A loop-to-base processing mechanism underlies the biogenesis of plant microRNAs miR319 and miR159

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The first step in microRNA (miRNA) biogenesis usually involves cleavage at the base of its fold-back precursor. Here, we describe a non-canonical processing mechanism for miRNAs miR319 and miR159 in *Arabidopsis thaliana*. We found that their biogenesis begins with the cleavage of the loop, instead of the usual cut at the base of the stem-loop structure. DICER-LIKE 1 (DCL1) proceeds then with three additional cuts until the mature miRNA is released. We further show that the conserved upper stem of the miR319 precursor is essential to organize its biogenesis, whereas sequences below the miRNA/miRNA* region are dispensable. In addition, the bulges present in the fold-back structure reduce the accumulation of small RNAs other than the miRNA. The biogenesis of miR319 is conserved in the moss *Physcomitrella patens*, showing that this processing mechanism is ancient. These results provide new insights into the plasticity of small-RNA pathways.

The EMBO Journal (2009) 28, 3646–3656. doi:10.1038/emboj.2009.292; Published online 8 October 2009

Subject Categories: RNA; plant biology

Keywords: *Arabidopsis*; microRNAs; miR319; processing

Introduction

MicroRNAs (miRNAs) are small RNAs, about 21 nucleotides (nts), that regulate multiple biological pathways in complex organisms. In *Arabidopsis* and other plants, miRNAs have been implicated in diverse functions such as development, hormone signalling and stress responses (Jones-Rhoades *et al*, 2006). MiRNAs are defined by their unique biogenesis, which involves precise excision from the stem of a fold-back precursor (Meyers *et al*, 2008). They are processed from longer primary transcripts (pri-miRNAs) by the type-III ribonuclease DICER-LIKE 1 (DCL1) (Park *et al*, 2002; Reinhart *et al*, 2002; Kurihara and Watanabe, 2004; Kurihara *et al*, 2006). DCL1 is assisted by the dsRNA-binding protein HYPONATIC LEAVES1 (HYL1) and the zinc-finger protein SERRATE, which improve the efficiency and precision of

miRNA biogenesis (Kurihara *et al*, 2006; Dong *et al*, 2008). These components are sufficient to process plant miRNA precursors *in vitro* (Dong *et al*, 2008). After excision from their precursors, miRNAs are 2'-O-methylated by HEN1 (Yu *et al*, 2005), exported to the cytoplasm (Park *et al*, 2005) and sorted out into ARGONAUTE complexes (Mi *et al*, 2008; Montgomery *et al*, 2008). Then, miRNAs guide these complexes to target RNAs that have partial complementarity to their own sequences. Plant miRNAs generally cause cleavage of their targets, although they can also inhibit translation (Voinnet, 2009).

In animals, miRNA biogenesis is spatially separated in two compartments. First, a nuclear microprocessor complex formed by DROSHA and the dsRNA-binding protein DGCR8/PASHA cleaves the pri-miRNA and releases the hairpin, called pre-miRNA (Denli *et al*, 2004; Gregory *et al*, 2004; Han *et al*, 2004). Then, the pre-miRNA is transported to the cytoplasm where DICER together with another dsRNA-binding protein produce the second cut to release the mature miRNA (Kim *et al*, 2009). Most of the specificity in the selection of the miRNA sequence from the precursor lies in the first cleavage performed by DROSHA, which acts as a molecular ruler cutting at approximately 11 nts, one helical turn, from the joint between the single-stranded RNA and the double-stranded stem (Han *et al*, 2006).

The canonical miRNA biogenesis pathway is prone to exceptions and modifications in animals. For some precursors, the DROSHA requirement can be bypassed by the splicing machinery (Okamura *et al*, 2007; Ruby *et al*, 2007). For the processing of others, the interaction of specific factors with the precursor loop is required (Guil and Caceres, 2007; Trabucchi *et al*, 2009). Furthermore, processing of the pre-miRNA can be specifically impaired in certain tissues or conditions (Obenosterer *et al*, 2006; Heo *et al*, 2008; Viswanathan *et al*, 2008).

In contrast to animal miRNAs, there is still little information about the structural requirements for miRNA processing in plants. So far, the evidence indicates that plant pri-miRNAs are first cleaved by DCL1 to release their fold-back precursors, which in turn are cut again by DCL1 to generate the miRNAs (Kurihara and Watanabe, 2004; Kurihara *et al*, 2006). Intriguingly, whereas animal precursors usually contain homogenous 70 to 80-nt fold-back structures, plant miRNAs comprise a heterogeneous collection of hairpins with variable size and shape (Reinhart *et al*, 2002; Zhang *et al*, 2006).

In *Arabidopsis*, the miR319 and miR159 miRNA families are encoded by six genes (Palatnik *et al*, 2007). They are widely conserved in plants, and copies of miR319 can even be found in mosses (Arazi *et al*, 2005; Axtell and Bartel, 2005; Axtell *et al*, 2007), indicating their ancient origin. Their precursors have large fold-back sequences, which are also highly conserved (Palatnik *et al*, 2003; Li *et al*, 2005; Axtell *et al*, 2007; Warthmann *et al*, 2008). These unusually long

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Received: 24 June 2009; accepted: 7 September 2009; published online: 8 October 2009

precursors have been shown to harbour three regions from which potentially small RNAs can be generated, although the miRNA sequence accumulates much more than any other (Rajagopalan *et al*, 2006; Talmor-Neiman *et al*, 2006; Axtell *et al*, 2007). MiR159 and miR319 are similar in their sequences, but they have distinct functions *in vivo*: miR159 is a highly abundant miRNA that regulates MYB transcription factors involved in stamen development (Achard *et al*, 2004; Schwab *et al*, 2005), while miR319, which is expressed at low levels, regulates TCP transcription factors that control cell proliferation and differentiation (Nath *et al*, 2003; Palatnik *et al*, 2003, 2007; Koyama *et al*, 2007; Ori *et al*, 2007; Efroni *et al*, 2008; Schommer *et al*, 2008).

Overexpression of miR319, as seen in the *jaw-D* mutant, causes extensive degradation of TCP transcription factors and loss of cell proliferation control, which in turn is responsible for the strongly altered leaf curvature of the *jaw-D* mutant (Nath *et al*, 2003; Palatnik *et al*, 2003; Schommer *et al*, 2008). Recently, the precursors of *Arabidopsis* miR319 and miR159 have been successfully manipulated to generate artificial miRNAs (Niu *et al*, 2006; Schwab *et al*, 2006; Duan *et al*, 2008; Haas *et al*, 2008; Lin *et al*, 2009; Park *et al*, 2009). Artificial miRNA sequences have also been expressed from the miR319 precursor in the moss *Physcomitrella patens* (Khraiweh *et al*, 2008).

Here, we have studied the biogenesis of miR319 and miR159, which we discovered to undergo a non-canonical processing pathway. Their biogenesis begins with a cut below the terminal loop instead of the usual cleavage at the base of the precursor, and continues with three more dicing events until the miRNA is released. These results provide new insights into the plasticity of miRNA biogenesis in plants.

Results

Definition of a functional pri-miR319 in plants

To study the biogenesis of miR319, we decided to first determine the minimal sequence requirements for the *in vivo* processing of its primary transcript. We divided the pri-miR319 sequence into four parts comprising a lower stem (LS), the miRNA/miRNA* (duplex M/M*), an upper stem (US) and the terminal loop (TL). We prepared a series of vectors harbouring primary transcripts of different length and expressed them in *Arabidopsis* under the control of the 35S viral promoter (Figure 1A). We then analysed the accumulation of miR319 and evaluated the generation of crinkled leaves, which is a known developmental defect caused by miR319 overexpression (Figure 1A and Palatnik *et al*, 2003).

Due to their sequence similarities miR319 and miR159 are detected simultaneously in small-RNA blot hybridization experiments; however, they are easily distinguished due to their different electrophoretic mobilities (Palatnik *et al*, 2007). Surprisingly, we found that miR319a primary transcripts with different lengths were processed with similar efficiencies *in vivo* (Figure 1A and C). Actually, even the complete removal of the bases belonging to the lower stem below the miRNA/miRNA* did not affect miR319 production (319LS3; Figure 1A–C). In all these transgenic lines, we observed large changes in leaf morphogenesis in at least 75 out of 100 independent transgenic plants (Figure 1A), which is in good agreement with a strong accumulation of miR319 (Figure 1C). Also, the expression levels of *TCP4*, a target gene efficiently

cleaved by miR319 (Palatnik *et al*, 2003), were severely reduced in the transgenic lines (Supplementary Figure S1). Therefore, the hairpin consisting of 186 nts was sufficient to fully drive miR319 expression *in vivo*.

These results contrasted with the current model for animal miRNA precursor processing, where a semi-structured segment below the miRNA/miRNA* is required for their biogenesis (Zeng *et al*, 2005; Han *et al*, 2006; Zeng and Cullen, 2006). Interestingly, when compared with other plant miRNA precursors, miR319 has an unusually long fold-back structure (Figure 1D). We then studied whether the precursor sequences below the miRNA/miRNA* were relevant for the processing of another miRNA, miR172a, which is known to regulate flowering time and flower architecture (Aukerman and Sakai, 2003; Chen, 2004). In this case, we found that they were required for its biogenesis (Figure 1E and Supplementary Figure S2; JL Mateos and JF Palatnik, unpublished results). Similar results were obtained for other precursors such as those corresponding to miR398a, miR165a and miR164c (JL Mateos and JF Palatnik, unpublished results).

To further explore the sequence requirements for pri-miR319a processing *in vivo*, we decided to test whether the conserved upper stem segment of the long miR319 precursor was necessary for its processing. When we deleted the upper stem region of the miR319a precursor (Figure 1B), we observed that all 100 independent transgenic lines overexpressing this mutant precursor had wild-type (wt) leaves, and neither accumulated the miRNA nor had a decrease in *TCP4* (319USΔ1; Figure 1B–C and Supplementary Figure S1A), indicating that this region was indispensable for miR319a biogenesis. The levels of pri-miR319 in these transgenic lines (319USΔ1) were higher than those in plants expressing the control primary transcript (319LS2), indicating that the precursor was transcribed but not able to be processed (Supplementary Figure S1B). In contrast, a partial deletion of the top of pri-miR172 did not affect the biogenesis of this miRNA (Supplementary Figure S2). Taken together, these results indicate that the precursor sequences below miR319/miR319* are not necessary for the processing of the pri-miRNA, but instead the upper stem segment is indispensable.

Loop-to-base processing of miR319 and miR159 precursors

The unusual sequence requirements for its primary transcript suggested that miR319 processing could be unusual. MiRNA biogenesis in plants is fast and intermediates are hardly seen in small-RNA blots, so we turned to more sensitive approaches. We first used a cycle RT-PCR protocol, which is based on the self-ligation of precursor intermediates and should render two products for canonical precursor processing: one corresponding to the pre-miRNA and the other to the excised loop (Basyuk *et al*, 2003). This technique allowed us to identify intermediates for miR172, however not giving results for miR319 (NG Bologna and JF Palatnik, unpublished data). We then turned to a different protocol based on 5' RACE-PCR to detect miR319 processing intermediates (Figure 2A and Kasschau *et al*, 2003). In this method, RNA is ligated to a specific RNA adaptor and then used for a 5' RACE-PCR with generic and miRNA-specific oligos (Supplementary Figure S3 and Figure 2A). If the precursors are processed first at the base, this technique should render

Figure 1 Sequence requirements for a minimal pri-miR319a in *Arabidopsis*. **(A)** Definition of a functional pri-miR319a *in vivo*. A scheme with the different pri-miR319a constructs used in the experiments is shown on the left. The miRNA is indicated in red and the miRNA* in blue. A picture of a representative transgenic plant overexpressing each construct is shown. Note that wt leaves are flat but become crinkled when miR319 is overexpressed. The right panels show the leaf shape distribution observed in at least 100 independent transgenic plants overexpressing each construct. Transgenic plants were classified according to the shape of their leaves: wt, moderately crinkled (+) and severely crinkled (++). **(B)** Deletion of a segment from the upper stem of miR319a precursor. The right panel shows the leaf shape distribution of at least 100 independent transgenic plants for each construct. **(C)** Small-RNA blot showing expression of miR319. Each sample is a pool of 25 independent transgenic plants. **(D)** Size distribution of miRNA precursors in *Arabidopsis*. The size distribution of *Caenorhabditis elegans* precursors is included for comparison. The precursors were considered as the sequence between the miRNA and the miRNA*. **(E)** Effects of a deletion of the lower sequences on pri-miR172a processing. A scheme with the different pri-miR172a versions used in the experiments is shown on the left. The blot on the right indicates the accumulation of miR172 in a pool of 25 independent transgenic plants. The miRNA is indicated in red and the miRNA* is indicated in blue.

only one PCR product; however, if the processing starts at the loop the successive intermediates can be detected (Figure 2A).

When we applied this approach to miR319, we detected four products of different size (Figure 2B, inset). Sequencing of the fragments revealed that they corresponded to four

Figure 2 Loop-to-base processing of miR319 precursor. (A) Scheme illustrating the 5' RACE-PCR strategy used to map processing intermediates and the expected results depending on the processing direction of the miRNA precursors. (B) Small-RNA sequences from publicly available data (<http://asrp.cgrb.oregonstate.edu/db/>; Backman *et al*, 2008) were incorporated into the scheme of the *Arabidopsis* miR319a precursor. Small RNAs cloned more than 100 times are indicated in red. Other small RNAs are indicated by thin grey lines. Left inset: Polyacrylamide gel showing four fragments after the 5' RACE-PCR method depicted in panel A. The positions of DCL1 cleavage sites determined after sequencing of the PCR bands are indicated by arrows. (B–F) Processing intermediates detected after performing 5' RACE-PCR method and cloning of the products (fragments were cloned without any further purification). The positions, as revealed by 5' RACE and the number of sequenced clones corresponding to each site are indicated by arrows. The length of the arrows indicates the relative cloning frequency of the intermediates. (C) *Arabidopsis* miR319b precursor. (D) *Arabidopsis* miR159a precursor. (E) *Physcomitrella patens* miR319a precursor. (F) *Arabidopsis* miR172a precursor.

distinct cleavage sites along the fold-back precursor of miR319a (Figure 2B and Supplementary Figure S3). The distance between the cuts was 20 to 22-nt long, which is the usual distance between two consecutive DICER cleavage sites. Therefore, these results are consistent with the precursor having a first cleavage below the terminal loop, which is then followed by three more DICER cuts towards the base of the precursor to release the mature miR319a (Figure 2A). This

is also in good agreement with our previous results that indicated a requirement of the upper stem of the precursor for miR319a biogenesis (Figure 1B).

We also used publicly available data from deep sequencing projects (<http://asrp.cgrb.oregonstate.edu/>) (Backman *et al*, 2008) to search for small RNAs derived from the miR319a precursor region. Most of the small-RNA sequences arising from the miR319a precursor corresponded to the actual

miRNA, which is excised at cleavage sites 3 and 4 according to our 5' RACE-PCR mapping studies (Figure 2B). Interestingly, at low frequency there are small-RNA sequences that matched other parts of the precursor (Figure 2B and Rajagopalan *et al*, 2006; Talmor-Neiman *et al*, 2006; Axtell *et al*, 2007). These small RNAs corresponded to the cleavage positions found in our 5' RACE analysis of processing intermediates (Figure 2B). Taken together, these results indicate that there is a good concordance between the cleavage map of miR319a precursor that we have obtained by 5' RACE-PCR and the small RNAs identified by deep sequencing methods.

We next analysed the processing intermediates of other members of the miR319 and miR159 families of *Arabidopsis* (Figure 2C and D). We found that miR319b and miR159a precursors had a processing pattern similar to miR319a, indicating that all these miRNA precursors are first cleaved close to the loop. We found that the cloning frequency of each fragment varied, which may represent the different stabilities of the corresponding intermediates, although a bias during the amplification process cannot be discarded (Figure 2C–F). In the case of miR319b precursor, we only detected the first three cleavage products, probably due to a low abundance of the last processing intermediate.

To test the conservation of the miR319-processing pathway, we turned to the phylogenetically distant moss *P. patens*, which also has miR319 (Arazi *et al*, 2005; Axtell *et al*, 2007). When we analysed the processing pattern of the miR319a precursor in the moss, we obtained results similar to those previously found in *Arabidopsis*, indicating that the origin of the miR319-processing mechanism is quite ancient (Figure 2E). Interestingly, the sequence along the upper stem of *Arabidopsis* and *Physcomitrella* miR319 precursors is partially conserved (Supplementary Figure S4 and Axtell *et al*, 2007), which might underlie the common processing mechanisms.

As control, we applied the same strategy to detect *Arabidopsis* miR172a processing intermediates. In this case, we only detected the DCL1 cut that separates the miR172a hairpin from the rest of the primary transcript, in good agreement with the first cleavage occurring at the base of the precursor (Figure 2F). Interestingly, through this method we also identified a pri-miR172a fragment that had been cleaved in the central region of miR172*, which is the expected hallmark of an active miR172 loaded into RISC (Figure 2F). Similar degradation signatures have been obtained for miR172b precursor by global analysis of RNA ends (German *et al*, 2008). This canonical base-to-loop processing of miR172 has previously been observed in other conserved *Arabidopsis* miRNAs such as miR164 and miR166 (Kurihara *et al*, 2006), and miR168 (Vaucheret *et al*, 2006). Also, global analysis of RNA ends from *Arabidopsis* has indicated that this is the most common mechanism in plants (German *et al*, 2008).

Sequence requirements for miR319 processing

The non-canonical biogenesis of miR319 prompted us to study the sequence requirements for its processing. Our first focus lied on the terminal loop itself, which is 16-nt long in wt miR319a. When we doubled the size of the loop to 33-nt by inserting a sequence predicted to be non-structured (319TL-large loop; Figure 3A), we observed an important

Figure 3 Sequence determinants for miR319a processing. (A) Scheme showing the stem loops of several mutant miR319 precursors. The cleavage sites analysed by 5' RACE method (see Figure 2 for details) are indicated by black lines on the right of each precursor when determined. 319 wt corresponds to 319LS2 in Figure 1. The miRNA is indicated in red and the miRNA* in blue. (B) Small-RNA blots showing accumulation of miR319. Two pools of 25 independent transgenic plants expressing the corresponding precursor from the 35S promoter were analysed in each case. (C) Analysis of leaf shape in at least 100 independent transgenic plants for each construct. See Figure 1 for details.

reduction of mature miR319 accumulation (Figure 3B). However, the levels of miR319 in transgenic plants over-expressing this construct were high enough to cause degradation of *TCP4* mRNA (Supplementary Figure S1C) and changes in leaf morphology (Figure 3C).

We then reduced the size of the terminal loop by replacing its 16 nts by U-A-C-G, a small tetraloop known to form a very stable structure (Molinaro and Tinoco, 1995). The over-expression of this vector in *Arabidopsis* resulted in large accumulation of miR319a, albeit to lower levels than the wt precursor (319TL-tetraloop; Figure 3B). The target *TCP4* mRNA was largely reduced in the same transgenic lines and leaf morphology was affected accordingly (Figure 3C and Supplementary Figure S1C). These results indicate that although the terminal loop is not essential for miR319 processing, its modification is detrimental for the biogenesis of the miRNA.

Next, we deleted a short 8-nt stretch from the upper stem (319USΔ2; Figure 3A). *Arabidopsis* plants expressing this pri-miR319a failed altogether to accumulate the miRNA

(Figure 3B). In good agreement, all transgenic plants carrying that construct had wt leaves (Figure 3C) and normal levels of *TCP4* mRNA (Supplementary Figure S1C). We determined the cleavage sites of this precursor (319USΔ2), and of the precursor with a larger deletion in the upper stem (319USΔ1; Figures 1C and 3A). In both cases we observed the same pattern: most of the cuts localized in the middle of the miRNA, whereas a few more were stochastically distributed along the precursor sequences (Figure 3A). These results suggest that the upper stem region of miR319 is required to organize the serial dicing cuts that finally release the miRNA. Interestingly, modifications in the stem segment close to the loop, where the first cut occurred, are sufficient to alter miR319 biogenesis, which is in good agreement with a loop-to-base processing mechanism.

Abortive processing of metazoan miR16 has been shown to occur *in vitro* by DROSHA/DGCR8 when the complex recognizes the pri-miRNA from the loop instead of from the base of the precursor (Han *et al*, 2006). Our results suggest that unproductive processing or degradation of miR319 may also exist in the mutant precursors with modifications in their upper part region, which cannot proceed through their normal biogenesis pathway.

We also generated a precursor version that only has the top segment of the pri-miR319 (upper stem and terminal loop), without the stem segment containing the miR319/miR319*. As expected, transgenic plants expressing this construct did not cause any phenotype (Figure 3A–C). However, when we mapped the processing of this pri-miRNA, we found that several cleavage sites were still located in regions similar to those of the wt precursor (319M/M*Δ; Figure 3A). These cuts were also consistent with the small-RNA sequences cloned from *Arabidopsis* plants (see Figure 2B). To test the importance of the middle region of the stem, we closed the large bulge located in the central part of the stem. We found that miR319 biogenesis was impaired (Supplementary Figure S5), indicating that the overall secondary structure of the upper stem is important for the processing of this long precursor. Taken together, these results show that the top region of pri-miR319 has determinants necessary to commence the biogenesis of miR319.

Productive loop-per-base exchange during miR319 precursor processing

Previous results have shown that miR319 biogenesis requires DCL1 (Palatnik *et al*, 2003). That miR319a precursor processing requires four successive cuts, prompted us to evaluate whether other DICER proteins apart from DCL1 were also recruited for miR319a processing. To test this possibility, we overexpressed miR319a in the context of a *dcl234* mutant. We observed that miR319 expression levels were not compromised in the triple mutant or were even slightly enhanced (Figure 4A). In contrast, when we overexpressed miR319 in *serrate* or *hyl1* mutants, we observed marked decrease in the levels of the miRNA (Figure 4A), indicating that known components of the miRNA-processing machinery were implicated in the multistep processing of this precursor.

Next, we decided to test the importance of the relative position of the miRNA sequence towards the ends of the fold-back structure. To do this, we generated a new variant of pri-miR319a where its orientation was reversed: the loop of the precursor was opened, while the base of the precursor was

closed to form a loop (319 B↔L; Figure 4B). As a consequence, miR319 localized in the 5' strand of the precursor, instead of the 3', as it is seen in the wt version.

When we analysed the phenotypes of 100 independent transgenic plants expressing this artificial pri-miRNA, we recovered 24 with appreciable changes in their leaf morphology (Figure 4C). In good agreement, we observed that miR319 levels were increased in this mutant, albeit to a lesser extent than in the wt version (Figure 4D). Interestingly, this mutant precursor where the wt loop is open (319B↔L) directed miR319 accumulation with an efficiency similar to the one with a large terminal loop (319TL-large loop; Figure 3A and B), further suggesting that the structure and conformation of the terminal part of the miR319 precursor is relevant for its processing. Therefore, while slight modifications in the upper stem completely abolished miR319a production (Figures 1 and 3), the relative position of the miRNA sequence regarding the precursor ends was less important.

Bulges present in miR319a precursor prevent the accumulation of other small RNAs

The four cleavage sites used during miR319a processing generate three potential birth regions for six small RNAs; however, only the miRNA accumulates to high levels (Figure 2A and Rajagopalan *et al*, 2006; Talmor-Neiman *et al*, 2006; Axtell *et al*, 2007). To study the mechanistic basis of this process, we focused on one potential small RNA, which we arbitrarily named 'small RNA #1' (Figure 5A).

We could not detect the small RNA #1 (Figure 5B) or other small RNAs potentially arising from the wt precursor other than the miRNA itself, when we performed small-RNA blots using the experimental conditions that detected miR319. We could, however, detect small RNA #1 in transgenic plants overexpressing the miRNA using six times more RNA and an LNA probe (Supplementary Figure S6), in good agreement with genome-wide experiments, which have sequenced this small RNA at very low frequency (Rajagopalan *et al*, 2006; Axtell *et al*, 2007; Backman *et al*, 2008). Interestingly, small RNA #1 was not detected in *hyl1* mutants, suggesting that its biogenesis also depends on previously known components of the plant miRNA-processing machinery (Supplementary Figure S6).

An analysis of the secondary structure of the miR319a precursor revealed that it has many bulges located along its structure outside the miRNA/miRNA* region (Figure 5A). Typical miRNA/miRNA* pairs have four or less mismatches (Meyers *et al*, 2008); however, the upper stem of miR319a precursor that can produce small RNA #1 has six mismatches and one asymmetric bulge (Figure 5A). Therefore, we thought that this high number of bulges could be important for the specific processing of the miR319a precursor.

Closing the uppermost two bulges (319-B1) or the next bulge (319-B2) did not have any effect on miR319a or small-RNA #1 accumulation (Figure 5A–C). However, when we closed the 2-nt bulge present in the middle of small-RNA #1 region (319-B3), we detected the presence of this small RNA (Figure 5C). Transgenic plants expressing this mutant pri-miRNA produced levels of miR319 similar to those harbouring the wt precursor (Figure 5B) and as expected, had similar changes in leaf morphology (Figure 5D).

We then closed the 3-nt bulge (319-B4; Figure 5A) and observed high accumulation of small RNA #1 (Figure 5C).

Figure 4 *In vivo* activity of a reversed miR319a precursor. (A) Small-RNA blots for miR319 in *serrate* (*se*)-1, *hyl1-2* and *dcl234* mutants. (B) Scheme showing the exchange of the loop and the base in the miR319 precursor. 319 wt corresponds to the 319LS2 from Figure 1. The miRNA is indicated in red and the miRNA* in blue. (C) Leaf morphology changes in transgenic plants. See Figure 1 for details. (D) Small-RNA blots showing the accumulation of miR319. A pool of 25 independent transgenic plants expressing the corresponding precursor from the 35S promoter was analysed in each case.

The two mutants that resulted in an increase in small-RNA #1 accumulation improved the pairing of this small RNA to its opposite strand. Therefore, these results suggest that the bulges present in the conserved fold-back precursor of miR319a prevent the accumulation of small RNAs other than the miRNA itself. It is then likely that during processing of the miR319a precursor the interaction between small RNA #1 and its complementing sequence is destabilized after the cut by DCL1 due to the high number of bulges, which in turn may diminish the stabilization by HEN1 methylation (Yu *et al*, 2005) or the incorporation into AGO complexes (Vaucheret *et al*, 2006).

Interestingly, miR319a accumulation was impaired in the mutant precursor that accumulated high levels of small RNA #1 (319-B4; Figure 5A–C). Therefore, we looked at the processing intermediates of this mutant and found major accumulation of fragments corresponding to the second cleavage site (Figure 5A). These results indicate that the processing of the 319-B4 mutant precursor stalled after the second cut, with the production of small RNA #1, further

supporting the loop-to-base processing model for the biogenesis of miR319.

Discussion

In this study, we showed that pri-miR319 and pri-miR159 are processed through a non-canonical pathway. Their biogenesis starts with a first cleavage that releases the loop from the fold-back structure in a manner opposite to that for other known miRNAs. Next, three more dicing events are performed by DCL1 until the miRNA is finally released (Figure 6). Although the miR319 precursor has three regions, which can potentially generate small RNAs, we propose that the high number of bulges located in the precursor upper stem diminishes the accumulation of small RNAs from this region (Figure 6).

Previous studies have shown that plant miRNAs are processed by DCL1 in a manner similar to animal precursors: a first cut at the base separates the hairpin from the rest of the transcript, while a second cleavage releases the mature

Figure 5 Prevention of small RNA #1 accumulation by precursor bulges. **(A)** Scheme of the different mutants where bulges were closed. Bulges were numbered arbitrarily from the top of the precursor. The sequence that gives rise to small RNA #1 from the top segment is highlighted in green (see Figure 2A for details). 319 wt corresponds to 319LS2 in Figure 1. The cleavage sites analysed by 5' RACE method (see Figure 2 for details) are indicated by black lines on the right of the 319-B4. **(B)** Small-RNA blot for miR319 in pools of 25 independent primary transformant plants. **(C)** Small-RNA blot for small RNA #1 in the same samples as in panel B. **(D)** Leaf morphology changes in transgenic plants. See Figure 1 for details.

miRNA (Kurihara *et al*, 2006; Vaucheret *et al*, 2006). Our analysis of miR172a confirmed that this precursor was also processed first at the base. Then, it is likely that at least two processing pathways for evolutionarily conserved miRNAs exist in plants.

The sequences underneath the miRNA/miRNA* region are necessary for the processing of the miR172a precursor. On the contrary, deletion of sequences below the miR319/miR319* region did not pose any major challenge to miRNA production. Actually, a miR319a precursor with no lower stem at all was processed efficiently *in vivo*. The biogenesis of miR319a was extremely susceptible to modifications at the top part of the precursor. Small deletions or modifications in the bulges of the upper stem were sufficient to compromise miR319a biogenesis, which is consistent with the loop-to-base directionality of its processing.

Figure 6 The proposed model for the biogenesis of miR319 and miR159. The miRNA precursor is first cleaved below the loop by DCL1. The processing then continues with three more cuts until the miRNA/miRNA* is released. The miRNA (indicated in red) is subsequently incorporated into an AGO complex. The other dsRNA segments and the miRNA* (indicated in blue) likely become destabilized by the presence of a high number of bulges.

Most miRNA precursors are only conserved along the miRNA/miRNA* sequences (Reinhart *et al*, 2002). In contrast, the fold-back of miR319 is largely conserved during evolution (Palatnik *et al*, 2003; Li *et al*, 2005; Axtell *et al*, 2007; Warthmann *et al*, 2008). So far, the evidence indicates that the only small RNA coming from the precursor with a biological function is miR319, which regulates *TCP* transcription factors (Palatnik *et al*, 2003, 2007; Schwab *et al*, 2005; Schommer *et al*, 2008). It is then likely that the conservation of the upper stem segment of miR319a has been, at least partially, preserved due to its role during miRNA biogenesis, which is, in turn, conserved between *Arabidopsis thaliana* and the moss *P. patens*.

MiR319 and miR159 accumulate much more than any other small RNA from their precursors as judged by deep sequencing studies from *Arabidopsis* plants (Figure 2A; Rajagopalan *et al*, 2006; Axtell *et al*, 2007; Backman *et al*, 2008). Our results showed that the large number of bulges present in the upper stem reduce the accumulation of these other small RNAs. This contrasts with the ta-siRNA pathway where many small RNAs with no biological function accumulate during the biogenesis of a few biologically relevant ta-siRNAs (Peragine *et al*, 2004; Vazquez *et al*, 2004; Allen *et al*, 2005; Williams *et al*, 2005). However, the small RNAs generated from miR319 and miR159 precursors are still present at low abundance in plants, and small RNA #1 has been shown to associate with AGO4 (Qi *et al*, 2006), so we cannot rule out completely that they do not have any biological activity.

Previous studies hypothesized that miRNA genes evolved from ancestral 'proto-MIRs', which were products of inverted gene duplication (Allen *et al*, 2004). Given their inverted-repeat origin, proto-MIRs would have tight stem structures with few bulges, making them susceptible to processing by multiple DCL enzymes (Rajagopalan *et al*, 2006; Vazquez *et al*, 2008; Voinnet, 2009). Selection for miRNA regulation would work against precursor mutations that abolish miRNA function but favour mutations that prevent off-target small-RNA biogenesis, perhaps even narrowing precursor processing to DCL1 alone. Our findings, which show that the bulges located in the miR319 precursor stem can prevent or diminish the accumulation of small RNA species other than the miRNA itself, are in good agreement with this model.

Interestingly, a precursor with an exchanged base and loop was still able to direct miR319 expression, indicating that the overall structure of the stem is important but not the relative position of the miRNA towards the end of the precursor. The

animal microprocessor can also produce a first cut to separate the loop from the stem in artificial miRNAs (Han *et al*, 2006). However, a loop-to-base processing pathway would be difficult to occur *in vivo* in animals due to sequence requirements during the pre-miRNA export to the cytoplasm (Yi *et al*, 2003). In contrast, a loop-to-base processing pathway could be a prerogative for plants, as pre-miRNA dicing is performed in the nucleus.

In animals, the DROSHA complex recognizes the joint between the lower stem of the precursor and the single-stranded RNA sequences, and then cuts 11 nts from the base. The mechanism underlying the processing of the base-to-loop miRNA precursors in plants is still unknown. Processing of miR319 and miR159 precursors seemed to require the same known protein components as other conserved miRNAs, although we cannot rule out the participation of other specific factors yet to be discovered. Recently, specific proteins usually associated with the splicing machinery have been implicated in the processing of some animal miRNAs (Guil and Caceres, 2007; Michlewski *et al*, 2008; Trabucchi *et al*, 2009).

The miR319 precursor has been successfully engineered to produce multiple artificial miRNAs (Schwab *et al*, 2006; Haas *et al*, 2008; Khraiweh *et al*, 2008; Park *et al*, 2009; <http://2010.cshl.edu/scripts/main2.pl>). It is tempting to speculate whether the unusual biogenesis of miR319 and miR159 contributes to their suitability for artificial miRNA production. During their biogenesis, the first cleavage site, which seems to be the most important for miRNA processing, occurs in the top region independently of the sequence of the miRNA itself. It is plausible that the physical separation of the processing determinants for the first cleavage from the miRNA sequence provides the miR319 precursor with a high flexibility for artificial manipulation.

Materials and methods

Plant material

Arabidopsis ecotype Col-0 was used for all experiments. Plants were grown in long days (16 h light/8 h dark) at 23°C. Seeds of *dcl2-1/dcl3-1/dcl4-2* line were provided by J Carrington (Oregon State University, USA). Seeds of *se-1* (CS3257) and *hyl1-2* (SALK_064863) were obtained from the Arabidopsis Biological Resource Center (ABRC).

Transgenes

Mutated versions of the miR319 precursor were generated by PCR. The loop-per-base mutant precursor was synthesized by Mr. Gene GmbH (Germany). See Supplementary Table 1 for a list of binary plasmids and the sequence of the mutant precursors used in this study.

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Small-RNA analysis

Total RNA was extracted from inflorescences using TRIzol reagent (Invitrogen). Between 4 and 8 µg of total RNA was resolved on 17% polyacrylamide gels under denaturing conditions (7 M urea). Antisense DNA oligos to miR319 or small RNA1 were end-labelled as probes with [γ -32P] ATP using T4 polynucleotide kinase (Fermentas). For miR172 detection, LNA probes were used (Exiqon, Denmark). Hybridizations were performed overnight at 38°C using Perfect Hyb buffer (Sigma). Small-RNA sequences were obtained from the ASRP database (<http://asrp.cgrb.oregonstate.edu/db/>; Backman *et al*, 2008).

Expression analysis

A 1 to 4-µg weight of total RNA was treated with RQ1 RNase-free Dnase (Promega). Next, first-strand cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen). PCR reactions were performed with a Mastercycler ep *realplex* thermal cycler (Eppendorf) using SYBRGreen I (Roche) to monitor dsDNA synthesis. qPCR for each gene was performed on at least three biological replicates with technical duplicates for each replicate. The relative transcript levels were determined for each sample by normalizing them to *PROTEIN PHOSPHATASE 2A* subunit cDNA level (Czechowski *et al*, 2005). A list of primers used for these assays is shown in Supplementary Table 2.

Cleavage site mapping of miRNA precursors

For construction of libraries, polyadenylated RNA molecules were isolated using PolyAT trackt kit (Promega). Ligation of an RNA adaptor, reverse transcription and 5' RACE were performed according to the procedure described by Palatnik *et al* (2007). Two nested gene-specific reverse oligonucleotides were used for 5' RACE for wt plants, while two vector-specific reverse oligonucleotides were used for 5' RACE for plants overexpressing mutated precursors. The PCR products were resolved on 10% polyacrylamide or 3% agarose gels and detected by ethidium bromide staining.

Prediction of RNA secondary structure

The secondary structures of pre-miRNAs were predicted using MFOLD 3.2 (<http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi>).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Carla Schommer, Javier Martinez and the members of JP laboratory for comments on the paper and Jim Carrington for the *dcl234* seeds. We also thank Lionel Imbert, Rodolfo Rasia, Ramiro Rodriguez and Jerome Boisbouvier for valuable discussions and help with miRNA precursor synthesis. NB and JM are CONICET fellows, EB was supported by an ASPB SURF fellowship and JP is a member of CONICET. The studies were supported by grants to JP (Human Frontier Science Program Organization Young Investigator Grant and the Howard Hughes Medical Institute). JP is an HHMI International Research Scholar.

Conflict of interest

The authors declare that they have no conflict of interest.

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