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The Nuclear dsRNA Binding Protein HYL1 Is Required for MicroRNA Accumulation and Plant Development, but Not Posttranscriptional Transgene Silencing

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Summary

MicroRNAs (miRNAs) are 21–24 nucleotides long molecules processed from imperfect double-stranded RNAs (dsRNAs). They regulate gene expression by targeting complementary mRNA for cleavage or interfering with their translation [1–6]. In *Arabidopsis*, point mutations in or short truncations of the nuclear DICER-LIKE1 (DCL1) or HEN1 protein reduce miRNA accumulation and increase uncleaved target mRNAs accumulation, resulting in developmental abnormalities [7–12]. Here, we show that miRNA accumulation also depends on the activity of HYL1, a nuclear dsRNA binding protein [13]. *hyl1* mutants exhibit developmental defects overlapping with that of *dcl1* and *hen1* mutants, suggesting that DCL1, HEN1, and HYL1 act together in the nucleus. We validate additional target mRNAs and show that reduced miRNA accumulation in *hyl1* correlates with an increased accumulation of uncleaved target mRNAs, including meristem- and auxin-related genes, providing clues for the developmental abnormalities of *hyl1* and for the previous identification of *hyl1* as a mutant with altered responses to phytohormones [13]. Lastly, we show that posttranscriptional transgene silencing occurs in *hyl1*, suggesting that HYL1 has specialized function in the plant miRNA pathway, whereas the HYL1-related RDE-4 and R2D2 proteins associate with DICER in the cytoplasm and act in the RNAi pathway in *C. elegans* and *Drosophila*, respectively [14–15].

Results and Discussion

Developmental Defects in *hyl1* Mutants Overlap with that of *dcl1* and *hen1* Mutants

Two proteins that are required for the accumulation of miRNAs in plants have been previously identified: DCL1 and HEN1 [6–12]. *dcl1* null alleles are embryolethal [16], suggesting that DCL1 is required for miRNA-mediated regulation of plant development. Partial loss-of-function mutants, *dcl1-7, dcl1-8*, and *dcl1-9*, that carry either point mutations in the RNA helicase domain or a truncation of one of the two dsRBD are viable [16]. However, these three mutants are sterile, confirming that plant reproduction depends on DCL1 activity. The four previously identified *hen1* mutants carry either point mutations or a small truncation due to a stop codon near the C terminus of the protein. These *hen1* mutants exhibit developmental defects that overlap with that of *dcl1* partial loss-of-function mutants, although the *hen1* mutants do produce a limited number of seeds [8, 10]. Because both DCL1 and HEN1 proteins contain nuclear localization signal (NLS) and dsRNA binding domains (dsRBD), we decided to investigate if other *Arabidopsis* proteins with putative NLS and dsRBD could also play a role in miRNA-mediated regulation of development [8, 11, 16]. We therefore looked at the phenotype of *Arabidopsis* transgenic lines from the SALK and INRA collections that carry T-DNA insertions in genes encoding proteins with NLS and dsRBD. We found that plants homozygous for a T-DNA insertion in the *Arabidopsis* gene At1g09700 (insertion SALK_064863) exhibited developmental defects overlapping with that of *dcl1* and *hen1* mutants. Plants homozygous for a Ds transposon insertion mutation in the same gene were previously identified during a phenotypic screen for mutants with altered response to phytohormones, and the corresponding mutant was called *hyl1* for *hy*ponastic *l*eaves [13]. This mutant is characterized by a shorter stature, delayed flowering, leaf hyponasty, reduced fertility, decreased rate of root growth, and an altered root gravitropic response [13]. It also exhibited less sensitivity to auxin and cytokinin and hypersensitivity to abscisic acid. The original *hyl1-1* allele and the SALK *hyl1-2* allele are disrupted by Ds and T-DNA insertions, respectively, that are nearly at the same position in the first fourth of the ORF. These two alleles are therefore assumed to be null alleles. When grown side by side, they exhibit similar developmental defects, although they derive from the No and Col ecotypes, respectively (data not shown). We also identified plants homozygous for a T-DNA insertion in the *HEN1* gene (insertion SALK_049197). This newly identified *hen1-5* allele (in Col) carries a T-DNA inserted in the first half of the coding sequence, so it is likely that it is a null allele. We grew wild-type Col plants and *hen1-4*, *hen1-5*, and *hyl1-2* mutants (all in Col) side by side to compare their phenotypes. Both *hen1* and *hyl1* mutants exhibited a short stature, leaf curling, delayed flowering, and reduced fertility (Figure 1). However, the intensity of these characteristics varies between the two mutants. Short stature and leaf curling were more pronounced in *hyl1*, while delayed flowering and reduced fertility were more pronounced in *hen1*. No differences were observed between *hen1-4* and *hen1-5* (data not shown).

hyl1 Has Reduced miRNA Accumulation

A reduction in the accumulation of miRNAs has been reported in *dcl1* and *hen1* mutants [7–12]. To determine

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Figure 1. Developmental Defects in *hen1* and *hyl1* Single Mutants and in the *hen1 hyl1* Double Mutant

From left to right, wild-type plants (Col), *hyl1-2* (SALK_064863), and *hen1-5* (SALK_049197) single mutants and the *hen1 hyl1* double mutant grown under long day conditions. From top to bottom, photographs show plants after 10 and 20 days of growth in vitro and 15 days after their transfer to greenhouse for further growth. Closeup of flowers and stems were taken 40 days (Col) or 60 days (*hyl1*, *hen1*, *hyl1hen1*) after transfer to the greenhouse. The *hen1* and *hyl1* single mutants had curling leaves and were late flowering. They showed abnormal flowers that were barely fertile (arrows indicate siliques containing seeds). The double mutant showed stronger developmental defects. It remained dwarf and no stem developed. It had sterile flowers and eventually died without producing any progeny.

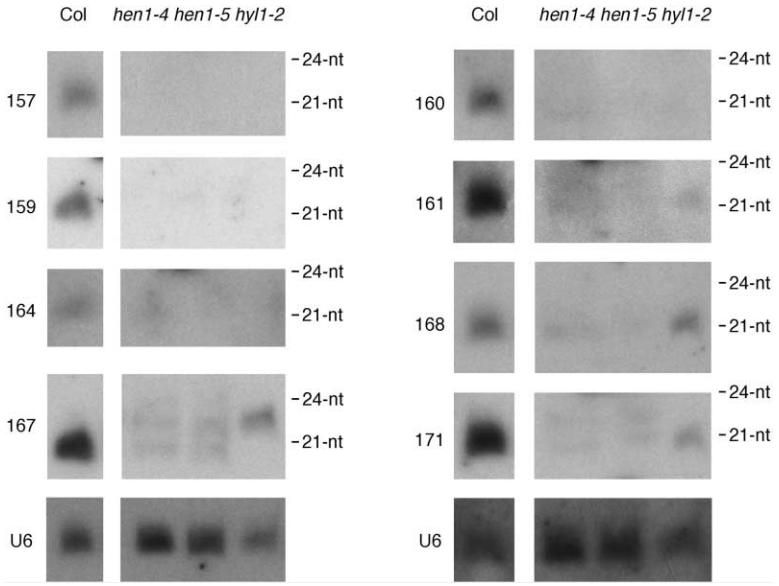

Figure 2. miRNA Accumulation in *hen1* and *hyl1* Mutants

RNA gel blot analysis of 10 μg of LMW RNA extracted from flowers of wild-type plants, *hen1-4*, *hen1-5*, and *hyl1-2* mutants (all in the Col ecotype). Two blots were made (left and right) that were successively hybridized, stripped, and rehybridized to specific miRNA probes. Blots were finally hybridized to a U6 probe. Rehybridization of the blot on the right with a probe complementary to miR167 revealed a shift to upper molecular weight in *hyl1* similar to that observed with the blot on the left.

if the reduction in *hen1-5* was similar to that in *hen1-4* and if the developmental abnormalities observed in the *hyl1-2* also correlate with a defect in miRNA accumulation, we extracted small RNAs from flowers of wild-type, *hen1-4, hen1-5*, and *hyl1-2* plants (all in the Col ecotype) and analyzed the accumulation of eight *Arabidopsis* miRNAs (Figure 2). A similar reduction in accumulation of every tested miRNA was observed in the *hen1-4* and *hen1-5* alleles (point mutation and T-DNA insertion respectively). This reduction was strong; however, low levels of miR161, miR164, miR167, miR168, and miR171 were still detectable in these mutants, as previously observed for miR167, miR171, miR172, and miR173 [8, 10, 12]. This suggests either that HEN1 is not as important as DCL1 for proper development (at least under nonstressful conditions of growth) or that other genes, like At4g20920 (which is predicted to encode a protein 82% homologous to HEN1), may partially compensate for the defect in HEN1. Furthermore, the presence of an additional band of higher molecular weight was observed for miR167 and miR171, as previously reported for these miRNAs in *hen1* mutants or in plants expressing the viral HC-Pro protein [10, 12, 17]. miR157, miR159, miR160, and miR164 were below detectable levels in the *hyl1-2* allele, whereas miR161, miR167, miR168, and miR171 can still be detected, thus possibly providing clues for the milder phenotype of *hyl1* compared with *hen1*. This limited reduction may be due to either a less drastic requirement for HYL1 in the miRNA pathway or to (partial) redundancy of HYL1 activity with some other protein(s). Interestingly, a band of upper molecular weight corresponding to miR167 was also reproducibly observed in *hyl1* (Figure 2 and data not shown), suggesting that HEN1 and HYL1 could participate in the control of the miRNA size.

At1g27370/*SPL10*, At1g06580/*PPR*, and At1g48410/*AGO1* Are Targets of miR156/157, miR161, and miR168, Respectively

The animal miRNAs that have been tested repress translation by binding to multiple segments of imperfect complementarity in the 3′UTR of target mRNAs, whereas plant miRNAs mostly trigger cleavage of target mRNAs by binding to unique segments of near-perfect complementarity within the coding region [3–6, 18] This cleavage was revealed by the identification of RNAs with 5′ ends that coincide with the middle of the miRNA sequence. This was found for 22 target miRNA corresponding to nine miRNAs [4–6]. To test if the reduced miRNA accumulation observed in *hyl1*, *hen1*, and *dcl1* mutants has an effect on the steady-state levels of uncleaved miRNA target mRNAs, we decided to perform real-time quantitative PCR (RT-qPCR) analysis on one target per miRNA analyzed. Although At5g43270/*SPL2* has been validated as a target of miR156/157, the increase in the accumulation of uncleaved target mRNA observed in *dcl1* mutants was very low [9]. In addition, targets have been predicted for miR161 and miR168, but they have not been validated yet [18]. We thus decided to perform 5′ RACE on At1g27370/*SPL10*, At1g06580/*PPR* and At1g48410/*AGO1* to validate targets for miR156/157, miR161, and miR168. Sequencing of 5′ RACE products clearly indicated that these three mRNAs are actual targets of the corresponding miRNAs (data available in the Supplemental Data), thus bringing the total number of validated miRNA targets to 25 and the number of miRNA families having validated targets to 11 out of 15 [4–6].

hyl1, *hen1*, and *dcl1* Mutants Show Increased Accumulation of Uncleaved Target mRNAs

RT-qPCR was performed on RNA extracted from flowers of wild-type Col plants and *hen1-4* and *hyl1-2* mutants (both in Col) using primers located on both side of the cleavage site on the mRNA so the steady-state level of uncleaved mRNA could be quantified. Flower RNA from wild-type Ler plants and *dcl1-9* mutants (in L*er*) was used as a control. RT-qPCR was performed on the following miRNA-mRNA pairs: miR156/157-At1g27370/*SPL10*, miR159-At3g11440/*MYB65*, miR160-At1g77850/*ARF17*, miR161-At1g06580/*PPR*, miR164-At5g53950/

CUC2, miR167-At5g37020/*ARF8*, miR168-At1g48410/*AGO1*, and miR171-At3g60630/*SCL6-III*. The entire experiment (RNA extraction, reverse transcription, and quantitative PCR) was done twice using the same plant material used for the miRNA accumulation analysis. For the eight targets analyzed, the steady-state level of uncleaved mRNA was higher in *dcl1-9* and *hen1-4* mutants compared with L*er* and Col wild-type plants, respectively (Figure 3), thus confirming that the strong decrease in miRNA accumulation observed in these mutants [7–12] reduces mRNA cleavage. The increase in the steady-state level of uncleaved target mRNA was always less in *hyl1* compared with *hen1* and *dcl1* (Figure 3), a result that is consistent with the limited reduction in miRNA accumulation (Figure 2) and the milder phenotype of this mutant (Figure 1). In the case of At1g06580/*PPR*, At5g37020/*ARF8*, and At1g48410/*AGO1*, there was no difference between Col and *hyl1*, suggesting that the residual amount of miR161, miR167, and miR168 observed in this mutant (Figure 2) is sufficient to maintain efficient cleavage of the corresponding targets. Interestingly, different steady-state mRNA levels of the meristem-related genes *SPL10, CUC2,*and *AGO1* were observed in the three mutants, providing clues for the range of flower phenotypes. In addition, the upregulation of *ARF17* mediating auxin response observed in *hyl1* provides clues for the previous identification of *hyl1* as a mutant with altered responses to phytohormones [13].

The *hyl1 hen1* Double Mutant Is Unable to Complete a Life Cycle

As opposed to *dcl1* null alleles that are embryolethal [16], *hen1* and *hyl1* null alleles are viable and can be propagated in a homozygous state, although their fertility is strongly compromised (Figure 1). We thus decided to look at the phenotype of the *hyl1 hen1* double mutant. Plants exhibited strongly reduced organ size. Leaf curling was enhanced compared to *hyl1* and *hen1* single mutants. Plants developed abnormal flowers with fused sepals and reduced or no petal and stamen formation or outgrowth. No stem developed and the plants remained dwarf. Unlike *hyl1* and *hen1* single mutants, the *hyl1 hen1* double mutants were sterile, so the plants eventually died without producing any progeny. Together, these results suggest that the coordinated action of *HEN1* and *HYL1* is required to complete a life cycle.

Posttranscriptional Transgene Silencing Occurs in *hyl1* Mutants

Through a forward genetic screen, we previously identified 44 *Arabidopsis* mutants deficient in sense posttranscriptional gene silencing (S-PTGS) triggered by the *35S-GUS* transgene carried at the L1 locus. These 44 mutants were classified in six complementation groups: *sgs1*, *sgs2*, *sgs3*, *ago1*, *hen1*, and *met1* [10]. We did not pick up *hyl1* mutants in this screen, suggesting that HYL1 is not required for S-PTGS. To test this hypothesis, the *hyl1* mutation was introduced into the L1 line (data available in the Supplemental Data). Plants homozygous for both the L1 locus and the *hyl1* mutation exhibited GUS activity and GUS mRNA levels similar to that of the

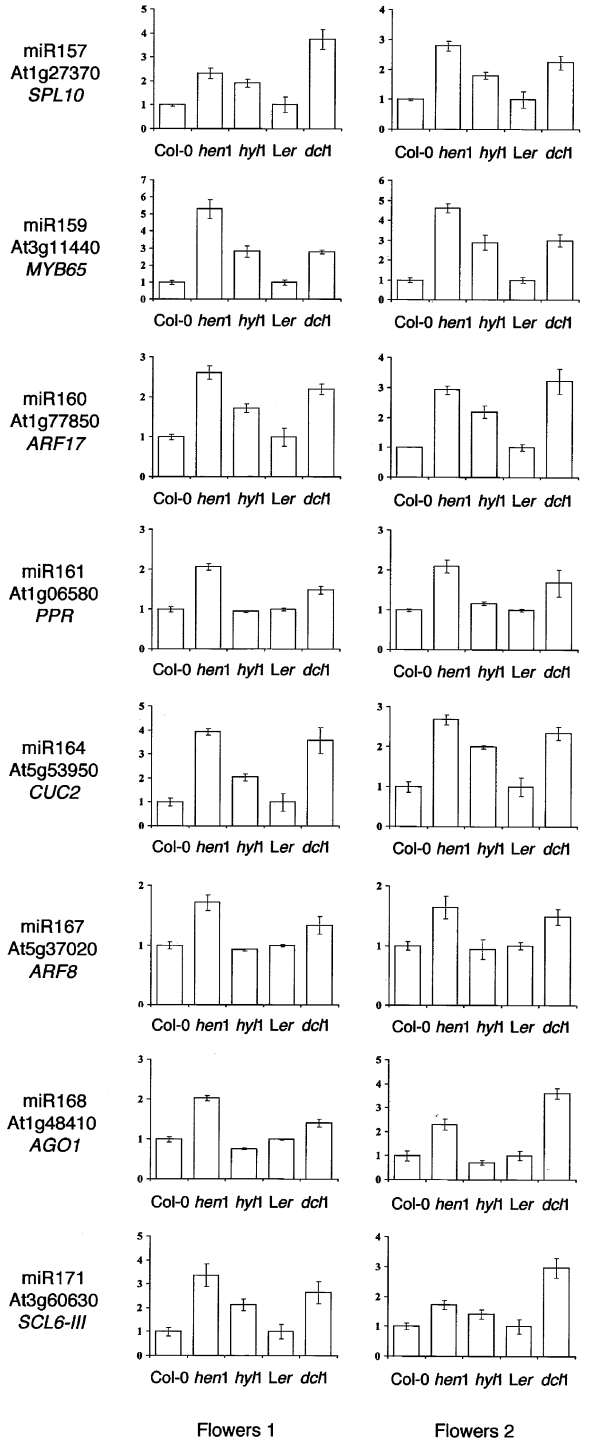

Figure 3. miRNA Target mRNA Accumulation in *hen1*, *hyl1*, and *dcl1* Mutants

HMW RNA extracted from flowers of Col, *hen1-4, hyl1-2*, L*er*, and *dcl1-9* was quantified for *SPL10, MYB65, ARF17, PPR, CUC2, ARF8, AGO1*, and *SCL6-III* mRNA relative accumulation by real-time quantitative PCR by using primers surrounding the cleavage site on two cDNA synthesis (Flowers 1 and Flowers 2). Quantifications are normalized with *ACTIN2*. The Col and L*er* values are arbitrarily fixed to one. miRNA numbers and gene names corresponding to each pair are given.

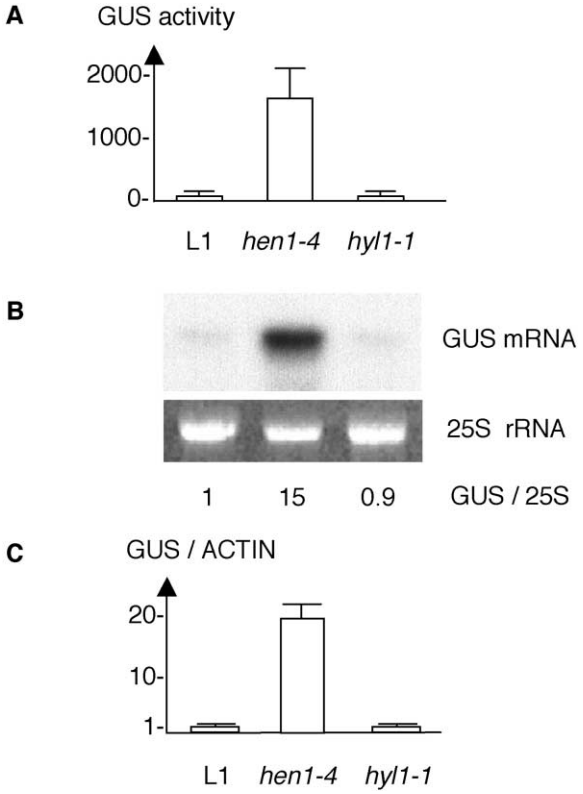

Figure 4. GUS Activity and *GUS* mRNA Accumulation in Wild-Type and Mutant Plants

(A) GUS activity in leaves of L1 plants triggering S-PTGS and of *hen1-4* and *hyl1-1* mutants homozygous for the L1 locus. The average activity of eight measurements in eight independent plants is given. Deviation bars correspond to standard errors.

(B) *GUS* mRNA relative accumulation estimated by Northern blot. mRNA extracted from leaves was hybridized with a *GUS* DNA probe. Ethidium bromide-stained gel is shown for standardization. The ratio between GUS and 25S signals is indicated.

(C) *GUS* mRNA relative accumulation estimated by RT-qPCR. Quantifications are normalized with *ACTIN2*. The L1 value is arbitrarily fixed to one.

silenced L1 line (Figure 4), indicating that HYL1 is not required to trigger efficient S-PTGS.

HYL1 Is Homologous to, but Not Functionally Similar to, *C. elegans* RDE-4 and *Drosophila* R2D2

Arabidopsis HYL1 shares homology with the dsRNA binding domain of *C. elegans* RDE-4 and *Drosophila* R2D2 proteins. RDE-4 and R2D2 associate with DICER in the cytoplasm and have been suggested to facilitate the loading of siRNAs into the RNA-initiated silencing complex (RISC) of the RNA interference (RNAi) pathway [14, 15]. Although the contribution of RDE-4 and R2D2 to the miRNA pathway has not been analyzed, it is likely that they have specialized functions in the RNAi pathway. Indeed, *rde-4* and *r2d2* mutants are defective in RNAi but do not exhibit developmental defects. Conversely, *hyl1* mutants trigger efficient PTGS but exhibit developmental defects that correlate with reduced accumulation of miRNAs and elevated levels of target mRNAs. It is therefore likely that HYL1 specifically functions in the miRNA pathway. Whether another protein with HYL1-like activity is required for PTGS in plants remains to be determined.

Supplemental Data

Supplemental Data including Experimental Procedures, as well as the diagrammatic representation of miRNA target cleavage sites determined by 5′ RLM-RACE, are available at http://www.current-biology.com/cgi/content/full/14/4/346/DC1/.

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Note Added in Proof

On January 13, 2004, Han and colleagues published new work indicating that the *hyl1-1* mutant has reduced accumulation of three miRNAs, while 35S-*HYL1* transgenic plants exhibit increased accumulation of two of these three miRNAs. They also reported that *hyl1-1* has no effect on PTGS triggered by dsRNA (Han, M.H., Goud, S., Song, L., and Fedoroff, N. (2004). The Arabidopsis double-stranded RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation. Proc. Natl. Acad. Sci. USA *101*, 1093–1998. Published online: January 13, 2004. 10.1073/pnas.0307969100).