

Exploring the landscape of miRNA production and the structural rules that shape it

Degradome sequencing of semi-active DCL1 mutants separates true miRNAs from DCL1-independent small RNAs in *Arabidopsis thaliana* and illustrates the processing pattern of 147 pri-miRNAs. In parallel, DMS-MaPseq decodes the in vivo secondary structures of pri-miRNAs, enabling a better understanding of cleavage modes and of the impact of DCL1 cofactors on cleavage.

This is a summary of:

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The mission

MicroRNAs (miRNAs) are 21–22 nucleotide (nt) non-coding RNAs that govern numerous biological processes in eukaryotes. Functional miRNAs are generated by precise cleavage from their hairpin-structured primary transcripts (pri-miRNAs) by Dicer-like 1 (DCL1), which works with SERRATE (SE) and HYPONASTIC LEAVES 1 (HYL1) in plants. In metazoans, pri-miRNAs have a relatively uniform structure and contain elements that are readily measured and processed by enzymes; however, pri-miRNA processing is more complex in plants owing to the structural diversity of their pri-miRNAs. Indeed, although the processing patterns and the structural rules that govern them have been predicted or studied for some plant pri-miRNAs^{1–3}, they largely remain unexplored. Additionally, the authenticity of numerous annotated miRNAs in plants remains to be confirmed. One strategy to elucidate how genuine miRNAs are uniformly produced from diverse substrates is to systematically pinpoint the first cut sites of pri-miRNAs and their structural determinants in vivo.

The solution

DCL1 contains two residues in its RNase III domains (E1507 in RIII a and E1696 in RIII b) that are pivotal for its enzymatic activity. E1507Q and E1696Q are semi-active DCL1 mutants that cleave only one strand of pri-miRNA duplexes, halting further processing and resulting in partially processed pri-miRNA intermediates⁴. These intermediates offer a chance to identify the true substrates of DCL1 and to pinpoint its initial cleavage sites in vivo. Thus, we performed degradome sequencing (degradome-seq) with the DCL1 mutant lines to identify genuine pri-miRNAs and their first cut positions. Next, using our earlier optimized strategy dimethyl sulfate (DMS) mutational profiling with sequencing (DMS-MaPseq) to probe RNA secondary structures (RSS)⁵, we systematically detected the structure of pri-miRNAs in wild-type *A. thaliana* plants and in *dcl1* mutant plants. Alignment of the RSS and the first cut sites of pri-miRNAs enabled us to elucidate the structures determining the cleavage patterns of pri-miRNAs in vivo.

We identified 147 bona fide DCL1 substrates from 326 previously annotated pri-miRNAs; the remaining pri-miRNAs were DCL1-independent (81) or remained unclassified (98). Based on the orientations of the first cleavage sites to the miRNA/* duplexes (where * indicates the complementary sequence), we re-sorted 147 pri-miRNAs into five processing modes (Fig. 1a) and revealed

28 new true miRNA species. In parallel, DMS-MaPseq (DRS) revealed that, in roughly 95% of detectable pri-miRNAs, the RSS deviated from that predicted in silico, providing a clearer interpretation of the initial cleavages by DCL1 at the standard distance (15–17 nt) away from reference loops and/or bulges. DRS also showed a wide presence of internal loops or bulges that are 9–11 nt away from the initial cleavage sites and thus might guide DCL1 in processing pri-miRNAs (Fig. 1b). We also found that DCL1 tended to cleave pri-miRNAs at unpaired (non-complementary) regions of RNA and that SE and HYL1 could synergistically and independently impact the processing patterns and the RSS of pri-miRNAs in vivo.

Future directions

We have reconstructed the landscape of miRNA production, providing an encyclopaedia for understanding miRNA biogenesis in *A. thaliana* and offering a strategy for designing artificial miRNAs according to the preference of DCL1 cleavage. We also demonstrated that the parallel use of degradome-seq and DRS is a powerful tool for elucidating the structural determinants and impact of DCL1 partners in pri-miRNA processing in *A. thaliana*. These ideas and approaches should be readily adopted to different organisms, enabling progress in basic studies and practical transformation beyond *A. thaliana*.

Our finding that 28 pri-miRNAs can produce new functional miRNA/* duplexes alongside canonical miRNAs in the same pri-miRNA backbones has physiological implications; it will be interesting to determine how these new miRNAs impact canonical miRNA homeostasis and function. Similarly, numerous pri-miRNAs can produce functional miRNAs while being subjected to abortive processing. How these opposite events are coordinated in response to varying physiological needs awaits in-depth investigation. Notably, while most pri-miRNAs are processed through the canonical molecular ruler (15–17 nt), here roughly half also harboured internal loops or bulges about 9–11 nt away from the initial cleavages. Whether these loops or bulges are new references for DCL1 will be exciting to explore.

Next we plan to determine the identities and first cleavage sites of the 98 annotated pri-miRNAs that remained undetermined due to low or absent reads in our degradome-seq.

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EXPERT OPINION

"This paper is an elegant and ingenious work that gives insights into the initial processing steps for the conversion of primary transcripts into miRNAs. The experiments utilise *dcl1* mutants that are defective in one of its two cutting domains and facilitate mapping of the first and subsequent cuts. Furthermore, DRS

determines the paired and unpaired regions of the hairpin structures of the pri-miRNAs that attract the DCL1 processing, giving excellent insights and real scientific advancement."

Peter Waterhouse, Queensland University of Technology, Brisbane, Queensland, Australia.

FIGURE

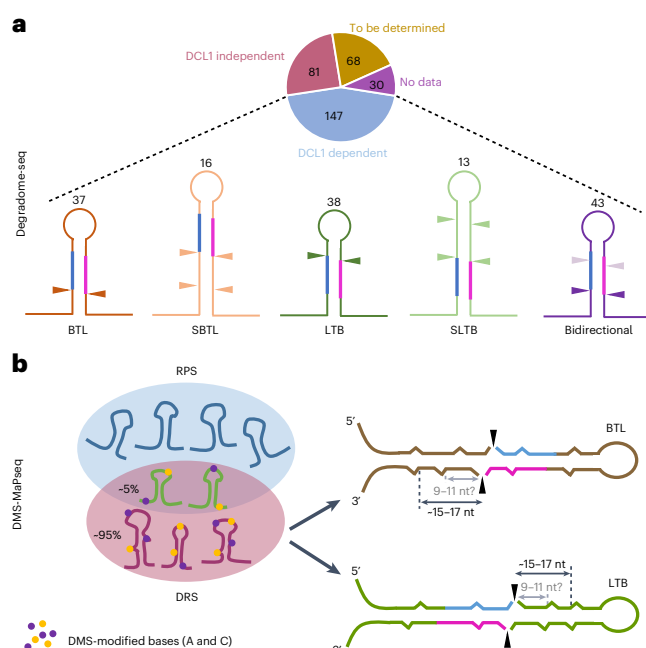


Fig. 1 | The miRNA biogenesis atlas in *A. thaliana*. **a**, Degradome-seq identified 147 bona fide pri-miRNAs from 326 previously annotated pri-miRNAs and reclassified them into five processing patterns, namely processing from base to loop (BTL), sequential base to loop (SBTL) processing, loop to base (LTB) processing, sequential loop to base (SLTB) processing or bidirectional processing. **b**, 95% of in vivo RSS for pri-miRNAs that were derived from our DRS were different from RNAfold Predicted Structures (RPS). The DRS better explains why DCL1 selects the first cut sites (black arrows) that are roughly 15–17 nt (black lines) away from the internal loops or bulges. DRS also detects additional internal loops or bulges that are roughly 9–11 nt (grey lines) away from the first cleavage sites by DCL1. © 2024, Yan, X. et al.

BEHIND THE PAPER

The inspiration behind this study traces back to research we conducted about 10 years ago⁴, which led us to fully employ DCL1 for in vitro and in vivo studies. Our initial attempt to secure funding for the study was unfruitful as the processing of metazoan pri-miRNA mechanisms had been extensively studied, and it was assumed that conclusions from animal studies could be applied directly to plants. With an improvement in our funding, our ideas to further study plant pri-miRNA processing were revived in 2018.

The most challenging period of our study coincided with the COVID-19 pandemic, which hindered our direct communication and impeded progress. However, we persevered through virtual meetings, and our discussions sparked inspiration for analysing extensive datasets generated in-house or sourced from public repositories, and for crafting figures. Overall, the success of this project was undoubtedly a result of effective teamwork between researchers. **X.Y. & X.Z.**

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FROM THE EDITOR

"The processing mechanism and sites are not clear for many pri-miRNAs. This study stands out because it comprehensively examines the cleavage sites and the secondary structures of pri-miRNAs by clever and innovative experimental design, providing good data resources and advancing our understanding of miRNA biogenesis." **Jun Lyu, Senior editor, Nature Plants.**