

Organellar and Secretory Ribonucleases: Major Players in Plant RNA Homeostasis^{1[OPEN]}

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During the last 20 years there has been an intense focus on posttranscriptional regulation of gene expression, and the role played by ribonucleases (RNases) in this process. Most research has centered on cytoplasmic and nuclear RNA decay enzymes, particularly those involved in mRNA regulation, and in proteins mediating nucleus-localized coding and noncoding RNA processing. These efforts have created a well-defined picture of RNA homeostasis in eukaryotes, including plants (Schmid and Jensen 2018; Sieburth and Vincent 2018; Towler and Newbury, 2018; Matsui et al., 2019). Thus, cytoplasmic degradation of mRNAs in plants (see Box 1), which involves deadenylation, decapping, and degradation by 3'-5' and 5'-3' exoribonucleases, has been extensively studied and reviewed (e.g. Zhang and Guo 2017; Sieburth and Vincent 2018; Sorenson et al., 2018; Lange et al., 2019). Similarly, different roles for the nuclear exosome and other RNases in nuclear RNA processing and decay have been established (Lange et al., 2014; Sikorska et al., 2017; Tomecki et al., 2017; Sáez-Vásquez and Delseny 2019). RNA decay mediated by small RNAs associated to RNA-induced silencing complexes and the processing and decay of small RNAs (sRNAs) have also been the focus of intense research efforts (Fukudome and Fukuhara 2017; Yu et al., 2017; Wang et al., 2019).

On the other hand, RNases that do not reside in the cytoplasm or nucleus have received significant less

attention, even though their activities are important for plant growth, development, and response to biotic and abiotic stimuli from the environment. As we will describe, organellar RNases are essential to maintain RNA homeostasis in chloroplasts and mitochondria. Plants also express members of the RNase T2 protein family, associated with the secretory pathway, which are involved in the maintenance of normal cellular homeostasis, with diverse roles, from stress responses to control of self-pollen rejection. This review will provide

ADVANCES

- Vacuolar RNases belonging to the RNase T2 family are essential for maintenance of nucleotide homeostasis through their function in the RNA salvage pathway.
- RNase T2 enzymes associated with the secretory pathway participate in the production of tRF and tRNA halves that mediate responses to abiotic and biotic stresses.
- Chloroplasts contain five major ribonucleases, which are involved in the processing and degradation of all kinds of RNAs, along with several other more substrate-specific RNases. Many are still waiting to be discovered and characterized.
- While some similarities exist between chloroplast and mitochondria, each organelle has also unique activities.
- The use of RNA-seq-based techniques now opens the way to a global understanding of the roles of RNases and identification of their targets.

¹This work was supported by the National Science Foundation (grant no. MCB-1714996 to G.C.M.) and Institute of Plant Sciences Paris-Saclay benefits from the Labex Saclay Plant Sciences (grant no. ANR-10-LABX-0040-SPS).

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G.C.M. wrote the secretory RNases section; B.C. wrote the organellar RNases section; both authors conceived the content and organization of the paper, and revised and edited the final text.

^[OPEN]Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.20.00076

a summary of the present knowledge on these RNases, from enzymatic characterizations to biological functions, and highlight questions that should be addressed in the future.

SECRETORY RIBONUCLEASES

Secretory RNases are enzymes targeted to the cellular secretory system and thus localized in organelles or the extracellular space. In plants, characterized secretory RNases belong to the RNase T2 family, a group of enzymes typified by the fungal RNase T2 purified from *Aspergillus oryzae* (MacIntosh 2011). Enzymes from the T2 family are endoribonucleases without sequence or base specificity that degrade single-stranded RNA through a 2',3' cyclic phosphate intermediate, in a reaction catalyzed by two absolutely conserved His residues that define the active site (Irie 1999). It has been proposed that their enzymatic mechanism involves a two-step (transphosphorylation and hydrolysis) general acid–base catalysis (Irie 1999; Thorn et al., 2012). Interestingly, fungal enzymes can complete both steps and yield 3' nucleotide monophosphates as final product, but bacterial enzymes end the reaction after the first step, resulting in the production of nucleotides (nt) with a 2',3' cyclic phosphate (Nicholson 1999; Fontaine et al., 2018). Although not many plant RNase T2 proteins have been characterized enzymatically, biochemical analyses of extracellular and vacuolar tomato enzymes indicated that plant RNase T2 enzymes generate 2',3' cyclic nucleotide monophosphates as primary product (Nürnberg et al., 1990; Löffler et al., 1992).

The RNase T2 family is conserved in almost all eukaryotes so far analyzed, with the exception of trypanosomes and *Schizosaccharomyces pombe* (Shang et al., 2018; Fricker et al., 2019) and is also found in a large number of bacteria and several viruses. In most non-plant organisms, only one gene belonging to this family is found in each genome (Hillwig et al., 2009; Ambrosio et al., 2014); however, in plants, the RNase T2 protein repertoire has expanded, and individual proteins have been adapted for a variety of functions. At least four genes are found in each seed plant genome that has been sequenced (MacIntosh et al., 2010; Ramanauskas and Igić 2017), and there is evidence of frequent duplications/gene losses that resulted in different number of RNase T2 gene in different species. In addition, gene expression and functional analyses have shown that these proteins have acquired a variety of biological roles, including participation in the cellular housekeeping salvage pathway, production of tRNA-derived sRNAs, defense activities, and a central role in gametophytic self-incompatibility in several plant families (Table 1). Phylogenetic analyses allowed separation of plant RNase T2 enzymes in three clades (Igić and Kohn 2001; MacIntosh et al., 2010; Ramanauskas and Igić 2017) that define three classes of proteins roughly associated with different functions: Class I are proteins associated with a variety of stress responses and show

evidence of gene duplications and gene sorting that provide large variability to this class, with variable number of class I proteins in individual species; Class II enzymes are conserved in all seed plants, in general with only one gene per genome, and their function has been related to RNA salvage; and Class III enzymes are mainly associated with self-incompatibility, although other functions have also been assigned to this class (Table 1). Historically, Class III enzymes were named S-RNases, whereas other plant T2 proteins are referred to as S-like RNases (McClure et al., 1990; Taylor et al., 1993). Class I and II proteins are found in all land plants, whereas Class III proteins are found only in core eudicots (Ramanauskas and Igić, 2017). Some plant RNase T2 proteins in the three classes have lost their catalytic activity (MacIntosh et al., 2010; Ramanauskas and Igić 2017). A monocot-specific class I subclade of proteins without RNase activity is particularly conserved (MacIntosh et al., 2010). Some of these proteins, for example OsRNS4 (Table 1), seem to have significant stress-related functions (Zheng et al., 2014), although their specific biological activity is not known.

For brevity, this review will focus on two plant RNase T2 functions for which significant advances have occurred in recent years: ribosomal RNA (rRNA) turnover and tRNA processing.

Housekeeping Role Recycling rRNA, the Ancestral Function of Eukaryotic RNase T2 Enzymes

The presence of RNase T2 enzymes in almost all eukaryote genomes suggests that these enzymes carry out an important biological function. Phylogenetic and gene expression analyses suggested that enzymes in Class II carry out a housekeeping function in plants, and that this role is likely the ancestral function of RNase T2 enzymes (Hillwig et al., 2009; MacIntosh et al., 2010). RNS2, the Class II enzyme present in *Arabidopsis* (*Arabidopsis thaliana*), is localized mainly in the vacuole (Hillwig et al., 2011a). Characterization of *rns2* mutants showed that the enzyme is necessary for normal rRNA turnover, because the half-life of 28S rRNA and 18S rRNA almost doubled in the null mutant *rns2-2* (Hillwig et al., 2011a), and rRNA accumulated in mutant vacuoles (Floyd et al., 2015). Plants lacking RNS2 activity showed a constitutive autophagy phenotype, indicating that housekeeping turnover of rRNA is an essential process to maintain cellular homeostasis (Hillwig et al., 2011a; Floyd et al., 2015). Characterization of *rns2-1*, a mutant expressing an active RNase missing the last 14 amino acids, determined that the C terminus of the protein contains a putative vacuolar localization signal, as the truncated RNS2 protein is mislocalized outside the vacuole and secreted outside of the cells. Moreover, *rns2-1* mutants present a constitutive autophagy phenotype identical to the *rns2-2* null mutant; thus, vacuolar activity of this enzyme is essential to maintain normal cellular functions (Floyd et al., 2017). Although the mechanism of ribosomal or rRNA transport from the cytoplasm to the vacuole is

BOX 1. Major RNases in Cytoplasmic mRNA**Decay in Plants***Deadenylation*

Deadenylation is a second order process. In mammals, the first phase is catalyzed by the PAN2-PAN3 deadenylase complex. No orthologs have been found in vascular plants.

CCR4 and CAF1: Catalytic subunits of the CCR4-NOT deadenylation complex. CCR4 belongs to the EEP nuclease superfamily and CAF1 has sequence similarity to the RNase D class in the DEDD superfamily. In plants, both CCR4 and CAF1 are encoded by multigene families, and different CCR4-NOT complexes with various combinations of deadenylases exist. In mammals, these enzymes catalyze the second phase of poly(A) removal. It is unknown whether they carry out both phases in plants.

PARN: Poly(A)-specific ribonuclease that localizes in cytoplasmic foci and suppresses PTGS. PARN also has a mitochondrial role (see "Mitochondrial 3' to 5' Exoribonucleases" section).

Decapping

DCP2: A decapping enzyme and member of the Nudix hydrolase family, DCP2 specifically removes the 5' m⁷G cap of mRNAs. It is part of a decapping complex that also includes DCP1 and VARICOSE (VCS).

3'-5' decay

RNA exosome: Multi-subunit complex with a 9-protein core (Exo9) and peripheral proteins. Six of the core subunits have sequence similarity to bacterial RNase PH. Unlike metazoan and fungal Exo9, the plant core exosome has exoribonuclease activity provided by RRP41. The exosome-associated RNase RRP44 is also part of the complex. Although metazoan RRP44 orthologs have endo- and exoribonuclease activity, *in vitro* characterization of plant RRP44 only detected exonuclease activity. Metazoan RNA exosome also associates with another RNase, RRP6. While RRP6 orthologs exist in plants, their association with the exosome is unproven. The RNA exosome localizes to the cytoplasm and nucleus and has various roles in RNA decay.

SOV: SUPPRESSOR OF VARICOSE (SOV)/DIS3L2, another cytoplasmic 3'-5' exoribonuclease with sequence similarity to RRP44, but acting independently of the RNA exosome. This enzyme contributes to the decay of a subset of cytoplasmic mRNAs.

5'-3' decay

XRN4: 5'-3' exoribonuclease that degrades uncapped mRNAs and 3' mRNA fragments resulting from endonucleolytic cleave (for example products of miRNA-dependent cleavage).

still not well understood, evidence suggests that a selective transport mechanism that uses part of the proteins necessary for the autophagy process, specifically ATG5 (Autophagy-Related Gene 5, a protein indispensable for autophagic vesicle formation; Le Bars et al., 2014), participates in the transport of ribosomes or rRNA to vacuoles for degradation (Floyd et al., 2015).

RNase T2-mediated RNA turnover is part of the salvage pathway that maintains normal cellular levels of nucleosides/nucleotides. Lack of vacuolar RNA turnover in the *rms2* mutant causes metabolic changes, primarily in carbon flux through the pentose phosphate pathway, as a way to generate ribose-phosphate for de novo nucleotide synthesis (Morriss et al., 2017). A similar role for RNase T2 enzymes has been described in other organisms, including yeast (*Saccharomyces cerevisiae*; Huang et al., 2015), zebrafish (*Danio rerio*; Haud et al., 2011), and nematodes (*Caenorhabditis elegans*; Liu et al., 2018). Although RNS2, and potentially RNS2 orthologs in other plants, are necessary for normal rRNA turnover and homeostasis maintenance, rRNA still decays in *rms2* mutants even if the rate is reduced (Hillwig et al., 2011a). This observation implies that other RNases can attack rRNA *in vivo*. Whether this

turnover occurs in the vacuole or the cytoplasm is not known, but because *rms2* plants have a constitutive autophagy phenotype, it is tempting to speculate that this process could transport extra RNase activities to the vacuole that could contribute to rRNA turnover. For example, in stress situations the presence of P-bodies, cytoplasmic complexes involved in mRNA translation repression and RNA decay, increases in plants. P-bodies contain several ribonucleases including the decapping complex, CCDR and CAF1 deadenylases and XRN4 (Chantarachot and Bailey-Serres, 2018), and it has been shown that P-bodies are processed through an autophagic process (granulophagy) in yeast and mammalian cells (Buchan et al., 2013; Frankel et al., 2017). It can thus be hypothesized that P-body-associated RNases could contribute to rRNA decay in the *rms2* mutants.

As mentioned, it has been hypothesized that the role in RNA salvage (and perhaps P scavenging) is the ancestral function of RNase T2 enzymes (MacIntosh 2011). However, despite their almost absolute conservation, these enzymes are not strictly essential, although significant cellular and developmental phenotypes have been observed in plants and animals defective in RNase

Table 1. Classification of plant RNase T2 proteins and proposed functions

Class and Functions	Examples	References
Class I		
Defense, antiviral	RNS1 (Arabidopsis), ZnRNase II (<i>Zinnia elegans</i>)	Kim et al., 2019; Trifonova et al., 2012
Defense, antimicrobial	RNase NE (<i>Nicotiana tabacum</i>)	Hugot et al., 2002
Defense, wounding	RNS1 (Arabidopsis), RNase LE (<i>Solanum lycopersicum</i>), OsRNS4 and OsRNS5 (<i>O. sativa</i>)	Hillwig et al., 2008; Gross et al., 2004; MacIntosh et al., 2010
Stress, drought/salinity	OsRNS4 (<i>O. sativa</i>), PvRNS3 (<i>Phaseolus vulgaris</i>)	Zheng et al., 2014; Diaz-Baena et al., 2020
Stress, P-starvation	RNS1 (Arabidopsis), EsRNS1 (<i>Eutrema salsugineum</i>), RNase LX and RNase LE (<i>S. lycopersicum</i>)	Bariola et al., 1999; Velasco et al., 2016; Köck et al., 1995
tsRNA synthesis	RNS1 and RNS3 (Arabidopsis)	Alves et al., 2017; Megel et al., 2019
Development/PCD	RNase LX (<i>S. lycopersicum</i>), ZRNase I (<i>Z. elegans</i>)	Lehmann et al., 2001; Lers et al., 2006; Fukuda 2000
Class II		
RNA/nucleotide salvage	RNS2 (Arabidopsis)	Hillwig et al., 2011a
Stress, P-starvation	RNS2 (Arabidopsis)	Bariola et al., 1999
Class III		
Self-incompatibility	S-RNases (Solanaceae, Plantaginaceae, Rubiaceae, Rosaceae)	Ramanauskas and Igić, 2017
Defense, antimicrobial	S ₁ -RNase and S _X -RNase (<i>Petunia hybrida</i>), SA2-RNase (<i>Nicotiana glauca</i>)	Hillwig et al., 2011b; Silva et al., 2020
Stress, P-starvation	NnSR1 and S _X -RNase (<i>N. glauca</i>)	Rojas et al., 2015; Rojas et al., 2018

T2 activity (see above). It is still clear that the presence of T2 enzymes may provide a significant advantage to the organism that results in the conserved presence of these enzymes in eukaryotes. It is important to note that although the “ancestral” biological role has been assigned to RNS2 in Arabidopsis, neofunctionalization and gain/loss of gene duplications may have shifted the function to other T2 homologs in other species. For example, the Class II RNase T2 in tomato, RNase LER, seems to have a prominent role in guard cells, and it seems to localize exclusively to the ER (Köthke and Köck, 2011), whereas class I enzymes are found in the tomato vacuole and could be in charge of the RNA/nucleotide salvage function in this plant (Abel and Köck, 2001). For a more detailed analysis of phylogenetic relationships and evolution of the RNase T2 family in plants, the analysis by Ramanauskas and Igić, (2017) is recommended.

A Shared Role in the Biosynthesis of tRNA-Derived Small RNAs

Among the ever-growing collection of small RNA species found in living organisms, tRNA-derived small RNAs (tsRNAs) are a recent addition. tsRNAs vary in size, ranging from 19 to 35 nt, and are derived from different parts of tRNAs. Two main categories can be found in all eukaryotes: (1) 5' tsRNAs that are likely the result of cleavage in the tRNA D-loop and 3' tsRNAs produced by cleavage of the T-loop, commonly referred as tRNA-derived fragments (tRFs); and (2) 5' tRNA halves and 3' tRNA halves (tRHs) that result from processing at the anticodon loop (Dou et al., 2019). Note that nomenclature for these tsRNAs is not fully

established, and some authors refer to all tsRNAs as “tRFs” and differentiate between “long tRFs” (~30–35 nt) and “short tRFs” (<28 nt; Megel et al., 2015). Although the function of tsRNAs is less well understood than that of other sRNAs, their association with Argonaute (AGO) proteins indicate participation in post-transcriptional regulation of gene expression, with miRNA-like activity (Kumar et al., 2014). In addition, AGO-independent functions have been proposed, including regulation of both positive and negative regulation of translation, competitive binding to RNA-binding proteins, and modulation of mRNA secondary structures (reviewed by Dou et al., 2019). Given the significant conservation among tRNA sequences, it is not surprising that some tsRNAs are also highly conserved among organisms as divergent as mammals and bacteria (Kumar et al., 2014).

Analyses of tsRNA populations have been characterized in a variety of plants and relative abundance of individual tsRNAs is tissue specific and also modulated by biotic and abiotic stress conditions (reviewed by Zhu et al., 2018). The biosynthetic pathway(s) responsible for production of tRFs and tRHs is controversial. Recently, Martinez et al. (2017) described tRFs in the 18 to 25 nt range in Arabidopsis, and identified 19 nt 5'tRF species that accumulate preferentially in pollen. These 5'tRFs also accumulate in pollen from rice (*Oryza sativa*) and maize (*Zea mays*), and a similar phenomenon can be observed in the gametophyte/sporophyte of *Physcomitrella patens* (Martinez et al., 2017). The authors found a similar increase in 19 nt 5'tRFs in the *ddm1* mutant that is defective in the DDM1 *swi/snf* family chromatin remodeler protein. Moreover, the accumulation of these 19 nt 5'tRFs depends on the Dicer RNase DCL1. These DCL1 products are found in AGO1

complexes and target transposon elements (TE). These results indicated that the tRFs are produced by a Dicer-dependent pathway and act in a miRNA-like manner to control TE expression. However, other authors have found that the overall population of 5'tRF and 3'tRF is only modestly affected in *dcl1-4* Arabidopsis mutants (Nowacka et al., 2013; Alves et al., 2017).

On the other hand, Alves et al. (2017) found that several 5'tRF and 3'tRFs accumulate to higher levels in Arabidopsis plants overexpressing RNS1, a Class I RNase T2, suggesting that this enzyme could be involved in tRF production in plants. A more definitive demonstration of the role of plant RNase T2 enzymes in tsRNA production was recently presented by Megel et al. (2019), who used RNA sequencing (RNA-seq) and in vitro tRNA cleavage assays to demonstrate that tRF abundance is not affected in the quadruple Arabidopsis mutant *dcl1234* and that protein extracts from this mutant are still able to process tRNAs in vitro to produce tRFs and tRHs. The same biochemical approach was used to demonstrate that RNS1 and RNS3, another class I RNase T2 protein from Arabidopsis, and to a minor extent RNS2, can produce tRF and tRHs in vitro. Expression analyses also indicated that *rns1* mutants fail to accumulate tRFs and tRHs that are normally induced after Pi-starvation (Megel et al., 2019).

These conflicting results could indicate that specific tRFs and tRHs may be produced by different pathways, and, hypothetically, alternative biosynthetic pathways may be associated with different biological roles and mode of action, although both Dicer-dependent and -independent tRFs associate with Argonaute proteins in plants (Alves et al., 2017; Martinez et al., 2017). Also intriguing is the fact that a large number of plastid-derived tRFs accumulate in the cytoplasm and can associate with AGO1, although the RNases involved in their processing are not known (Cognat et al., 2017). Similarly, at least two pathways have been proposed for the formation of tsRNAs in human (*Homo sapiens*) cells. Cole et al. (2009) identified a large number of tsRNAs in HeLa cells, and determined that accumulation of a 19-nt 5'tsRNA derived from tRNA^{Gln} is Dicer dependent and showed that purified Dicer can cleave this tRNA in vitro. On the other hand, Yamasaki et al. (2009) identified stress-induced tRHs in human U2OS cells that depend of the activity of angiogenin, a member of the vertebrate-specific RNase A family, and overexpression of this enzyme increases accumulation of the tsRNAs in the absence of stress. Moreover, Rny1 is also responsible for the production of stress-induced tsRNAs in *S. cerevisiae* (Thompson and Parker 2009), several RNase T2 enzymes contribute to the production of tsRNAs in the ciliate *Tetrahymena thermophila* (Andersen and Collins 2012), and purified human RNASET2 can process tRNAs in vitro (Megel et al., 2019), suggesting that RNase T2 enzymes may have a conserved role in tsRNA production in eukaryotes.

Connecting Secretory RNases with their Substrates

Strong data support a role for plant RNase T2 proteins in the turnover of rRNA, and increasing evidence indicate that they also participate in the production of tsRNAs. However, the enzymes and their substrate are not normally found in the same subcellular location. Both tRNAs and rRNAs accumulate in the cytoplasm, but RNS2 is a vacuolar enzyme (Bariola et al., 1999; Hillwig et al., 2011a; Floyd et al., 2017) and RNS1 (and likely RNS3, given its similarity to RNS1) is mainly secreted outside of the cell (Bariola et al., 1999). As mentioned, autophagy-dependent mechanisms participate in the transport of rRNA to the vacuole where it can be attacked by RNS2, but data regarding the location where tRNA processing to produce tsRNAs takes place is missing. Whether tRNAs are transported to an organelle or whether the RNases are released into the cytoplasm to act on tRNAs (and/or rRNAs?) is not known. If the latter were true, the mechanisms that prevent cytotoxic effects of releasing RNases with little substrate specificity into the cytoplasm should also be investigated (see Outstanding Questions).

Many class I RNases are also associated with responses to abiotic and biotic stresses (Table 1). In some cases, it is possible to envision a substrate directly related to a biological function, for example, in defense against RNA viruses, or during phosphate starvation. But in other cases, their potential substrates and the specific role in stress responses is less evident. It is tempting to hypothesize that the role of RNase T2 enzymes in stress responses may also be connected to their ability to generate tsRNAs, thus having a regulatory role in these processes.

PLASTIDIAL RNASES

Both exo- and endo-ribonucleases have a dominant role in creating the chloroplast functional transcript population. They are responsible for intercistronic RNA cleavage along with 5' and 3' processing and RNA decay (Stoppel and Meurer 2012; Germain et al., 2013). Although seventeen chloroplastic RNases were predicted by mining the Arabidopsis genome for RNase annotations (Stoppel and Meurer, 2012), this figure certainly needs to be revised. Some predicted RNases were indeed shown to carry other functions (see, for example, the L-PSP/RidA enzyme; Niehaus et al., 2014), and new RNases keep being discovered in bacteria, some of them with a chloroplast homolog awaiting characterization (see, for example, the Rae1/YacP endoribonuclease; Condon et al., 2018). Indeed, as direct evidence of its cyanobacterial origin, nearly all chloroplast RNase homologs are present in bacteria (Stoppel and Meurer, 2012; Germain et al., 2013; dos Santos et al., 2018). Based on their chlorotic or embryo-lethal phenotypes and the associated disruption in RNA patterns, there are five major RNases whose putative roles are discussed below. Following

transcription, the 5'-3' exoribonuclease RNaseJ processes the 5' ends, whereas two 3'-5' exoribonucleases, polynucleotide phosphorylase (PNPase) and RNR1, cooperatively mature the 3' ends. Intercistronic cleavage is performed by endoribonucleases, but the division of labor between RNaseJ, RNaseE, and CSP41 is unclear (Fig. 1A; Germain et al., 2013 for a model of chloroplast RNA maturation). These RNases are not specific and act on a wide variety of transcripts.

Exoribonucleases

Absence of either of the two prominent exoribonucleases, PNPase and RNase R, leads to defects in 3' end

maturation for both mRNAs and rRNAs, and depletion of both causes embryo lethality in Arabidopsis (Germain et al., 2011; Germain et al., 2012).

PNPase

PNPase was the first discovered enzyme able to synthesize RNA in vitro (Grunberg-Manago et al., 1955). Its main role is, however, to degrade RNA through a phosphorolytic 3'-5' exoribonuclease activity, and the balance between the two activities depends on the relative concentration of nucleoside diphosphates and Pi: the addition of Pi stimulates degradation, whereas nucleoside diphosphates promote degradation

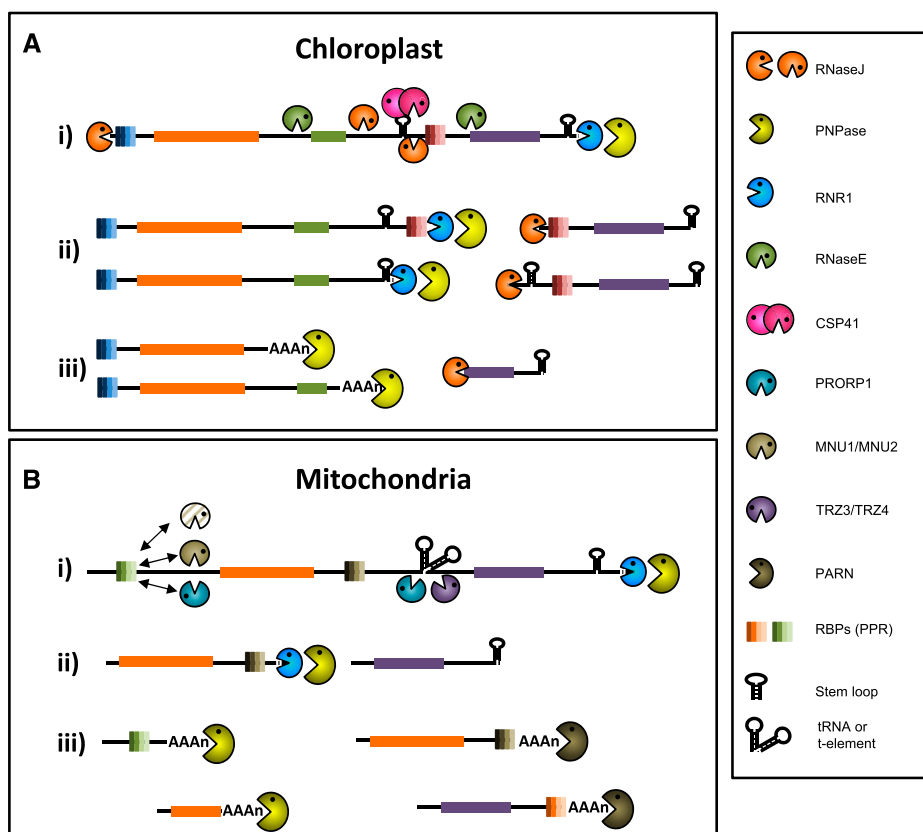


Figure 1. The roles of ribonucleases in chloroplast and mitochondrial mRNA metabolism. A, (i) In chloroplasts, RNase J processes the 5' end of polycistronic mRNAs, whereas PNPase and RNR1 cooperatively mature the 3' ends. The transcript is also subject to inter-cistronic cleavage by several endoribonucleases, but the division of labor between RNase J, RNase E, and CSP41 is unclear. A, (ii) Another round of exoribonuclease digestion gives the final termini. Mature transcript ends are protected from exoribonuclease degradation by sequence-specific RNA binding proteins (for example, PPR proteins) and secondary structures. A, (iii) Illegitimate, partially, or misprocessed transcripts are then digested at the 3' end by PNPase following the addition of a poly(A) tail. RNase J also participates in RNA degradation and surveillance. Its major role preventing the formation of double stranded RNA is not represented. B, (i) In mitochondria, endoribonucleases have a major role in defining 5' ends through their interactions with PPR proteins that specifically bind RNA targets. Cleavage by PRORP1, MNU1/2, or yet unknown endoribonucleases occurs downstream of the PPR binding site. Alternatively, PRORP1 and TRZ3/4 can cleave tRNAs or t-elements between two genes and release mature 5' and 3' ends. As in chloroplasts, PNPase and RNR1 cooperatively mature the 3' ends. B, (ii) Mature 3' ends are protected from exoribonuclease degradation by RBPs and/or secondary structures. B, (iii) Illegitimate, partially, or misprocessed transcripts are then digested at the 3' end by PNPase, following the addition of a poly(A) tail. Additionally, the 3'-5' exoribonuclease PARN appears to regulate the poly(A) status of correctly processed transcripts; however, the significance of this activity is unclear. RNase, RNase; PNPase, polynucleotide phosphorylase; RNR1, RNase R homolog 1; CSP41, chloroplast stem loop binding protein of 41 kD; PRORP1, PROTEINACEOUS RNASE P1; MNU, mitochondrial nuclease; TRZ, RNase Z; RBP, RNA binding protein. Figure inspired by Germain et al. (2013).

(Yehudai-Resheff et al., 2001). In vivo, the polymerase activity is responsible for the addition of short heteropolymeric A/U-rich tails to RNAs, but their biological role is still unclear (Germain et al., 2011). Different from the *Escherichia coli* enzyme, the active form purified from chloroplast is a complex of three PNPase monomers that does not seem to physically interact with other ribonucleases (Baginsky et al., 2001; Yehudai-Resheff et al., 2003). Extensive in vitro research has shown that PNPase is necessary to process RNA 3' extensions and that its activity is inhibited by stem loops structures or tightly bound RBPs (Hayes et al., 1996; Yehudai-Resheff et al., 2001; Yehudai-Resheff et al., 2003; Prikryl et al., 2011). PNPase has a high affinity for poly(A) sequences, making it a major player in poly(A)-assisted RNA decay in chloroplast (Lisitsky et al., 1996; Yehudai-Resheff et al., 2003; Schuster and Stern, 2009). Arabidopsis PNPase mutants have chlorotic young leaves and are smaller than wild type but can still grow to full maturity (Marchive et al., 2009; Germain et al., 2011). Under phosphorus deprivation, Arabidopsis mutants fail to initiate lateral roots, whereas PNPase-depleted *Chlamydomonas* cells cannot survive in the same condition (Yehudai-Resheff et al., 2007; Marchive et al., 2009). PNPase was the first chloroplastic RNase whose influence on RNA maturation was investigated using RNA-seq (Hotto et al., 2011; Castandet et al., 2013; Castandet et al., 2019). This analysis, in association with traditional molecular biology techniques, showed that PNPase-deficient Arabidopsis plants accumulate numerous RNAs with 3' extensions along with additional defects in RNA splicing and editing. Studies in Arabidopsis and maize determined that all types of RNAs are affected, including mRNAs, rRNAs, tRNA precursors, antisense RNAs, and spliced intron products (Walter et al., 2002; Marchive et al., 2009; Williams-Carrier et al., 2010; Germain et al., 2011; Hotto et al., 2011; Castandet et al., 2013; Ruwe et al., 2013; Castandet et al., 2019).

RNR1

In bacteria, members of the RNaseII/R 3'-5' exoribonuclease superfamily mainly involved in mRNA metabolism are named RNaseII, whereas the ones displaying significant activity on rRNA are named RNR (Li and Deutscher, 2004). This interpretation of division of labor is, however, likely to be an oversimplification (Cheng and Deutscher, 2005). The Arabidopsis RNaseII/R homolog, RNR1, is dually localized to chloroplast and mitochondria (Kishine et al., 2004; Perrin et al., 2004b; Bollenbach et al., 2005) and combines the two activities described in bacteria (Germain et al., 2012). The *rnr1* null mutants can only germinate on Suc, have white cotyledons that accumulate anthocyanins, and show retarded growth and pale green rosette leaves (Kishine et al., 2004; Bollenbach et al., 2005). Photosynthesis is impaired in the mutants, and electron microscopy revealed a lack of stacked thylakoids (Bollenbach et al., 2005). *rnr1* mutants overaccumulate

rRNA precursors at the expense of the mature forms (Kishine et al., 2004; Bollenbach et al., 2005; Germain et al., 2012). Additionally, mature rRNAs contain short 3' extensions consistent with the ability of the recombinant enzyme to process the 3' end of the *rnr5S* precursor in vitro (Bollenbach et al., 2005). It was noticed that the impaired maturation of the *rnr5S* transcript in the mutant is associated with the accumulation of an antisense RNA (asRNA), and this defective maturation was recapitulated in transplastomic plants artificially overexpressing the asRNA (Hotto et al., 2010; Sharwood et al., 2011b). Based on the ability of the *E. coli* enzyme to unwind RNA duplexes in vitro, it can be speculated that RNR1 might degrade the asRNA in vivo (Sharwood et al., 2011b; Chu et al., 2017). A more thorough investigation of the *rnr1* RNA patterns also demonstrated the essential role of the enzyme in mRNA 3' maturation, in cooperation with PNPase. Most mRNAs in the *rnr1* mutant accumulate short 3' extensions, a feature probably explained by the ability of the enzyme to come closer than PNPase to the secondary structures and RBP binding sites marking the mature mRNA 3' ends (Germain et al., 2012). Precursor mRNAs accumulate in nonpolysomal fractions and are more stable in the *rnr1* mutant than wild type, suggesting a close link between RNA maturation and degradation (Germain et al., 2012).

The Endo- and Exoribonuclease RNase J

RNase J belongs to the metallo- β -lactamase family of ribonucleases (Condon and Gilet 2011), and its role in chloroplast RNA metabolism has recently been reviewed (Hotto et al., 2020). Initially described as an endoribonuclease in *Bacillus subtilis*, most members harbor both endo- and 5'-3' exoribonuclease activity (Even et al., 2005; Mathy et al., 2007; Condon and Gilet 2011). As an illustration of its essential role in plant and chloroplast development, RNase J null mutants are embryo lethal (Tzafrir et al., 2004; Chen et al., 2015). Virus-induced gene silencing (VIGS) was therefore used to deplete RNase J in both Arabidopsis and tobacco chloroplasts (Sharwood et al., 2011a; Luro et al., 2013), and it showed that RNase J matures the 5' end of several transcripts, with RBPs acting as a barrier to its activity (Luro et al., 2013). This result is in agreement with earlier in vitro studies that used commercial 5'-3' exoribonucleases and recombinant RBPs (Prikryl et al., 2011; Hammani et al., 2012). The most striking effect in VIGS plants, however, was the accumulation of RNA species antisense to genic transcripts that could form duplexes preventing translation (Sharwood et al., 2011a). Because RNase J degrades preferentially single-stranded over double-stranded RNA in vitro, it is likely that its role in vivo is to degrade asRNAs before the formation of duplexes (Sharwood et al., 2011a; Halpert et al., 2019), making it an essential player in chloroplast RNA quality control. Interestingly, VIGS plants did not accumulate longer or unprocessed polycistronic

precursors in place of mature transcripts, suggesting that RNase J does not mediate endonucleolytic processing (Sharwood et al., 2011a). This is in contrast with *in vitro* results where, similar to its bacterial homologs, Arabidopsis RNase J displays both activities (Halpert et al., 2019). On the other hand, although *Chlamydomonas* chloroplasts likely contain a 5'-3' exoribonuclease activity (Drager et al., 1998; Drager et al., 1999), its RNase J acts only as an endoribonuclease *in vitro* (Liponska et al., 2018). One of the peculiarities of the plant enzyme is that it contains a C-terminal extension with high similarity to the GT-1 DNA binding domain usually found in transcription factors. It has been proposed that this domain could confer substrate specificity to the enzyme (Hotto et al., 2020). Finally, the possibility that chloroplast RNase J might also be involved in transcription termination of stalled RNA polymerase complexes, as recently described for the *Bacillus* enzyme (Šiková et al., 2020), has not been explored yet.

Endoribonucleases

CSP41

All photosynthetic organisms contain the CSP41 (Chloroplast Stem loop binding Protein of 41 kD) endoribonuclease whose elusive functions have recently been reviewed (Leister 2014). CSP41 proteins are highly abundant RNA binding proteins that display strong endoribonuclease activity on stem loops *in vitro* (Yang et al., 1996; Yang and Stern 1997; Bollenbach and Stern 2003a, b), a structure they preferentially bind (Yang et al., 1996). According to this characteristic, it was proposed that CSP41 cleaves the *psbT-psbH* transcript intergenic region (Chevalier et al., 2015). Chloroplasts contain two homologous proteins, CSP41A and CSP41B, which can form homo- and heterodimers (Bollenbach et al., 2009; Qi et al., 2012) and bind ribosomes (Beligni and Mayfield 2008; Bollenbach et al., 2009). Although the *csp41a* null mutant does not display any obvious phenotype, the *csp41b* null mutant shows mild chlorosis that is stronger under stress. Moreover, the absence of CSP41B also destabilizes CSP41A, resulting in similar phenotypes for the *csp41b* mutant and the *csp41ab* double mutant (Bollenbach et al., 2009; Qi et al., 2012). RNA immunoprecipitation-microarray (RIP-Chip) analyses more recently showed that CSP41 proteins are able to bind a large variety of transcripts and that their role is more likely to be RNA stabilization rather than cleavage (Qi et al., 2012). It has been proposed that the endoribonuclease activity could be regulated *in vivo*, making active forms behave like ribonucleases and inactive forms like stabilization factors (Leister 2014). If true, this hypothesis could reconcile the conflicting results obtained *in vitro* and *in vivo*.

RNase E

Chloroplast-localized homologs of *E. coli* RNase E can be found in most plants and green algae with the

notable exception of *Chlamydomonas* (Schein et al., 2008). It is an essential endoribonuclease in *E. coli*, involved in both mRNA maturation and degradation (Ait-Bara and Carpousis, 2015). *In vitro* RNA cleavage assays confirmed that the plant enzyme is an endoribonuclease with a preference for single-stranded RNA with A/U rich sequences and 5' monophosphate, a result in line with the described behavior of the bacterial enzyme (Mudd et al., 2008; Schein et al., 2008). RNase E is found in high M_r complexes in the soluble fraction of the chloroplast stroma, probably in an oligomeric state (Schein et al., 2008). Null *rne* mutants grow extremely slowly compared with wild type, are chlorotic, and contain smaller chloroplasts, demonstrating the essential role of RNase E in plants (Mudd et al., 2008; Walter et al., 2010). They accumulate very long transcripts precursors at the expense of processed, mature products, suggesting that the enzyme could be involved in intercistronic cleavage of the long mRNA precursors (Walter et al., 2010; Stoppel et al., 2012). It was indeed proposed that the strong phenotype observed for *rne* mutants was the consequence of the mutant inability to properly express some of the chloroplast-encoded ribosomal proteins transcripts (Walter et al., 2010). It is, however, important to note that mature transcripts still accumulate in the mutant, albeit at a lower extent than in wild type, suggesting that other enzymes can partially complement RNase E deficiency (Walter et al., 2010; Stoppel et al., 2012). Coimmunoprecipitation showed that RNase E forms a ~800 kD complex with an RNA binding protein, RHON1, which has been hypothesized to confer endonucleolytic cleavage specificity (Stoppel et al., 2012). RHON1 seems particularly important for the processing of the *rbcl-accD* and *rrn23S-4.5S* intergenic area, but the exact role of the interaction with RNase E is still unclear. It was indeed shown that RHON1 is involved in transcription termination downstream of *rbcl* independently of RNase E (Chi et al., 2014) and that it interacts with the plastid-encoded RNA polymerase to prevent the formation of detrimental R-loops during transcription-replication-head-on conflicts (Yang et al., 2020).

Other Ribonucleases

Besides the five major RNases previously described, chloroplasts contain additional RNases that are more specific to their substrate. Chloroplast tRNAs are processed at the 5' end by a protein-only RNase P enzyme named PRORP1 (PROTEINACEOUS RNASE P1; Gobert et al., 2010; Gutmann et al., 2012) that is dually localized to both the mitochondria and the chloroplast (see "Mitochondrial Endoribonucleases") and at the 3' by the RNase Z TRZ2 (Canino et al., 2009). Several other ribonucleases are involved in the processing of the rRNA transcriptional unit.

Mini-RNase III

Most plants contain one homolog of the *Bacillus subtilis* mini-III endoribonuclease. These enzymes are

active on double-stranded RNA (dsRNA) and only contain the catalytic domain of the RNase III enzyme, without the dsRNA binding domain (Redko et al., 2008; Hotto et al., 2015). Arabidopsis plants contain two paralogs, RNC3 and RNC4, whose null mutants do not display any macroscopic phenotype. Double *rnc3/4* mutants are, however, smaller than wild type and display reduced chlorophyll accumulation in mature leaves. Both gene-specific and genome-wide approaches (RNA-seq) demonstrated that mini-III processes the 5' end of the 23S rRNA and the 3' of the 4.5S rRNA. The enzyme is also involved in the processing of an RNA antisense to the 4.5S rRNA and plays a role in intron degradation following splicing (Hotto et al., 2015).

YbeY

In both *E. coli* and *B. subtilis*, rRNA 16S 3' end is matured by an endoribonuclease of the YbeY family (Baumgardt et al., 2018). In vitro, the Arabidopsis homolog AtYbeY preferentially cleaves rRNA substrates but is not able to recapitulate the correct rRNA 16S 3' end processing. Null mutants do not survive on soil, and plants with a reduced amount of YbeY grow slowly, have pale-green cotyledons and leaves, and completely altered chloroplast development. Mutants contain 5' and 3' extensions on the 16S, 23S, and 4.5S rRNA species, but mRNAs accumulate normally. AtYebY is therefore likely to be an endoribonuclease specific to the rRNA operon maturation (Liu et al., 2015).

ERIL1

ERI-1 proteins are conserved eukaryotic 3'-5' exoribonucleases from the DEDDh family that participate in small RNA pathways (Thomas et al., 2014). The Arabidopsis homolog, ERIL1, is conserved in all sequenced plant genomes and targeted to the chloroplast. Knock-down mutants in Arabidopsis and tobacco showed mild chlorosis and smaller leaves compared with wild type. Recombinant ERIL1 possess a 3'-5' exoribonuclease activity on single-stranded but not double-stranded RNA in vitro. The mutants showed incorrect processing of the 23S to 4.5S rRNA precursor and a decrease in mature 4.5S and 5S rRNA, but the exact molecular defects remain unclear (Mermigka et al., 2016).

PPR proteins

Present in all eukaryotes, the pentatricopeptide repeat (PPR) protein family has greatly expanded in plants and is involved in every step of plastidial gene expression (Barkan and Small, 2014). Molecular analyses of the *crr2* (*CHLORORESPIRATORY REDUCTION2*) Arabidopsis mutant showed an accumulation of the unprocessed *rps7-ndhB* dicistronic transcript (Hashimoto et al., 2003). The CRR2 protein has 9 PPR

domains and characteristic E and DYW domains found in many PPR proteins. Because some DYW domains (including the one present in CRR2) have endoribonucleolytic activity in vitro, it was proposed that CRR2 was a sequence-specific endoribonuclease responsible for the precise *ndhB* 5' end processing, the specificity being conferred by the PPR repeats (Nakamura and Sugita 2008; Okuda et al., 2009). Recent analyses, however, suggest that CRR2 role in *rps7-ndhB* processing is probably to protect the *ndhB* 5' end from exoribonucleases (Ruwe et al., 2019). The PPR SMR is another subfamily of PPR proteins that potentially contain sequence specific endoribonucleases (Zhang and Lu 2019). Arabidopsis mutant for the PPR SMR SOT1 (SUPPRESSOR OF THYLAKOID FORMATION1) protein has retarded growth, virescent leaves, and abnormal chloroplast structures, whereas maize mutants for the PPR53 ortholog are chlorotic and have an impaired NDH complex (Zoschke et al., 2016; Zhou et al., 2017). Molecularly, *sot1* and *ppr53* mutants accumulate incorrectly processed 23S to 4.5S rRNA precursor and mature forms, a possible explanation to the lower chloroplast protein synthesis observed (Wu et al., 2016; Zoschke et al., 2016; Zhou et al., 2017). Recombinant SOT1/PPR53 proteins can specifically bind the 5' end of the 23S rRNA through their PPR domain, protecting the transcript from RNase degradation. Additionally, the SMR domain is necessary for correct rRNA maturation in vivo, and it can cleave the 5' region of the 23S rRNA in vitro. Interestingly, the SMR domain expressed alone can cleave both Arabidopsis DNA and total RNA, whereas the full-length recombinant SOT1 specifically cleaves in the 5' region of the 23S rRNA, around 19 to 20 nt downstream of its binding site (Zhou et al., 2017). It is, therefore, likely that the addition of a sequence-specific PPR domain to a nonspecific endonucleolytic SMR domain created a sequence-specific endoribonuclease in chloroplast. Arabidopsis chloroplasts contain at least five additional PPR SMR whose role as ribonucleases have not been explored yet (Liu et al., 2013; Zhang and Lu 2019).

Toward a Plastome View of RNase Action?

So far, analyses of RNA phenotypes for RNases mutants or silenced (VIGS) plants have been done with tedious and time-consuming gene by gene molecular techniques, leading to a general focus on a few heavily studied transcripts or clusters along with the rRNA operon. A consequence is that the knowledge has often been built on conflicting results from in vitro and/or in vivo analyses. For example, the relevance of strong in vitro endonuclease activity for CSP41 (Yang et al., 1996) does not easily reconcile with roles suggested by in vivo mutant analyses (Bollenbach et al., 2003; Baligni and Mayfield 2008; Bollenbach et al., 2009; Qi et al., 2012). Additionally, mutants for the endoribonuclease RNase E, its specificity partner RHON1, RNase J, and others, accumulate unique and apparently

unprocessed transcripts, but the precise sites of cleavage are still unknown (Walter et al., 2010; Stoppel et al., 2012; Luro et al., 2013). These limitations have recently started to be overcome with the use of RNA-seq based approaches that have been instrumental in understanding the global role of two chloroplast ribonucleases, the exoribonuclease PNPase and the endoribonuclease mini-III (Hotto et al., 2011; Castandet et al., 2013; Ruwe et al., 2013; Hotto et al., 2015; Castandet et al., 2019). Combining a transcriptome-wide view to a single-nt resolution map of the chloroplast RNA processing sites is likely to speed up any future functional analysis of poorly studied chloroplastic ribonucleases whose RNA targets are still unknown.

MITOCHONDRIAL RNASES

Similar to chloroplast, 3' ends of mitochondrial transcript are mainly matured through the cooperative action of the two 3'-5' exoribonucleases, PNPase and RNR1. The major difference between the two organelle lies in the generation of mature 5' ends: whereas the RNase J 5'-3' exoribonuclease activity is essential in the chloroplast, there is no evidence of such an activity in plant mitochondria. Mature 5' ends are, rather, generated through the action of various endoribonucleases whose identities have only begun to be revealed (Fig. 1B).

Mitochondrial 3' to 5' Exoribonucleases

As evidence of its essential role in plant and mitochondria physiology, mitochondrial PNPase null mutants are embryo lethal (Perrin et al., 2004b). In vivo studies using plants where the enzyme was down-regulated showed the accumulation of unprocessed transcripts containing long 3' extensions (Perrin et al., 2004a; Perrin et al., 2004b). These plants also accumulate misprocessed, truncated, and cryptic polyadenylated transcripts that are not, or barely, detectable in wild-type plants. This is consistent with the known role of PNPase in the polyadenylation-stimulated degradation pathway that is conserved from bacteria to organelles. No heteropolymeric tails could, however, be identified in these plants, suggesting that the mitochondrial PNPase might not function as a polymerase in vivo (Holec et al., 2006). Although plant mitochondria contain their own PNPase, RNR1 is dually targeted to both the chloroplast and mitochondria (Bollenbach et al., 2005; Perrin et al., 2004b). Mutants for RNR1 showed the accumulation of partially processed mitochondrial transcripts containing short 3' tails that could be removed by the enzyme in vitro. This suggests that PNPase first removes large 3' extensions, whereas RNR1 processes the remaining 3' tails to produce mature 3' ends. Like in chloroplast, secondary structures and RBPs can specify the correct site of processing by acting like barriers against 3' to 5' degradation (Forner et al., 2007; Häili et al., 2013; Ruwe et al., 2016). It is

important to note that such a model has so far only been validated for a few mitochondrial transcripts and still awaits global validation.

Plant mitochondria contain a third 3' to 5' exoribonuclease, the eukaryotic deadenylase PARN [poly(A)-specific RNase; Hirayama et al., 2013]. PARN contains three Exo domains characteristics of the RNase D family, and its homologs are widely distributed among eukaryotes (Chiba et al., 2004; Reverdatto et al., 2004). Null mutants are embryo lethal, and a weak allele is dwarf, with short roots, and displays hypersensitivity to the plant hormones abscisic acid and salicylic acid (Chiba et al., 2004; Reverdatto et al., 2004; Nishimura et al., 2005). The partially defective mutant accumulates 3' polyadenylated mitochondrial mRNAs (Hirayama et al., 2013). It was shown in vitro that PARN only degrades the poly A tail and not the rest of a substrate RNA; however, it is unclear whether PARN is involved in vivo in the removal of poly A tract only or more largely in RNA degradation and/or processing (Reverdatto et al., 2004). Different from PNPase, the PARN mutant did not accumulate defective or misprocessed transcripts, suggesting that these two RNases have distinct roles in poly(A)-dependent RNA metabolism. The PARN role is, however, conserved in embryophytes as the enzyme from *Marchantia polymorpha* can complement the Arabidopsis mutant (Kanazawa et al., 2020).

Mitochondrial Endoribonucleases

Endoribonucleases have a major role in generating mitochondrial RNA termini, especially at the 5' end (Binder et al., 2016). Termini mapping of mRNAs revealed that some 5' and 3' ends were located directly at the processing sites of adjacent tRNAs and t-elements (for tRNA-like elements), suggesting the involvement of RNase P and RNase Z endonucleases (Forner et al., 2007). The RNase P PRORP1 is a member of the PPR protein family and also contains a conserved NYN (for Nedd4-BP1, YacP Nuclease) metallo-nuclease domain (Matelska et al., 2017). Null mutants are lethal, but VIGS plants depleted for PRORP1 showed an altered mitochondrial structure with disorganized cristae. The enzyme can process in vitro a mRNA containing a t-element similarly to what is observed in vivo, and the same transcript is unstable in VIGS plants (Gobert et al., 2010; Gutmann et al., 2012). tRNAs and t-elements 3' ends are processed by TRZ3 and TRZ4, two of the four RNase Z homologs encoded in the Arabidopsis genome. Single knock-out lines are viable but the double null mutant is lethal, pointing to a functional redundancy. Recombinant TRZ3 and TRZ4 correctly process tRNA and t-elements 3' ends in vitro, at the exact mRNA site described in vivo (Forner et al., 2007; Canino et al., 2009).

Most mRNAs are, however, independent from tRNAs or t-elements and generated through the action of endoribonucleases. Mitochondrial nuclease1 (MNU1) and MNU2, two other members of the NYN family, also

play a role in mitochondrial transcript maturation. Although single and/or double mutants did not display any obvious macroscopic phenotypes, large, 5'-extended mRNA precursors accumulated at the expense of some mature mRNAs in the mutants. This was, however, not true for most mRNAs and the endoribonuclease activities have not been tested in vitro (Stoll and Binder 2016). Coimmunoprecipitation and yeast two-hybrid assays recently showed that PRORP1 physically interacts with MNU2, and the latter also plays a role in tRNA accumulation (Bouchoucha et al., 2019). Because several sequence specific PPR proteins are known or predicted to bind upstream of mitochondrial mRNA 5' mature ends (Ruwe et al., 2016), the current model is that they direct unspecific endoribonucleases to the correct site of processing (Binder et al., 2016). This mode of action is convincingly supported by an experiment with the RNA binding protein RPF2 that was altered to bind to a new transcript, different from its natural cognate. This induced a new endoribonuclease cleavage site downstream of the modified RPF2 binding site (Colas des Francs-Small et al., 2018). Similarly, binding of the PPR protein RFL2 to the mitochondrial *orf291* transcript induces a cleavage by PRORP1 around 70 nt downstream of the binding site (Fujii et al., 2016).

CONCLUSION

RNA homeostasis is essential to maintain proper cellular function. In addition to the "usual suspects" that participate in cytoplasmic and nuclear RNA metabolism, it is clear that organellar and secretory RNases have an important role in this homeostatic regulation. RNases located in plastids and mitochondria maintain a functional transcriptome, affecting not only mRNA accumulation but also adequate synthesis of rRNA and other noncoding RNA species essential for

their function. Similarly, secretory RNases connect RNA levels to primary metabolism, and have also been recruited for a variety of stress response mechanisms. Despite their importance, we still do not have a full understanding of their activities and the actual number of RNases that are located in organelles. More important, in some cases we do not know the in vivo substrate of these RNases and whether the substrates are recruited to the RNase location or RNases translocate to carry out their specific functions. Finally, although it is clear that many of these RNases have to coordinate their activity to achieve the desired RNA outcome, either in a synthetic or catabolic pathway, very little information exists on partners of organellar and secretory RNases, and posttranslational regulatory mechanisms that could control their activity (see Outstanding Questions). This is best exemplified by the unresolved question of the RNA quality control in chloroplast, where the same ribonucleases in charge of transcript processing also function in RNA degradation: How do they "know" whether to preserve or degrade RNA? In addition to the known polyadenylation-assisted degradation (Schuster and Stern, 2009; Germain et al., 2013) and the protective action of secondary structures and RNA binding proteins (Hayes et al., 1996; Perrin et al., 2004b; Manavski et al., 2018), three main hypotheses have been proposed. First, biochemical evidence showed that RNases do not act alone and are part of high M_r complexes, where they interact with multiple unknown partners that support their functions (Hayes et al., 1996; Stoppel et al., 2012). It is easy to speculate that some of these partners could play a role in cleavage specificity. Specific RNA binding proteins could also direct a RNase to a specific RNA target, similarly to what has been described in mitochondria. Second, proteomics studies have shown that RNases can be post translationally modified, for example through phosphorylation. As proposed for CSP41, this could represent a way to regulate the RNase specificity and/or activity (Leister 2014). Third, RNase activity could be regulated through the spatial localization of both enzymes and substrates, similarly to what has been described in bacteria (Redder 2016). It was for example proposed that the cooperation between PNPase and RNR1 could be based on their preferential localization in the chloroplast nucleoid and stroma, respectively (Germain et al., 2012). These questions should drive research in the near future if we are to have a systems understanding of RNA homeostasis in plants.

OUTSTANDING QUESTIONS

- What are the interacting partners of the secretory and plastidial ribonucleases?
- How many RNases are in the chloroplast and the plant mitochondrion?
- How are chloroplastic and mitochondrial RNases directed to preserve or degrade RNA?
- Are there other RNases in the vacuole?
- Where in the cell do RNase T2 enzymes process tRNAs?
- Are the defense roles of RNase T2 enzymes directly related to activity on pathogen RNAs, or are they the result of the regulatory function of tRF and tRNA halves produced by these enzymes?

ACKNOWLEDGMENTS

We thank all the members of our respective past and present laboratories for support.

Received January 21, 2020; accepted May 31, 2020; published June 8, 2020.

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