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Enhanced microRNA accumulation through stemloop-adjacent introns

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MicroRNAs (miRNAs) originate from stemloop-forming precursor RNAs found in longer primary transcripts that often contain introns. We show that in plants, those introns, when located 3' of the stemloop, can promote mature miRNA accumulation, through a mechanism that likely operates at the level of miRNA processing or stability. Reversely, when miRNA production is reduced such as in *dicer-like 1* mutants, splicing of introns that promote miRNA processing is considerably increased, pointing to a tight physical and temporal coordination of intron splicing and miRNA processing in plants. Our findings further suggest that miRNA transcripts without introns generated through alternative polyA-site usage might contribute to the differential adjustment of miRNA levels, possibly at a tissue-specific level.

Keywords: miRNA; intron; *Arabidopsis*

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INTRODUCTION

MicroRNAs (miRNAs), a family of small non-coding RNAs, regulate many biological processes by base-pairing to complementary target transcripts and impose a post-transcriptional control that reduces the rate of target protein production [1]. As target regulation is generally of quantitative nature, increased miRNA abundance normally correlates with decreased target levels. Rapid changes in miRNA accumulation, for example on perception of an external stimulus or stress, thus allow the effective adjustment of the levels of often several targets that might participate in the control of the same biological pathway.

Adjustment of miRNA levels can occur at several different steps. Apart from varying the activities of promoters that drive the expression of *MIRNA* genes, processing of the stemloop-forming precursor transcripts and the stability of mature miRNAs can be modified in several ways. Most of the known proteins with roles in

miRNA processing bind to the loop of the *MIRNA* stemloop and either inhibit (for example, LIN-28A) or promote (for example, KSRP) precursor processing by Dicer enzymes [2]. Stability of miRNAs is often reduced when their Argonaute protein partners are not available, or by exonucleolytic degradation, for example, by SDN proteins in *Arabidopsis* [3].

Like other RNA PolII transcripts, primary miRNA transcripts often contain introns. These might be part of the host gene when miRNA precursors are encoded within an intron in animals, or they might be adjacent to the miRNA stemloop in plants, where most *MIRNA* genes form independent transcription units. It had been established early on that sequences outside the miRNA stemloop were largely dispensable for miRNA production [4]. Therefore, it remained mysterious why most plant *MIRNA* genes are rather long [5–7] and contain elements such as introns, which attract elaborate RNA-modifying activities including via splicing.

RESULTS AND DISCUSSION

To investigate the possible function of introns in plant miRNA transcripts, we selected two representative *MIRNA* loci: *Ath-MIR172a*, producing a conserved miRNA present in many monocot and dicot species [8], and *Ath-MIR163*, which has only been found in the genus *Arabidopsis* and is thus considered a recently evolved *MIRNA* gene [9]. Transcripts mapped from these loci include variants with retained and variants with spliced introns (for example, *MIR172a* variants 2 and 4, Fig 1A [5,6]). *MIR163* additionally produces transcripts that do not contain any intron sequences at all owing to an alternative polyadenylation site (*MIR163* variant 2, Fig 1A [10]). Similar to most introns in *Arabidopsis MIRNA* genes, introns in *Ath-MIR172a* and *Ath-MIR163* are located 3' of the miRNA stemloop [5,6].

Higher miRNA levels from intron-containing precursors

We generated transgene variants of the two mapped *MIR163* isoforms, 163-1 with a spliceable intron, and 163-2 without intron, and expressed them transiently in *Nicotiana benthamiana* leaves. We consistently observed higher levels of mature miR163 generated from the longer, intron-containing form (Fig 1B bottom panel). Similarly, expressing a transgenic version of *MIR163* in which the entire intron sequence was omitted (163-4) yielded much lower levels of miRNA compared to a transgene containing

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Fig 1 | Introns in miRNA precursors correlate with increased miRNA levels. (A) Endogenous transcripts from *MIR163* and *MIR172a* loci. Grey bars indicate transcripts without intron, black bars those with intron. Introns are depicted in thinner lines. (B) *MIR163* transgenes (top) and resulting miRNA levels in stable transgenic lines (middle) and *N. benthamiana* leaves (bottom). (C) *MIR172a* transgenes (top) and resulting miRNA levels in *N. benthamiana* leaves (middle) and stable transgenic lines (bottom). (D) Effect of different 3' introns (blue) and GFP (green) on miR163 accumulation in transient assays. Transgenes are sketched on top. For transient experiments, independent biological replicates are shown in light and dark grey bars, respectively, error bars indicate standard errors of the means of technical triplicates. A pool of 40–60 plants was processed from stable transgenic lines. Div, diverse; GFP, green fluorescent protein; miRNA, microRNA; Rep, replicate.

the intron in its endogenous position (163-3). To rule out any artifactual effects due to transient expression, we introduced these transgenes in an *Arabidopsis* *MIR163* T-DNA knockout background accumulating no mature miR163 (SALK_034556; 'KO' in Fig 1B). Analysis of stable transgenic lines showed that miR163 accumulated at higher levels from the intron-containing precursor transcript (Fig 1B, middle panel), confirming the transient expression results.

We then investigated *MIR172a*, which harbours two introns 3' of the miRNA stemloop, by generating transgenes terminating downstream of either the first (172a-3 and 172a-4) or the second intron (172a-1 and 172a-2), respectively. In both instances, the intron-containing precursors produced higher levels of mature miRNA compared to their intron-less counterparts (Fig 1C). Because the primary transcript produced from the short *MIR172a* variant with intron (172a-3) was longer than the transcript from the

long variant lacking introns (172a-2), we exclude a positive effect of transcript length on mature miRNA accumulation, and we therefore conclude that the presence of an intron in both the *miR172a* and *miR163* primary transcripts correlates with increased mature miRNA accumulation.

To test if introns in miRNA transcripts contained special features, such as transcriptional enhancers, that would increase miRNA accumulation, we engineered other, unrelated, intron sequences of various lengths (see supplementary Table S1 online for details). These introns were added at the 3' end of the intron-less *MIR163* variant (163-4), a site at which the *MIR163* intron could restore mature miRNA accumulation (Fig 1D, i163). As shown in Fig 1D, both the *Petunia* chalcone synthase-A intron (iCHS; chromdb.org) and the potato LS1 intron (also known as 'GUS (β -glucuronidase) intron', iGUS; [11]) could increase miRNA accumulation; this was also observed with *MIRNA*

introns isolated from the *MIR172b* locus (i172b3, i172b5). Engineering the green fluorescent protein (GFP) coding sequence at the same position had no effect. The levels of mature miRNA were neither correlated with intron length nor with the AT content of the engineered pieces of DNA or U content of introns (see supplementary Table S1, supplementary Fig S1 online). We therefore conclude that increased miRNA accumulation is likely mediated by defined elements that are not specific to introns normally found in *MIRNA* genes.

A similar positive effect of introns had been previously observed when engineering longer hairpins as the triggers of RNA interference in transgenic plants. Separating the two arms of the hairpin by a spliceable intron indeed correlated with the highest rates of target-gene silencing compared to silencing achieved from constructs in which non-intronic or reverse-intron spacer sequences formed the loop between the two hairpin arms [12,13]. We thus decided to investigate the mechanisms underlying these positive intron effects, focusing on *MIRNA* genes.

Influence of intron position

Most introns in endogenous *Arabidopsis* *MIRNA* genes localise 3' relative to the miRNA stemloop, including in cases when primary transcripts comprise longer stretches of 5' sequence [6]. Therefore, we tested if intron position could influence their competence to increase mature miRNA accumulation. First, we generated *MIR172b* transgenic variants including or lacking the endogenous 5' intron found in the *miR172b* transcript (172b-2 and 172b-3, respectively; Fig 2A). We found that the presence of this 5' intron had only a minor effect on mature miRNA accumulation. To further investigate a possible intron position effect, we re-engineered three introns that effectively boosted miRNA accumulation when located 3' of the miRNA stemloop (iCHS, iGUS and i163; Fig 1D), but placed them 5' as opposed to 3' relative to the stemloop in the context of the intron-less *MIR163* transgene (163-4). In all cases, the 5' introns had a much weaker effect on mature miRNA accumulation (Fig 2B), suggesting that introns are less effective when located 5' of the miRNA stemloop.

Introns are generally removed from nascent transcripts co-transcriptionally by the spliceosome, and removal of different introns occurs with different efficiencies [14]. Processing of miRNA precursors (hereafter called 'dicing') and splicing are tightly coupled processes when miRNA precursors reside within the introns of protein-coding genes. In human cells, the two processes are not strictly interdependent [15,16], yet alternative splicing events can affect the production of intronic miRNAs in plants [17]. To test if the splicing of adjacent introns affects plant miRNA accumulation, we introduced point mutations at the splice donor sites in both the *MIR163* and *MIR172a* precursor RNAs, which completely abolished splicing of the respective introns (163Δ, 172aΔ; Fig 2C). Mature miRNA levels were slightly reduced in transient assays, but unchanged or rather upregulated in transgenic lines, even if acceptor and putative branch sites were mutated in addition (data not shown). This suggests that the positive effect of introns on mature miRNA accumulation does not strictly require splicing of the respective intron, but is more likely mediated by one or several elements within intronic sequences. The position effect of introns further suggests that such elements should be located 3' relative to the miRNA stemloop in order to exert their positive effects on miRNA accumulation and that these

effects might thus entail a defined spatial and/or temporal order of stemloop and intron production during transcription.

Motifs and mechanisms mediating the intron effect

In plants and animals, several introns have been shown to increase steady-state levels of their host protein-coding transcripts in a poorly understood process termed 'intron-mediated enhancement of gene expression' (IME) [18]. Similar to our observations, IME is independent of intron splicing [19] but correlates with the over-representation of short sequence motifs within introns, known to increase gene expression in *Arabidopsis* and rice [20].

To determine whether increased primary transcript levels could account for higher miRNA accumulation from intron-containing precursors, we introduced *MIR163* transgenes with and without intron into *dicer-like1* (*dcl1*) mutants, which are impaired in miRNA processing. The rationale of this approach was that precursor processing would not interfere with primary transcript level measurements. We carried out semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) using oligonucleotides amplifying the nascent transgene *MIR163* transcripts before processing ('stemloop' in Fig 3A, supplementary Fig S2 online), and also oligonucleotides amplifying a short product in the transcribed terminator region, possibly stabilized after processing. As shown in Fig 3A, the precursor levels were only slightly increased when produced from the intron-containing transcripts (163-5). This suggests that an IME-like mechanism that acts to promote transcript accumulation, is unlikely to account, at least significantly, for the observed increased miRNA levels.

To further exclude an IME-type of regulation, we used three introns that had been shown to strongly (iUBQ, At4g05320), moderately (iTRP1, At5g17990) or weakly (iTCH3, At2g41100) increase *PAT1:GUS* mRNA accumulation through IME [19]. As in the previous experiments, these introns were engineered at the 3' end of the otherwise intron-less 163-4 transcript. As shown in Fig 3B, both the *UBQ* and *TCH3* introns could boost *MIR163* accumulation, while the *TRP1* intron had no effect, indicating no correlation between the ability of introns to cause IME, on the one hand, and to promote miRNA accumulation on the other. Furthermore, the *MIR163* and *MIR172a* introns produced very low scores in IMEter tests, which estimate the ability of an intron to stimulate gene expression [20] (supplementary Table S2 online), yet both increased miRNA production very effectively. In addition, IME is most effective from introns located close to the transcription start [21], whereas in our case, 5' introns were less effective (Fig 2B). Therefore, increased miRNA accumulation from 3' localised introns is likely mediated by elements distinct from those involved in IME in protein-coding transcripts.

Having established that 3'-adjacent introns can affect mature miRNA accumulation, we tested whether the converse was also true, that is, that miRNA precursor processing could affect splicing of those *MIRNA* introns. We carried out semiquantitative RT–PCR with oligonucleotides flanking the introns of several endogenous *MIRNA* genes and monitored the relative accumulation of spliced versus retained intron in two distinct *dcl1* *Arabidopsis* mutants, *dcl1-7* and *dcl1-11*, which display strongly reduced miRNA processing. As shown in Fig 3D, the 3' introns in *MIR163*, *MIR172a* (first intron), and *MIR172b*—all of which could boost mature miRNA accumulation when located 3' of a miRNA

Fig 2 | Introns enhance miRNA levels when located 3' of the miRNA stemloop. (A) Endogenous (top) and transgenic (middle) *MIR172b* variants, and the accumulation of mature miRNA in *N. benthamiana* leaves and stable transgenic lines (bottom). (B) Effect of 5' introns (light blue labels, right-most bars) on miR163 accumulation in transient expression assays. The same introns engineered at the 3' end of *MIR163* (dark blue labels) are shown as a control. (C) Effect of splice donor site mutations on miRNA accumulation. Transgenes are sketched on top, including sequences of the 5' splice junctions and the position of oligonucleotides used for RT-PCR (middle). Red asterisks illustrate the donor site mutation. Northern blots (bottom) were from stable transgenic lines. For transient experiments, independent biological replicates are shown in light and dark grey bars, respectively, error bars indicate standard errors of the means of technical triplicates. A pool of 40–60 plants was processed from stable transgenic lines. miRNA, microRNA; RT-PCR, reverse transcription polymerase chain reaction; Rep, replicate.

stemloop (Fig 1)—were spliced more efficiently in *dcl1* mutants compared to WT plants. The 5' intron in *MIR172b*, which did not affect mature miRNA accumulation remained, by contrast, largely unaffected (Fig. 3D). Slight alterations in spliced miRNA transcript levels were also observed when *HYL1*, a DCL1 co-factor, was mutated [6].

These findings collectively suggest a negative effect of dicing on 3' adjacent intron splicing, similar to the situation in mammals, where miRNA-containing introns are spliced less effectively when pri-to-pre-miRNA processing takes place simultaneously [15,16]. Our data thus support a model in which proteins carrying out the miRNA processing reaction could slow down or hinder splicing reactions that occur in the vicinity [15].

While dicing seems unfavourable to adjacent intron splicing, we did not find convincing evidence suggesting that splicing of adjacent introns is required for their effect on miRNA accumulation. Rendering those introns that could boost mature miRNA levels unspliceable did not abolish their effects or even increased miRNA accumulation in transgenic lines (Fig 2C). Likewise, we identified spliceable introns that were unable to affect miRNA levels, and also found that a similar increase in mature miRNA accumulation could be mediated by introns with very different splicing efficiencies (Fig 3C). We presume, therefore, that factors mediating the intron effect on miRNA accumulation might operate before or during spliceosome recruitment. This hypothesis also takes into account the fact that introns were less effective

Fig 3 | miRNA transcript levels in transgenic WT and *dcl1* mutant plants. (A) RT-PCR analysis of RNA extracted from transgenic *Arabidopsis dcl1* mutant plants expressing *MIR163* variants (bottom); the positions of oligonucleotides are indicated on top. 163-5 and 163-6 transgenes correspond to 163-3 and 163-4, but contain an additional GFP at their 5' side, which does not alter the intron effect (supplementary Fig S2 online). (B) Effect of IME introns on miRNA accumulation in *N. benthamiana* (middle). Histograms show independent biological replicates in light and dark grey bars, error bars indicate standard errors of the means of technical triplicates. Northern blots are from an independent experiment. Semiquantitative RT-PCR analyses indicate precursor levels (bottom); miR173 served as a control for equal infiltration. (C) RT-PCR analyses employing oligonucleotides flanking the cloned intron fragments indicate the degree of splicing. (D) RT-PCR analyses conducted with oligonucleotides spanning introns in WT and *dcl1* mutants. Quantifications indicate the ratio of unspliced versus spliced products and represent a single biological experiment, independent of the one shown as a gel picture on the left side. A pool of 15–20 plants was processed from *dcl1* and control plants. GFP, green fluorescent protein; IME, intron-mediated enhancement; miRNA, microRNA; RT-PCR, reverse transcription–polymerase chain reaction; WT, wild type.

when located 5' of the miRNA stemloop, as those factors might no longer be accessible when splicing or spliceosome assembly has already been initiated, before the miRNA stemloop is fully folded.

Bielewicz *et al*, in an accompanying paper [22], observed a much more pronounced reduction of mature miRNA levels when mutating the splice donor site in *MIR163* transgenes. This discrepancy might be due to the different point mutations that were engineered to render those introns unspliceable: while both successfully abolished completion of the splicing reaction, they might have affected recruitment of binding factors differently. Alternatively, we suspect that the use of different promoters from

which the transgenes were expressed (35S versus endogenous *MIR163* promoter) might have contributed to this effect, possibly also by affecting the spectrum of recruited proteins. Bielewicz *et al* observed that miR161 levels were affected only to a milder degree when expressing splice donor-site mutated precursor transcripts from the 35S promoters, compared to miR163 versions expressed from their own promoter elements, which supports the latter explanation. Nevertheless, further experiments will be required to clarify this issue.

Introns have been shown to increase transgene stability by protecting them from the recruitment of RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and subsequent degradation of the

resulting double-stranded RNA [23]. Protection correlates with the efficiency of intron splicing and therefore likely employs a set of factors that differ from the ones involved in intron-mediated miRNA increase. Nonetheless, in a similar way that splicing might alter the relative composition of factors acting on nascent RNAs, which might disfavour the recruitment of RDR6 [23], the presence or absence of factors binding to intronic motifs might alter the availability of miRNA-producing or -stabilizing proteins.

We have shown that the specific location of some, but not all, introns 3' of miRNA stemloops in precursor transcripts can increase the levels of the respective mature miRNA. Using the *dcl1* background, we have ruled out an effect on miRNA primary transcript stability, suggesting that increased processing of miRNA precursor or reduced turnover of mature miRNA might underpin these properties of intron-containing miRNA transcripts. Although the precise mechanisms involved are yet to be elucidated, at least one biological role can be foreseen for the phenomenon reported here. Indeed, use of alternative polyadenylation sites, such as those within introns, has been observed for a number of *Arabidopsis* MIRNAs [6], generating transcripts of different length. We have shown that in the case of *MIR163*, this can have a principal effect on mature miRNA accumulation. Tissue-specific or stress-induced variations of polyA-site usage could thus modulate the extent of miRNA-mediated regulation of gene expression in a spatiotemporal manner.

METHODS

Strains and growth conditions. All *Arabidopsis thaliana* plants used in this study were of the Col-0 ecotype. *dcl1-7*, and *dcl1-11* mutants have been described [24,25]. Plants were grown in growth chambers in long-day conditions (16 h light, 8 h dark) at 21 °C.

Transgenes and expression assays. Transgenes were engineered into the pB2GW7 binary plasmid containing a 35S promoter [26] and mobilised into *Agrobacterium* strain GV3101. Oligonucleotides and templates are listed in supplementary Table S4 online. Transient assays in *N. benthamiana* leaves (labelled 'Transient expression' in figures) were performed as described [4] with a starting OD₆₀₀ of 0.5; leaves were harvested after 3 days. *MIR163* (163-3) was co-infiltrated and served as a control for equal infiltration for all experiments analysing miR172 levels, 172a-3 served as a control for miR163 experiments except in Fig 3B bottom panel where miR173 was used. Stable *Arabidopsis* transformants were generated for selected lines (labelled 'Transgenic lines').

RNA analyses. RNA was extracted from pooled inflorescences (*Arabidopsis*) ($n=15-20$ in *dcl1* experiment in Fig 3D; rest $n=40-60$) or four infiltrated leaves (*N. benthamiana*) using Trizol. Reverse transcription was performed with Superscript III (Invitrogen) or RevertAid (Thermo). MiRNA abundance was quantified by RT-PCR with oligo-dT and specific stemloop primer using SYBR-Green (Thermo) on a CFX system (Bio-Rad). Relative expression was normalised to *MIR163* and *MIR172a* respectively (see above). Experiments were repeated and two biological replicates are represented as independent experimental series in the figures. The means of technical triplicates are shown ($\pm$ standard error of the means). Details are listed in supplementary Methods online. Semiquantitative RT-PCR was performed in Fig 3 with standard reagents. Band intensities were quantified with ImageJ in Fig 3D.

Small RNA northern blots were performed as described [4]. Oligonucleotides used for probes and PCRs are listed in supplementary Table S4 online.

Supplementary information is available at EMBO reports online (<http://www.emboports.org>).

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Author contributions: R.S. designed and performed the experiments and wrote the paper; C.S. performed experiments; S.L. and O.V. participated in fruitful discussions and helped in writing the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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