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Identification of Nuclear Dicing Bodies Containing Proteins for MicroRNA Biogenesis in Living *Arabidopsis* Plants

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Abstract

MicroRNAs (miRNAs) are important for regulating gene expression in muticellular organisms. MiRNA processing is a two step process, in animal cells the first step is nuclear and the second step cytoplasmic, whereas in plant cells both steps occur in the nucleus via the enzyme Dicer-like1 (DCL1) [1,2] and other proteins including the zinc finger domain protein Serrate (SE) [3,4] and a double-stranded RNA (DsRNA) binding domain protein Hyponastic Leaves1 (HYL1) [5–7]. Furthermore, plant miRNAs are methylated by Hua Enhancer (HEN1) at their 3' ends [8] and loaded onto Argonuate1 (AGO1) [9]. However, little is known about the cellular basis of miRNA biogenesis. Using live-cell imaging, we show here that DCL1 and HYL1 colocalize in discrete nuclear bodies in addition to being present in a low level diffuse nucleoplasmic distribution. These bodies, which we refer to as nuclear dicing bodies (D-bodies), differ from Cajal bodies [10,11]. A mutated DCL1 with impaired function in miRNA processing fails to target to D-bodies, and an introduced pri-miRNA transcrpt is recruited to D-bodies. Furthermore, bi-molecular fluorescence complementation (BiFC) shows that DCL1, HYL1 and SE interact in D-bodies. Based upon these data we propose that D-bodies are crucial for orchestrating pri-miRNA processing and/or storage/assembly of miRNA processing complexes in the nuclei of plant cells.

Results and discussion

To determine the subnuclear organization of proteins involved in miRNA biogenesis, we generated transgenic *Arabidopsis* plants expressing yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) -tagged SE, DCL1 and HYL1, each under the control of their endogenous promoters. The functionality of each fusion protein was determined by complementation of mutant phenotypes (Figure 1A) and restoration of mature miR173 level (Supplementary Figure S1). DCL1 was enriched in round nuclear bodies measuring 0.2–0.8μm in diameter as well as being diffusely distributed throughout the nucleoplasm. The number of nuclear bodies present in each nucleus ranged from 0 (12%), 1 (58%), 2 (23%), 3 (8%) to 4 (1%) (total nuclei = 350). A population of DCL1 bodies (~60%) localizes in close proximity to, but not within, nucleoli (Figure 1B). HYL1 displayed a similar localization pattern to DCL1, except that its signal was normally stronger in old plants. A previous study also showed that transiently expressed HYL1 localized in discrete nuclear foci as well as forming a ring structure [5]. Unlike DCL1 and HYL1, SE was distributed in a heterogeneous subnuclear pattern in a cell-type dependent manner (Figure 1B). This pattern was reminiscent of nuclear speckles or interchromatin granule clusters (IGCs) in which serine/arginine (SR)

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splicing factors are enriched [10,12]. To further determine the nature of the subnuclear domains of SE localization, we co-infiltrated HcRed-SE and SR33-YFP [12], and a colocalization of these two proteins was observed (Figure 1E).

To more precisely examine the relative localization of DCL1, HYL1 and SE we generated transgenic plants co-expressing DCL1-YFP/HYL1-CFP or YFP-SE/ HYL1-CFP by crossing corresponding complemented lines. DCL1 bodies were found to colocalize fully with HYL1 bodies indicating that they are one and the same structure (total nuclei = 165) (Figure 1C). In contrast, the SE signal was present in HYL1 bodies in only a small population of cells (~20%, total nuclei = 125), in addition to its nucleoplasmic distribution (Figure 1D), implicating a more transient role of SE in DCL1/HYL1 containing bodies. SE predominantly localizes to nuclear speckles and apart from the miRNA pathway, SE mutants exhibit phenotypes similar to a mutant of the mRNA cap-binding protein gene *abh1* (Figure 1A) [13], implicating SE in a broader role in RNA metabolism.

The round morphology, size, and the average number per nucleus of the DCL1/HYL1 containing bodies are largely different from previously identified cyclophilin, phytochrome or abscisic acid-activated protein kinase-containing domains and nuclear speckles [10,12]. However, the DCL1/HYL1 containing bodies resemble Cajal bodies (CBs) in their round shape and frequent perinucleolar distribution [10,11]. CBs contain components involved in small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) processing/assembly [14,15]. Interestingly, plant CBs have also been shown by immunolocalization to contain components involved in small interfering RNA (siRNA) directed cytosine methylation at endogenous DNA repeats [16,17]. To compare the localization between DCL1/HYL1 containing nuclear bodies and CBs, we generated a signature marker for CBs by tagging the *Arabidopsis* coilin homologue Atcoilin [11] with a fluorescent protein and expressing it under the control of its endogenous promoter. The tagged Atcoilin was able to restore the formation of CBs in a non-Cajal body mutant, *ncb-1*, in which a single base change at a splice site of Atcoilin leads to total disassembly of CBs [11] (Figure 2A). In co-tranformed plants expressing HYL1-CFP and Atcoilin-YFP, HYL1 nuclear bodies were observed to be clearly distinct from CBs in several different cell types (Figure 2B). Moreover, in the background of *ncb-1*, HYL1-CFP forms nuclear bodies distributed among a diffuse nucleoplasmic U2B”-GFP signal (Figure 2C). Based upon these data we conclude that DCL1/HYL1-containing bodies are not CBs and we refer to them as nuclear dicing bodies or D-bodies. Time-lapse microscopy of D-bodies in living *Arabidopsis* plants shows that D-bodies move in the nuclei in a constrained manner (Supplementary Figure S2 and videoS1). Recently, Song et al. reported by transient expression that HYL1 and DCL1 colocalize in a nuclear body distinct from the Cajal Body in *Arabidopsis* protoplasts [18].

Since we identified several proteins essential for miRNA processing to concentrate in D-bodies, we were interested in determining if D-bodies are involved in miRNA biogenesis. First, we compared the localization pattern of wild-type DCL1 with the mutated DCL1: DCL1–9, in which the C-terminal 73 amino acids were truncated by T-DNA insertion, this region corresponds to the second DsRNA binding domain [19]. This short truncation results in severe defects in biogenesis of most miRNAs [20]. DCL1-YFP (~240 KDa) and DCL1–9-YFP (~230 KDa) were expressed as fusion proteins that exhibited mobilities similar to their predicted sizes as indicated by immunoblotting using an anti-GFP antibody (Figure 3A, lower panel, more intense upper band). We observed that DCL1–9 fails to localize to D-bodies, but instead is diffusely distributed in the nucleoplasm upon transient expression (Y. F. & D.L.S., unpublished) and in transgenic lines (Figure 3A), demonstrating that the second DsRNA binding domain of DCL1, which is involved in protein-protein interaction [20], is required for its localization to D-bodies.

To directly track a specific pri-miRNA in the nucleus, an amplified 245 bp genomic DNA fragment flanking mi173 was fused upstream to 24 tandem MS2 translational operators (MS2 repeats) and introduced into tobacco cells by co-infiltration with MS2 coat protein-YFP (MS2-YFP) [21–23] and DCL1-CFP fusion proteins. The introduced pri-miR173 was processed to a mature miR173 as indicated by a small RNA blot (Supplementary Figure S3A). To control the expression level of the MS2-YFP protein in plant nuclei, an ethanol-inducible gene expression system was used in vector construction [24]. Upon induced expression of MS2-YFP, its binding with the MS2 repeats allows the pri-miRNA to be localized in the cells, while DCL1-CFP under the control of its endogenous promoter was used as a marker for D-bodies. Pri-miR173 was observed in D-bodies in addition to being present in a diffuse distribution in the nucleoplasm (Figure 3B). In contrast, when DNA fragments containing only MS2-YFP or MS2-YFP with MS2 repeats were infiltrated into tobacco leaf cells, MS2-YFP was not enriched in D-bodies (Figure 3B) (See Supplementary Table 1 for quantitative analysis). The expression of MS2 repeats under the control of the CaMV 35S promoter was confirmed by reverse transcriptase (RT)-PCR (Supplementary Figure S3B). These data indicate that the pri-miRNA is recruited to D-bodies where the machinery for their processing is enriched.

The protein-protein interaction between DCL1 and HYL1 has been shown to be important for pri-miRNA processing [20]. In addition, interaction between SE and HYL1 was previously identified in a yeast two-hybrid assay [3,4]. Native gel electrophoresis detected HYL1 in a ~300 KDa complex [5], while biochemical fractionation identified DCL1 in a larger complex of >660 KDa [25]. If D-bodies are the nuclear sites for the dicing reaction of DCL1, protein-protein interaction of the respective proteins (DCL1 and HYL1) should occur within D-bodies. We used bimolecular fluorescence complementation (BiFC) to determine the locations in the cell nuclei where these proteins interact [26]. Protein partners were fused to N- or C-terminal fragments of YFP respectively, and introduced into tobacco cells by infiltration. Protein-protein interaction between the tester proteins results in the proper folding of YFP and its subsequent fluorescence. However, one cannot exclude the possibility of interaction between proteins based on negative BiFC results. BiFC signals between DCL1, HYL1 and SE were observed in discrete foci while the signal in the nucleoplasm was very low (Figure 3C), suggesting that these proteins interact in the nuclear D-bodies. In addition, DCL1 and HYL1 self-interact in the D-bodies, while SE self-interacts in nuclear speckles. By contrast, DCL1–9 showed no BiFC signals with SE, HYL1 or DCL1 (Figure 3C) (See Supplementary Table 2 for quantitative data). Together, these data implicate a role of D-bodies in pri-miRNA processing. However, given that DCL1, HYL1 and the MS2 repeats-tagged pri-miRNA is also distributed diffusely in the nucleoplasm, although at low levels, we cannot exclude the possibility that D-bodies may serve as storage/assembly sites for miRNA processing proteins or protein complexes. In this scenario, these proteins would be recruited from D-bodies to sites of miRNA processing.

Plant miRNA/miRNA* duplexes generated by DCL1 are methylated by HEN1 at their 3’ ends [8], and then the miRNA strand is preferentially loaded onto the RNA-induced silencing complex (RISC) containing the slicer AGO1 to degrade target RNAs [1,9]. HEN1 and AGO1 localize both in the nucleus and the cytoplasm (Supplementary Figure S4A). When HEN1-YFP and YFP-AGO1 were transiently coexpressed with HYL1-CFP, HEN1 and AGO1 exhibited some localization to D-bodies, however, a large nucleoplasmic signal is observed (Supplementary Figure S4B). It is of future interest to determine in living plants if HEN1 and AGO1 are recruited to D-bodies to methylate miRNA/miRNA* duplexes and load mature miRNAs. In *Drosophila*, the asymmetry of a siRNA duplex is sensed by the Dicer2–R2D2 heterodimer [27]. We observed BiFC between HYL1 (a R2D2 homlog) and AGO1 (Figure 3D) (See Supplementary Table 3 for quantitative data), raising the possibility that an analogous mechanism, involving DCL1 and HYL1, operates to load the plant miRNAs onto AGO1 to form the initial RISC complex in D-bodies.

Mutation of a single component of D-bodies (DCL1, HYL1 or SE) does not result in a disruption of these bodies (Supplementary Figure S5). Given that DCL1, HYL1 and SE interact with each other in D-bodies, it is reasonable that depletion of one component of the pri-miRNA processing machinery doesn't affect the integrity of D-bodies. Moreover, potential redundancies of HYL1-like and Dicer-like proteins make it difficult to fully elucidate the formation of D-bodies. Additionally, it is presently unclear if D-bodies are involved in other small RNA pathways in plants. In this regard, we have observed full colocalization between HYL1 and its homolog DRB4 in D-bodies in DRB4-YFP/HYL1-CFP co-transformed lines (Y. F. & D.L.S., unpublished). DRB4 is known to interact with DCL4 *in vivo* [28], and is involved in the biogenesis of trans-acting siRNAs and virus-induced siRNAs [28–33]. Apart from miRNAs, DCL1 has also been shown to be involved in the processing of endogenous inverted repeats and endogenous siRNAs induced by bacterial pathogens [34,35]. We anticipate that D-bodies may be involved in these small RNA pathways, and serve an important role for plant development and defense.

In summary, we have characterized the localization and in vivo interaction of a series of proteins involved in miRNA processing and found that DCL1 and HYL1 colocalize in discrete nuclear bodies in living *Arabidopsis* plants that we refer to as D-bodies. Based upon protein-protein interaction and the localization of an introduced pri-miRNA transcript, we suggest that D-bodies are involved in pri-miRNA processing. Our results provide significant insight into the organization of pri-miRNA processing machinery in the cell nucleus of living plants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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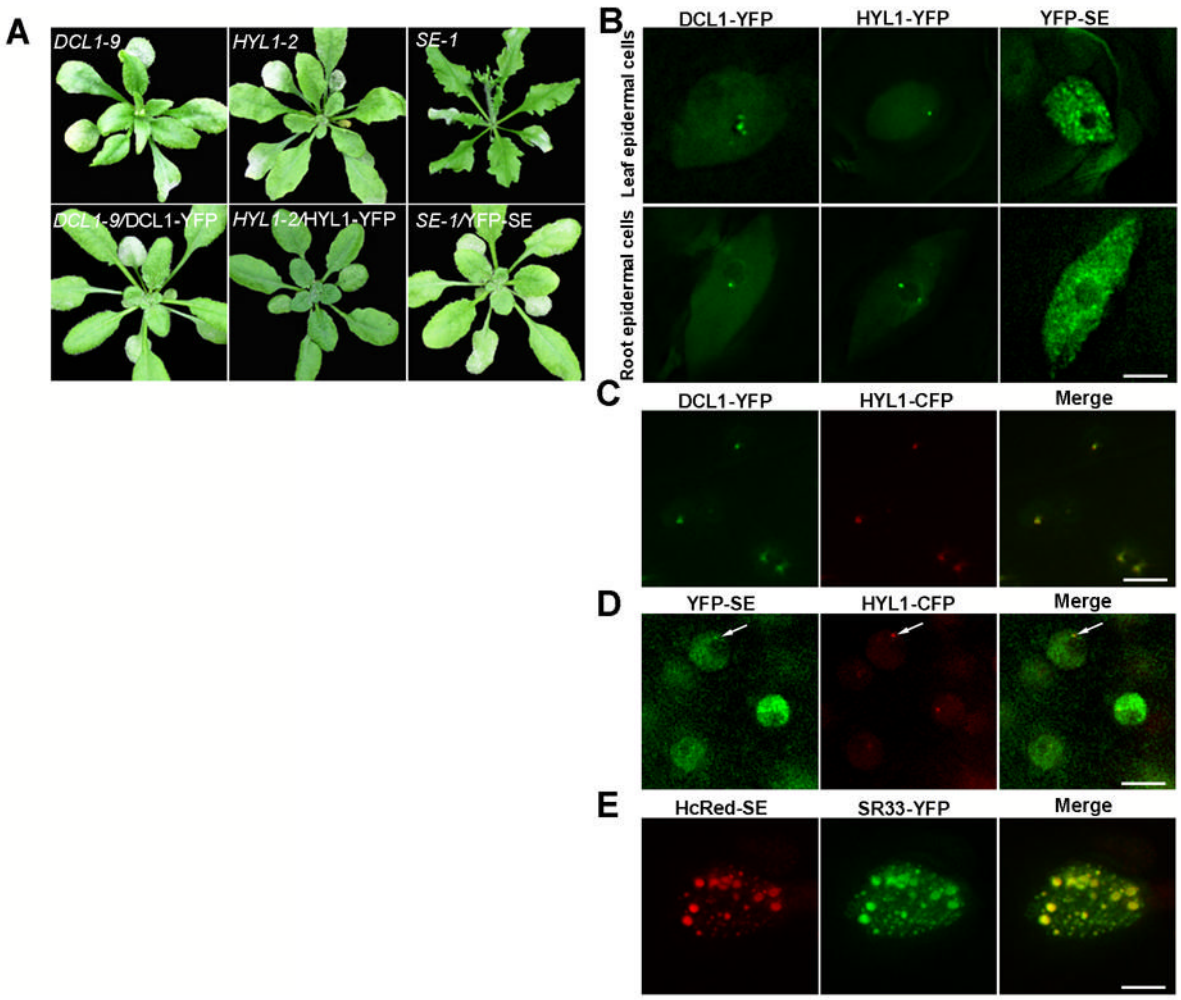

Figure 1. Subnuclear localization and colocalization of DCL1, HYL1 and SE

(A) Introduced fusion proteins of DCL1-YFP, HYL1-YFP and YFP-SE complemented *DCL1–9*, *HYL1–2* and *SE-1* mutants, respectively.

(B) DCL1 and HYL1 localize in discrete nuclear foci both in leaf and root cells, while SE localizes in nuclear speckles.

(C) DCL1-YFP (green) fully colocalizes with HYL1-CFP (red), cells in root tip are shown.

(D) YFP-SE (green) partially colocalizes with HYL1-CFP (red), cells in root tip are shown.

(E) HcRed-SE and SR33-YFP were transiently co-expressed in tobacco leaf cells. Colocalization of HcRed-SE (red) with SR33-YFP (green) was observed in nuclear speckles. Scale bars, 5μm.

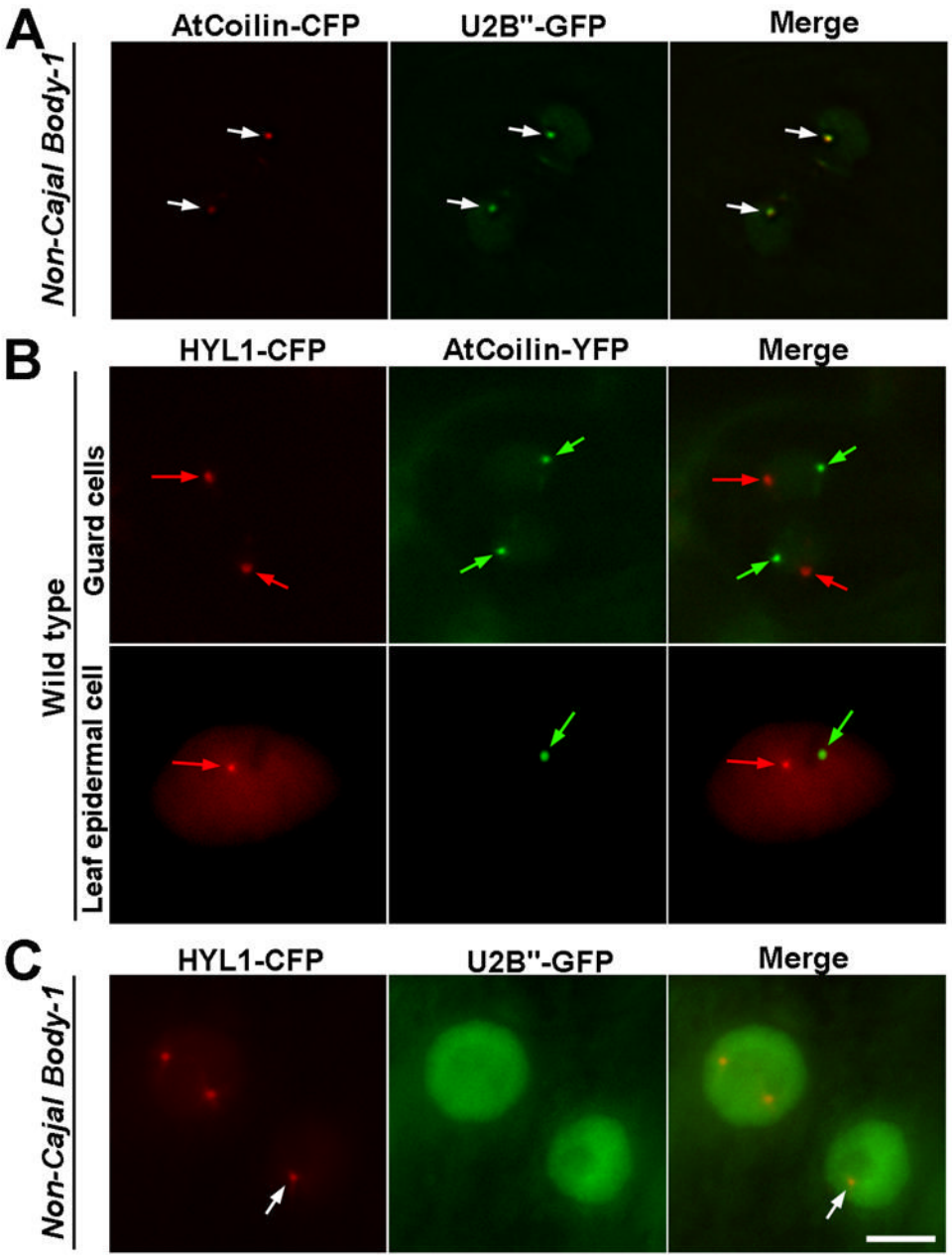

Figure 2. DCL1 and HYL1 containing nuclear bodies do not correspond to Cajal bodies

(A) Restoration of Cajal bodies by introduction of an AtCoilin-CFP (red) fusion into *non-Cajal bodies-1* mutant expressing U2B"-GFP (green). A pair of guard cells is shown in the image.

(B) DCL1and HYL1-containing nuclear bodies were labeled with HYL1-CFP (red) and Cajal bodies were labeled with AtCoilin-YFP (green), HYL1 foci do not colocalize with Cajal bodies as shown in guard cells (upper panel) and a leaf epidermal cell (lower panel).

(C) In *non-Cajal bodies-1* mutant, U2B"-GFP (green) is diffusely distributed in the nucleoplasm, while HYL1-CFP (red) is localized to discrete nuclear bodies. Scale bar for all images, 5μm.

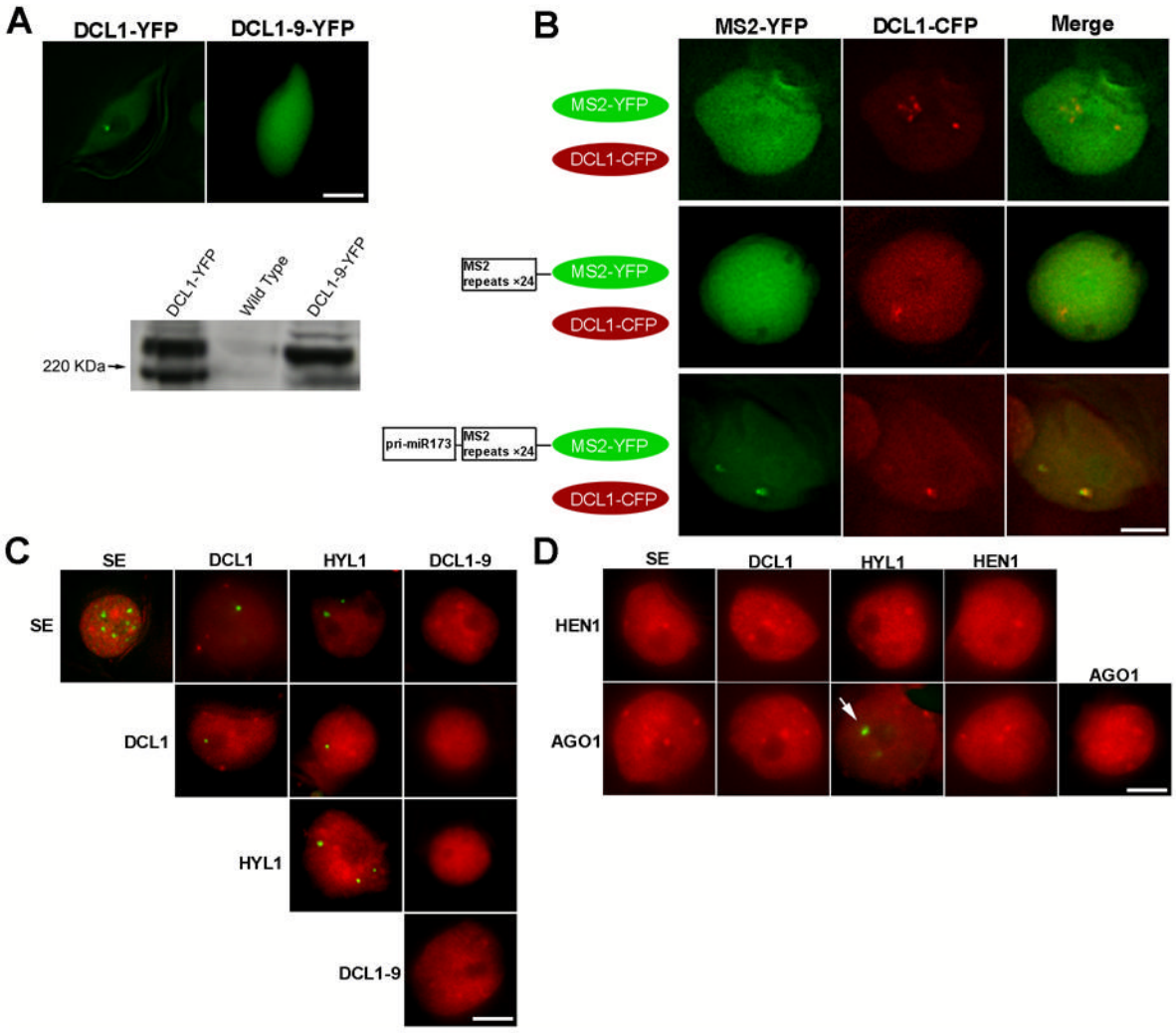

Figure 3. D-bodies are involved in pri-miRNA processing

(A) DCL1-YFP localizes to D-bodies, while DCL1-9-YFP is diffusely distributed in the nucleoplasm. Representative nuclei of leaf epidermal cells are shown in upper panel. Lower panel shows anti-GFP immunoblot of extracts from plants expressing DCL1-YFP and DCL1–9-YFP (upper more intense bands), and wild type plants with no tagged protein. The lower band in lanes 1 and 3 may represent a commonly observed degradation product.

(B) Genomic DNA flanking miR173 was fused to MS2 repeats, and coinfiltrated to tobacco leaves with MS2-YFP and DCL1-CFP. The miR173 transcript tracked by MS2-YFP (green) was observed to be recruited to DCL1-CFP foci (red, lower panel). In contrast, in nuclei expressing only MS2-YFP (upper panel) or MS2-YFP and MS2 repeats (middle panel), DCL1-CFP bodies (red) were distributed among a diffuse MS2-YFP signal (green).

(C) Pair-wise BiFC experiments between SE, DCL1, HYL1, and DCL1–9. Protein partners was fused to an N-terminal fragment or C-terminal fragment of YFP, respectively, and co-infiltrated into tobacco leaves. Hoechst 33342 was used to label the nuclei (red). BiFC signals between SE, DCL1 and HYL1 were observed in D-bodies (green). No BiFC signals were observed between DCL1–9 and SE, DCL1 or HYL1.

(D) Pair-wise BiFC experiments between SE, DCL1, HYL1, and HEN1, and AGO1. Interaction between HYL1 and AGO1 is observed in the D-body (arrow). Scale bars, 5μm.