Review

Special Issue: Noncoding and small RNAs

Intronic noncoding RNAs and splicing

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The gene organization of small nucleolar RNAs (snoRNAs) and microRNAs (miRNAs) varies within and among different organisms. This diversity is reflected in the maturation pathways of these small noncoding RNAs (ncRNAs). The presence of noncoding RNAs in introns has implications for the biogenesis of both mature small RNAs and host mRNA. The balance of the interactions between the processing or ribonucleoprotein assembly of intronic noncoding RNAs and the splicing process can regulate the levels of ncRNA and host mRNA. The processing of snoRNAs – both intronic and non-intronic – is well characterised in yeast, plants and animals and provides a basis for examining how intronic plant miRNAs are processed.

Noncoding RNAs in eukaryotes

The existence of noncoding RNAs (ncRNAs), such as tRNAs and rRNAs, has been known for many decades. Other small, stable RNAs are relatively well characterised and involved in, for example, splicing, ribosome biogenesis, translation and chromosome replication. These RNAs include small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), RNase P and mitochondrial RNA processing (MRP) RNA, signal recognition particle (SRP) RNA, and telomerase RNA (see Glossary for list of RNA species discussed in this review). Recently, developments in high-throughput sequencing and bioinformatic approaches have helped to identify a vast number of ncRNAs, particularly with the discovery that a significant proportion of the eukaryotic genome outside of the protein-coding genes is expressed as ncRNAs. These ncRNAs include the many thousands of small regulatory ncRNAs (e.g. microRNAs [miRNAs] and small interfering RNAs [siRNAs]), transcripts such as natural antisense transcripts (NAT), and those derived from transposon- or retrotransposon-rich regions [1–4]. The range of functions of these ncRNAs in regulating transcription, RNA stability and translation, and how their pathways of synthesis and action interact with one another in regulatory networks are currently some of the primary questions in biology. The genomic organization of ncRNAs varies greatly, being expressed both from non-protein-coding intergenic regions and from host protein-coding genes. In particular, many ncRNAs are encoded in introns and, in this review, we examine specifically how the intronic organization of two classes of ncRNAs – snoRNAs and miRNAs – potentially influences the production of host mRNA and the ncRNAs themselves.

snoRNAs and their gene organization

The two major classes of snoRNA (box C/D and box H/ACA snoRNAs; see Glossary) are responsible for directing 2′-O-ribose methylation and pseudouridylation of target RNAs, most commonly rRNA and snRNAs. snoRNAs of each class associate with different sets of core proteins to form small nucleolar ribonucleoprotein particles (snoRNPs), which function in modification [5–7]. The organization of snoRNA genes is extremely diverse, both within and among different eukaryotes (Figure 1) [7]. The majority of animal and some yeast snoRNAs are intron-encoded. Processing of snoRNAs is largely splicing-dependent, and the snoRNP is released by exonucleolytic trimming from the 5′ and 3′ ends of the linearised intron lariat (Figure 1a) [5,6]. Intron-encoded snoRNA genes depend on the transcription of their host genes – most of which encode proteins involved in ribosome biogenesis and nucleolar function – and this organization provides co-ordinated expression of RNA and protein components [8]. The majority of animal snoRNAs depend on this intron context; this is further highlighted by the examples of human and *Drosophila* snoRNAs that are encoded in numerous introns of genes that do not encode proteins [9,10].

Glossary

Box C/D: Box C/D snoRNAs contain conserved sequences: box C (GUGAUGA) and D (CUGA), near their 5′ and 3′ ends, respectively.
Box H/ACA: Box H/ACA snoRNAs fold into two stem–loop structures in the 5′ and 3′ halves of the RNA. These structures are adjacent to the conserved internal box H (ANANNA) and the 3′-terminal box ACA (ACANNN).
miRNAs: microRNAs, small regulatory RNAs.
ncRNA: noncoding RNA, does not code for protein.
pre-miRNA: precursor miRNA transcript produced by initial processing of pri-miRNA by Drosha (in animals) or DCL1 (in plants).
pri-miRNA: primary transcript containing miRNA(s).
RNase P and RNase MRP: RNA moiety of RNP endonucleases involved in tRNA and rRNA processing, respectively.
RNP: ribonucleoprotein complex.
siRNAs: small interfering RNAs, small regulatory RNAs.
snoRNA: small nucleolar RNAs that guide modification of rRNAs and cleavage of pre-rRNAs.
snRNA: small nuclear RNAs (U1, U2, U4, U5 and U6), which function in pre-mRNA splicing.
Spliceosome: large RNA–protein complex responsible for removal of introns (splicing) from pre-mRNAs.
SRP RNA: RNA component of the signal recognition particle. It targets specific proteins to the endoplasmic reticulum.
Telomerase RNA: RNA component of telomerase RNP. It adds multiple tandem repeats of a short sequence at the ends of each chromosome.

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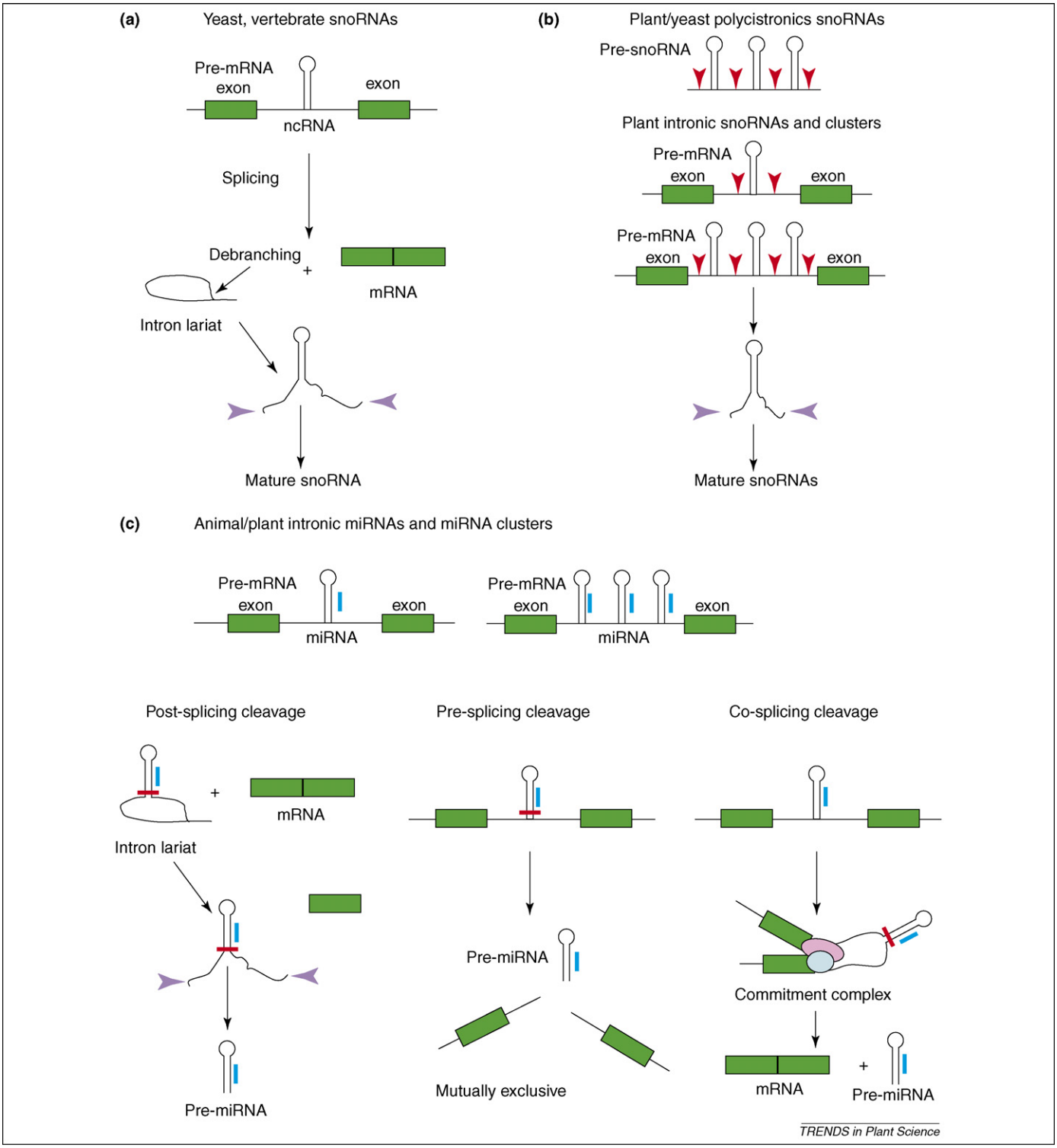

Figure 1. Processing of intronic ncRNAs. Schematic diagrams of different modes of processing of snoRNAs and miRNAs. **(a)** Animal and yeast snoRNAs are released from linearised introns by exonucleolytic trimming. This processing is splicing-dependent. **(b)** Yeast and plant polycistronic snoRNAs and plant intronic snoRNAs (both single and clusters) are released by endonucleolytic cleavage (red arrowheads), which is followed by exonucleolytic trimming. This processing is splicing-independent. **(c)** Animal and plant intronic miRNAs (single) and animal intronic miRNA clusters. Cleavage of the pre-mRNA by Drosha or DCL1 (red line) can occur, after splicing, on the intron lariat or de-branched intron. Cleavage of the unspliced pre-mRNA can be mutually exclusive – producing only the miRNA – or can occur after the splicing commitment complex has formed – producing both the miRNA and spliced host mRNA. Key: blue line, mature miRNA sequence; green box, exon; black lines, intron; purple arrowhead, exonucleolytic processing; red arrowhead, endonucleolytic cleavage; red line, cleavage by Drosha (animals) or DCL1 (plants); stem–loop, snoRNA or miRNA.

The majority of plant snoRNAs are organized as polycistronic clusters of genes and expressed as precursor snoRNAs (pre-snoRNAs; Figure 1b) [11]. Polycistronic snoRNAs occur in plants and yeast, and a mechanism is required to separate the individual snoRNAs from the pre-snoRNA transcripts. In yeast, this is achieved by Rnt1p (RNase III). The spacer regions between the yeast snoRNAs contain stem–loop structures, with bulged nucleotides that are cleaved by Rnt1p. After this cleavage, the exosome, Xrn1p and Rat1p catalyze exonucleolytic

trimming to generate the mature snoRNA (Figure 1b) [12,13]. In plants, the spacer regions of pre-snoRNAs vary in size from 17 nt to approximately 200 nt and are often U-rich, with little sequence conservation. To date, there is little evidence for the formation of stem–loop structures that could represent typical RNase III substrates, and it is unknown which endonuclease cleaves between individual snoRNAs. Recently, the *Arabidopsis thaliana* orthologue of the yeast *Rnt1p* gene was described, but disruption of this gene does not affect polycistronic snoRNA processing [14]. In addition, plants have snoRNAs encoded within introns, either individually – as in yeast and animals – or as intronic snoRNA clusters – found only in plants and, uniquely among metazoan, in *Drosophila* [7,10,15]. *Arabidopsis* has approximately 20 single or clustered intronic snoRNAs (J. Brown and S.H. Kim, unpublished). The production of plant snoRNAs from introns is not splicing-dependent, because mature snoRNAs are released from transcripts in which the snoRNA is in either an intronic or a non-intronic context [11,16]. The processing of individual snoRNAs from polycistronic clusters within introns is again consistent with the requirement for an endonucleolytic activity (Figure 1b) [16].

miRNAs and their gene organization

miRNAs are small ncRNAs of approximately 21–23 nt in length. They regulate gene expression at the post-transcriptional level in animals and plants by base-pairing to target mRNAs [1,17–21]. miRNAs are produced from primary miRNA (pri-miRNA) transcripts, which fold to form extensively double-stranded stem–loop structures. In animals, processing of the pri-miRNA by the RNase III Drosha occurs in the nucleus and produces an intermediate precursor miRNA (pre-miRNA), which is then processed by a Dicer activity in the cytoplasm. The mature miRNA is incorporated into the RNA-induced silencing (RISC) complex [1,17]. In plants, processing of both the pri-miRNA and pre-miRNA occurs in the nucleus and requires DICER-LIKE 1 (DCL1), which liberates the mature miRNA from the precursor [1,19–21].

In humans, the hundreds of known miRNAs are predicted to regulate up to 30% of the protein-coding genes [22–24]. In plants, recent deep-sequencing of small RNAs has complemented more classical approaches to identifying miRNAs [25–30]. *Arabidopsis* contains approximately 60 different miRNA families, members of which are located in at least 155 miRNA loci [29]. The families are classified as being conserved (such families are also found in other plant species, such as rice [*Oryza sativa*] and poplar [*Populus* spp.]) or non-conserved (i.e. apparently species-specific) [29,31].

miRNAs have complementarity with target mRNAs and regulate their expression by cleaving the mRNA or through translational repression [1,17–21,32]. Besides their function in growth and development, miRNAs are also part of the cell response to stresses; and, in some conditions, instead of inhibiting translation, miRNAs can activate translation [33–35]. In plants, in which miRNAs generally show almost perfect complementarity, most target mRNAs are cleaved and degraded [1,19–21]. Plant miRNAs typically regulate developmental processes and responses to stress. This is reflected by the observations that they are expressed in specific tissues and developmental stages, and because their expression levels change under different environmental or growth conditions or in response to phytohormones [1,19–21,36]. A recent study regarding the regulation of phosphate levels by stress-induced expression of *miR-399* provided an elegant example of how miRNA activity can be fine-tuned [37]. The activity of miR399 was modulated through base-pairing to an ncRNA, *IPS1*, which sequestered the miRNA and blocked its interaction with and cleavage of its normal target mRNA.

In animals, the majority (~80%) of miRNAs are intron-encoded within both protein-coding and noncoding genes. As with intronic snoRNAs, there is some evidence for coordinated expression of miRNAs and their host mRNAs [38,39]. However, in contrast to animal intronic snoRNAs, which are only found individually in any particular intron, animal intronic miRNAs can occur as clusters within introns (Figure 1c). This essential difference in organization suggests that the lack of an endonucleolytic activity able to cleave between individual snoRNAs in animal cells does not restrict the processing of intronic miRNA clusters, presumably because cleavage of individual pri-miRNAs is carried out by Drosha. In plants, until recently, the majority of miRNAs were found as monocistronic or polycistronic transcription units with few intronic miRNAs [20,40]. The recent description of novel *Arabidopsis* miRNAs [29], and the analysis of their genomic organization has now identified 11 intronic miRNAs found individually in introns of both protein-coding and noncoding genes (Figure 2).

Splicing and intronic snoRNAs

As described above, intron sequences sometimes contain miRNAs and snoRNAs with extensive secondary structures that are recognized by proteins or assemble proteins to form RNPs. The existence of such sequences raises the question of whether RNA folding or RNP formation affects the splicing process or vice versa. Secondary structures in plant and animal introns can affect both splicing efficiency and splice-site selection [41–43], and therefore snoRNAs or pri-miRNAs have the potential to influence the splicing process.

snoRNP assembly and processing of some vertebrate box C/D snoRNAs occur relatively late in splicing, and the association of the core box C/D snoRNP proteins on the snoRNA is tightly dependent on the splicing process [44,45]. In particular, intron-binding protein 60 (IBP160), a splicing factor that binds to introns upstream of the branch-point in the C1 splicing commitment complex, might recruit the core 15.5 kDa protein, which directs assembly of the functional C/D snoRNP, or it might aid folding of the snoRNA [46]. In addition, the position of the C/D box snoRNA relative to the branch-point and the 3′ splice-site in the host intron is crucial for their processing [45]. In yeast, altering the position of an intronic snoRNA reduces the efficiency of production of both the snoRNA and the host mRNA, suggesting that there is competition between the forming spliceosome (see Glossary) and snoRNP assembly [47]. By contrast, there is no evidence for interactions between intronic H/ACA snoRNP assembly and splicing in vertebrates, and recognition of the intronic H/ACA and assembly of the pre-snoRNP is an early event

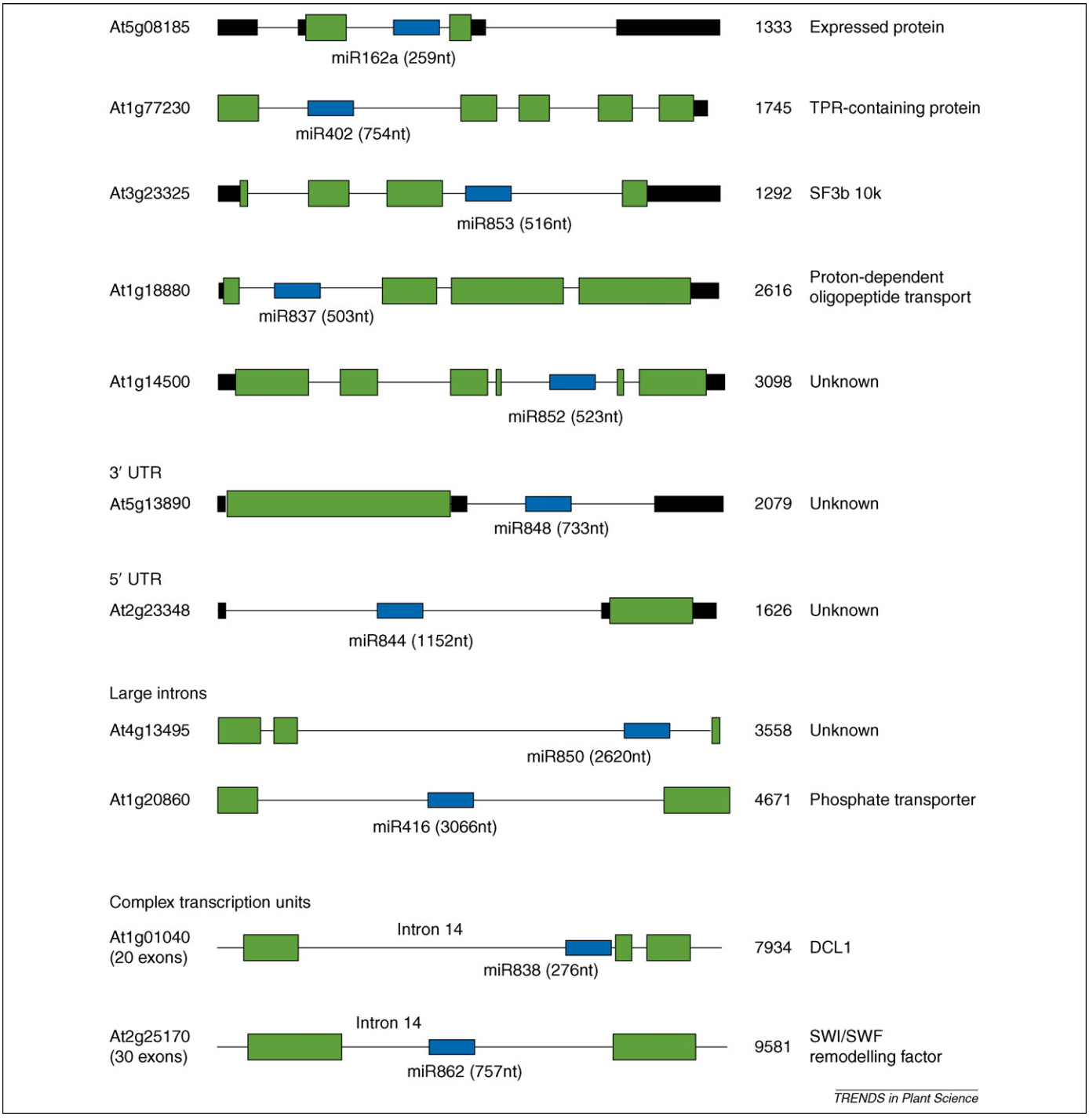

Figure 2. Intronic miRNAs in *Arabidopsis*. Schematic diagrams of the structure of *Arabidopsis* genes containing intronic miRNAs. Genes can be arranged in one of several ways: relatively simple transcripts (few exons and introns); genes in which the miRNA is in introns within the 3′ or 5′ untranslated regions (UTRs) or in long introns (80% of plant introns are in the range of 80–120 nt); or genes with complex structures with multiple exons and introns (only part of the gene structure is shown). The At gene identifier, the length of gene transcript, and the gene function are given. Black boxes, 3′ and 5′ UTR exons; black lines, intron; blue box, miRNA; green box, exon.

tightly associated with transcription [48,49]. Thus, depending on specific protein–protein interactions, the assembly of the different snoRNP types may or may not be influenced by the splicing process, and the position of snoRNAs in an intron has the potential to affect the efficiency of splicing of the host intron.

Splicing and intronic miRNAs

In animals, several routes for the generation of pri-miRNAs from introns in pre-mRNAs have been described. Processing might occur in a manner that is essentially the same as the processing of intronic snoRNAs, wherein the pri-miRNA is released from the excised and linearised intron lariat (Figure 1a). Alternatively, Drosha might cleave the pre-miRNA from the excised lariat, from the linearised intron or, indeed, from the unspliced pre-mRNA (Figure 1c). Drosha-mediated cleavage of the pri-mRNA from the unspliced pre-mRNA would also cleave the intron (pre-mRNA), and thus production of the spliced mRNA – and, therefore, the protein – and the miRNA would be

mutually exclusive (Figure 1c). However, it was recently shown that cleavage of the pri-mRNA from an intron does not necessarily lead to the mutually exclusive production of the miRNA and mRNA if this cleavage occurs after the intron has already committed to splicing [50]. That is, if spliceosomal commitment complexes tether the exons flanking the intron, cleavage of the pri-mRNA does not inhibit splicing (Figure 1c) [50]. Another route through which miRNAs are produced from animal introns reflects the extreme organization found in *Drosophila*, in which some pre-miRNAs are effectively introns – called 'mirtrons'. These short introns, which are a few dozen nucleotides in length, are liberated by the splicing reaction, and thus Drosha is not required for production of the miRNA [51]. This organization might reflect an evolutionary origin of intronic microRNAs, wherein intron length, which varies in different organisms and can be very short in *Drosophila*, determines the probability that mirtrons will occur. Finally, alternative splicing can determine whether an miRNA is intronic or exonic, as seen for some human miRNAs [38], and splicing is required to produce some rice miRNAs that can only be formed once splicing has brought together miRNA sub-sequences located in different regions of a precursor noncoding RNA [52].

Interestingly, of the 11 currently known plant intronic miRNAs (Figure 2), miR162a and miR838 are involved in the regulation of *Arabidopsis DCL1*. First, miR162a, the gene for which resides in the second intron of At5g08185, targets *DCL1* mRNA for degradation [53]. Second, miR838, encoded from intron 14 of the *DCL1* gene, might autoregulate *DCL1* mRNA by processing miR838 from the pre-mRNA and thus disrupting the mRNA [29]. The detection of *DCL1* mRNA fragments cleaved at the position of miR838 implies that there is direct competition between splicing and miRNA production; however, by analogy to findings in animal cells [50], it is possible that splicing occurs if miR838 processing takes place once a commitment complex has formed on intron 14. In addition, *miR853* is found in an intron of the gene encoding the 10 kDa protein of splicing factor SF3b.

The relationship between splicing and processing of an intronic pri-mRNA in plants has been studied in the case of miR162a and its host gene [54] (J. Brown and S.H. Kim, unpublished). Transcripts from this gene display a complex pattern of alternative splicing, with at least six different transcript variants (Figure 3a). Only some of the transcripts contain the pri-miRNA and can give rise to mature miRNAs; the others would be non-productive. Close examination of the position of the variant splice sites demonstrates clearly the potential competition between splicing and miRNA production or, more accurately, between assembly of splicing factors on splice sites and folding of the pri-mRNA into its secondary structure (Figure 3a). For example, removal of part of the intron from the authentic 5′ splice-site to an AG at the base of the pre-miRNA generated an mRNA transcript with an intact pri-miRNA that can be cleaved by DCL1 (Figure 3a: AS2). In contrast, removal of an intron from a cryptic 5′ splice-site located in the terminal loop of the pri-miRNA stem–loop to a cryptic 3′ splice-site (3 nt downstream of the authentic 3′ splice-site) (Figure 3a: AS3) removed the 3′ arm of the pri-mRNA containing the miR162a sequence. The resultant mRNA contained fragments of the intron and the 5′ half of the pri-miRNA and could not be folded into a secondary structure to enable processing of the miRNA. It was therefore non-productive. The effect that the secondary structure of the pri-mRNA has on splicing is already apparent when considering these splicing events. In the first event, the use of the cryptic 3′ splice-site at the 5′ side of the base of the stem–loop suggests that this event occurred after folding of the pri-miRNA, which also sequesters the putative branch-point sequence in the stem, inhibiting selection of the authentic 3′ splice-site. The second event uses a cryptic 5′ splice-site adjacent to the terminal stem-loop. This would be unlikely to occur after folding of the pri-miRNA, because the region complementary to U1snRNA is in competition with base-pairing during stem formation (Figure 3b). Thus, splicing is affected by miRNA secondary structures, consistent with previous analyses showing that secondary structures in plant introns affect the efficiency of splicing and the accuracy of splice-site selection [41]. It is also conceivable that splicing events are influenced by the pre-assembly of the pri-miRNA processing complexes, as has been clearly shown for snoRNAs.

miRNAs and splicing, and in particular alternative splicing, have important roles in development and stress responses in both animals and plants [55,56]. Splicing itself is regulated by the levels and activity of numerous *trans*-acting factors that alter splicing patterns in response to cellular and extracellular stimuli and signalling pathways [57,58]. Although this review has concentrated on intronic ncRNAs, many plant miRNAs are expressed from ncRNA transcripts that are spliced. Thus, secondary structures or RNP assembly can influence splicing; furthermore, altered splicing of host transcripts in response to cellular conditions can also determine the integrity of the ncRNA, providing another level of regulation of the ncRNA and host mRNA. The final outcome in terms of production of the host mRNA and the ncRNA will depend on the position of the ncRNA in the intron, the dynamics of folding of these RNAs during and after transcription, the dynamics of splice-site selection and spliceosome formation, and positive interactions with or interference by the competing complexes.

We have drawn parallels between the intronic organization of snoRNAs and miRNAs and their processing schemes in animal and plant systems. These ncRNAs are similar in various ways: some are encoded in introns; snoRNAs contain regions of complementarity to rRNAs or snRNAs; miRNAs contain regions of complementarity to mRNAs; and they require secondary structure for stability, processing and association with proteins. The discovery of plant and animal 'orphan' snoRNAs, which have targets in mRNAs and can affect, for example, alternative splicing, demonstrates the potential that these RNAs have for crossover functions [59–61]. snoRNA evolution and miRNA evolution also have parallels; duplication and diversification events have occurred in both lineages, as revealed by the clusters of ncRNAs in introns and noncoding regions [7,62]. Indeed, it is also possible that some snoRNAs and miRNAs are related evolutionarily or that the same gene might have evolved features and functions of both types of

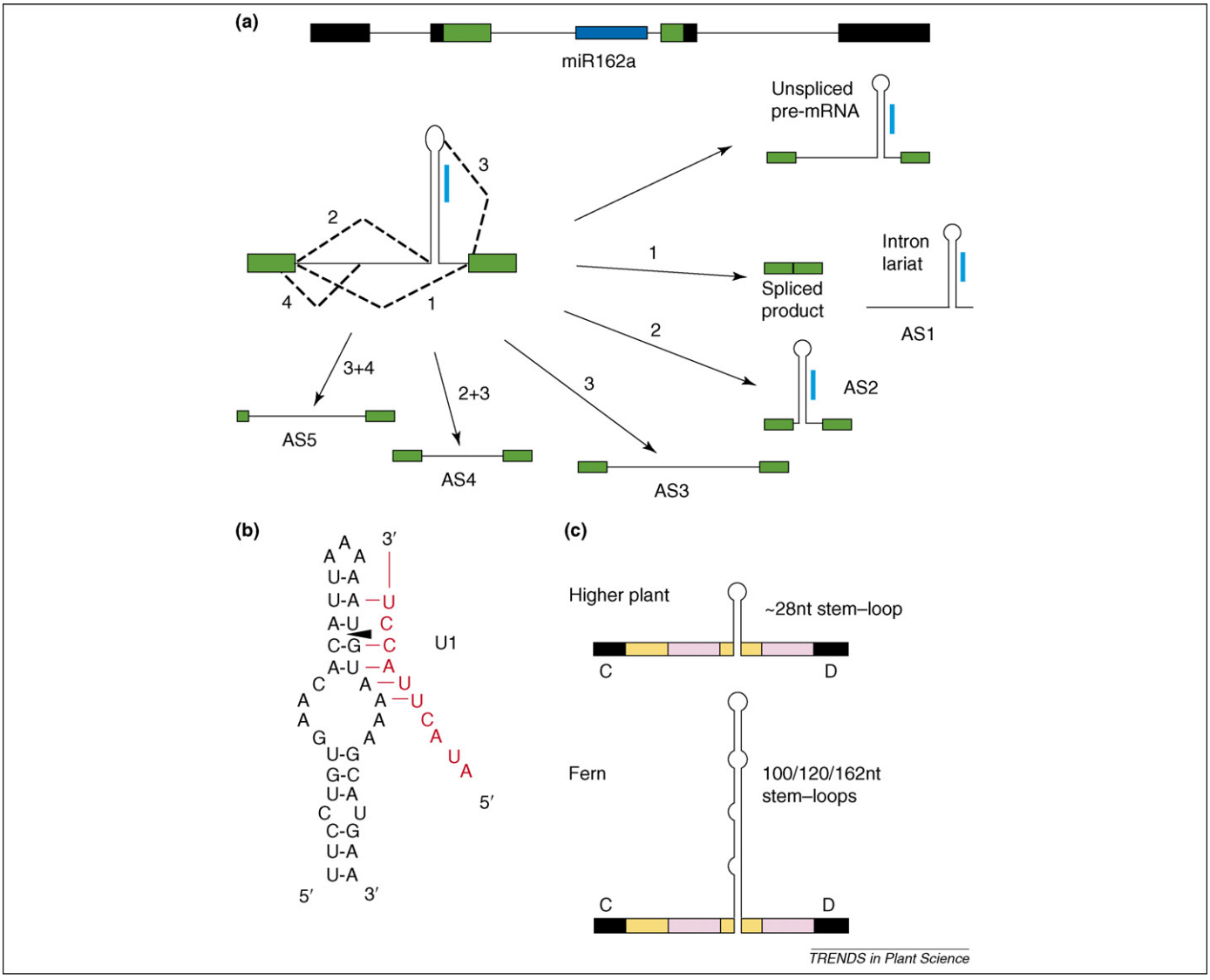

Figure 3. Alternative splicing of At5g08185, which contains miR162a; and the structure of fern U14snoRNAs. **(a)** Four different splicing events (1–4) give rise to five alternatively spliced transcripts (AS1–AS5) and the unspliced pre-mRNA. Only the unspliced pre-mRNA, AS1 and AS2 contain the pri-miRNA. Black box, 3′ and 5′ UTR exons; blue line, miRNA; dashed lines, splicing events; green box, exon; solid lines, intron; stem-loop, pri-miRNA. **(b)** Sequence of terminal stem–loop region of pri-miRNA, showing competition between stem–loop formation and base-pairing to U1snRNA to permit selection of the GU 5′ splice-site in splicing event 3. Arrowhead, 5′ splice-site; red sequence, 5′ end of U1snRNA. **(c)** Comparison of structures of U14snoRNAs from higher plants and the three U14snoRNA variants of the fern *A. nidus*, showing extensive stem–loops in the latter [63]. Black boxes labelled C and D, conserved terminal box C/D snoRNA sequences; orange boxes, U14 sequences; pink boxes, U14 sequences conserved from yeast to human.

RNA. Intriguingly, the structure of three U14snoRNAs from a primitive plant (the fern, *Asplenium nidus*) contains all the features of a box C/D snoRNA, along with extensive secondary structures reminiscent of a pri-miRNA (Figure 3c) [63].

Intronic sequences are under less stringent selection pressure than protein-coding exons, and they have the potential to tolerate sequence deletion, insertion and duplication. Rapid intron evolution can thereby generate sequences and/or secondary structures that are recognized by endonucleases or RNA-binding proteins. Introns have a great potential for evolving novel sequence combinations to develop new intron-encoded ncRNA species. The primary constraints of this aspect of intron evolution are the interactions with the splicing process, which can also drive changes in intron–exon structure of host genes. We anticipate that the rapid development and reduction in the costs of high-throughput sequencing, which is dramatically increasing the available genomic and transcriptome sequence resource in plants, will lead to the discovery of more intronic ncRNAs. Together with the powerful tools of comparative genomics, in particular the analysis of intron conservation, we will also gain new insights into the evolution and functional dynamics of intronic ncRNAs.

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