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DAWDLE Interacts with DICER-LIKE Proteins to Mediate Small RNA Biogenesis[1]

Shuxin Zhang,[a,2] Yongchao Dou,[b,3] Shengjun Li,[b] Guodong Ren,[c] David Chevalier,[d] Chi Zhang,[b] and Bin Yu[b,2]

[a]State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, Shandong 271018, China
[b]Center for Plant Science Innovation and School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68588-0666
[c]State Key Laboratory of Genetic Engineering and Collaborative Innovation Center for Genetics and Development, School of Life Sciences, Fudan University, Shanghai 200438, China
[d]Department of Biological Sciences, East Georgia State College, Swainsboro, Georgia 30401

ORCID IDs: 0000-0003-2577-0522 (S.Z.); 0000-0003-0469-3222 (S.L.); 0000-0002-1895-7578 (G.R.); 0000-0002-4763-177X (B.Y.) (B.Y.)

DAWDLE (DDL) is a conserved forkhead-associated (FHA) domain-containing protein with essential roles in plant development and immunity. It acts in the biogenesis of microRNAs (miRNAs) and endogenous small interfering RNAs (siRNAs), which regulate gene expression at the transcriptional and/or posttranscriptional levels. However, the functional mechanism of DDL and its impact on exogenous siRNAs remain elusive. Here, we report that DDL is required for the biogenesis of siRNAs derived from sense transgenes and inverted-repeat transgenes. Furthermore, we show that a mutation in the FHA domain of DDL disrupts the interaction of DDL with DICER-LIKE1 (DCL1), which is the enzyme that catalyzes miRNA maturation from primary miRNA transcripts (pri-miRNAs), resulting in impaired pri-miRNA processing. Moreover, we demonstrate that DDL interacts with DCL3, which is a DCL1 homolog responsible for siRNA production, and this interaction is crucial for optimal DCL3 activity. These results reveal that the interaction of DDL with DCLs is required for the biogenesis of miRNAs and siRNAs in Arabidopsis (*Arabidopsis thaliana*).

Small RNAs (sRNAs) including microRNAs (miRNAs) and small interfering RNAs (siRNAs) play multiple roles in regulating many biological processes by mediating transcriptional or posttranscriptional gene silencing (Baulcombe, 2004; Axtell, 2013). miRNAs and siRNAs are chemically indistinguishable (Zhang et al., 2015). However, miRNAs are processed from primary miRNA transcripts (pri-miRNAs) that contain one or more miRNA-residing imperfect stem-loops, while siRNAs originate from long perfect double-stranded RNAs (dsRNAs) that are often the products of the RNA-dependent RNA polymerase (RDR; Voinnet, 2009). Upon production, miRNAs and siRNAs join the effector protein called ARGONAUTE to silence genes containing complementary sequences through cleavage of a target transcript, translational inhibition, and/or chromatin modification (Baulcombe, 2004; Vaucheret, 2008; Voinnet, 2009).

Based on their origin, endogenous siRNAs can be further classified into three different classes: siRNAs generated from repeated DNAs such as transposons (ra-siRNAs), phased-siRNAs (pha-siRNAs), and natural antisense transcript siRNAs (nat-siRNAs) (Baulcombe, 2004; Axtell, 2013; Borges and Martienssen, 2015). During ra-siRNA biogenesis, the plant specific DNA-dependent RNA polymerase IV uses repeated DNAs as templates to produce RNA precursors, which are then converted to dsRNAs by RDR2. The resulting dsRNAs are mainly processed by DCL3, a homolog of DCL1, to generate ~24-nucleotide ra-siRNAs (Law and Jacobsen, 2010; Castel and Martienssen, 2013; Matzke and Mosher, 2014; Xie and Yu, 2015). Pha-siRNAs including trans-acting siRNAs (ta-siRNAs) are secondary siRNAs. Their production begins with miRNA-mediated cleavage of target transcripts (Fei et al., 2013). RDR6 then uses the resulted cleavage products as templates to synthesize dsRNAs, which are processed by DCL4 to generate ta-siRNAs (Allen et al., 2005; Yoshikawa et al., 2005; Axtell et al., 2006). Compared with ra-siRNAs and pha-siRNAs, the precursor dsRNAs of nat-siRNAs are formed by independently transcribed complementary RNAs (Zhang et al., 2013b). Besides endogenous

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[2]Address correspondence to sxzhang@sdau.edu.cn or byu3@unl.edu.

[3]Current address: Department of Molecular and Human Genetics and Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, TX 77030.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Shuxin Zhang (sxzhang@sdau.edu.cn).

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siRNAs, plants also generate exogenous siRNAs from transgenes such as sense transgenes (s-siRNAs) or transgenes of inverted repeats (IR-siRNAs; Vaucheret and Fagard, 2001; Ossowski et al., 2008).

In plants, the DNA-dependent RNA Polymerase II transcribes most miRNA-coding genes (MIRs) to pri-miRNAs, with assistance from the Mediator complex (Kim et al., 2011), the Elongator complex (Fang et al., 2015), Negative on TATA less 2 (NOT2; Wang et al., 2013), and CELL DIVISION CYCLE5 (CDC5; Zhang et al., 2013a). Following transcription, the RNase III enzyme DICER-LIKE1 (DCL1) cleaves pri-miRNAs at least two times to release a miRNA/miRNA* (passenger strand) in the nucleus (Baulcombe, 2004). Many DCL1-associated proteins are also required for pri-miRNA processing, such as HYL1 (a dsRNA-binding protein; Dong et al., 2008), SERRATE (a zinc-finger protein; Grigg et al., 2005), TOUGH (an RNA-binding protein; Ren et al., 2012), the Elongator complex (Fang et al., 2015), NOT2 (Wang et al., 2013), CDC5 (Zhang et al., 2013a), PLEIOTROPIC REGULATORY LOCUS1 (Zhang et al., 2014), MAC3 (Li et al., 2018), and CBP20/80 (two cap-binding proteins; Gregory et al., 2008; Laubinger et al., 2008). Moreover, some proteins may also regulate the accumulation of miRNAs by modulating the levels or activities of the DCL1 complex or promoting the recruitment of pri-miRNAs to the DCL1 complex. Examples of such proteins include MODIFIER OF SNC1,2 (an RNA-binding protein; Wu et al., 2013), the ribosome protein STV1 (Li et al., 2017), the Pro-rich SICKLE (Zhan et al., 2012), RECEPTOR FOR ACTIVATED C KINASE1 (Speth et al., 2013), the pre-mRNA processing factor 6 homolog STABILIZED1 (Ben Chaabane et al., 2013), REGULATOR OF CBF GENE EXPRESSION3 (also known as HOS5 and SHI1; Chen et al., 2015; Karlsson et al., 2015), and the Gly-rich RNA-binding protein GRP7 (Köster et al., 2014).

DAWDLE (DDL) is a conserved forkhead-associated domain (FHA)-containing protein that is required for proper development and immune responses (Morris et al., 2006; Feng et al., 2016). Recently, DDL was shown to regulate the biogenesis of several miRNAs and ra-siRNAs (Yu et al., 2008). However, its global effect on miRNA accumulation still is not known. Furthermore, the effect of DDL on transgene-induced gene silencing is not defined. In addition, the impact of DDL on the precision of miRNA processing is unknown. Moreover, DDL interacts with DCL1 through its FHA domain and stabilizes pri-miRNAs (Machida and Yuan, 2013), yet the biological significance of this interaction in miRNA biogenesis remains elusive.

Here, we report that DDL globally influences the accumulation of miRNAs and ra-siRNAs and is also required for the biogenesis of s-siRNAs and IR-siRNAs. We further show that a mutation abolishing the DDL-DCL1 interaction disrupts pri-miRNA processing, demonstrating that the DDL-DCL1 interaction is required for miRNA biogenesis. Interestingly, this mutation also disrupts the interaction of DDL with DCL3 and impairs the activity of DCL3. These results suggest that DDL may participate in small RNA biogenesis by modulating the activity of DCLs.

RESULTS

DDL Globally Affects the Accumulation of miRNAs and siRNAs

To determine the global effect of DDL on the accumulation of miRNAs and siRNAs, we compared the small RNA profile in *ddl-1* with that in Wassilewskija (Ws; wild type). Small RNA libraries prepared from inflorescences of *ddl-1* and Ws were subjected to Illumina deep sequencing analyses. Many miRNAs were reduced in abundance in *ddl-1* relative to Ws in two biological replicates (Fig. 1A). RNA-blot analyses of several miRNAs (miR164, miR162, miR163, miR158, miR159/miR319, miR173, and miR390) further validated the deep sequencing results (Fig. 1B), suggesting that DDL is required for miRNA biogenesis. We also determined the effect of DDL on precision of pri-miRNA processing. If precisely processed, miRNAs or miRNA*s should fall within ±2 bases of the annotated mature miRNA(s) or miRNA*(s) positions (Liu et al., 2012). We focused our analyses on those with high readings because evaluation of miRNA precision relies on sequencing depth. We did not detect significant differences of miRNA precision between Ws and *ddl-1* (Supplemental Data Set S1).

Next, we analyzed the effect of DDL on siRNAs at global levels as described (Shahid and Axtell, 2014). We obtained short read counts to various siRNA loci using the software tool ShortStack, normalized reading numbers at various loci to those of miR163, whose abundance was not changed between *ddl-1* and Ws (Yu et al., 2008), and compared the abundance of siRNAs in *ddl-1* with that in Ws. The abundance of siRNAs from most loci was reduced in *ddl-1* relative to Ws in two biological replicates (Fig. 1A). Several siRNAs [Cluster4, TR2558, TAS3-5′D8(+), TAS2-siR1511, FWA, Copia, and Simple hat2] were further examined using RNA blots (Fig. 1C). As observed in RNA-seq, the abundance of these examined siRNAs was reduced in *ddl-1* compared with Ws. These results demonstrated that DDL plays essential roles in siRNA accumulation. We further analyzed the abundance of siRNAs in *ddl-1* and Ws based on their sizes (21, 22, and 24 nucleotides). The abundance of 21-, 22-, and 24-nucleotide siRNAs was reduced in *ddl-1* compared with Ws (Supplemental Fig. S1), suggesting that DDL may have a general role in siRNA biogenesis.

DDL Is Required for the Accumulation of Transgene-Induced siRNAs

Next, we investigated if DDL also affects the production of transgene-induced siRNAs. We first examined the effect of *ddl-1* on the sense transgene-induced siRNAs. *ddl-1* (from the Ws genetic background) was

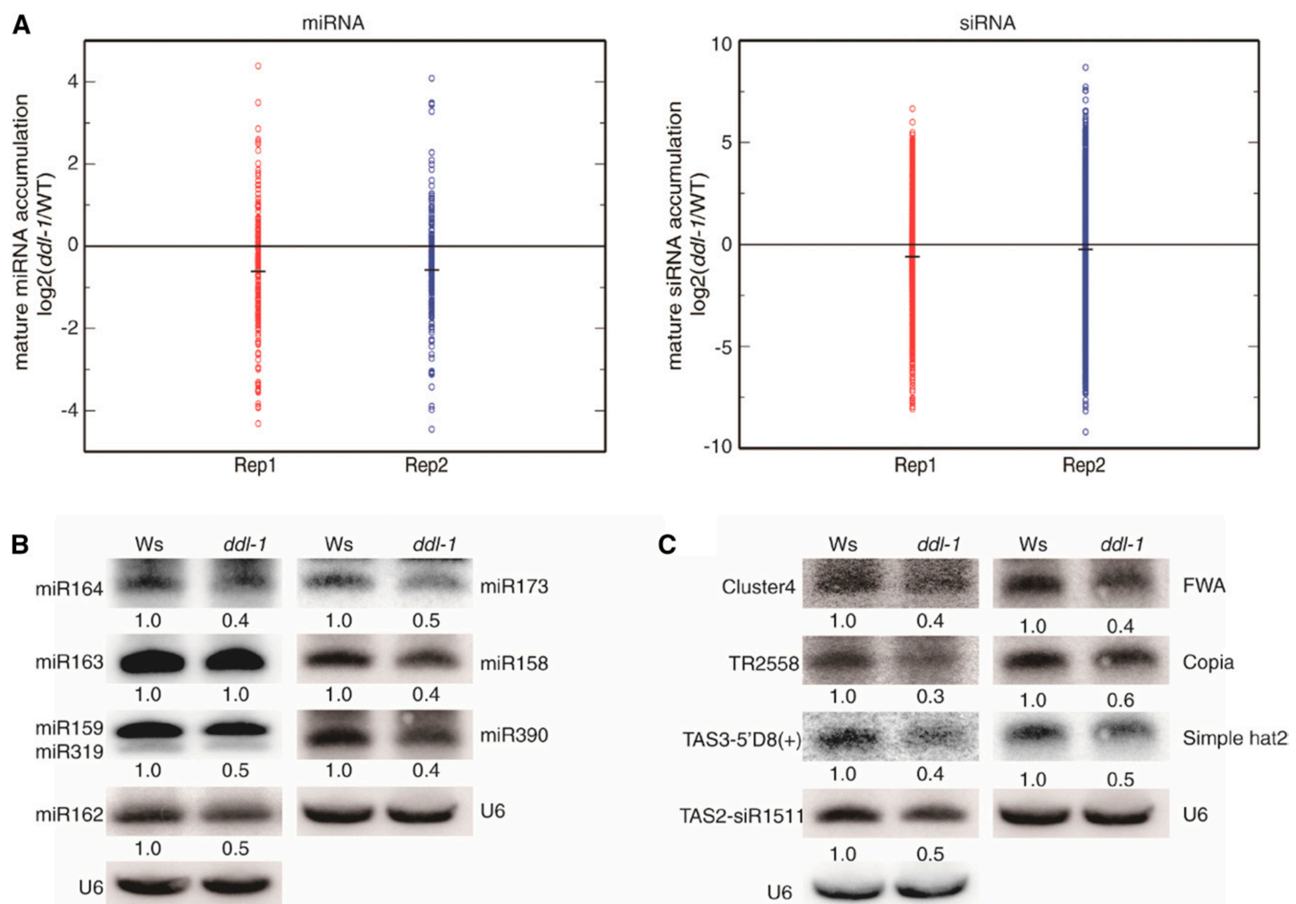

Figure 1. DDL regulates the accumulation of miRNAs and siRNAs. A, Deep sequencing analysis of mature miRNA and siRNA accumulation in *ddl-1*. Libraries of small RNA were produced from inflorescences of *ddl-1* and Ws. Each circle represents a small RNA calculated as reads per million, and a $\log_2$-transformed ratio of *ddl-1*/Ws was plotted. The thick lines in the middle of circles indicate median values. Rep1 and Rep2 were two biological replicates of sequencing. B, miRNA abundance in inflorescences of *ddl-1* and Ws. C, siRNA abundance in inflorescences of *ddl-1* and Ws. Ws, wild-type control of *ddl-1*; U6, spliceosomal RNA U6, used as loading control. Small RNAs were detected by RNA blot. Radioactive signals were detected with a phosphor imager and quantified with ImageQuant (v5.2). The amount of miRNA or siRNA in *ddl-1* was normalized to U6 RNA and compared with that in Ws (set as 1) to determine the relative abundance of small RNAs in *ddl-1*. The number below *ddl-1* indicates the relative abundance of miRNAs or siRNAs, which is the average value of three replicates. $P < 0.05$. For miR159/319, upper band, miR159; lower band, miR319. The numbers represent the relative abundance quantified by three replicates (*t* test, $P < 0.05$).

crossed to the L1 line (Col genetic background; Mourrain et al., 2000), which harbors a silenced 35S promoter-driven GUS sense transgene and is often used as a reporter line of sense transgene-induced gene silencing. To rule out the potential effect of different genetic backgrounds, we generated a recombined inbred line through repeated self-crossing of DDL/*ddl-1* harboring the L1 locus obtained from the original cross for five generations. In the sixth generation, we obtained wild-type (*DDL*$^+$) and *ddl-1* containing the *L1* locus and examined the effect of *ddl-1* on GUS expression. GUS histochemical staining and RNA-blot analyses revealed that the expression of the GUS transgene was recovered in *ddl-1* compared with *DDL*$^+$ (Fig. 2, A and B). We further examined GUS-derived siRNAs (GUS-siRNAs) from *DDL*$^+$ and *ddl-1* using RNA blots. The accumulation of GUS-siRNAs was reduced in *ddl-1* relative to *DDL*$^+$ (Fig. 2C). These results demonstrate that DDL is required for sense transgene-induced siRNA silencing.

We then crossed *ddl-1* to the *AP1-IR* line (Landsberg *erecta* background; Chuang and Meyerowitz, 2000), in which the expression of an inverted repeat transgene produces siRNAs targeting the AP1 gene that encodes a transcription factor controlling flower development, resulting in AP1 silencing. The resulting DDL/*ddl-1* containing the *AP1-IR* locus was self-crossed for five generations to produce a recombined inbred line. In the sixth generation, we examined the effect of *ddl-1* on the accumulation of AP1 siRNAs. The levels of AP1

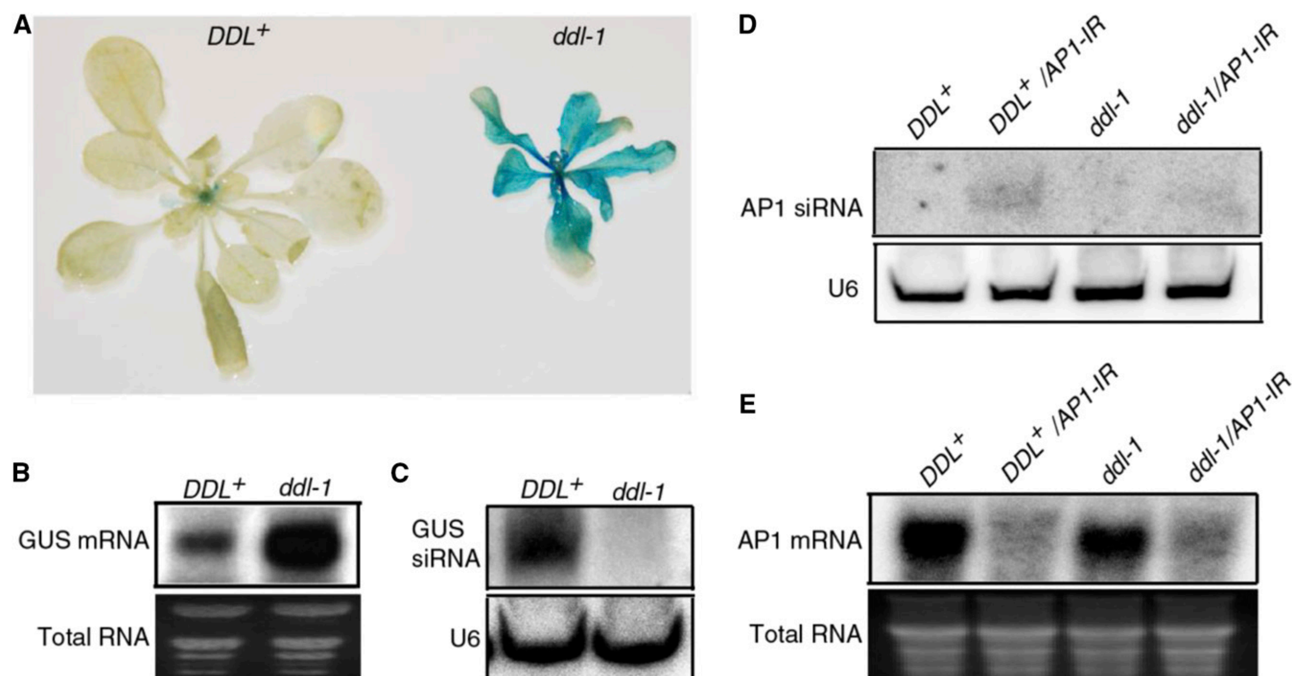

Figure 2. DDL is required for transgene induced siRNA accumulation. A, Histochemical staining of GUS in plants containing the *L1* locus. Wild-type (*DDL*+) and *ddl-1* containing the *L1* locus were segregated from the sixth generation of a recombined inbred line through repeated five generations of self-crossing of DDL/*ddl-1* harboring the *L1* locus. Twenty plants containing *GUS* were analyzed for each genotype. B, *GUS* mRNA levels in *DDL*+ and *ddl-1* detected by RNA blot. Equal total RNAs were loaded for RNA blot. C, The accumulation of *GUS* siRNA in *DDL*+ and *ddl-1*. D. The accumulation of AP1 siRNAs in *DDL*+, *DDL*+*/AP1-IR*, *ddl-1*, and *ddl-1/AP1-IR* plants detected by RNA blot. U6 RNA was used as loading control. E, *AP1* expression levels in *DDL*+, *DDL*+*/AP1-IR*, *ddl-1*, and *ddl-1/AP1-IR*. *DDL*+, *DDL*+*/AP1-IR*, *ddl-1*, and *ddl-1/AP1-IR* plants were segregated from a recombined inbred line that was produced by five generations of repeated self-crossing of DDL/*ddl-1* containing the *AP1-IR* locus. Equal total RNAs were loaded for the RNA blot.

siRNAs were reduced in *ddl-1* compared with the wild type (*DDL*+; Fig. 2D), suggesting that DDL may be required for IR siRNA production. However, the levels of AP1 mRNAs were similar in *ddl-1* and the wild type harboring the *AP1-IR* lines (Fig. 2E). It is possible that the reduction of AP1 siRNA in *ddl-1* may be not sufficient to repress AP1 silencing. Alternatively, DDL may have additional function in promoting *AP1* transcript levels since many FHA-containing proteins can regulate multiple biological processes (Chevalier et al., 2009). The reduction of the *AP1* mRNA levels in *ddl-1* relative to the wild type (Fig. 2E) strongly supports this notion.

DDL Interacts with DCL3 But Not DCL4

Next, we examined how DDL affects the accumulation of various sRNAs. We have shown that DDL interacts with DCL1, the major enzyme producing miRNAs. By analogy, we hypothesized that DDL might interact with DCL3 that generates ra-siRNAs and DCL4 that produces ta-siRNAs and transgene-induced siRNAs. To test the DDL-DCL3 interaction and to determine the protein domains of DCL3 that interacts with DDL, we coexpressed N-terminal MYC-fused full-length DCL3 and four DCL3 fragments (Fig. 3A), namely, MYC-DCL3, MYC-F1 (amino acids 1–350, covering amino terminus to helicase domain 1), MYC-F2 (amino acids 351–700; helicase domain 2), MYC-F3 (amino acids 701–980; Piwi/Argonaute/Zwille domain), and MYC-F4 (amino acids 971–1580) with C-terminal GFP-fused DDL in *Nicotiana benthamiana*. After coexpression, anti-GFP antibodies conjugated with protein G agarose beads were used to perform immunoprecipitation (IP) of DDL-GFP. The full-length DCL3 and F3 fragment, but not other fragments of DCL3, were detected in the DDL-GFP IP (Fig. 3B), revealing that DDL may interact with the PAZ domain of DCL3. We also examined the interaction of DDL and DCL4. However, we did not detect the DDL-DCL4 interaction using coimmunoprecipitation (co-IP; Supplemental Fig. S2). Bimolecular fluorescence complementation was performed to confirm the DDL-DCL3 interaction. In this assay, DDL was fused with the N-terminal fragment of Venus (DDL-nVenus) and DCL3 was fused with C-terminal fragment of cyan fluorescent protein (cCFP-DCL3). The interaction between DDL and DCL3 resulted in the production of functional yellow florescent protein (YFP; Fig. 3C, green) in the leaf cells of *N. benthamiana* coexpressing nVenus-DDL and cCFP-DCL3.

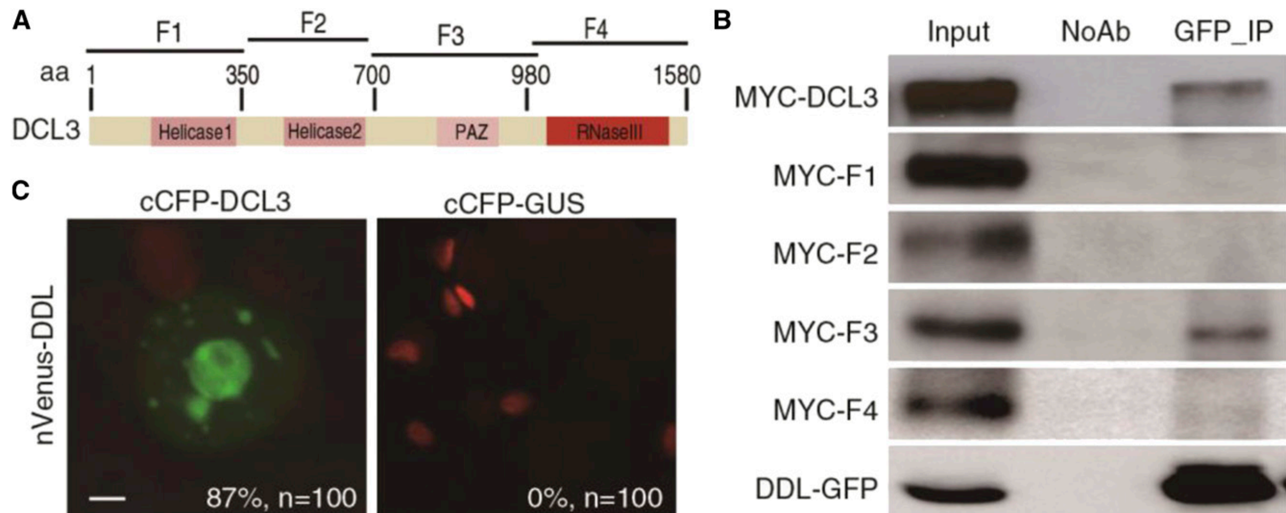

Figure 3. DDL interacts with DCL3. A, Schematic diagram of DCL3 domains and truncated DCL3 fragments used for protein interaction assay. B, The interaction between DDL and various DCL3 fragments detected by co-IP. Full-length and truncated DCL3 proteins fused with a MYC tag at their N terminus were expressed in *N. benthamiana* leaves. For DDL, protein extract from plants expressing *35S::DDL-GFP*. The protein pairs in the protein extracts were indicated by the labels on the left side and above the picture. An anti-MYC antibody was used to detect MYC fusion proteins in immunoblots. Labels on left side of picture indicate proteins detected by immunoblot. Five percent input proteins were used for MYC-tagged proteins, while 20% inputs were used for *DDL-GFP*. C, Bimolecular fluorescence complementation analysis of the interaction DDL with DCL3. Paired nVenus and cCFP-fused proteins were coinfiltrated into *N. benthamiana* leaves. Yellow fluorescence (green in image) signals were examined at 48 h after infiltration by confocal microscopy. The red spots were autofluorescence from chlorophyll. One hundred nuclei were examined randomly for each pair of proteins and one image is shown; the percentage of cells with fluorescence is shown. Bar = 20 μm.

In contrast, the negative control pair DDL/GUS did not produce the YFP signal (Fig. 3C). These results demonstrate the DDL-DCL3 interaction.

DDL Is Required for the Optimal Activity of DCL1 and DCL3

Because the reduction of various sRNAs could be caused by impaired DCL activity, we used an in vitro pri-miRNA processing assay to evaluate the effect of DDL on DCL1 activity (Qi et al., 2005; Ren et al., 2012). We generated a [^{32}P]-labeled RNA fragment containing the stem-loop of pri-miR162b flanked by 6-nucleotide arms at each end (*MIR162b*) using in vitro transcription and examined its processing in the protein extracts from inflorescences of Ws or *ddl-1*. *ddl-1* reduced the production of miR162 from *MIR162b* at various time points (Fig. 4A). At 80 min, the amount of miR162b generated in *ddl-1* was ~60% of that produced in Ws (Fig. 4B). It was also known that DCL3 catalyzes the production of 24-nucleotide siRNA in the in vitro dsRNA-processing assay (Qi et al., 2005). We adapted this assay to test the impact of DDL on DCL3 activity. The amount of 24-nucleotide siRNAs generated from a radioactive labeled dsRNA (460 bp) in the *ddl-1* protein extracts was lower than that in Ws (Fig. 4, C and D). Because the protein levels of DCL1 and DCL3 in the protein extracts of *ddl-1* were similar to those in Ws (Fig. 4E), these results suggest that the activity of DCL1 and DCL3 is impaired by *ddl-1*.

The FHA Domain of DDL Is Required for miRNA Biogenesis

DDL interacts with the helicase and RNase III domains of DCL1 and the PAZ domain of DCL3. Because these domains are critical for DCL1/DCL3 activity, we reasoned that DDL may promote DCL1/DCL3 activity through its interaction with DCL1/DCL3. To test this hypothesis, we examined a *ddl-3* allele (Narayanan et al., 2014), which is in the Col genetic background and carries a Gly-to-Arg (G222R) change at amino acid position 222 of DDL (Fig. 5A). This Gly is highly conserved both in the sequence and the crystal structure of the FHA domain (Machida and Yuan, 2013). It localizes at the protein-protein interaction face of the FHA domain (Fig. 5A) and is required for phospho-Thr recognition (Machida and Yuan, 2013). We suspected that this G222R mutation might disrupt the DDL-DCL1 interaction and thereby affect miRNA biogenesis. We compared the accumulation of miRNAs in *ddl-3* with that in wild-type plants (Col er-105 genotype). RNA blots revealed that the abundance of examined miRNAs was reduced in *ddl-3* (Fig. 5B), suggesting that the G222R mutation impaired the activity of DDL in miRNA biogenesis. We also expressed the *ddl-3* cDNA in *ddl-1* to examine its effect on miRNA biogenesis in

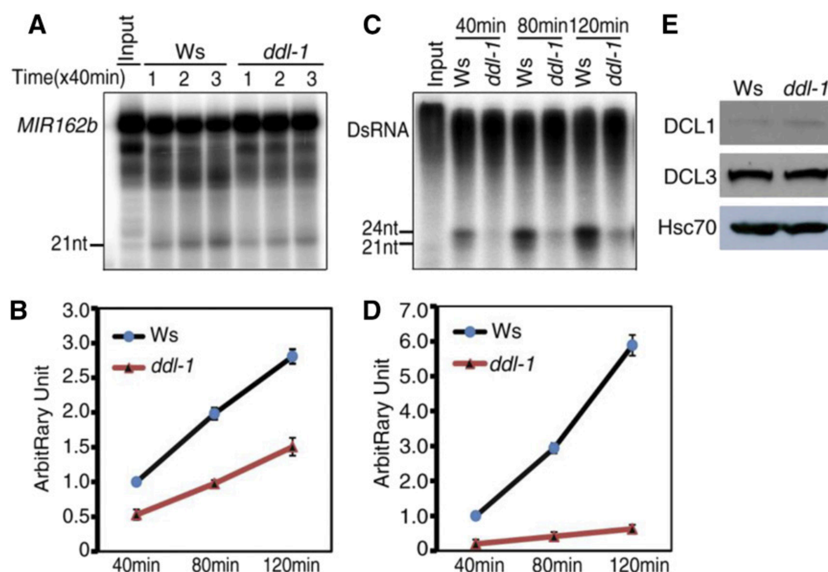

Figure 4. *ddl-1* reduces the accumulation of miR162 and siRNA in an in vitro processing assay. A, miRNA production from pri-miR162b in the protein extracts of *ddl-1* and Ws. [^{32}P]-labeled *MIR162b* that contains the stem-loop of pri-miR162b flanked by 6-nucleotide arms at each end was generated through in vitro transcription. B, Quantification of miRNAs generated from pri-miR162b in *ddl-1* compared to those in Ws at 40, 80, and 120 min. C, siRNA production from dsRNAs in the protein extracts isolated from inflorescences of *ddl-1* and Ws. dsRNAs were synthesized through in vitro transcription of a DNA fragment (5′ portion of *UBQ5* gene, ~460 bp) under the presence of [α-^{32}P]UTP. D. Quantification of siRNAs produced from dsRNAs in *ddl-1* compared to those in Ws at 40, 80, and 120 min. The amounts of miRNAs or siRNAs produced at 40 min from Ws were set as 1, and means ± SD were calculated from three biological replicates. E, The protein levels of DCL1 and DCL3 in protein extracts of Ws and *ddl-1* detected by immunoblot. Hsc70 was used as loading control.

the Ws background. The *ddl-1* plants harboring the *ddl-3* cDNA still displayed growth defects compared to Ws (Fig. 5C). In agreement with this observation, the levels of miRNAs in *ddl-1* expressing the *ddl-3* cDNA were much lower than those in Ws (Fig. 5D). These results demonstrate that the FHA domain is required for the proper function of DDL in miRNA biogenesis.

The G222R Mutation Disrupted the DDL-DCL1 and DDL-DCL3 Interactions and the Activity of DCLs

We further tested if the G222R mutation disrupted the DDL-DCL1 interaction by the co-IP assay. We coexpressed GFP, C-terminal GFP fused *ddl-3* (*ddl-3*-GFP), or DDL-GFP with N-terminal MYC fused DCL1 (MYC-DCL1) in *N. benthamiana.* We then performed IP with anti-GFP antibodies. As expected, DDL-GFP, but not GFP, coimmunoprecipitate with MYC-DCL1 (Fig. 6A). However, MYC-DCL1 was not detected in the *ddl-3*-GFP IPs (Fig. 6A). These results demonstrate that the G222R mutation abolished the interaction between DDL and DCL1. We also examined the *ddl-3*-DCL3 interaction by co-IP. Compared with the DDL-DCL3 interaction, a much weaker *ddl-3*-DCL3 interaction was detected (Fig. 6B). These results demonstrate that the FHA domain is required for the interaction of DDL with DCLs.

Furthermore, we tested if *ddl-3* impaired the activity of DCLs using in vitro processing assay. We compared the processing of *MIR162b* and dsRNA in the protein extracts of *ddl-3* with that in er-105. The generation of miR162 and siRNAs from *MIR162b* and dsRNAs, respectively, was reduced in *ddl-3* (Fig. 6, C and D), demonstrating that the G222R mutation impaired the activity of DCL1 and DCL3.

DISCUSSION

In summary, we demonstrated that DDL plays a general role in sRNA biogenesis. This is evidenced by reduced accumulation of miRNAs, ta-siRNAs, ra-siRNAs, and transgene-induced siRNAs in *ddl-1*. DDL may have global effect on miRNA and ra-siRNA biogenesis since many miRNAs and ra-siRNAs are reduced in abundance in *ddl-1*.

DDL is able to positively regulate the accumulation of miRNAs through promoting DCL1 activity, which is supported by the observation that *ddl-1* and *ddl-3* reduced processing of pri-miRNAs. DDL likely enhances the activity of DCL1 through its interaction with DCL1 since the disruption of the DDL-DCL1 interaction impairs DCL1 activity. The FHA domain of DDL is a phospho-Thr-binding domain, interacting with the helicase and RNase III domains of DCL1 (Machida and Yuan, 2013). Interestingly, these two domains of DCL1 contain the predicted pThr+3(Ile/Val/Leu/Asp) motif (Machida and Yuan, 2013), which raises the possibility

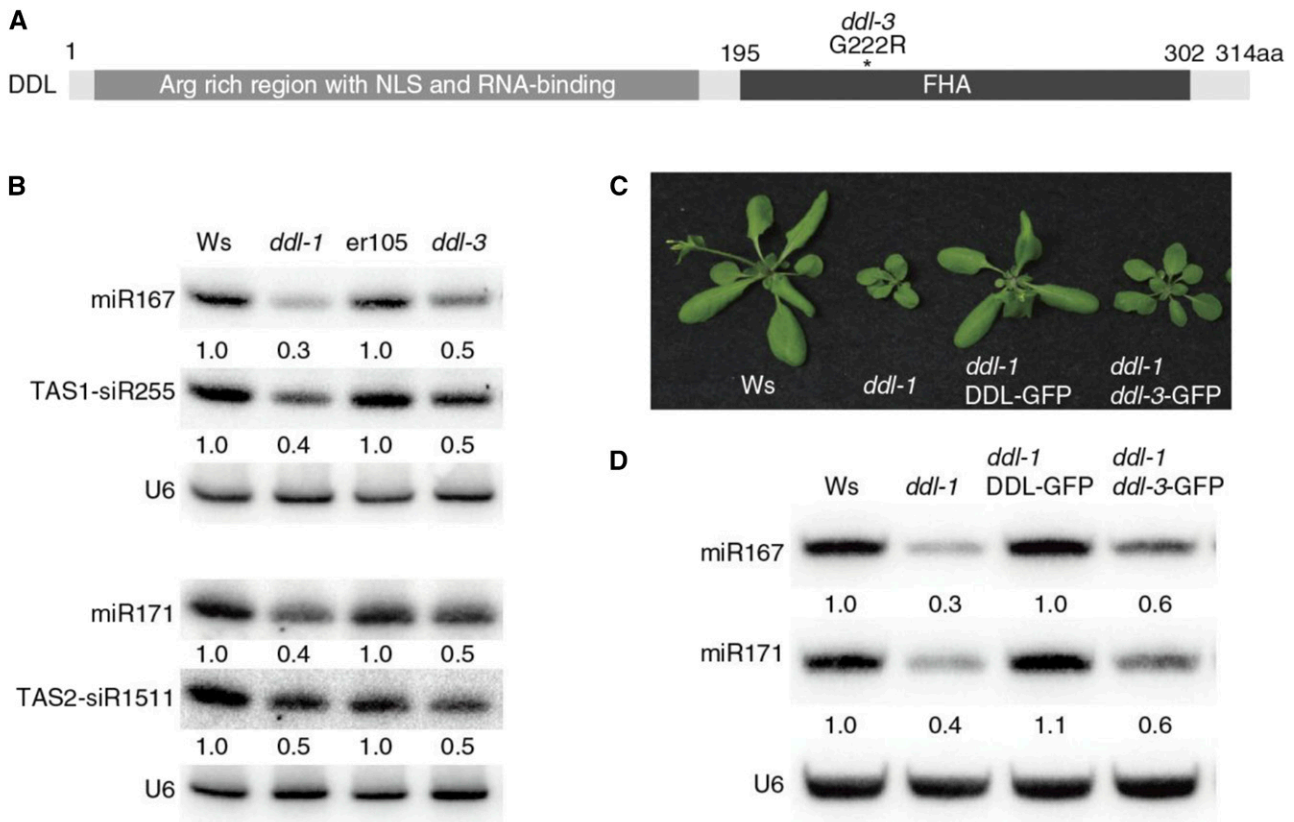

Figure 5. The FHA domain of DDL is required for miRNA biogenesis. A, Schematic diagram of DDL protein domains and the G222R mutation of *ddl-3*. NLS, nuclear localization signal. B, miRNA and siRNA abundance in inflorescences of Ws, *ddl-1*, er105, and *ddl-3* detected by RNA blotting. C, The phenotypes of *ddl-1* harboring the *DDL-GFP* or the *ddl-3-GFP* transgenes. D, miRNA abundance in various genotypes detected by RNA blotting. The radioactive signals were detected with a phosphor imager and quantified with ImageQuant (v5.2). The amount of a small RNA was normalized to U6 RNA and compared with Ws. The numbers represent the relative abundance quantified in three replicates (*t* test, $P < 0.05$). Ws, background control of *ddl-1*; er-105, control of *ddl-3*.

that plants use phosphorylation to control DDL-DCL1 interaction and thereby modulate miRNA biogenesis. Consistent with this hypothesis, DCL1 is indeed phosphorylated in vivo (Engelsberger and Schulze, 2012). Expression of *ddl-3* partially recovered miRNA levels and developmental defects of *ddl-1* (Fig. 5, C and D), suggesting that DDL may have other functions in miRNA biogenesis besides its effect on DCL1 activity. In fact, DDL is an RNA-binding protein and stabilizes pri-miRNAs (Yu et al., 2008). Based on these analyses, we propose that DDL may affect miRNA biogenesis through its combined effect on DCL1 activity and pri-miRNA stability.

DDL is required for the accumulation of 21-, 22-, and 24-nucleotide siRNAs. Because DCL4, DCL2, and DCL3 are responsible for the generation of 21-, 22-, and 24-nucleotide siRNAs, respectively, our results suggest that DDL may affect the activity of all DCLs in Arabidopsis (*Arabidopsis thaliana*). It likely promotes ra-siRNA biogenesis as a cofactor of DCLs, given the observation that DDL interacts with DCL3 and facilitates its activity. DDL binds the PAZ domain of DCL3. In contrast, it interacts with the helicase and RNase III domains of DCL1 (Machida and Yuan, 2013). This may have arisen from a different molecular structure or phosphorylation patterns as a result of different DCL proteins. DDL functions in ta-siRNA biogenesis as well. Since the production of ta-siRNAs depends on miRNAs, we believe that the reduced miRNA accumulation in *ddl-1* may be at least partially responsible for ta-siRNA biogenesis. DDL may also contribute to ta-siRNA biogenesis through its interaction with DCL4. However, we did not detect the DDL-DCL4 interaction through co-IP, although it is still possible that DDL transiently or weakly interacts with DCL4. Besides endogenous sRNAs, the accumulation of transgene-induced siRNAs is also reduced in *ddl-1*. Given the fact that multiple DCLs are responsible for the production of transgene-induced siRNAs, it is reasonable to speculate that DDL may contribute to the biogenesis of transgene-induced siRNA through its interaction of different DCLs. Alternatively, DDL may bind and stabilize the precursors of transgene-induced siRNAs and

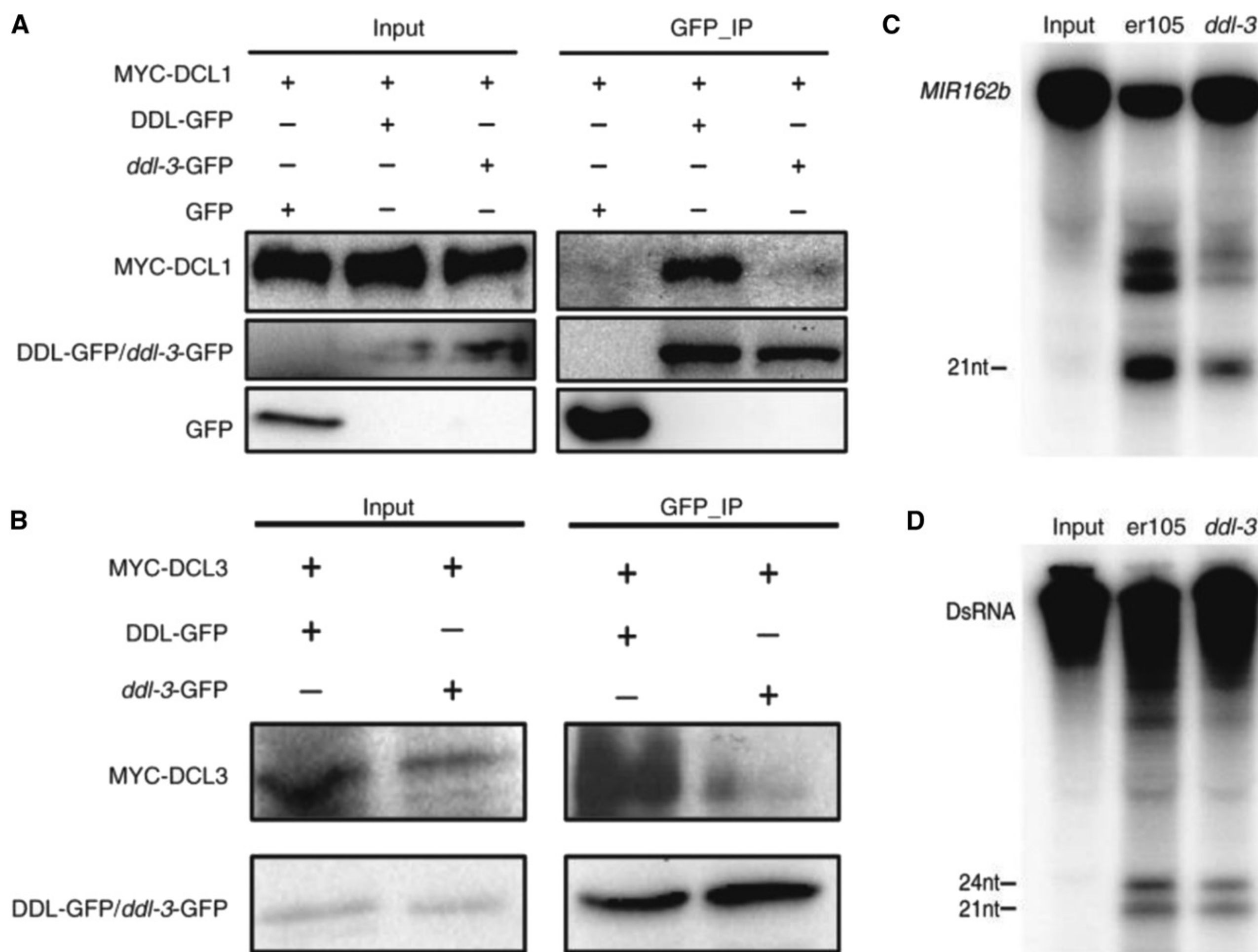

Figure 6. The G222R mutation disrupts the DDL-DCL1 and DDL-DCL3 interactions and the activity of DCLs. A and B, Interactions of *ddl-3* with DCL1 (A) and DCL3 (B) examined by co-IP. GFP, *ddl*-3 fused with GFP at its C terminus (*ddl-3*-GFP) or DDL-GFP were coexpressed with N-terminal MYC-fused DCL1 (MYC-DCL1) or DCL3 (MYC-DCL3) in *N. benthamiana*. Anti-GFP antibodies were used to perform IP, and anti-MYC antibody was used to detect MYC fusion proteins in immunoblots. Five percent input proteins were used for immunoblots. C and D, *MIR162b* (C) and dsRNA (D) processing by protein extracts of *ddl-3* and er-105, respectively. [^{32}P]-labeled *MIR162b* that contains the stem-loop of pri-miR162b flanked by 6-nucleotide arms at each end was generated through in vitro transcription. dsRNAs were synthesized through in vitro transcription of a DNA fragment (5′ portion of *UBQ5* gene, ~460 bp) under the presence of [α-^{32}P]UTP. Proteins were isolated from inflorescences of *ddl-3* or er-105. The in vitro processing reactions were stopped at 80 min and RNAs were extracted for gel running. Radioactive signals were quantified with ImageQuant (v5.2).

thereby contribute to the accumulation of transgene-induced siRNAs.

MATERIALS AND METHODS

Plant Materials

The *ddl-1* (CS6932) mutant is in the Ws genetic background. An L1 line (Mourrain et al., 2000) and AP1-IR line (Chuang and Meyerowitz, 2000) were crossed to *ddl-1.* In the F2 generation, L1, *ddl-1*, and *AP1-IR* were identified by PCR genotyping of *GUS*, *ddl-1*, and *AP1-IR*, respectively. The resulting *DDL/ddl-1* plants harboring *L1* or *AP1-IR* were self-crossed for five generations. In the sixth generation, the *DDL*$^{+}$ (wild type) and *ddl-1* plants containing *L1* or *AP-IR* were identified through PCR genotyping for *ddl-1*, *GUS*, or *AP1-IR*, respectively. *ddl-3* that contains a missense point mutation is in a Columbia er-105 background (Narayanan et al., 2014). Constructs harboring DDL-GFP and *ddl-3*-GFP were transformed into *ddl-1* or *ddl-3* for complementation assay.

Small RNA-Seq and Data Analysis

RNA-blot analysis of small RNAs and RT-qPCR analysis of pri-miRNA transcription levels were performed as described (Ren et al., 2012). Small RNA libraries from inflorescences were prepared and sequenced using Illumina Genome Analyzer IIx following the standard protocol. Adapters from the 3′ end of reads were trimmed off and trimmed reads shorter than 12 nucleotides or longer than 30 nucleotides were excluded from further analysis. Clean reads were mapped to either the Arabidopsis (*Arabidopsis thaliana*) genome (TAIR 9.0, for both miRNA and siRNA abundance) or miRNA hairpin sequences (from miRBase v1.8, for miRNA imprecision) using Bowtie with up to one mismatch (Langmead et al., 2009). EdgeR with TMM normalization method was used for differential analysis (Robinson et al., 2010; Robinson and Oshlack, 2010). The

miRNA/miRNA* imprecision ratios were determined and analyzed using the same method described by Liu et al. (2012). Shortstack (Shahid and Axtell, 2014) was used to find the siRNA clusters. The cluster read counts are reads mapped to each cluster (±150 bp) and normalized to counts of miR163, whose abundance is not changed in *ddl-1*.

Plasmid Construction

DDL cDNA and *DCL3* cDNA were cloned into pSAT4-C-nVenus and pSAT4-C-cCFP, respectively (Ren et al., 2012). The *DDL-C-nVenus* and *DCL3-C-cCFP* fragments were then released by I-*Sce*I restriction enzyme digestion and subsequently cloned into the pPZP-ocs-bar-RCS2-2 vector. The truncated DCL3 (F1 to F4)-MYC fragments were cloned into vector pGWB520. cDNAs of DDL and *ddl-3* were cloned into vector pMDC83 to obtain constructs harboring DDL-GFP and *ddl-3*-GFP, respectively. The primers used for plasmid construction are listed in Supplemental Table S1.

Co-IP Assay

For DDL-DCL3 co-IP, MYC-DCL3 and truncated MYC-DCL3 (F1 to F4) were expressed in *Nicotiana benthamiana* and protein extract from plants expressing *35S::DDL-GFP* was incubated with anti-GFP (Clontech) antibody coupled to protein G-agarose beads for 4 h at 4°C. After washing five times, the proteins in the immunoprecipitates were subjected to immunoblot analysis using anti-GFP antibody and anti-MYC antibodies, respectively.

Dicer Activity Assay

Pri-miR162b and DsRNA were prepared by in vitro transcription under the presence of [α-^{32}P]UTP. In vitro dicer activity assay was performed (Qi et al., 2005; Ren et al., 2012). Radioactive signals were quantified with ImageQuant version 5.2.

Bimolecular Fluorescence Complementation Assay

Paired nVenus-DDL with cCFP-DCL3 or cCFP-GUS was coinfiltrated into *N. benthamiana* leaves. After 48 h, yellow fluorescence signals and chlorophyll autofluorescence signals were excited at 488 nm and detected by confocal microscopy (Fluoview 500 workstation; Olympus) with a narrow barrier filter (BA 505–525 nm).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GSM2809457, GSM2809458, GSM2809459, and GSM2809460.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Deep sequencing analysis of mature siRNAs in different size accumulation in *ddl-1*.

Supplemental Figure S2. DDL does not interact with DCL4.

Supplemental Table S1. DNA oligos used in this study.

Supplemental Data Set S1. The effect of DDL on the precision of miRNA processing.

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