Loss of Function of *OsDCL1* Affects MicroRNA Accumulation and Causes Developmental Defects in Rice[1][w]

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MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are two types of noncoding RNAs involved in developmental regulation, genome maintenance, and defense in eukaryotes. The activity of Dicer or Dicer-like (DCL) proteins is required for the maturation of miRNAs and siRNAs. In this study, we cloned and sequenced 66 candidate rice (*Oryza sativa*) miRNAs out of 1,650 small RNA sequences (19 to approximately 25 nt), and they could be further grouped into 21 families, 12 of which are newly identified and three of which, OsmiR528, OsmiR529, and OsmiR530, have been confirmed by northern blot. To study the function of rice DCL proteins (OsDCLs) in the biogenesis of miRNAs and siRNAs, we searched genome databases and identified four OsDCLs. An RNA interference approach was applied to knock down two OsDCLs, *OsDCL1* and *OsDCL4*, respectively. Strong loss of function of *OsDCL1IR* transformants that expressed inverted repeats of *OsDCL1* resulted in developmental arrest at the seedling stage, and weak loss of function of *OsDCL1IR* transformants caused pleiotropic developmental defects. Moreover, all miRNAs tested were greatly reduced in *OsDCL1IR* but not *OsDCL4IR* transformants, indicating that *OsDCL1* plays a critical role in miRNA processing in rice. In contrast, the production of siRNA from transgenic inverted repeats and endogenous CentO regions were not affected in either *OsDCL1IR* or *OsDCL4IR* transformants, suggesting that the production of miRNAs and siRNAs is via distinct OsDCLs.

Most eukaryotes have two classes of short (21–25 nt) noncoding RNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs), which are involved in RNA-silencing pathways (Baulcombe, 2004). The two classes of small RNAs are related but differ in their biogenesis from their origins, initiation, to their subsequent assembly into different RNA-induced silencing complexes (RISCs; Tang, 2005). miRNAs are processed from single-stranded RNAs with imperfect stem-loop structure, whereas siRNAs are derived from double-stranded RNAs (dsRNAs). Both miRNA and siRNA precursors are cut into double-stranded duplexes by Dicer, a multidomian enzyme of the RNase III family. Then the duplexes are unwound and one strand is subsequently assembled into miRISC or siRISC, respectively. Both miRNAs and siRNAs down-regulate gene expression via sequence complementarity to their target mRNAs by either cleavage or translational repression mechanism. Although the function of miRNAs and siRNAs is interchangeable depending on the extent of base pairing between the small RNAs and their mRNA targets in cleaving RISCs, miRNAs and siRNAs have distinct target functions in cells (Hutvagner and Zamore, 2002; Doench et al., 2003; Tang et al., 2003; Zeng et al., 2003; Bartel, 2004). For example, miRNAs play important roles in regulating gene expression and cell differentiation for normal development (Bartel and Bartel, 2003; Kidner and Martienssen, 2005), while siRNAs are involved in genome management of transposon and retrotransposon activity at the chromatin level (Lippman et al., 2004) and in protecting against virus infection or transgene invasion (Hamilton and Baulcombe, 1999; Xie et al., 2004).

In plants, several components that are involved in miRNA biogenesis and metabolism have been experimentally characterized. In Arabidopsis (*Arabidopsis thaliana*), miRNA genes are transcribed into long precursors, pri-miRNAs, which are first processed into pre-miRNAs and then into miRNAs by Dicer-like (DCL)1 (Kurihara and Watanabe, 2004). It has been shown that ARGONAUTE1 (AGO1), a key component of RISC, is necessary for the function of miRNAs (Vaucheret et al., 2004). Moreover, HEN1 plays a broad role in small RNA metabolism that is required for the accumulation of both miRNA and siRNA (Park et al., 2002; Boutet et al., 2003; Xie et al., 2004). Further study revealed that HEN1 is a small RNA methyltransferase that methylates ribose of the last nucleotide of both

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mRNAs and siRNAs (Yu et al., 2005). Furthermore, HYL1 encodes a double-stranded RNA-binding protein, and a subset of miRNAs is reduced in the *hyl1* background (Han et al., 2004). In addition, an ortholog of exportin-5, known as HASTY, might play a role in the nuclear export of most miRNAs in Arabidopsis (Park et al., 2005). All these components are involved in miRNA biogenesis or metabolism; therefore, they affect multiple aspects of normal development.

Dicer, a multidomain endonuclease, plays essential roles in RNA-silencing pathways (Tijsterman and Plasterk, 2004). First, Dicer initiates the process of RNA interference (RNAi) by cutting dsRNA into 21 to approximately 24-nt siRNA duplexes, which form RISC-loading complexes. Then RISC-loading complexes mature into RISC in an asymmetric way such that only one strand of the duplex is chosen to be assembled (Hannon, 2002; Pham et al., 2004; Tomari et al., 2004a, 2004b). In metazoan, miRNAs are processed first by Drosha and Pasha in the nucleus and then by Dicer in cytoplasm (Lee et al., 2003; Denli et al., 2004). In plants, evidence suggests that miRNAs are likely processed by Dicer in the nucleus (Kurihara and Watanabe, 2004; Park et al., 2005).

An N-terminal helicase domain, two tandemly repeated RNase III domains, and one or more C-terminal dsRNA-binding domains are highly conserved among all Dicers and DCL proteins. However, the number of DCL proteins varies among different organisms. For example, in mouse (*Mus musculus*) and human (*Homo sapiens*), a single Dicer gene is responsible for the generation of both miRNAs and siRNAs, whereas in other organisms, such as flies and plants, multiple DCL proteins exist.

In organisms with multiple DCL genes, these genes may have distinct roles in the RNA-silencing pathways (Kadotani et al., 2004; Lee et al., 2004; Xie et al., 2004). For example, in Drosophila, Dicer-1 is responsible for miRNA maturation, whereas Dicer-2 is required for siRNA accumulation in the initiation step of siRNA pathways (Lee et al., 2004). Furthermore, both Dicer-1 and Dicer-2 are involved in the formation of RISC in the effector step. In the filamentous fungus *Magnaporthe oryzae*, one of the two DCL proteins, MDL-2, is responsible for siRNA accumulation and gene silencing, whereas MDL-1 function is unknown to date (Kadotani et al., 2004).

Arabidopsis has four DCL proteins, and distinct functions have been observed for three of them. *DCL1*, also named *SHORT INTEGUMENTS1/SUSPENSOR1/CARPEL FACTORY* (Robinson-Beers et al., 1992; Jacobsen et al., 1999; McElver et al., 2001; Schauer et al., 2002), is required for normal plant development. Partial loss-of-function alleles of *dcl1* show pleiotropic phenotypes due to a reduction of miRNA accumulation (Park et al., 2002). In Arabidopsis, maturation of miRNAs requires at least three cleavage steps, and *DCL1* is involved in processing pri- and pre-miRNAs (Kurihara and Watanabe, 2004). In contrast, *DCL2* and *DCL3* have no effect on plant development, but are important for resistance to a specific viral pathogen and the accumulation of different types of endogenous siRNAs, respectively (Xie et al., 2004).

Rice (*Oryza sativa*), one of the most important crop species in the world, has become the model monocot species for genomic and molecular analysis. Twenty miRNA families have already been identified in rice through computational approaches based on conservation with known miRNAs from Arabidopsis (Llave et al., 2002; Park et al., 2002; Reinhart et al., 2002; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Wang et al., 2004; Adai et al., 2005), whereas direct experimental evidence was only recently reported (Sunkar et al., 2005). In addition, multiple DCL proteins (OsDCLs) have been predicted in rice. However, which OsDCL is involved in the miRNA biogenesis is unknown. Moreover, how OsDCLs affect rice development is also unclear. In this study, we identified miRNAs from rice by direct-cloning method. We also evaluated the roles of *OsDCL1* and *OsDCL4* in miRNA/siRNA biogenesis and rice development. Loss of function of *OsDCL1IR* and *OsDCL4IR* transformants were generated by RNAi approach. We confirmed that *OsDCL1* and *OsDCL4* were knocked down specifically. We further demonstrate that *OsDCL1*, but not *OsDCL4*, is important for miRNA accumulation. In contrast, the production of siRNAs from transgenic inverted repeats and endogenous CentO regions are not affected in either *OsDCL1IR* or *OsDCL4IR* transformants, suggesting that the production of miRNAs and siRNAs is via distinct OsDCLs.

RESULTS

Analysis of Small RNAs from Rice

To identify novel miRNAs from rice, we constructed a small RNA library (see ''Materials and Methods''). Clones from the above library were sequenced. Nineteen- to 25-nt-long RNA molecules with perfect match to noncoding regions of the rice genome were collected and analyzed. Upstream and downstream genomic sequences of each miRNA candidate (with 20–200 nt for each side) were extracted and screened for hairpin-like secondary structure using an m-fold RNA-folding program. Among all sequenced small RNAs, 66 were derived from the stem region of hairpin-structured precursors. Therefore, these small RNAs could be candidate miRNAs according to the current miRNA annotation criteria (Ambros et al., 2003). These cloned candidates could be divided into 21 subfamilies, including nine previously identified rice miRNAs (Supplemental Table I). Among them, 12 are newly identified families that do not have phylogenetic conservation with known Arabidopsis miRNAs (Fig. 1A and Supplemental Fig. 1; Llave et al., 2002; Park et al., 2002; Reinhart et al., 2002; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Wang et al., 2004; Xie et al., 2004; Adai et al., 2005). Further investigation indicated that three of them, OsmiR528, OsmiR529,

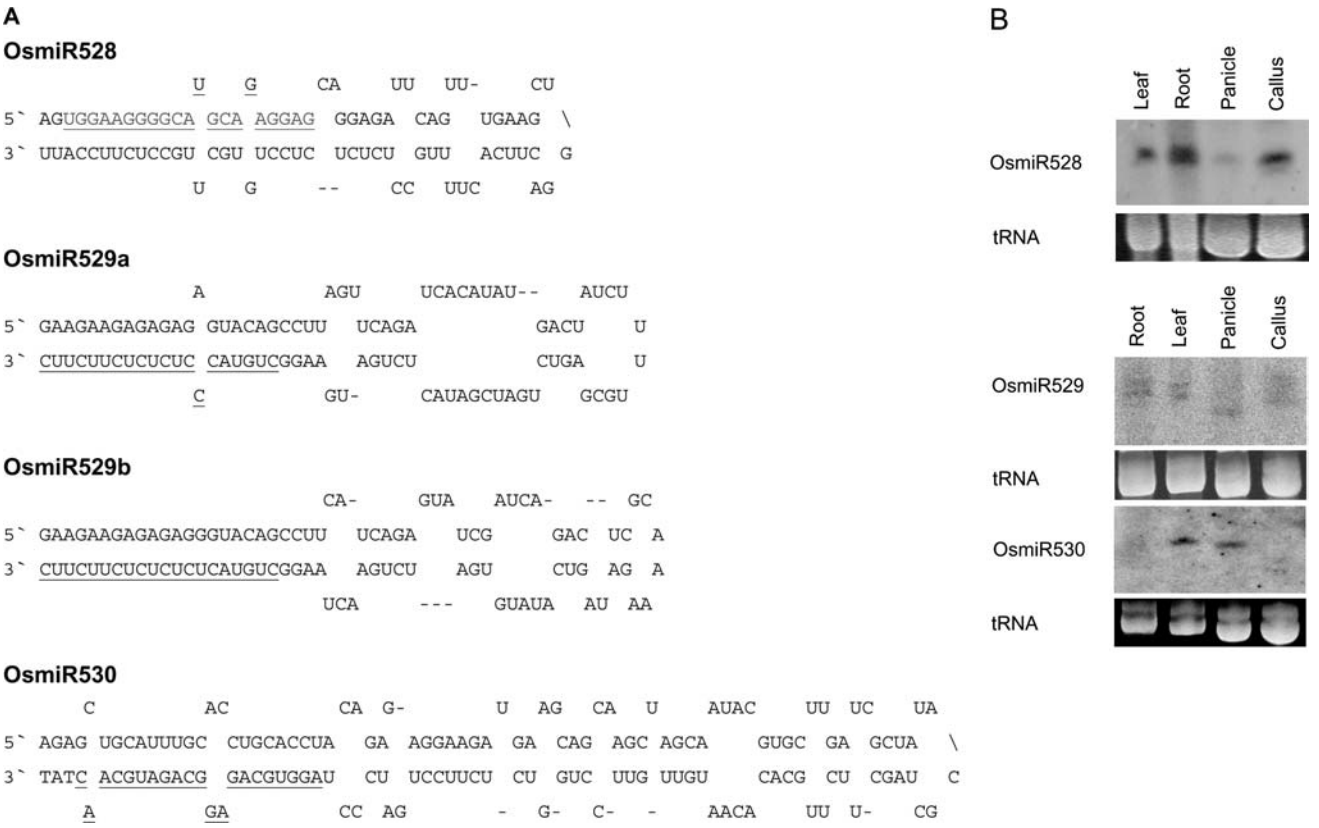

Figure 1. Representatives of newly identified miRNAs from rice. A, Predicted fold-back structure of OsmiR528, OsmiR529a, OsmiR529b, and OsmiR530 precursors from rice. B, Expression patterns of novel rice miRNAs. The samples from different tissues of wild-type rice, including root, leaf, panicle, and callus, are indicated above. The tRNA bands were visualized by ethidium bromide staining of gels and served as internal loading controls.

and OsmiR530 (Fig. 1A), are bona fide miRNAs since they can be detected by northern-blot hybridization and the expression patterns from different tissues were observed (Fig. 1B). Therefore, we conclude that OsmiR528, OsmiR529, and OsmiR530 are three novel miRNAs from rice (Fig. 1, A and B). In addition, the three novel miRNAs did not have targets of known proteins (Supplemental Table II). This may be due to the incomplete annotation of the rice genome. Comparison of precursor sequences of the cloned miRNAs with rice cDNAs showed half of the putative miRNAs had primary transcripts in full-length cDNA clones. Since the cloning of the cDNAs relied on the presence of 5′ CAP structure (Kikuchi et al., 2003), these miRNA precursors were likely transcribed by RNA polymerase II (Supplemental Table III).

Rice Has Four DCL Proteins

To study the function of OsDCLs in the biogenesis of miRNAs and siRNAs, we searched rice genome databases using the protein sequence of Arabidopsis DCL1. Four putative rice proteins, named OsDCL1 to OsDCL4, were identified from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the Plant Chromatin Database (http://www.chromdb.org). Sequences of these OsDCLs were aligned with selected plant and animal Dicer or DCL proteins to explore their relationships.

Phylogenetic analysis of DCL proteins from different species showed that Dicer-1 in Drosophila, mouse, and human were grouped together (Fig. 2). Dicer-2 from Drosophila was separated from other animal Dicers reflecting its unique function in siRNA processing (Lee et al., 2004). In plant DCLs, three subfamilies could be defined with high bootstrap value. The OsDCLs and DCLs did not show one-to-one relationships, except for OsDCL4 and DCL4. Instead, OsDCL1 was grouped with both DCL1 and DCL2, whereas DCL3 had two rice orthologs, OsDCL2 and OsDCL3. Each subgroup may have similar or related functions.

Knock Down of *OsDCL1* and *OsDCL4* by RNAi

To understand the roles of OsDCLs in the RNA-silencing pathways in rice, especially in miRNA biogenesis, we applied the RNAi approach to knock down rice *OsDCLs*. Phylogenetic analysis showed that among all OsDCLs, OsDCL1 was the closest to DCL1, which is responsible for miRNA processing in Arabidopsis. In order to knock down *OsDCLs*, we used Pfam (http://pfam.wustl.edu/) to predict the

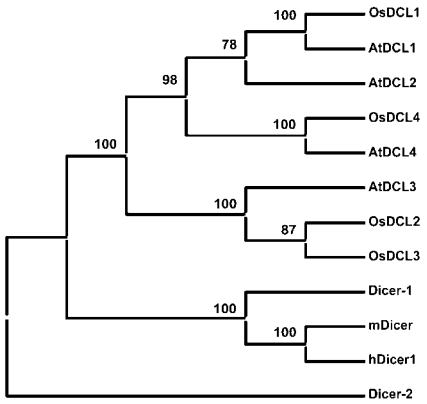

Figure 2. Phylogenetic relationships of DCL proteins in higher plants and animals. Full-length protein sequences were used for phylogenetic analyses. Abbreviations and accession numbers were as follows: OsDCL1 to OsDCL4 are four putative DCL proteins from rice. The sequences were derived from http://www.chromdb.org/ and partially confirmed by RT-PCR. DCL1 to DCL4 are four Arabidopsis DCL proteins. DCL1 (NM_099986), DCL2 (NM_111200), DCL3 (NM_114260), and DCL4 (NM_122039) correspond to predicted protein sequences from Arabidopsis At1g01040, At3003300, At3g43920, and At5g20320 genes, respectively. Dicer-1 (NM_079729), *Drosophila melanogaster* DCR-1; Dicer-2 (NM_079054), *D. melanogaster* DCR-2; hDicer (NM_177438), human Dicer-1; mDicer (NM_148948), mouse Dicer1.

domain structure of OsDCLs (Fig. 3A). OsDCL4 is more similar to OsDCL1 among the four OsDCLs (Figs. 2 and 3A). Therefore, a less conserved region between the DUF283 and PAZ domain of OsDCL1 and OsDCL4 was selected to ensure the specificity of the RNAi experiment (Fig. 3A). dsRNAs were generated under the rice *Actin1* promoter (Fig. 3B).

We evaluated whether the RNAi approach resulted in knockdown of *OsDCL1* and *OsDCL4*, respectively. A significant reduction in *OsDCL1* expression was observed by reverse transcription (RT)-PCR in loss-of-function transformants of *OsDCL1IR* compared to that of wild type (Fig. 4A, top section). To rule out the possibility that the RNAi approach also affected other *OsDCL* genes, we detected the expression of *OsDCL2* and *OsDCL4* using RT-PCR. As expected, no difference was observed for the expression of *OsDCL2* and *OsDCL4* between loss of function of *OsDCL1IR* transformants and wild-type plants (Fig. 4A, middle sections). The *Actin* gene was used as an internal control in the RT-PCR reactions (Fig. 4A, bottom section). A similar approach was applied to *OsDCL4IR* transformants, and the expression of *OsDCL4*, but not *OsDCL1* and *OsDCL2*, was greatly reduced in *OsDCL4IR* transformants compared to that of control plants (Fig. 4B). These results indicated that *OsDCL1* and *OsDCL4* had indeed been knocked down specifically by RNAi.

OsDCL1 Is Required for Rice Development

In contrast with loss of function of *OsDCL4IR* transformants that did not show developmental defect at vegetative stage, the regenerated transgenic plants containing *OsDCL1* RNAi construct showed various degrees of developmental defects compared to control plants (Fig. 5). Strong loss of function of *OsDCL1IR* transformants showed overall shoot and root abnormalities such as severe dwarfism and dark green color. These plants often produced rolled leaves and malformed shoots with tortuousness. Root elongation was also greatly reduced in *OsDCL1IR* transformants (Fig. 5A). During further development, the shoots of strong loss of function of *OsDCL1IR* transformants were greatly enlarged transversely and rolled leaves wilted and senesced. These plants showed developmental arrest during rooting or at the young seedling stage and eventually died either on sterile medium or in soil (Fig. 5B).

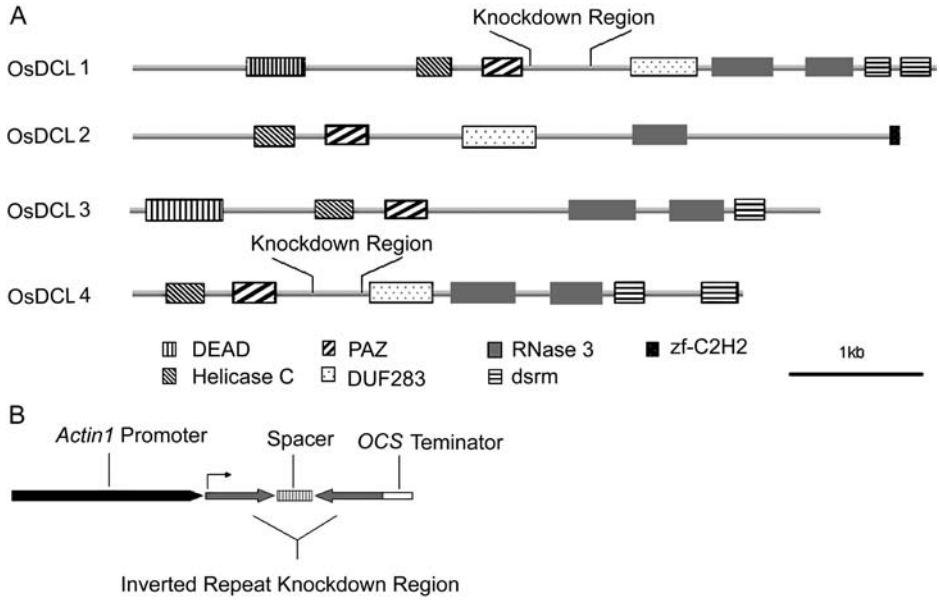

Figure 3. A, Schematic representation of conserved motifs among four DCL proteins in rice. Regions used in the RNAi knockdown are labeled. B, Diagram of *OsDCL* RNAi constructs. Fragments containing respective *OsDCL* genes in sense and antisense orientations separated by an unrelated intron were cloned under the rice *Actin1* promoter.

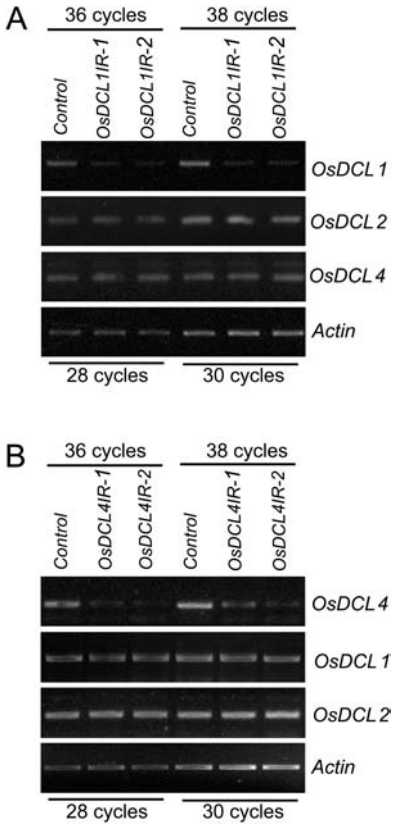

Figure 4. Specificity of RNAi in *OsDCL1IR* and *OsDCL4IR* transformants. RT-PCR analyses were performed for the *OsDCL1*, *OsDCL2*, and *OsDCL4* loci in a control plant and two *OsDCL1IR* (A) and *OsDCL4IR* (B) transformants, respectively. Equal amount of cDNAs was determined by RT-PCR with 28 and 30 cycles at the *Actin* locus; the same amount of cDNAs was used to amplify 36 and 38 cycles at *OsDCL1*, *OsDCL2*, and *OsDCL4* loci, respectively.

Weak loss of function of *OsDCL1IR* transformants displayed a dark green color and dwarfism with different leaf and root phenotypes, including narrow, rolled, and outward-folded leaves (Fig. 5C). They also had fewer adventitious roots compared to wild type (Fig. 5C). This is consistent with the exhibition of *ago1* in Arabidopsis which regulates adventitious root emergence through mRNA mediate-regulation pathway (Sorin et al., 2005). Furthermore, the adventitious roots of weak loss of function of *OsDCL1IR* transformants were short and radically swollen when grown on sterile medium (Fig. 5, D and E). The short adventitious roots phenotype was partially restored after transferring the plants into soil. Since the selection medium contained 0.5 mg/L naphthylacetic acid, the altered root phenotypes of *OsDCL1IR* transformants might be due to altered sensitivity to auxin.

To further investigate if cellular pattern of adventitious roots had been affected, we made transverse sections at the differentiated region of fresh root tissue. Ectopically developed chloroplasts were found in *OsDCL1IR* transformants roots; however, the cell number and overall cellular organization of *OsDCL1IR* transformants roots did not change (Fig. 5, F and G).

OsDCL1 Is Essential for miRNA Processing in Rice

Because *dcl1* loss-of-function alleles resulted in a reduced miRNA population and caused developmental defects in Arabidopsis, we investigated whether

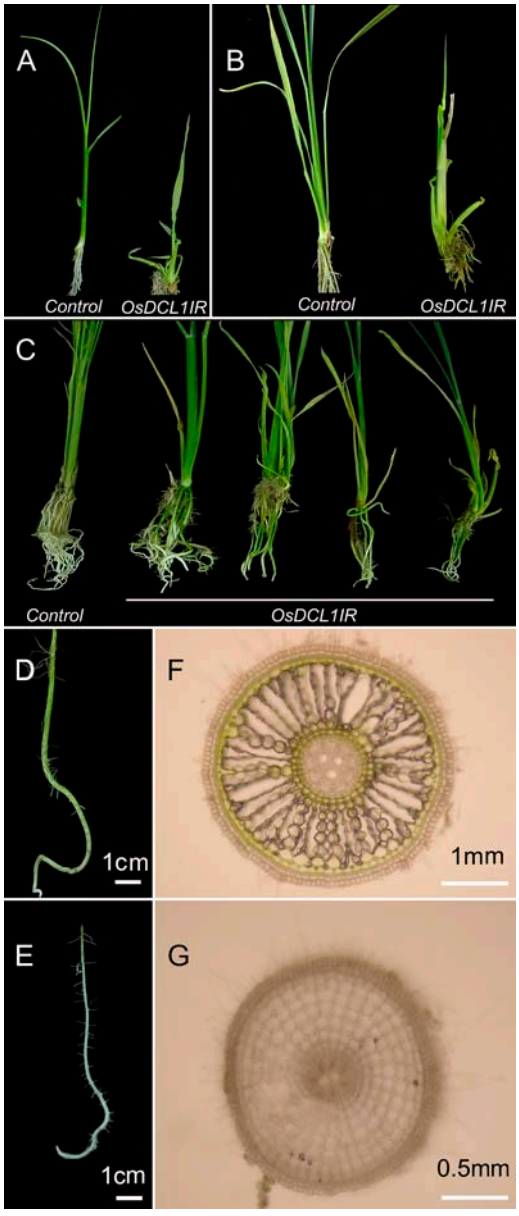

Figure 5. Morphology of *OsDCL1IR* transformants showing pleiotropic phenotypes. A, Wild type and strong loss of function of *OsDCL1IR* transformants. The *OsDCL1IR* transformant showed severe dwarfism, rolled and curly leaves, and tortuous shoots. B, The strong loss of function of *OsDCL1IR* transformant showed developmental arrest at the young seedling stage. C, The weak loss of function of *OsDCL1IR* transformants displayed pleiotropic phenotypes at the seedling stage. D and E, Roots from *OsDCL1IR* transformants (D) and control (E) plants. The weak *OsDCL1IR* transformants had fewer adventitious roots. F and G, Cross sections of roots from *OsDCL1IR* transformants (F) and control plants (G). Ectopically developed chloroplasts were found in *OsDCL1IR* transformants roots; however, the cell number and overall cellular organization of weak loss of function of *OsDCL1IR* transformant roots did not change.

OsDCL1IR transformants also caused a reduction of miRNA in rice. We validated the miRNA level in leaves of *OsDCL1IR* transformants and different tissues of wild-type plants (Fig. 6). Hybridization results showed that all miRNAs accumulation in leaves of *OsDCL1IR* transformants was abolished or greatly reduced at the tested loci compared to that of wild-type leaves. These loci included OsmiR156, OsmiR159, OsmiR166, OsmiR167, OsmiR168, OsmiR168*, OsmiR396, and OsmiR528 (Fig. 6). In contrast to *OsDCL1*, loss of function of *OsDCL4IR* transformants did not display altered miRNA accumulation from both leaf and flower samples (Fig. 7, A and B, top sections). These results suggest *OsDCL1* plays an essential role in miRNA accumulation.

Neither OsDCL1 nor OsDCL4 Is Essential for the Production of siRNAs from Inverted Repeats of Transgene and Endogenous CentO Satellites in Rice

RNAi is an evolutionarily conserved process in which double-stranded RNAs are converted into 21- to 25-nt siRNAs that trigger the degradation of homologous mRNAs. In Arabidopsis, it has been shown that different DCL proteins participate in the biogenesis of siRNAs and miRNAs, respectively (Xie et al., 2004). To determine whether *OsDCL1* is required for siRNA processing in rice, we first analyzed siRNA accumulation corresponding to RNAi regions in *OsDCL1IR* transformants. By using α-^{32}P-UTP-labeled RNA probes specific for *OsDCL1* inverted repeats region, we detected both 24-nt and a large amount of 21-nt siRNAs from *OsDCL1IR* transformants but not from control plants and *OsDCL4IR* transformants (Fig. 7A). Twenty-four- and 21-nt siRNAs were also observed in *OsDCL4IR* transformants, if RNA probes specific for *OsDCL4* inverted repeats region were applied (Fig. 7A). These results were consistent with the fact that *OsDCL1* and *OsDCL4* mRNA levels were reduced in loss of function of *OsDCL1IR* and *OsDCL4IR* transformants, respectively (Fig. 4). From these results, we conclude that neither *OsDCL1* nor *OsDCL4* is essential for the production of siRNAs derived from transgenic inverted repeats.

In order to determine if endogenous siRNAs production was also affected in loss of function of *OsDCL1IR* and *OsDCL4IR* transformants, we performed northern-blot hybridization using a probe corresponding to CentO satellites, which is similar to 165-bp repeated sequences in *Oryza punctata* (Zhang et al., 2005). The same blot was hybridized sequentially with probes corresponding to inverted repeat regions of *OsDCL1IR* or *OsDCL4IR* transformants to verify each sample. We observed that the production of endogenous CentO siRNAs was not decreased in either *OsDCL1IR* or *OsDCL4IR* transformants compared to that in wild type, which suggests that neither OsDCL1 nor OsDCL4 is the key enzyme for the production of CentO satellites related to endogenous siRNAs (Fig. 7B). This result also suggests that the production of miRNAs and siRNAs is via distinct OsDCLs.

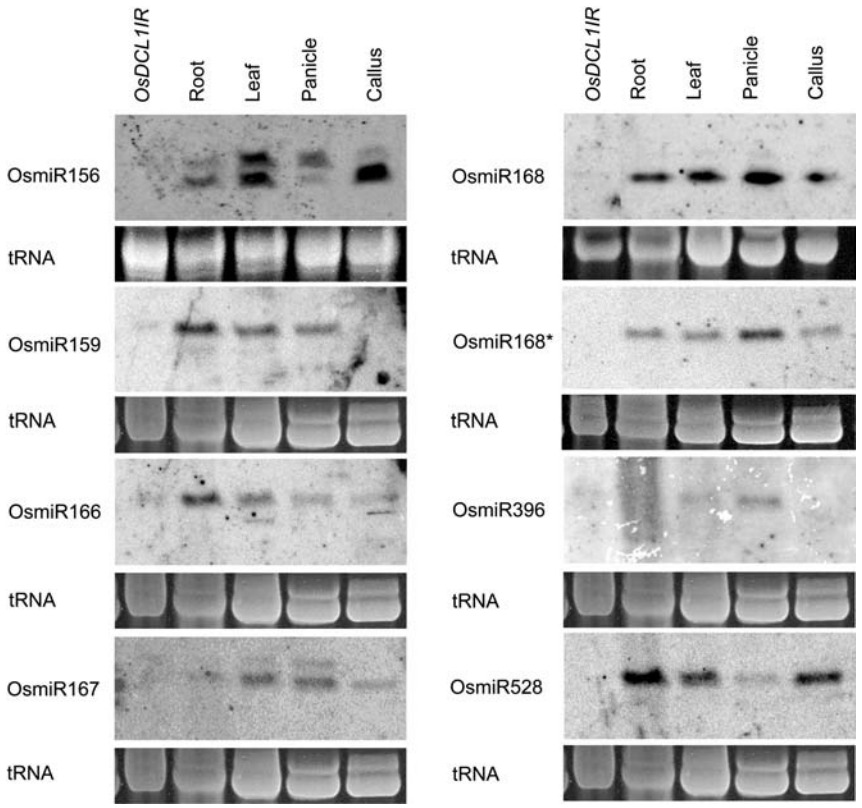

Figure 6. The *OsDCL1* gene is essential for miRNAs accumulation. Small-sized RNAs from different tissues were separated in polyacrylamide gels, and blots were probed with antisense oligenucleotides complementary to mRNA sequences. The samples from leaf tissue of *OsDCL1IR* transformants and different tissues of wild type, including root, leaf, panicle, and callus are indicated. The tRNA bands were visualized by ethidium bromide staining of gels and served as internal loading controls. Each probe is listed.

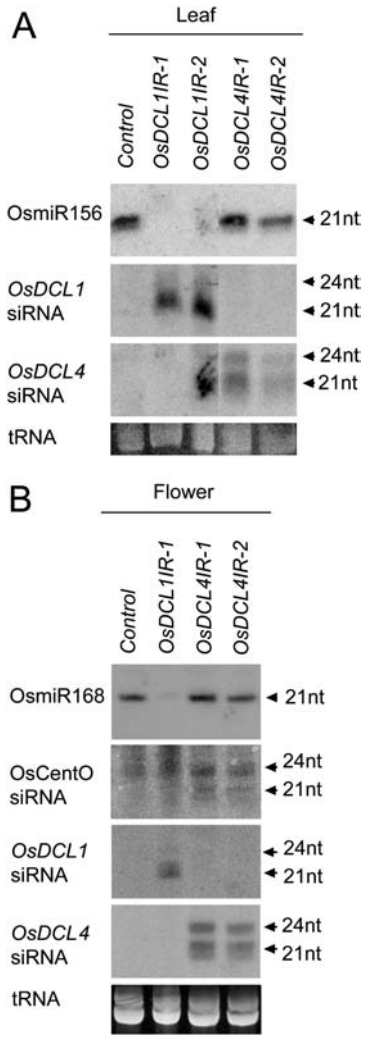

Figure 7. siRNA and miRNA accumulation in *Osdcl1IR* and *Osdcl4IR* transformants. To detect siRNA, each *OsDCL* inverted repeats region and endogenous CentO regions was labeled by T7 RNA polymerase, and miRNA was detected as previously described. Hybridization was performed sequentially to the same blot. The tRNA bands were visualized by ethidium bromide staining of gels and serve as internal loading controls. Leaf and flower RNA samples were isolated from control plants, *OsDCL1IR*, and *OsDCL4IR* transformants as indicated.

DISCUSSION

Rice miRNAs have been identified based on the conserved hairpin structure of miRNA precursor. Yet the experimental evidence of OsmiRNA identification is only recently reported (Sunkar et al., 2005), and how OsmiRNAs are produced and further affect rice development are unclear. In this paper, we experimentally identified three novel miRNA families and nine candidate miRNAs from rice. Functional study of OsDCLs demonstrated that OsDCL1 is required for miRNA processing and rice normal development.

Specificity of DCLs in Higher Plants

The Dicer family has relatively more genes in higher plants compared to animals. Different functions were found for different DCL proteins that participate in the biogenesis process of different types of small RNAs. In Arabidopsis, DCL1 is responsible for miRNA but not siRNA accumulation. Impaired miRNA production in *dcl1* mutants causes pleiotropic developmental defects but does not affect gene silencing (Jacobsen et al., 1999; Xie et al., 2004). In contrast, DCL2 is important for some siRNA accumulation related to viral resistance. Loss of function of *dcl2* mutants reduces the accumulation of viral siRNA and causes more severe disease symptoms compared to that of wild type after infection with turnip crinkle virus (Xie et al., 2004). In addition, DCL3 is involved in 24- to 25-nt siRNA accumulation (Xie et al., 2004). Impaired siRNA accumulation in *dcl3-1* mutants caused plants to lose the ability to maintain characteristic heterochromatic features, specifically in DNA methylation and histone methylation at H3Lys-9. The *dcl3* as well as *drm2*, *sde4*, *ago4*, and *rdr2* mutant alleles all lost the ability of de novo methylation of the newly transformed *FWA* transgene (Cao and Jacobsen, 2002; Chan et al., 2004). Recently, *SDE4* gene has been identified to encode the largest subunit of RNA polymerase IV (Herr et al., 2005; Onodera et al., 2005). Moreover, DCL3, RNA polymerase IV, and RNA-dependent RNA polymerase 2 (RDR2) are all essential for the accumulation of endogenous 24-nt siRNAs (AtSN1, 1003, 02, and cluster 2), which are important for the maintenance of silencing at corresponding loci. Therefore, it is likely that DCL3, RDR2, and RNA polymerase IV act together in the RNA-silencing pathways. These results suggest DCL3 is important in RNA-directed gene silencing and chromatin modification.

Similar to Arabidopsis, the rice genome also contains four OsDCLs. In this paper, we demonstrated that OsDCL1 is essential for miRNA processing. Loss of function of *OsDCL1IR* transformants greatly reduced miRNA accumulation resulting in pleiotropic phenotypes, but these plants did not affect siRNA production either from inverted repeats of transgene or endogenous CentO satellite repeats. The rice OsDCL4 shares the highest homology to OsDCL1, but it did not affect the accumulation of either miRNA or siRNA from transgenic inverted repeats and endogenous CentO satellites DNA. These results indicate OsDCL1 is the ortholog of DCL1 from Arabidopsis. Moreover, the specificity of OsDCL1 for miRNA biogenesis is conserved between monocots and dicots. We were able to detect both 21-nt and 24-nt-long siRNAs in *OsDCL1IR* and *OsDCL4IR* transformants suggesting either OsDCL2 or OsDCL3 or both are required for short and long siRNA accumulation in rice. Although from our limited data OsDCL4 did not affect either siRNAs from its own inverted repeat of transgenes or endogenous CentO siRNAs, we cannot rule out the possibility that OsDCL4 is involved in siRNAs biogenesis at specific loci or at certain types of uncharacterized

siRNAs. How siRNAs affect rice at the developmental or physiological level is unclear. The exact roles of OsDCL2, OsDCL3, and OsDCL4 are under investigation.

miRNAs and Rice Development

miRNAs play an important role in plant development. In this paper, we showed that weak loss of function of *OsDCL1IR* transformants cause pleiotropic phenotypes including narrow, rolled, and outward-folded leaves. A similar effect was also observed in miRNA-defective mutants such as *dcl1*, *hen1*, *ago1*, and *hyl1* in Arabidopsis (Jacobsen et al., 1999; Park et al., 2002; Kidner and Martienssen, 2004; Vazquez et al., 2004). In Arabidopsis, the expression of *PHABULOSA*, *PHAVOLUTA*, and *REVOLUTA*, the class III HD-Zip mRNAs, was developmentally down-regulated by miR165/166 to determine abaxial fate. In maize (*Zea mays*), the expression of HD-Zip III family member *ROLLED LEAF 1* was also regulated spatially through miR166 that determine adaxial/abaxial polarity in developing leaves. The rolled-leaf phenotype of *OsDCL1IR* transformants could be due to less expressed miR165/166. This is consistent with conserved function of miR165/166 in establishment of leaf polarity and morphology both in Arabidopsis and maize (Emery et al., 2003; Tang et al., 2003; Juarez et al., 2004; Kidner and Martienssen, 2004; Mallory et al., 2004).

RNAi Approach to Study Proteins Involved in RNAi Pathway

The RNAi approach has been widely used to study gene functions since dsRNAs have been recognized to trigger mRNA degradation (Fire et al.,1998). In this study, we used RNAi approaches to investigate the functions of OsDCLs in RNA-silencing pathways. If any OsDCL protein is essential for siRNA accumulation, siRNA accumulation derived from the double-stranded regions of RNAi will not be detected and silencing may not occur. Alternatively, some siRNA can be detected due to partial loss of function. However, in *OsDCL1IR* and *OsDCL4IR* transformants, siRNAs with 21 nt and 24 nt in size corresponding to each RNAi region can be easily detected suggesting that both *OsDCL1* and *OsDCL4* are not required for the production of siRNAs from inverted repeats of transgene and endogenous CentO siRNAs. This result also suggests distinct pathways of miRNA and siRNA in higher plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Rice (*Oryza sativa*) plants used in this study were *japonica* cv Nipponbare. Leaves and young panicles were harvested from plants grown in the field before the primary panicle branches grew out from the axils. Roots were collected from 14-d-old seedlings grown in a growth chamber at 25°C with 16 h light and 8 h dark. Callus tissues were induced from embryogenic calli on Murashige and Skoog (with 2 mg/L 2,4-dichlorophenoxyacetic acid) medium for 28 d before harvesting.

Small RNA Cloning

Rice panicles and callus cultures were used for small RNA isolation. Total RNA was extracted and enriched for small-sized RNAs using polyethylene glycol precipitation as described previously (Mette et al., 2000; Hamilton et al., 2002). Cloning of small-sized RNAs was performed as described (Elbashir et al., 2001; Park et al., 2002). Briefly, using the Decade Marker System (Ambion) as a size marker, a band of small RNAs between 10 and 30 nt was excised from 15% polyacrylamide-7 M urea-gel/0.225XTBE. Approximately 200 μg of enriched small-sized RNA was loaded and then eluted with 0.3 M of NaCl at 4°C overnight followed by ethanol precipitation. The RNA was then dephosphorylated and ligated with T4 RNA ligase (New England Biolabs) to a 3′ adapter (pUUUctgtaggcaccatcaat-it: uppercase, RNA; lowercase, DNA; p, phosphate; it, inverted deoxythymidine). The ligated product was recovered, 5′ phosphorylated by T4 Polynucleotide kinase (New England Biolabs), and ligated with 5′ adapter (5′atcgtaggcaccUGAAA; uppercase, RNA; lowercase, DNA). The second ligated product was gel purified and eluted in the presence of RT primer (attgatGGTGGCtacagaaa: uppercase, *Ban*I site), used as carrier. RT was performed by SuperScript II Reverse Transcriptase (Invitrogen) and subsequently followed by PCR using the forward (atcgtaGGCACCtgaaa: uppercase, *Ban*I site) primers and Taq DNA polymerase. The PCR product was digested with *Ban*I and concatamerized with T4 DNA ligase (New England Biolabs) at 22°C for 5 h. Concatamers of a size of 500 to 1,000 bp were recovered from 2% of agarose gel and ends blunted with Taq DNA polymerase before cloned into pCR4-TOPO (Invitrogen) cloning vector for DNA sequencing.

miRNA Prediction

Nineteen- to approximately 25-nt fragments were extracted from all of the sequenced small RNAs and BLASTed against the nucleotide database downloaded from ftp://ftp.ncbi.nlm.nih.gov/blast/db/. Fragments of noncoding RNAs (such as rRNAs, tRNAs, and snoRNA) or those having mismatches to database sequences were discarded as contaminating species. The remaining sequences were regarded as putative small RNAs and subjected to blast analysis against *japonica* genome sequences downloaded from ftp://ftp.dna.affrc.go.jp/pub/Rice_Seq_DB/. Fragments localized at the genome sequences with upstream 200 bp plus downstream 20 bp and vice versa were extracted as putative precursors, which were analyzed for hairpin structure using the m-fold program (Zuker, 2003). The small RNAs whose putative precursors have hairpin structure were defined as putative miRNAs and further verified using expression and biogenesis criteria for mRNA annotation (Ambros et al., 2003; Griffiths-Jones, 2004).

Phylogenetic Analysis

Alignments of full-length Dicer protein sequences were performed using ClustalX version 1.81 with default parameters (Tian et al., 2004). A bootstrapping phylogenetic tree was constructed by the MEGA2 program with the Unweighted Pair Group Method with Arithmetic Mean method. OsDCL1 through OsDCL4 are four putative DCL proteins from rice. These sequences are derived from http://www.chromdb.org/ and partially confirmed by RT-PCR. Domain structures were predicted by Pfam (http://pfam.wustl.edu/).

Construction of RNAi Vector

pCam23ACT:OCS, a derivative of pCambia 2300, carrying the rice *Actin1* promoter and the *OCS* terminator, was used for plant transformation. The first-strand cDNA from rice panicle was used as a template and the primer pairs used were as follows: *OsDCL1* (CX0020: 5′ccgctcGAGCAGAATGATGAAGGTGAA 3′; CX0021: 5′ATGCTTTTGCGGGATCCCAA3′); *OsDCL4* (CX0026: 5′cgcggaTCCCATACCAGAAGATAGGC3′; CX0027: 5′ccgctcGAGGCATGCACAGACACATCT3′). The PCR fragments were sequentially cloned into *Xho*I/BglII and *Bam*HI/*Sal*I sites of pUCC-RNAi vector to target the gene in both the sense and antisense orientations. The whole-stem loop fragment was further cloned into pCam23ACT:OCS between the rice *Actin1* promoter and *OCS* terminator sequence, yielding the binary *OsDCL1* and *OsDCL4* RNAi vector. pCam23ACT:OCS plasmid without *OsDCL* insertion was used as control for transformation.

Plant Transformation and Detection

Rice transformation and regeneration were based on a previously published protocol with some modifications (Hiei et al., 1994). The regenerated plants were further confirmed by PCR using primers corresponding to the neomycin phosphotransferase II gene. The primer sequences were as follows: CX637 (5′GATTGAACAAGATGGATTGCACGCAGGTT3′) and CX638 (5′CAGAAGAACTCGTCAAGAAGGCGATAGAA3′).

RT-PCR Analysis of Gene Expression

For confirmation of gene expression, leaves were harvested from plants grown in soil and flash frozen in liquid nitrogen. Tissues were stored at −80°C until RNA extraction. Total RNAs were isolated from leaves using Trizol, treated with RNase-free DNase I (Roche), quantified with a GeneQuant (Amersham) spectrophotometer, and visualized on a 1.2%-formaldehyde agarose gel. For the RT-PCR reaction, 2 μg total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and equal amounts of RT products were used to perform PCR as described previously (Zilberman et al., 2003). For the *Actin* gene, reactions proceeded for 28 to 30 cycles, using the following primer pairs: CX0151 (5′CTTCGTCTCGACCTTGCTGGG3′) and CX0152 (5′GAGAAACAAGCAGGAGGACGG3′). The primer pairs were CX0020 and CX0290 (5′GCTCCTACAGGGACAAAGAGGT3′) for *OsDCL1*, CX0022 (5′cgcgGATCCAGTTCCAGAAATTGTA3′) and CX0274 (5′CACTCGCAGTGTTCCACACAAA3′) for *OsDCL2*, and CX0026 and CX0276 (5′GCCAACTACATCGGTTTTACT3′) for *OsDCL4*. Reactions proceeded for 36 and 38 cycles. Control reactions without RT were used to assess the presence of any contaminating DNA.

RNA Filter Hybridization

Total RNAs were isolated from rice tissues (panicles, leaves, roots, and callus cultures) and small RNAs were enriched by using polyethylene glycol precipitation as described by Mette et al. (2000). The enriched small-sized RNAs were dissolved in 0.2% of SDS in RNase-free water. For RNA filter hybridization, 50 μg of enriched small-sized RNAs was loaded per lane and separated on a 15% polyacrylamide-7 M urea-gel/0.225XTBE. The RNAs were then transferred electrophoretically to Bio-Rad Zeta-Probe GT nylon membrane at 10 V for 1 h. The nylon membrane was cross-linked under UV transillumination followed by vacuum drying at 80°C for 2 h to fix the RNA onto the membrane. The complementary sequences corresponding to miRNAs were used as probes labeled with γ-^{32}P-ATP using T4 polynucleotide kinase or α-^{32}P-UTP by T7 RNA polymerase (Ambion). The probe corresponding to endogenous CentO siRNAs, which contained two 165-bp tandem repeats between 12061625 to 12061954 positions of BAC clone AP008218, was amplified. Primers used for amplification were CX0759 (5′GATTTTTGGACATATTGGAGTGTATTGGGT3′) and CX0760 (5′ATGTTTTGGTGCTTTTGAAAACCTTTTCA3′). Hybridization was performed as previously described (Zilberman et al., 2003).

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