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Title

DAWDLE interacts with DICER-LIKE proteins to mediate small RNA biogenesis

Shuxin Zhang[1]*, Yongchao Dou[2,#], Shengjun Li[2], Guodong Ren[3], David Chevalier[4], Chi Zhang[2] and Bin Yu[2]*

[1]: State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, Shandong 271018, China

[2] :Center for Plant Science Innovation & School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, Nebraska 68588–0666, USA

[3]: State Key Laboratory of Genetic Engineering and Collaborative Innovation Center for Genetics and Development, School of Life Sciences, Fudan University, Shanghai 200438, China

[4]: Department of Biological Sciences, East Georgia State College, Swainsboro, Georgia 30401, USA

[#]: Current Address: Department of Molecular and Human Genetics & Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, TX 77030

*: Corresponding author:
Shuxin Zhang
Tel: 86-538-8244008
Email: sxzhang@sdau.edu.cn

Bin Yu
Tel: 402-472-2125
Email: byu3@unl.edu

One-sentence summary: DAWDLE modulates small RNA processing through its interaction with DCL proteins.

Author Contributions

S.Z. and B.Y. designed the experiments and prepared the manuscript. S.Z., S.L., Y.D., G.R., D.C., and C.Z. performed the experiments. S.Z. and B.Y. analyzed the data.

Short title: DAWDLE in small RNA biogenesis

Key word: DAWDLE, miRNA, siRNA, protein-protein interaction, Arabidopsis

Abstract

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 post-transcriptional 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.

Introduction

Small RNAs (sRNAs) including microRNAs (miRNAs) and small interfering RNAs (siRNAs) play multiple roles in regulating many biological processes by mediating transcriptional or post-transcriptional 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 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 (AGO) 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 *et al.*, 2015). During ra-siRNA biogenesis, the plant specific DNA-dependent RNA polymerase IV (Pol 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 nt ra-siRNAs (Law & Jacobsen, 2010; Castel & Martienssen, 2013; Matzke & Mosher, 2014; Xie & 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., 2013). Besides endogenous siRNAs, plants also generate exogenous siRNAs from transgenes such as sense transgenes (s-siRNAs) or transgenes of inverted repeats (IR-siRNAs) (Vaucheret & Fagard, 2001; Ossowski *et al.*, 2008).

In plants, the DNA-dependent RNA Polymerase II (Pol 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 CYCLE 5 (CDC5) (Zhang *et al.*, 2013). 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 double stranded RNA-binding protein) (Dong *et al.*, 2008), SERRATE (SE, a Zinc-finger protein) (Grigg *et al.*, 2005), TOUGH (TGH, an RNA-binding protein) (Ren *et al.*, 2012), the Elongator complex (Fang *et al.*, 2015), NOT2 (Wang *et al.*, 2013), CDC5 (Zhang *et al.*, 2013), PLEIOTROPIC REGULATORY LOCUS 1 (PRL1) (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 (MOS2, an RNA-binding protein) (Wu *et al.*, 2013), the ribosome protein STV1 (Li *et al.*, 2017), the proline-rich SICKLE (SIC) (Zhan *et al.*, 2012), RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1) (Speth *et al.*, 2013), the pre-mRNA processing factor 6 homolog STABILIZED1 (STA1) (Ben Chaabane *et al.*, 2013), REGULATOR OF CBF GENE EXPRESSION 3 (RCF3, also known as HOS5 and SHI1) (Chen *et al.*, 2015; Karlsson *et al.*, 2015) and the glycine-rich RNA-binding protein GRP7 (Koster *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 & 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 Ws (wild type, WT). 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 (Figure 1A). RNA blot analyses of several miRNAs (miR164, miR162, miR163, miR158, miR159/miR319, miR173 and miR390) further validated the deep-sequencing results (Figure 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* (DataSet S1).

Next, we analyzed the effect of DDL on siRNAs at global levels as described (Shahid & 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 (Figure 1A). Several siRNAs (Cluster4, TR2558, TAS3-5'D8(+), TAS2-siR1511, FWA, Copia and Simple hat2) were further examined using RNA blots (Figure 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-nt). The abundance of 21-, 22-, and 24-nt siRNAs was reduced in *ddl-1* compared with Ws (Figure 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 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*$^+$ (Figure 2A and 2B). 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*$^+$ (Figure 2C). These results demonstrate that DDL is required for sense transgene-induced siRNA silencing.

Then we crossed *ddl-1* to the *AP1-IR* line (Ler background) (Chuang & 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 siRNAs were reduced in *ddl-1* compared with WT (*DDL*$^+$) (Figure 2D), suggesting that DDL may be required for IR siRNA production. However, the levels of AP1 mRNAs were similar in *ddl-1* and WT harboring the *AP1-IR* lines (Figure 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 WT (Figure 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 co-expressed N-terminal MYC-fused full-length DCL3 and four DCL3 fragments (Figure 3A), namely MYC-DCL3, MYC-F1 (aa 1–350, covering amino terminus to helicase domain 1), MYC-F2 (aa 351–700; helicase domain 2), MYC-F3 (aa 701-980; Piwi/Argonaute/Zwille domain), and MYC-F4 (aa 971–1580) with C-terminal GFP-fused DDL in *Nicotiana benthamiana*. After co-expression, anti-GFP antibodies conjugated with protein G agarose beads were used to perform IP of DDL-GFP. The full-length DCL3 and F3 fragment, but not other fragments of DCL3, were detected in the DDL-GFP IP (Figure 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 co-IP (Figure S2). Bimolecular fluorescence complementation (BiFC) 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 florescence protein (YFP) (showing in green, Figure 3C) in the leaf cells of *N. benthamiana* co-expressing nVenus-DDL and cCFP-DCL3. In contrast, the negative control pair DDL/GUS did not produce the YFP signal (Figure 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-nt 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 (Figure 4A). At 80 min, the amount of miR162b generated in *ddl-1* was ~60% of that produced in Ws (Figure 4B). It was also known that DCL3 catalyzes the production of 24-nt 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 nt siRNAs generated from a radioactive labeled dsRNA (460 bp) in the *ddl-1* protein extracts was lower than that in Ws (Figure 4C and 4D). Because the protein

levels of DCL1 and DCL3 in the protein extracts of *ddl-1* were similar to those in Ws (Figure 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 Glycine-to-Arginine (G222R) change at amino acid position 222 of DDL (Figure 5A). This G is highly conserved both in the sequence and the crystal structure of the FHA domain (Machida & Yuan, 2013). It localizes at the protein-protein interaction face of the FHA domain (Figure 5A) and is required for phospho-threonine recognition (Machida & 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 (WT; Col er-105 genotype). RNA blots revealed that the abundance of examined miRNAs was reduced in *ddl-3* (Figure 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 the Ws background. The *ddl-1* plants harboring the *ddl-3* cDNA still displayed growth defects compared to Ws (Figure 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 (Figure 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 co-expressed 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, co-IPed with MYC-DCL1 (Figure 6A). However, MYC-DCL1 was not detected in the *ddl-3*-GFP IPs (Figure 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 (Figure 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* (Figure 6C and 6D), demonstrating that the G222R mutation impaired the activity of DCL1 and DCL3.

Discussion

In summary, we have 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-threonine-binding domain, interacting with the helicase and RNase III domains of DCL1 (Machida & Yuan, 2013). Interestingly, these two domains of DCL1 contain the predicted pThr+3(Ile/Val/Leu/Asp) motif (Machida & Yuan, 2013), which raises the possibility 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 & Schulze, 2012). Expression of *ddl-3* partially recovered miRNA levels and developmental defects of *ddl-1* (Figure 5C and 5D), 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-nt siRNAs. Because DCL4, DCL2 and DCL3 are responsible for the generation of 21-, 22- and 24-nt siRNAs, respectively, our results suggest that DDL may affect the activity of all DCLs in Arabidopsis. It likely promotes ra-siRNA biogenesis as a co-factor 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 & 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 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 & 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.

303 **Small RNA-seq and Data Analysis**

304 RNA blot analysis of small RNAs and RT-qPCR analysis of pri-miRNA transcription levels
305 were performed as described (Ren *et al.*, 2012). Small RNA libraries from inflorescences were
306 prepared and sequenced using Illumina Genome Analyzer IIx following the standard protocol.
307 Adapters from the 3′ end of reads were trimmed off and trimmed reads shorter than 12
308 nucleotides or longer than 30 nucleotides were excluded from further analysis. Clean reads were
309 mapped to either the Arabidopsis genome (TAIR 9.0, for both miRNA and siRNA abundance) or
310 miRNA hairpin sequences (from miRBase v1.8, for miRNA imprecision) using Bowtie with up
311 to 1 mismatch (Langmead *et al.*, 2009). EdgeR with TMM normalization method was used for
312 differential analysis (Robinson *et al.*, 2010; Robinson & Oshlack, 2010). The miRNA/miRNA*
313 imprecision ratios were determined and analyzed using the same method described by Liu *et al.*
314 (Liu *et al.*, 2012). Shortstack (Shahid & Axtell, 2014) was used to find the siRNA clusters. The
315 cluster read counts are reads mapped to each cluster (+/-150bp) and normalized to counts of
316 miR163, whose abundance is not changed in *ddl-1*.
317
318 **Plasmid Construction**

319 *DDL* cDNA and *DCL3* cDNA were cloned into pSAT4-C-nVenus and pSAT4-C-cCFP,
320 respectively (Ren *et al.*, 2012). The *DDL-C-nVenus* and *DCL3-C-cCFP* fragments were then
321 released by I-SceI restriction enzyme digestion and subsequently cloned into the pPZP-ocs-bar-
322 RCS2-2 vector. The truncated DCL3 (F1 to F4)-MYC fragments were cloned into vector
323 pGWB520. cDNAs of DDL and *ddl-3* were cloned into vector pMDC83 to obtain constructs
324 harboring DDL-GFP and *ddl-3*-GFP, respectively. The primers used for plasmid construction are
325 listed in Table S1.
326
327 **Co-Immunoprecipitation (Co-IP) assay**

328 For DDL-DCL3 co-IP, MYC-DCL3 and truncated MYC-DCL3 (F1 to F4) were expressed in *N.*
329 *benthamiana* and protein extract from plants expressing *35S::DDL-GFP* was incubated with
330 anti-GFP (Clontech) antibody coupled to protein G-agarose beads for 4 hours at 4 °C. After
331 washing five-times, the proteins in the immunoprecipitates were subjected to immunoblot
332 analysis using anti-GFP antibody and anti-MYC antibodies, respectively.
333

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.

BiFC Assay

Paired nVenus-DDL with cCFP-DCL3 or cCFP-GUS was co-infiltrated into *N. benthamiana* leaves. After 48 hours, 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 (BA505–525 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

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

Figure S2. DDL does not interact with DCL4.

Table S1. DNA oligos used in this study.

Data Set S1 (XLS) The effect of DDL on the precision of miRNA processing.

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Figure legends

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* indicated 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, $P < 0.05$).

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.

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-immunoprecipitation (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 twenty percent inputs were used for *DDL-GFP*. **C**. BiFc analysis of the interaction DDL with DCL3. Paired of nVenus and cCFP fused proteins were co-infiltrated into *N. benthamiana* leaves. Yellow fluorescence (green in image) signals were examined at 48 h after infiltration by confocal microscopy. The red spots were autoflorescence from chlorophyll. One hundred nuclei were examined randomly for each pair of proteins and one image was shown, the percentage of cells with fluorescence was shown in the images. Scale bar, 20 μm.

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-nt 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 min, 80 min 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 min, 80 min 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.

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*. Arg rich region: Arginine rich domain, NLS: Nuclear Localization Signal, FHA: Forkhead Associated Domain. **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*.

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-terminal (*ddl-3*-GFP) or DDL-GFP were co-expressed 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-nt 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).

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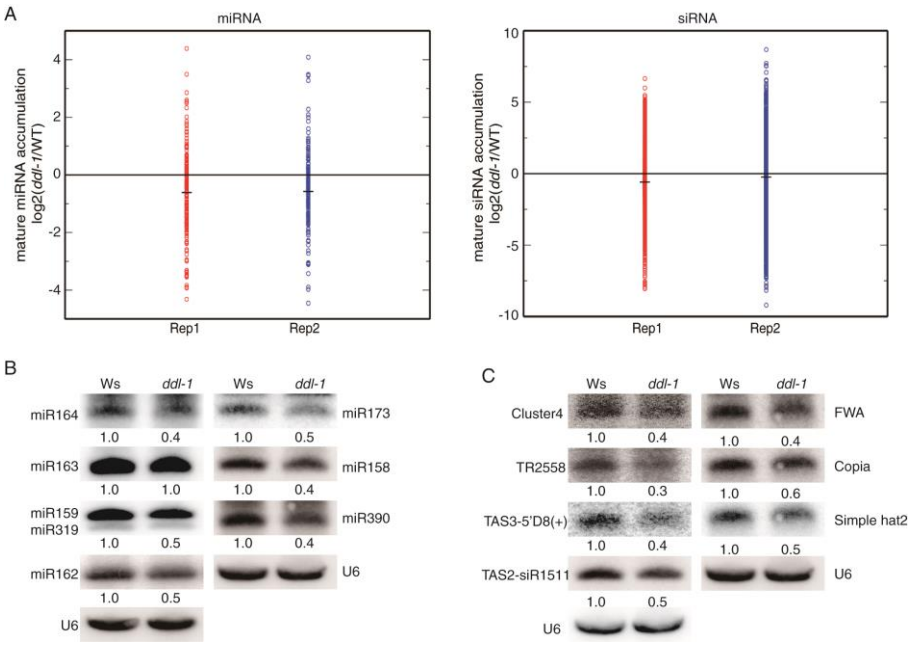


Figure 1. DDL regulates the accumulation of miRNAs and siRNAs. A. Deep sequencing analysis of mature miRNA and siRNA accumulation in *ddl-1*. Libraries of small RNA were produced from inflorescences of *ddl-1* and Ws. Each circle represents a small RNA calculated as reads per million, and a $\log_2$-transformed 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* indicated 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, $P < 0.05$).

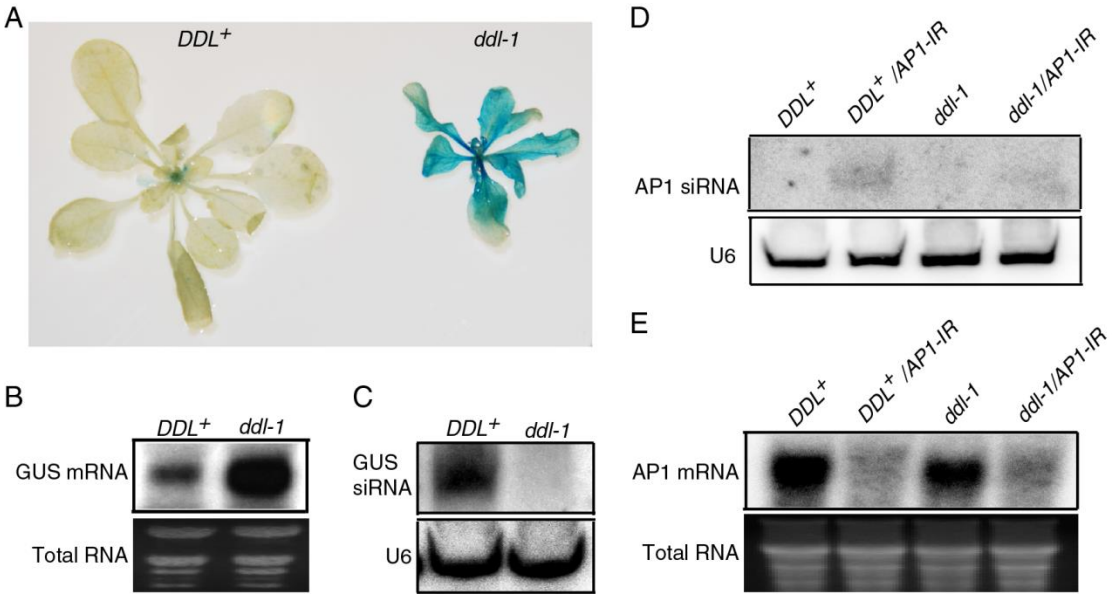
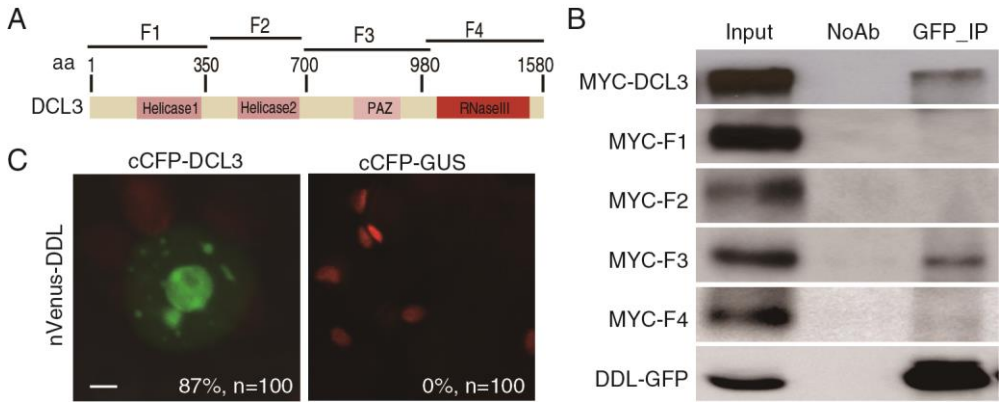


Figure 2. DDL is required for transgene induced siRNA accumulation. A. Histochemical staining of GUS in plants containing the *L1* locus. Wild-type (*DDL*$^+$) and *ddl-1* containing the *L1* locus were segregated from the sixth generation of a recombined inbred line through repeated five generations 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.



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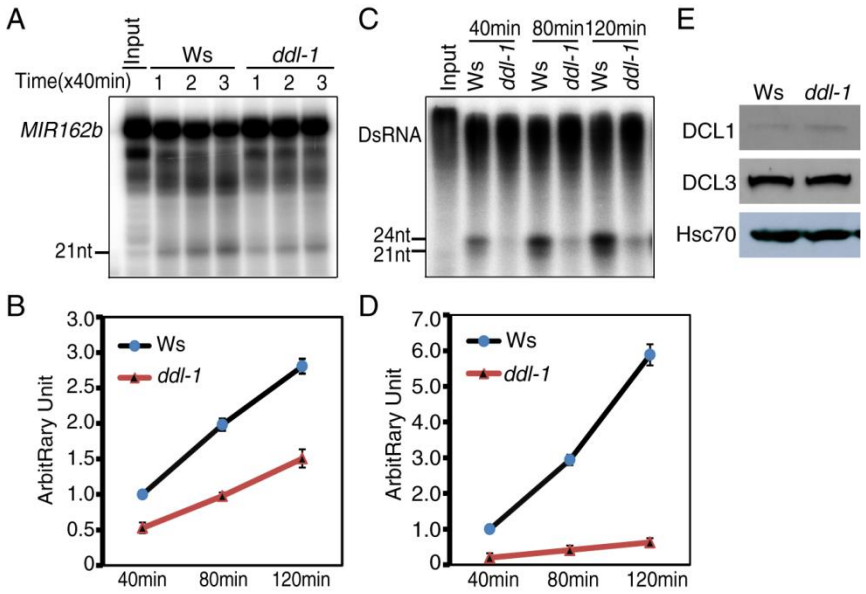

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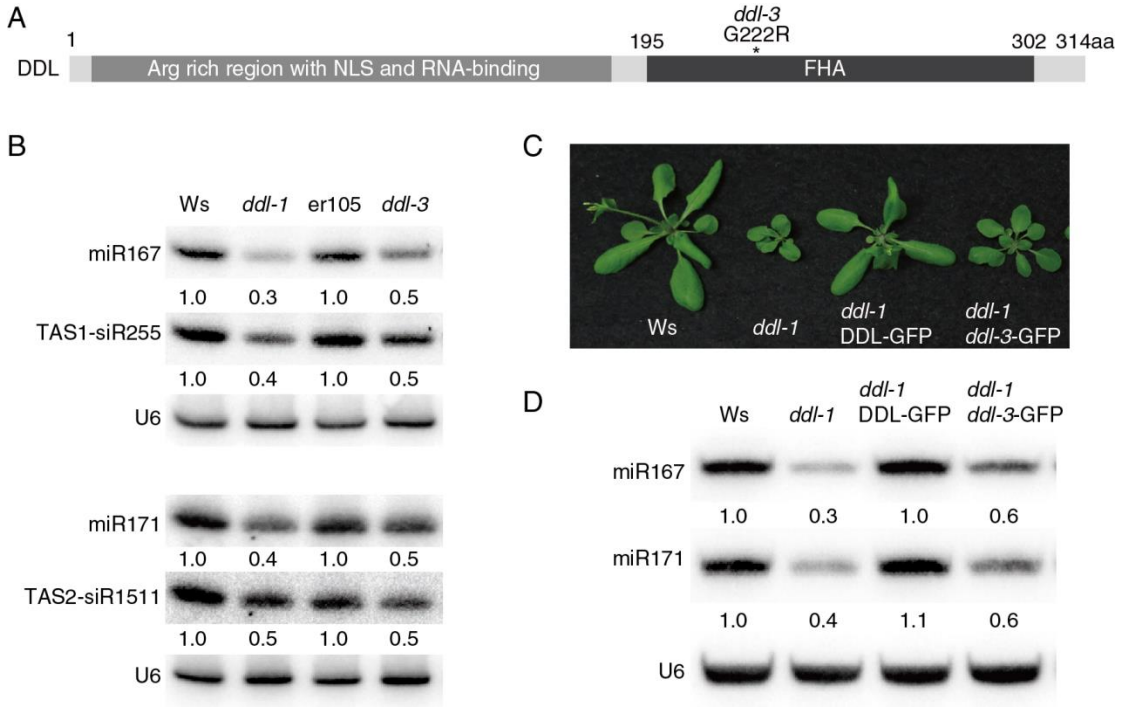

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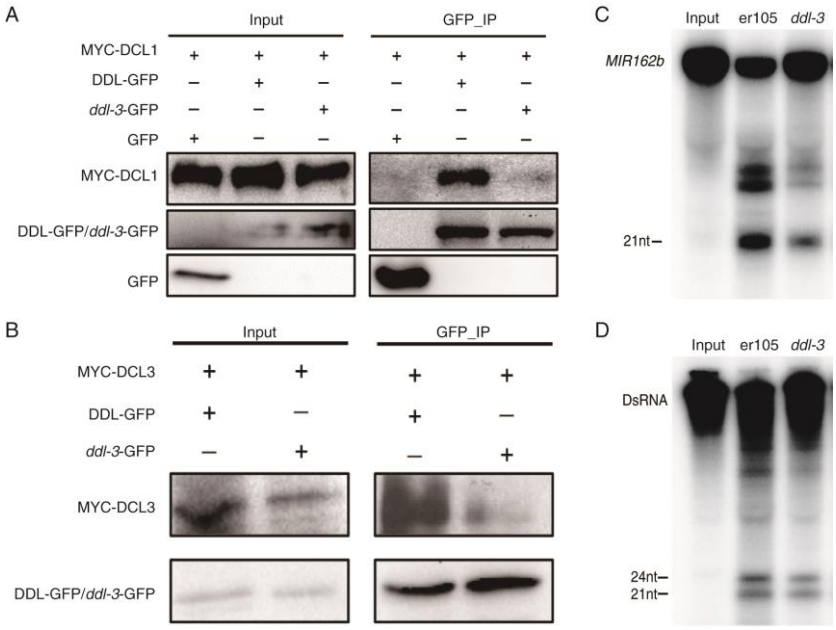

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