Reconstituting plant miRNA biogenesis

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The genomes of higher eukaryotes encode not only proteins but also diverse noncoding RNAs, particularly small (20- to 30-nt) regulatory RNAs (1–3). The small RNAs include microRNAs (miRNAs), siRNAs, and piwi-interacting RNAs (piRNAs) (4, 5). These small RNAs repress gene expression at the transcriptional or posttranscriptional levels and have critical functions in genome defense, growth, development, diseases, and stress responses (1, 3, 6–8). Small RNAs are classified largely on the basis of their biogenesis requirements. miRNAs arise from single-stranded primary miRNA transcripts (pri-miRNAs) that can form imperfect stem–loop structures (6) (Fig. 1). In animals, pri-miRNAs are processed in the nucleus into shorter hairpin RNAs of ≈65 nt (premiRNAs) by the microprocessor complex containing the RNaseIII enzyme Drosha and its cofactor DGCR8/Pasha, a dsRNA-binding protein (5, 9). The premiRNA is then exported to the cytoplasm, where it is further processed by another RNaseIII enzyme, Dicer, to release an ≈22-nt miRNA/miRNA* duplex (5, 9). Dicer function also requires a dsRNA-binding protein, TRBP, as a cofactor. The miRNA is loaded into the effector complex, known as RISC, to direct complementary or partially complementary mRNAs for cleavage or translational repression (5, 6). In plants, the two-step processing of pri-miRNAs into mature miRNAs occurs entirely in the nucleus and is carried out by a single RNaseIII enzyme, DCL1 (Dicer-like 1) (6). In addition to DCL1, genetic analysis revealed that HYL1, a dsRNA-binding protein, and SE, a C2H2-type zinc finger, are also required for processing pri-miRNAs and for accumulation of mature miRNAs (10–12) (Fig. 1). However, whether DCL1 alone is active in processing pri-miRNAs into miRNAs and how HYL1 and SE may function in the processing steps are not known. In this issue of PNAS, Dong *et al.* (13) reconstituted the processing of pri-miRNAs *in vitro* by using recombinant proteins and thereby provided much-needed biochemical data to explain the genetic roles of DCL1, HYL1, and SE in miRNA biogenesis in plants.

Dong *et al.* (13) constructed tagged DCL1, HYL1, and SE proteins and expressed them in insect cells by using a baculovirus vector. Unlike the animal protein Drosha, which is not active by itself on pri-miRNAs (9), purified DCL1 alone could process pri-miRNAs, premiRNAs, and dsRNAs into 21-nt small RNAs. This difference between the animal and plant enzymes may be explained by the fact that DCL1 contains two dsRNA-binding domains, whereas Drosha has only one. When the authors (13) added HYL1 or SE or both to the *in vitro* processing assay, they found that either protein could enhance the activity of DCL1 and that the two proteins have a synergistic stimulating effect. Although DCL1 alone could generate 21-nt small RNAs from pri-miRNAs at a low rate *in vitro*, when the small RNAs were cloned, only a small fraction of the small RNAs were miRNAs, whereas the rest were sequences from other parts of the pri-miRNA structure. This interesting result suggested that DCL1 is inaccurate in catalyzing the release of miRNAs from pri-miRNAs. Remarkably, HYL1 or SE could improve the accuracy. When both were present with DCL1, the accuracy was increased greatly, resulting in ≈80% of the *in vitro* processing products being miRNAs.

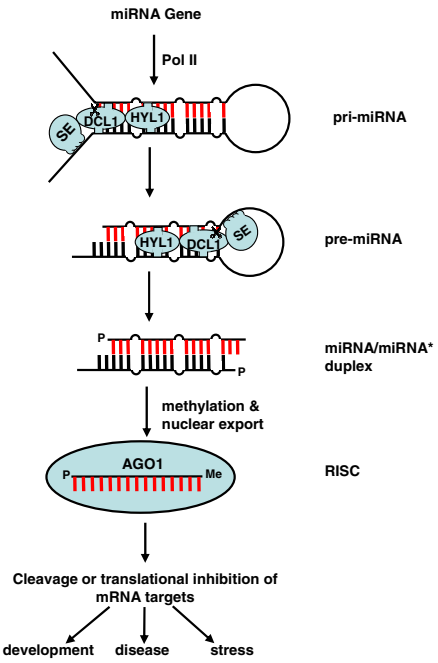

Fig. 1. miRNA biogenesis and function in plants. Primary miRNA transcript is processed by the RNaseIII enzyme DCL1 (containing two double-stranded RNA-binding domains) and its associated RNA-binding cofactors HYL1 (containing two double-stranded RNA-binding domains) and SE (a C2H2-type zinc finger) to generate a miRNA, which is then methylated, exported to the cytoplasm and incorporated into the Agonaute 1 (AGO1)-containing RNA-induced silencing complex (RISC) to silence mRNA targets important for development, diseases, and stress responses.

The importance of DCL1, HYL1, and SE in plant growth and development was evident early on from the severe and pleitropic plant phenotypes exhibited by their loss-of-function mutant alleles (10–12, 14). Only in the last 6 years has each of these genes been found to be necessary for miRNA accumulation in plants. In the *dcl1*, *hyl1*, or *se* mutants, pri-miRNA levels increase, whereas mature miRNAs are reduced (10–12). Many, if not all, of the pleitropic phenotypes of these mutants can be explained by miRNA biogenesis defects. Recently, several groups discovered that these three proteins can interact with each other *in vitro* and *in vivo*, and at least part of them colocalize in the same subnuclear bodies that also contain pri-miRNAs (15–17). The exciting work of Dong *et al.* (13) nicely explains why HYL1 and SE are required for miRNA biogenesis.

It appears that HYL1 and SE participate in both steps of pri-miRNA processing, i.e., from pri-miRNA to premiRNA and from premiRNA to miRNA (Fig. 1), although this is difficult to test vigorously, because the premiRNA intermediate does not appear to accumulate and is quickly processed to release mature miRNA. Unlike animal pri-miRNAs, which have an ≈70-nt stem–loop structure where the miRNA is always located ≈11 nt from the base of the stem–loop (9), the stem–loop structures of plant pri-miRNAs vary greatly in length (from ≈100 to >1,000 nt) (6, 18). So how does the DCL1-HYL1-SE trimeric complex recognize where the miRNA is within the stem–loop and ensure that DCL1 makes appropriate cleavages to release the correct miRNA? Gel mobility-shift assays performed by Dong *et al.* (13) suggest that each of the proteins is capable of binding pri-miRNAs (Fig. 1). However, where each protein binds and

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which sequence and/or structural features they recognize are not known. Crystal structures of each of the proteins bound to a pri-miRNA, and eventually the structures of the trimeric complex with bound pri-miRNA and premiRNA will be needed to understand the precise mechanism of accurate miRNA production.

The ≈80% miRNA processing accuracy achieved by the DCL1-HYL1-SE trimeric complex *in vitro* is still lower than the near 100% accuracy observed for most miRNAs *in vivo*. This may be because the *in vitro* conditions are not optimal, or the tagged recombinant proteins are not fully active. It is also possible that other as-yet-unidentified components may be needed to attain 100% miRNA processing accuracy. The *in vitro* reconstituted miRNA processing assay will be a powerful tool for dissecting the functional domains of each of the three proteins and for testing the biochemical function of other miRNA biogenesis components, such as the mRNA cap-binding protein ABH1 (19). This *in vitro* assay could be extended to study the other steps of the miRNA pathway, such as miRNA methylation by HEN1 (20) and miRNA loading into the effector complex. The elegant work of Dong *et al.* (13) should inspire others to reconstitute *in vitro* the processing of other types of small RNAs. *In vitro* reconstitution of miRNA- or siRNA-directed mRNA cleavage and small RNA-directed DNA methylation should also be attempted.

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