Feed backwards model for microRNA processing and splicing in plants

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MicroRNAs (miRNAs) are a class of small RNAs, ranging in size from 19–24 nucleotides that mediate gene silencing at the post-transcriptional level. As in animals, plant primary miRNA transcripts (pri-miRNAs) contain imperfect, self-complementary stem-loop regions. Yet, there are some fundamental differences between animal and plant miRNAs including (i) genomic location of the primary miRNA transcripts—animal pri-miRNAs are generally encoded within introns of protein-coding genes whilst plant pri-miRNAs are more often intergenic and comprise independent protein-non-coding transcription units; and (ii) miRNA biogenesis—animal pri-miRNAs are first processed by Drosha in the nucleus and then exported from the nucleus and cleaved in the cytoplasm by Dicer [1], but plants have no Drosha homologue. The plant Dicer homologue Dicer-like 1 (DCL1) carries out processing events in the nucleus, typically resulting in an approximately 21-nucleotide mature mRNA/miRNA passenger strand duplex [2].

Plant pri-miRNA genes have a typical exon–intron structure resembling a protein-coding gene. Most plant pri-miRNA stem-loops are exonic, but surrounded by introns that more often occur towards the 3' adjacent side. It has been thought that the region outside the stem-loop is largely dispensable and lacks any regulatory role. However, in this issue of *EMBO reports*, two back-to-back articles from the groups of Voinnet and Jarmolowski indicate a clear connection between dicing and splicing.

The Voinnet group [3] investigated two representative miRNA loci: *MIR163* and *MIR172a*, both are expressed in *Arabidopsis thaliana* and have 3' end-located introns. To determine the influence of intronic sequences on miRNA expression, they expressed several transgene variants either transiently in *Nicotiana benthamiana* leaves or as stable transgenic lines in an *Arabidopsis* MIR T-DNA knockout background using the viral CaMV 35S promoter.

Fig 1 | Plant introns enhance miRNA production. (A) The intron (green line) is shown to positively regulate processing of the miRNA embedded in the upstream exon. DCL-1 cleaves the miRNA stem-loop. (B) According to Schwab *et al*, MIR genes that have exonic miRNA located upstream from adjacent introns are placed under the CaMV 35S promoter (pink box). Under these conditions, unknown factors within introns, in a potentially splicing-independent manner, enhance miRNA production. Microprocessor-deficient *dcl-1* mutants result in enhanced intron splicing, suggesting that the microprocessor is in competition with splicing. (C) According to Bielewicz *et al*, MIR genes that have exonic miRNA located upstream from adjacent introns are placed under the native MIR promoter (pink box). Here, a functional 5' splice site has a major role in miRNA enhancement. Additionally, the 5' splice site suppresses intronic polyA site activation. These events, which lead to normal mRNA maturation, also result in miRNA enhancement. DCL-1, Dicer-like 1; miRNA, micro RNA; PAS, polyA site.

In detail, four *MIR163* isoforms were tested: with and without an intron. Consistently, higher levels of mature miR163 were generated from the intron containing forms as compared with the intron-less ones. Adding more generality, *MIR172a* was investigated and found to harbour two introns interrupted by an exon downstream from the miRNA stem-loop. Consistently, intron-containing isoforms produced higher levels of miRNA compared with their intron-less counterparts. Overall, this points to a positive correlation between the presence of an intron in the primary transcript and increased miRNA production. To test if this behaviour is specific to introns of miRNA genes, unrelated intron sequences of various lengths—*Petunia chsA* and potato LS1—were placed at the 3' end of intron-less *MIR163-4* and *MIR172b* transgenes. Interestingly, these exogenous introns have a similar activating effect to native introns, which is position-dependent

as placing them 5' to the miRNA stem-loop is less effective.

To better understand the crosstalk between splicing and dicing, first point mutations that inactivate splicing were introduced at the 5' splice site in both the *MIR163* and *MIR172a* genes. Only a slight reduction in miRNA levels was observed, suggesting that miRNA accumulation does not require splicing of introns, but rather is mediated by as yet unidentified intronic elements. The elusive phenomenon of intron-mediated enhancement (IME) of gene expression was investigated. To test this, a clever trick of introducing *MIR163* transgenes with or without an intron into *dcl-1* mutants was carried out. Precursor processing in this situation will not reduce primary transcript levels, which can therefore be measured directly. Only a slight increase in transcript level from the intron-containing construct was observed,

suggesting that IME is unlikely to be important in miRNA levels. Second, two *dcl1* (*dcl1-7* and *dcl1-11*) mutants were tested for their effect on splicing. Interestingly, enhanced splicing of 3'-adjacent introns was observed, suggesting a negative effect of miRNA processing on 3' intron splicing. These results [3] establish that splicing acts in competition with miRNA processing for these miRNA genes. This is in accordance with studies on mammalian introns showing that miRNA-containing introns are spliced less effectively when pri-miRNA processing occurs [4].

The Jarmolowski group [5] also addressed the role of 3' introns in plant miRNA production. The relationship between splicing and miRNA production was tested to determine the significance of introns on miRNA levels. Wild-type (WT) and intron-less (Δ IVS) miR163 constructs transcribed from the native miR163 promoter were used to rescue *MIR163-2* mutants. The WT *MIR163* transgene restored levels of mir163 completely. By contrast, the Δ IVS lines expressed three times lower levels of miRNA than WT lines. Interestingly, the level of pri-mir163 in Δ IVS lines was significantly higher than in WT lines. This observation was extended to miR161 with similar conditions. These results not only highlight the positive influence of introns on miRNA levels but also suggest that processing of pri-miRNA is enhanced by introns.

To investigate whether splicing of the intron or an unknown stimulatory feature within the intron influences mir163 levels, variants were generated in which splice sites (5', 3' or both) were mutated. Mutating the 5' splice site alone reduced the levels of miRNA to one-third of WT levels, without any change in the levels of pri-mir163. By contrast, mutating the 3' splice site alone did not change miRNA levels. These results suggest a far bigger effect of mutating the 5' splice site as compared with the 3' splice site, although intron splicing is equally affected. Moreover, 5' and 3' splice site double mutants resulted in an even greater decrease in mir163 accompanied by a strong reduction in pri-miRNA levels. This might be due to the unstable nature of resulting transcripts. However, it cannot be excluded that, on splice site inactivation, splicing factors such as U1 snRNP, which are known to enhance transcription in human cells [6], might also affect transcription of plant miRNA genes. Interestingly, in human cells interaction between U1

snRNP and the 5' splice site is stimulated by 3' splice site-associated factors such as U2AF65 [7]. This could explain the additive effect of 3' splice site mutation on miRNA levels in 5' splice site mutants. Even so, the authors conclude that the 5' splice site is a major regulator of miRNA.

The influence of splice site mutation on polyA site (PAS) usage was also tested. By using 3' RACE analysis, use of a proximal PAS, which is embedded in the 3' intron was detected. In 5' splice site mutants, with reduced levels of miRNA, the proximal PAS was used at twice the levels seen in WT. Interestingly, in 5' and 3' splice site double mutants, there was an almost exclusive usage of the proximal PAS. A correlation between usage of the proximal PAS and reduced miRNA production was made. This is similar to mammals in which it has been shown that U1 snRNP protects pre-mRNAs from premature cleavage and polyadenylation [8].

To test whether the positive influence of introns is modulated by intron excision, a battery of mutants of positive splicing factors called SR proteins were used. Mir163 biogenesis was tested in different SR protein mutants (sr null mutants). Only subtle changes in mir163 levels were observed, correlating with the effect on splicing of pri-mir163. Nevertheless, the effects were minor as compared with 5' splice site or double mutants, suggesting that the link between intron removal and miRNA production mainly relies on 5' splice site recognition rather than on intron excision. Furthermore, endogenous targets of mir163 were identified by using 5' RACE in WT and *xrn4-3* mutant plants that stabilize the 3' end-cleaved target mRNA. An *in silico* predicted mir163 target gene *At1g6690*, which encodes an S-adenosyl-L-methionine-dependent methyl transferase, was identified. As expected, the steady-state level of this mRNA in various miRNA biogenesis mutants was higher than in WT plants. As mir163 accumulates under various types of biotic stress, the stimulatory effect of the intron on miRNA biogenesis was tested on bacterial infection. Interestingly, in WT the mir163 levels were increased as per the physiological requirement and obtained a longer sustained response. By contrast, mir163 accumulation in splice site double mutants was low and not induced by infection. These results highlight an important role for the *MIR163* intron and its functional splice sites in the

regulation of mir163 biogenesis during bacterial infection.

It is clear that both studies [3,5] report a similar positive effect of introns on miRNA accumulation. However, differences lie in the effect of 5' splice site mutations, which in the first study did not affect mir163 accumulation. This discrepancy might be due to the nature of the mutations introduced to inactivate the 5' splice site, which, might not have completely abrogated U1 snRNP binding to the 5' splice site. The type of promoter might affect the 5' splice site-dependency of miRNA production by influencing the nature of engagement of splicing factors to the transcribed RNA. Even so, it is clear from these two studies that plant miRNA maturation cross-talks with associated transcript splicing. It is noteworthy that previous examples in which the 5' splice site has had a positive role in miRNA processing have been shown for intronic miRNAs within protein-coding host genes of plants and animals. In animals, the 5' splice site influences positively the co-transcriptional engagement of microprocessor with the pri-miRNA [7]. On the other hand, in plants, intronic miRNA (*MIR400*) processing depends on how efficiently the intron is spliced out of the host mRNA. It seems that in this case miRNA maturation occurs post transcriptionally once the miRNA-containing intron has been spliced [8]. Clearly, in future work on plant miRNA biogenesis, further clarification of how dicing and splicing are coordinated will be required.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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