5 ml of scintillation fluid. Radioactivity was counted with the scintillation counter.

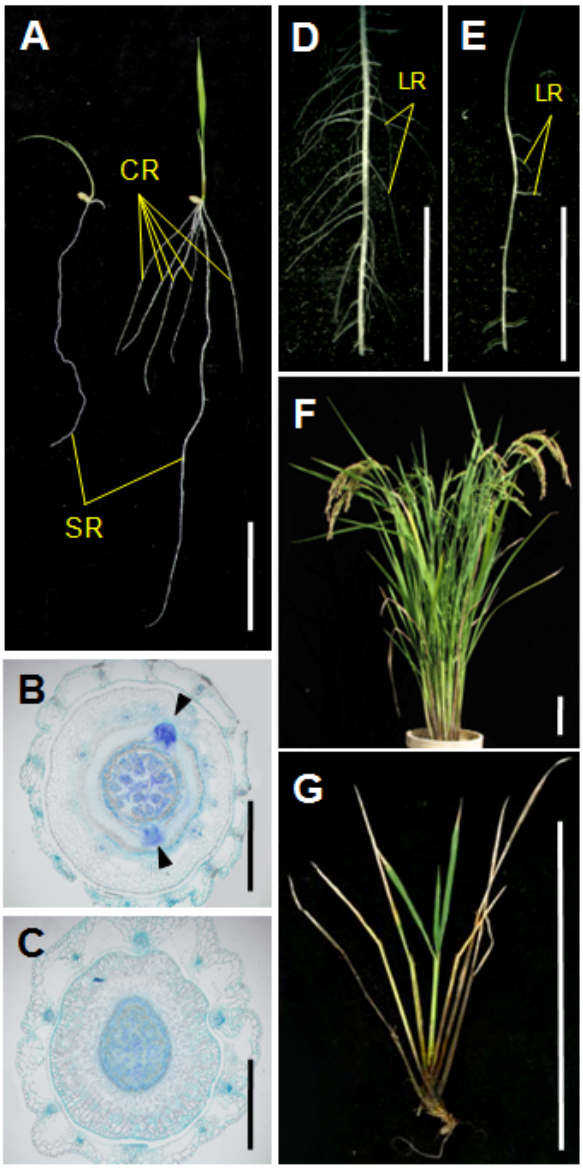

Fig. 1. Phenotypes of *crl4*. (A) 2-week-old wild-type, Nipponbare (right) and *crl4* (left) seedlings. CR, crown root; SR, seminal root. Bar = 10 cm. (B) and (C) Cross sections through the nodes in 5-day-old wild-type (B) and *crl4* (C) seedlings. The arrowheads indicate the crown root primordia. Bars = 500 µm. (D) and (E) Lateral roots in 2-week-old wild-type (D) and *crl4* (E) seedlings. LR, lateral roots. Bars = 1 cm. (F) and (G) 4-month-old wild-type (F) and *crl4* (G). Bars = 10 cm.

Results

Characterization of the crl4 mutant

Two-week-old wild-type plants (Nipponbare) formed several crown roots in comparison to *crl4* mutants, in which crown root production was scarce at the same developmental stage (Fig. 1A). We cross-sectioned the nodes in 5-day-old wild-type and *crl4* mutant seedlings to determine the cause of crown root reduction in *crl4* mutants. In wild-type, several crown root primordia were formed on the outside, adjacent to the peripheral vascular cylinder (Fig. 1B). In contrast, *crl4* mutants did not produce any crown root primordia (Fig. 1C). Besides the reduced crown root number, the number of lateral roots that were obtained from a seminal root of the *crl4* mutant was clearly lower than that of the wild-type (Fig. 1D and 1E). The seminal root in the *crl4* mutant was shorter than that of the wild-type (Fig. 1A), although there were no significant differences in the timing of the emergence of the seminal root. The *crl4* mutant produced the higher number of tillers (data not shown) and their growth angles were wider than those of wild-type (Fig. 1F and 1G). Some *crl4* mutants survived for more than 4 months. Four-month-old wild-type plants could reach the grain filling stage, but *crl4* mutants with tiny shoots and a single seminal root could not make the transition from the vegetative to the reproductive phase (Fig. 1F and 1G).

It has been reported that the reduction of crown and lateral root number are commonly seen in auxin-related phenotypes in rice (Inukai et al. 2005, Liu et al. 2005). The abnormality in gravitropic response has also been reported in several auxin-related mutants in *Arabidopsis* (Leyser et al. 1996, Tian and Reed 1999, Nagpal et al. 2000, Fukaki et al. 2002, Yang et al. 2004). We examined the root gravitropic response in *crl4* mutants by measuring the curvature after gravistimulation at 90° to the vertical. Wild-type roots responded sharply to the change in the gravity vector, whereas the response of *crl4* roots was impaired (Fig. 2A). The root tip angle of wild-type and *crl4* roots was compared (θ in Fig. 2B); all wild-type

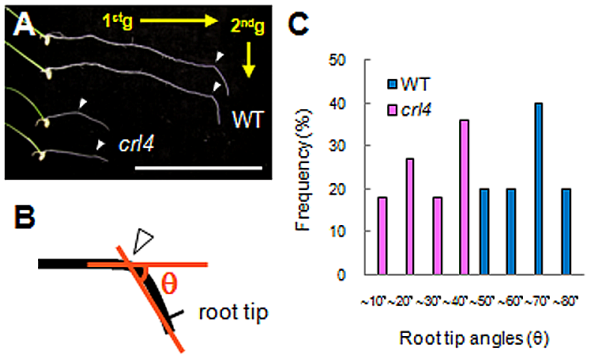

Fig. 2. Gravitropic response in a seminal root of *crl4*. (A) Wild-type and *crl4* seedlings were grown vertically (arrow of 1st g) and then rotated 90° (arrow of 2nd g). Bar = 10 cm. (B) and (C) The root tip angles (θ) in (B) was measured 24 hours after reorientation (C).

roots had root tip angles of over 50°, while all *crl4* mutants had angles of below 50° (Fig. 3C). The gravitropic response was therefore defective in *crl4* mutants. These results suggest that *CRL4* has an auxin-related function.

CRL4 encoded a gene similar to GNOM

We employed a map-based cloning approach to isolate the *CRL4* gene. The *CRL4* locus was mapped on the long arm of chromosome 3 around 101.9cM. By comparing the nucleotide sequences of the *crl4* mutant and wild-type, we found a single nucleotide substitution in Os03g46330 on the BAC clone OSJNBa0056E06, which resulted in a nonsense mutation (Fig. 3A). Introduction of a 9.2-kbp genomic DNA fragment containing the entire candidate gene (Fig. 3B) to the *crl4* mutant complemented the abnormal *crl4* mutant phenotype (Fig. 3C), produced crown roots and lateral roots same as the redifferentiated plant of wild-type (data not shown). Shoot of the complemented *crl4* plant was normal without abnormalities such as the higher number and wider growth angles of tillers than those of wild-type. Therefore, we concluded that *crl4* phenotypes are caused by the loss-of-function mutation in the predicted *CRL4* gene.

The *CRL4* gene encodes a protein of 1176 amino acid residues in one exon with the Sec7 domain characteristic of GNOM (Fig. 3A). It has been reported that GNOM is a membrane-associated guanine-nucleotide exchange factor on the ADP-ribosylation factor G protein (ARF-GEF) and that ARF-GEFs regulate specific endosomal trafficking pathways (Steinmann et al. 1999, Geldner et al. 2003a). GNOM is required for the recycling of PIN1, which mediates polar auxin transport in *Arabidopsis* between the endosome and plasma membrane by vesicle trafficking (Geldner et al. 2003a). Thus, it is

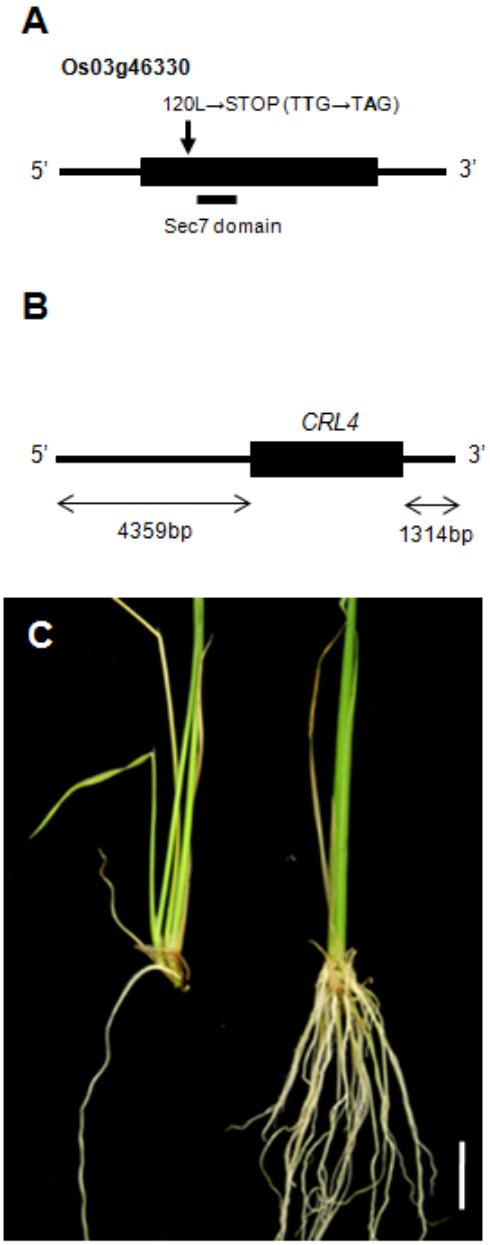

Fig. 3. Map-based cloning and phenotypic complementation by the introduction of *CRL4*. (A) Structure of *CRL4* gene. Black box indicated the exon, the arrow represented a single nucleotide mutation, resulted in the change into stop codon. (B) Structure of the genomic DNA fragment encompassing the entire *CRL4* gene. (C) The *crl4* mutant plant containing the empty vector (left) and the genomic DNA fragment encompassing the entire *CRL4* gene (right) were shown. Bar = 1 cm.

believed that GNOM plays an important role in polar auxin transport through the establishment of coordinated polar localization of PIN1, which in turn highly depends on the directed vesicle trafficking (Steinmann et al. 1999, Geldner et al. 2003a). We performed a BLAST search using the amino acid sequence of CRL4 and found its homologous gene *CRL4-like*

(Os02g22090) on chromosome 2 in rice (Fig. 4A). CRL4-like shared 83% amino identity in Sec7 domain with CRL4, while GNOM shared 90% (Fig. 4A). Then we drew dendogram of CRL4, CRL4-like and *Arabidopsis* GNOM family members; GNOM, GNOM-like1 (GNL1) and GNOM-like2 (GNL2). It was constructed based on the amino acid sequence of the Sec7 domains and GBF1, which is human ARF-GEF, was used as outgroup. It therefore appears that CRL4 is more homologous with GNOM in *Arabidopsis* than CRL4-like in rice (Fig. 4B).

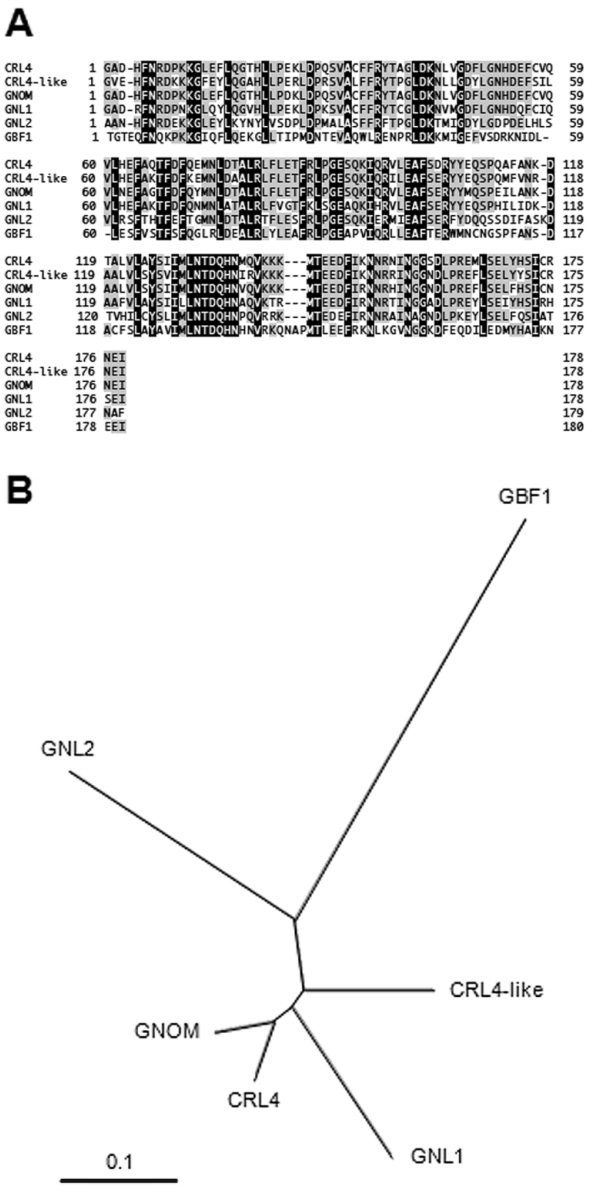

Fig. 4. Structure of CRL4. (A) Comparison of amino acid sequence of Sec7 domain in CRL4 with that in CRL4-like, GNOM, GNL1 and GNL2. (B) Dendogram of the GNOM family proteins of Sec7 domain in *Arabidopsis* (GNOM, GNL1 and GNL2) and rice (CRL4 and CRL4-like).

Analysis of CRL4 expression and auxin distribution

We examined the expression of *CRL4* in various organs. To estimate *CRL4* transcript levels, we performed semi-quantitative RT-PCR analysis (Fig. 5A). *CRL4* was expressed in all the examined organs; high levels of *CRL4* transcripts were found to be accumulated in leaf blades and roots. *CRL4-like* was also expressed in all the organs and the expression pattern was similar to that of *CRL4* (Fig. 5A).

We also examined the *CRL4* expression pattern by *in situ* hybridization. In the stem, signals were observed at the steles of the crown root primordia (Fig. 5B and 5C), the vascular bundles and the parenchyma cells adjacent to the peripheral vascular cylinder of the stem where the crown root primordia were formed (Fig. 5D and 5E). In addition, we compared the GUS staining controlled by *DR5* promoter between wild-type and *crl4* mutant. *DR5* is often used as an artificial promoter responding to auxin signals in *Arabidopsis* (Sabatini et al. 1999). This promoter was also used as a marker to visualize the in vivo distribution of auxin in rice (Scarpella et al., 2003). The staining was observed at the tip and middle of leaf blade, the leaf sheath and the basal portion of the shoot in wild-type plant (Fig. 5F). In the *crl4* mutant, faint staining was observed at the tip of leaf blade and the basal portion of the shoot (Fig. 5F and 5G). Then we made the cross section of the stem of *pDR5::GUS* transgenic plant. The GUS staining was localized at the vascular bundles and the parenchyma cells adjacent to the peripheral vascular cylinder in wild-type (Fig. 5H). On the other hand, the GUS staining was scarcely observed at the vascular bundles and faint staining spread the whole parenchyma cells in *crl4* mutant (Fig. 5I). The regions where the localized GUS staining of *pDR5::GUS* in wild-type was observed coincided with the regions where *CRL4* was expressed (Fig. 5D and 5H). It has previously been reported that *OsPIN1* is also expressed in these regions (Xu et al. 2005). GNOM coordinates the localization of PIN1, which is responsible for establishing the auxin gradient in *Arabidopsis* (Steinmann et al. 1999), and *CRL4* was expressed in the vascular bundles and the predicted region where the crown root primordia were formed in rice. These observations indicate that the similarity between CRL4 function in rice and GNOM function in *Arabidopsis*.

Defective auxin transport in crl4 mutants

It is well known that polar auxin transport is involved in vascular formation (Berlenth and Mattsson 2000) and that impaired auxin flow adversely affects vascular development (Koizumi et al. 2000, Geldner et al. 2003b, Sieburth et al. 2006). Abnormal vascular

structure was observed in the seminal root of the *crl4* mutant. Metaxylem elements were scattered in a regular pattern around the steles in wild-type, whereas they were found to be increased and arranged in a radial array around the steles in *crl4* mutants (Fig. 6A and 6B).

As mentioned above, *CRL4* encodes a protein highly homologous with *Arabidopsis* GNOM, which mediates auxin-dependent plant growth by coordinating the polar localization of PIN1. In addition, it is reported that wild-type rice plants rarely produce crown roots with 1-*N*-naphthylphthalamic acid (NPA) treatment, an inhibitor of polar auxin transport (Liu et al. 2005, Morita and Kyozuka 2007). Phenotypes of wild-type rice seedlings treated with NPA mimicked those of *crl4* mutants. These reports indicate that the polar auxin transport is indispensable for crown root formation in rice and that the abnormalities in the *crl4* mutant are a result of impaired polar auxin transport.

We compared the auxin transport kinetics in *crl4* mutants with those in wild-type using radioactive auxin [^{3}H]-IAA and found that the transport efficiency of [^{3}H]-IAA in *crl4* mutant shoots was reduced to 17% of that of wild-type shoots (Fig. 6C). Next, we assessed the acropetal (to root tip) and basipetal (from root tip) transport systems in a seminal root and found that in *crl4* mutants, acropetal and basipetal transport activities were 41% and 48%, respectively, compared with those observed in wild-type (Fig. 6C). We also investigated the accumulation of [^{3}H]-IAA at seminal root and node where crown roots are produced and found that the accumulation in *crl4* seminal root was reduced to 13% of that in wild-type (Fig. 6D). It was also revealed that [^{3}H]-IAA itself was scarcely transported to the root in *crl4* mutants. Meanwhile, the accumulation in the nodes of *crl4* mutants was also decreased to 53% of that of wild-type (Fig. 6D). These results suggest that auxin transport is defective in *crl4* mutants and distorted auxin transport results in the reduced auxin accumulation in the node and the seminal root.

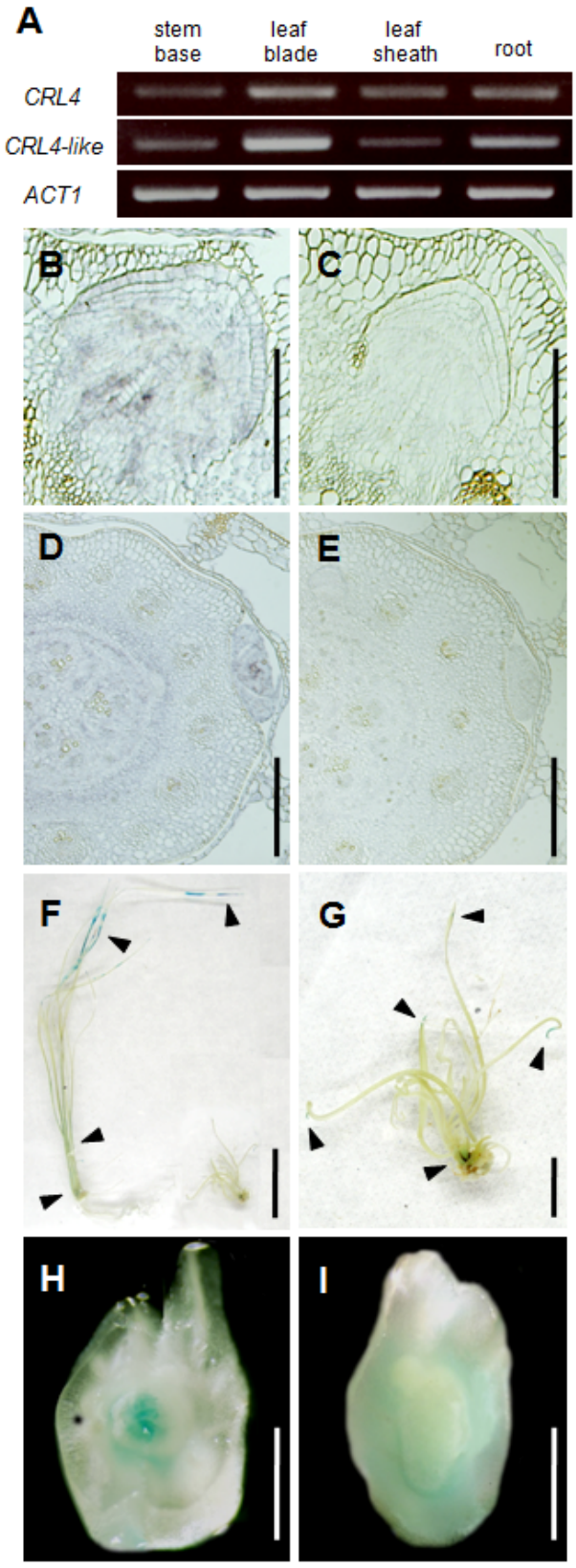

Fig. 5. Expression patterns of *CRL4* and auxin distribution. (A) *CRL4* and *CRL4-like* expression in various organs of wild-type rice. Semiquantitative RT-PCR was concluded, and *ACT1* was used as a control. Stem base includes shoot apical meristem and nodes. (B) to (E) *In situ* hybridization with cross sections through the nodes in 7-day-old wild-type seedling. Localization of *CRL4* in the crown root primordia with the antisense probe (B) and with the sense probe (C). Bars = 100 µm. Localization of *CRL4* in the node developing crown root primordia with antisense probe (D) and with sense probe (E). Bars = 200 µm. No signal was detected with sense probe of *CRL4*. (F) to (I) Localized GUS staining controlled by *DR5* promoter. Whole plant of wild-type (left) and *crl4* mutant (right) (F). Bar = 5 cm. Magnified the whole *crl4* mutant in Fig. 5E (G). Bar = 1cm. The black arrowheads indicate the localized GUS stainings. Cross sections through the nodes in wild-type (H) and *crl4* (I) transgenic plant. Bars = 1 mm.

Discussion

Effect of crl4 mutation in rice

CRL4 protein is highly homologous with the *Arabidopsis* GNOM protein, which has been shown contribute to the apical-basal pattern formation in *Arabidopsis* embryogenesis (Mayer et al. 1993). Strong alleles of *gnom* show loss of cell-to-cell alignment along the embryonic axis, lack an embryonic root, and display variable levels of fusion or deletion of cotyledons and hypocotyls (Mayer et al. 1993). Weak alleles of *gnom* have fused cotyledon with lost lateral veins and clustered tracheary elements, and rosette leaves whose venations become more complicated with the increase in number of freely ending veinlets (Koizumi et al. 2000, Geldner et al. 2003b, Sieburth et al. 2006). These reports indicate that *gnom* mutation causes serious defects in plant development. Nonsense mutation in *CRL4* occurred before the Sec7 domain characteristic of GNOM, suggesting that CRL4 lost its function in *crl4* mutants.

The *crl4* mutant showed several abnormalities such as loss of crown roots, aberrant gravitropic response, and increase of metaxylem-like structures in the seminal root. In addition, it could not make the transition from the vegetative to the reproductive phase. However, these abnormalities in *crl4* mutants did not seem as severe as those observed in severe alleles of *gnom* (Mayer et al. 1993, Shevell et al. 1994). The phenotypes of *crl4* mutant was similar to weak alleles of *gnom* (Geldner et al. 2003b), nevertheless the *crl4* mutant shows a loss in most of the CRL4 functions. *GNOM* has two homologous genes, *GNL1* and *GNL2*, in *Arabidopsis*, but it is reported that GNL1 does not contribute much to the polar auxin transport (Richter et al. 2007, Teh and Moore 2007). Therefore, the absence of genes with redundant function may result in severe mutant phenotypes of *gnom*. In rice, we hypothesized that a homologous gene of *CRL4*, *CRL4-like*, plays a redundant role with *CRL4* and can partially compensate for *CRL4* function because the expression pattern of *CRL4-like* is identical to that of *CRL4* (Fig. 5A). Besides, *CRL4-like* was also considered to play a redundant role in embryogenesis because of the absence of abnormalities in the mature embryo of the *crl4* mutant (data not shown).

Defective polar auxin transport in crl4 mutants

It is believed that GNOM plays an important role in polar auxin transport through the establishment of coordinated polar localization of PIN1 in *Arabidopsis* (Steinmann et al. 1999, Geldner et al. 2003a). Since polar auxin transport is essential for plant organization,

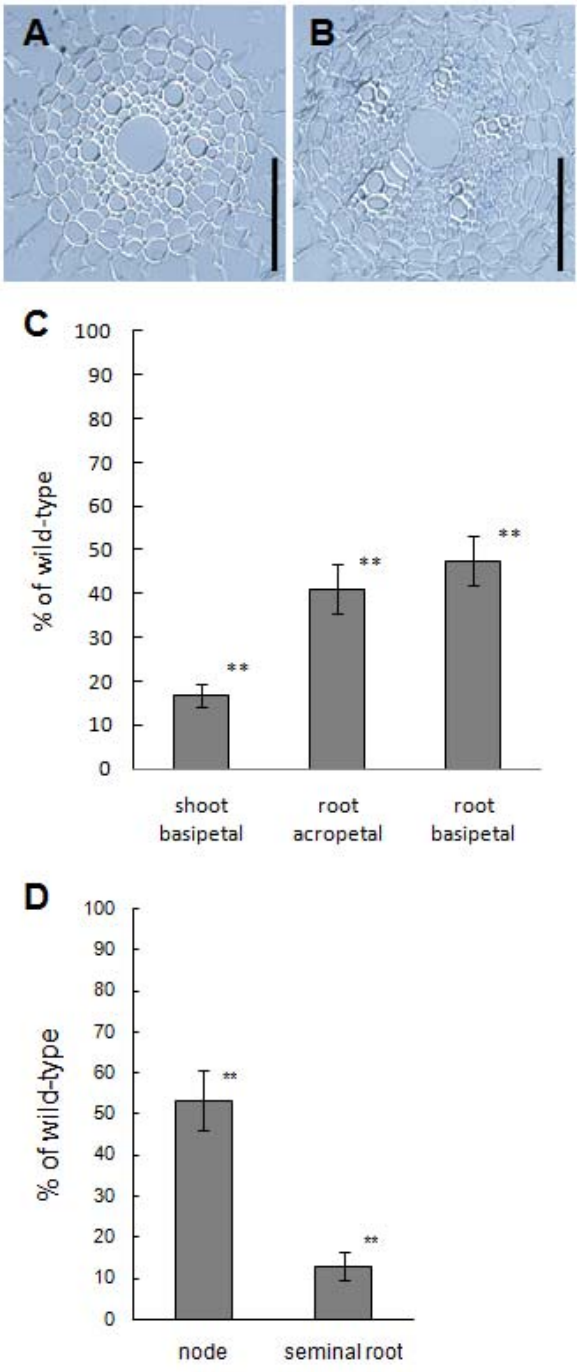

Fig. 6. Comparison of auxin transport of *crl4* with that of wild-type. (A) and (B) Cross section of the basal part of the 7-day-old wild-type seminal root (A) and *crl4* (B). Bars = 50 μm. (C) Comparison of the ability of [^{3}H]-IAA transport. Shoot besipetal (from apex to base) transport, root acropetal (from base to root tip) transport and root basipetal (from root tip to base) transport were measured. The measured value of [^{3}H]-IAA was expressed as a percentage relative to wild-type. ** indicates significantly different from wild-type at 1%. (D) Comparison of the accumulation of transported [^{3}H]-IAA from shoot to node and seminal root. The measured value of [^{3}H]-IAA was expressed as a percentage relative to wild-type. ** indicated significantly different from wild-type at 1%.

gnom mutants show several defects in cell polarity and embryo axis formation (Steinmann et al. 1999). Formation of lateral roots is regulated by auxin transported acropetally from above-ground tissues to the root tip and basipetally from the root tip (Geldner et al. 2003b, Smet et al. 2007). Lateral root formation was found to be inhibited in the weak alleles of *gnom* (Geldner et al. 2003b).

Both basipetal transport in the shoot and acropetal transport in the seminal root of *crl4* mutants were lower than those observed in wild-type. Because it has been reported that these polar transport mechanisms are mainly mediated by auxin efflux carriers (Jacobs and Gilbert 1983), these findings appear reasonable with regard to the predicted CRL4 function, which suggests that CRL4 might be related to auxin efflux carriers such as GNOM in *Arabidopsis*. Basipetal transport in the seminal root tip of *crl4* mutants was also lower than that of wild-type, suggesting that CRL4 is also required for auxin basipetal transport in root tips. The lateral root number was decreased in *crl4* mutants (Fig. 1D and 1E) and this fact is consistent with the finding that weak alleles of *gnom* fail to produce lateral roots due to insufficient basipetal transport in the seminal root tip (Geldner et al. 2003b). These results indicate that CRL4 is necessary for the normal development of roots and shoots through mediation of a viable auxin transport system.

Compared with wild-type, [^{3}H]-IAA accumulation in the basal portion of shoots was reduced to 53% in the *crl4* mutant (Fig. 6D). From the GUS staining controlled by *DR5* promoter, the staining was observed at the tip and middle of leaf blade, the leaf sheath and the basal portion of the shoot in wild-type plant (Fig. 5F). On the other hand, faint staining was only observed at the tip of leaf blade and the basal portion of the shoot in the *crl4* mutant (Fig. 5F and 5G). Since the tip of leaf blade is one of the auxin biosynthesis sites, it was conceivable that the absent of the GUS staining in the middle of leaf blade and the leaf sheath in *crl4* mutant resulted from the auxin transport inhibition in it. At the stem, auxin was localized at the vascular bundles and the parenchyma cells adjacent to the peripheral vascular cylinder in wild-type (Fig. 5H) but not in *crl4* mutant; the GUS staining was scarcely observed at the vascular bundles and faint staining spread the whole parenchyma cells in *crl4* mutant (Fig. 5I). When the auxin gradient is lost due to auxin transport inhibition and auxin is distributed equally, lateral root initiation ceases to occur in *Arabidopsis* roots (Smet et al. 2007). That is, it indicated that CRL4 plays an important role in crown root initiation through the establishment of appropriate auxin accumulation and gradient. In addition, formation of the lateral root primordia necessitates the re-establishment of cell division and elongation patterns orthogonal to the axis of the primary root in *Arabidopsis* (Geldner et al. 2003b). It is necessary for PIN1 to shift its polarity orthogonally in the daughter cells of stage I primordia in order to make the axis of the lateral root primordia orthogonal to that of the primary root. It is suggested that GNOM is also critical for the switching of PIN1 re-localization in the daughter cells of stage I primordia (Geldner et al. 2003b). The regions expressing *CRL4* coincided with the regions stained by *pDR5::GUS* and *pOsPIN1::GUS* in rice (Fig. 5D and 5H, Xu et al. 2005), indicating that CRL4 also plays a role in crown root development after the initiation. Besides, lateral root development was impaired in *crl4* mutants (Fig. 1D and 1E). Therefore, CRL4 is considered to regulate both root initiation and development by similar way to GNOM function. In conclusion, these findings indicate that an appropriate auxin accumulation and gradient mediated through CRL4 function is essential for the formation of crown and lateral roots in rice.

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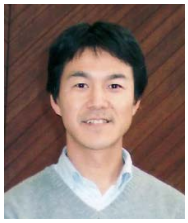

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