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► **To cite this version:**

Marjorie Chery, Laurence Drouard. Plant tRNA functions beyond their major role in translation. Journal of Experimental Botany, In press, 10.1093/jxb/erac483 . hal-03920414

**HAL Id: hal-03920414**

**<https://hal.science/hal-03920414v1>**

Submitted on 3 Jan 2023

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# Plant tRNA functions beyond their major role in translation

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## Highlight

Besides their crucial role in protein synthesis, tRNAs were shown to have other equally important functions. This review aims at recalling the other functions attributed to tRNAs in the plant kingdom.

## Abstract

Transfer RNAs (tRNAs) are well-known for their essential function as adapters in delivering amino acids to ribosomes and making the link between mRNA and protein according to the genetic code. Besides this central role in protein synthesis, other functions are attributed to these macromolecules or their genes in all living organisms. Here, this review focuses on these extra functions in photosynthetic organisms. For example, tRNAs are implicated in Tetrapyrrole biosynthesis, mRNA stabilization or transport, priming reverse transcription of viral RNAs, and tRNA-like structures play important roles in RNA viral genomes. Another important function of tRNAs in regulating gene expression is related to their cleavage allowing the production of small non-coding RNAs named tDRs for tRNA-derived RNAs. Here, we examine in more detail the biogenesis of tDRs and their emerging functions in plants.

**Keywords:** genome organization, mRNA stabilization, organelle, regulation of gene expression, Sine element, tetrapyrrole biosynthesis, photosynthetic organisms, tRNA derived RNA

Accepted Manuscript

## Introduction

Transfer RNAs (tRNAs) are likely among the most ancestral and the most well-known small non-coding RNAs. With a few exceptions, where they are shorter (Wende *et al.*, 2013), tRNAs have a size of 76 to 93 nucleotides. They have a characteristic cloverleaf secondary structure (Fig. 1) and adopt a L-shaped tertiary structure (Schimmel, 2018). In eukaryotes, the CCA triplet added post-transcriptionally at the 3'-extremity is the site of aminoacylation. Aminoacylated tRNAs can then play their major role, the incorporation of amino acids into the nascent peptide chains within the ribosome. In plants, protein synthesis takes place in three compartments, the cytosol, the plast, and the mitochondrion. In land plants, the plastidial genome encodes a full set of tRNA genes (*i.e.* 37 tRNA genes corresponding to 30 isoacceptor tRNAs in *Arabidopsis thaliana*) to ensure translation in this organelle. Mitochondrial DNA lacks several tRNA genes (*e.g.* 22 tRNA genes corresponding to 17 isoacceptor tRNAs in *A. thaliana*), thus several nucleus-encoded tRNAs have to be imported from the cytosol into mitochondria to get an active mitochondrial protein synthesis process. In the nuclear genomes, the number of tRNA genes varies from one species to the other. For instance, the *A. thaliana* nuclear genome has 585 tRNA genes that code for 46 isoacceptor tRNAs (Michaud *et al.*, 2011) (Cognat *et al.*, 2022). With the exception of 124 clustered tRNA genes that are epigenetically silenced in this model plant (Hummel *et al.*, 2020), it is believed that the others are expressed and active in protein synthesis in *A. thaliana*.

Besides the canonical role of tRNAs in the translation process, it is more and more evident that these macromolecules, aminoacylated or not, are important players in many other biological processes. This has already been emphasized in excellent reviews (*e.g.* (Raina and Ibba, 2014) (Katz *et al.*, 2016) (Schimmel, 2018)). Non exhaustively, we can cite the implication of charged tRNAs in the formation of the bacterial cell walls, the biosynthesis of antibiotics, the aminoacylation of membrane lipids in bacteria and fungi (Yakobov *et al.*, 2020), or the targeting of proteins for degradation (Kwon *et al.*, 1999) (Rai and Kashina, 2005) (Graciet *et al.*, 2006). Uncharged tRNAs have a regulatory role during amino acid starvation in yeast and mammals (Hinnebusch, 2005) (Avcilar-Kucukgoze and Kashina, 2020) or during cell death. Finally, another crucial regulatory function of tRNAs is the role played by tRNA fragments, which we will further name tDRs for tRNA-derived RNAs (Holmes *et al.*, 2022). First believed to be non-functional degradation by-product molecules, tDRs are now largely accepted as essential non-coding RNAs (Liu *et al.*, 2021) (Chen *et al.*, 2021). For instance, they were shown to inhibit translation in human under stress conditions (Yamasaki *et al.*, 2009), to regulate the RNA decay pathway in *Thermus thermophila* (Couvillion *et al.*, 2012), to regulate apoptosis in mouse (Saikia *et al.*, 2014), to be engaged in the RNA silencing pathway in various organisms to degrade specific mRNAs (Kumar *et al.*, 2014) (Kuscu *et al.*, 2018), or to be used as primers for viral reverse transcriptase (Ruggero *et al.*, 2014). The implication of tDRs in these molecular processes likely has important biological consequences such as the regulation of haematopoiesis (Goncalves *et al.*, 2016), cancer cell proliferation (Honda *et al.*, 2015) (Yu *et al.*, 2020) or epigenetic inheritance (Chen *et al.*, 2016) (Sharma *et al.*, 2016). Many more examples of tDR functions are emerging regularly and represent a very rapidly evolving field whatever the organism studied, including plants.

Concerning photosynthetic organisms, apart from the translation process, what are the other functions of tRNAs or tRNA-like structures? This review highlights our current knowledge in the field. The first part concerns the extra functions of tRNA or tRNA-like structures, the second part focuses on tDRs.

## Roles of tRNA and tRNA-like structure apart from translation

### *Production of organic compounds*

With the discovery of the genetic code and the key role played by tRNAs in decoding it, the understanding of tRNA biology was thought to be well covered. Unexpectedly, novel functions were attributed to certain tRNAs outside of translation. Some of them, the production of tetrapyrroles and of cytokinins, are crucial for plant growth and are detailed below.

Plant tetrapyrroles (heme, chlorophyll, siroheme, and phytychromobilin) constitute essential signaling molecules (Larkin, 2016). Their biogenesis is performed from the same precursor, the 5-aminolevulinic acid (ALA). The pathway of ALA synthesis is located in the stroma of plants and involves the glutamyl tRNA<sup>Glu</sup> charged with glutamate (Glu-tRNA<sup>Glu</sup>) (**Fig. 1a**). The plastidial genome of land plants encodes a single tRNA<sup>Glu</sup>(TTC). After its aminoacylation with glutamate, Glu-tRNA<sup>Glu</sup> is not only used for the incorporation of glutamate into proteins during plastidial translation but was shown, 35 years ago (Schon *et al.*, 1986), to be also the substrate of glutamyl-tRNA reductase (GluTR). This enzyme catalyzes the conversion of Glu into Glu-1-semialdehyde, the latter being then transformed in ALA by a second enzyme, the Glu-1-semialdehyde aminotransferase. Recent work interestingly demonstrated that GluTR activity is inhibited by unprocessed tRNA<sup>Glu</sup> (Agrawal *et al.*, 2020).

Plant hormones such as auxin and cytokinin are essential for plant growth and development. Their homeostasis is thus tightly regulated (Hrtyan *et al.*, 2015). Cytokinins are derivatives of adenine with either an isoprene or an aromatic side chain attached at the N<sup>6</sup>-terminus. The presence of isoprene cytokinins-containing tRNAs was observed in different organisms, from bacterial to mammals (Juhling *et al.*, 2009). Cytokinins found in plants, zeatin and isopentenyladenine residues, are located in position 37 of tRNAs. Although *de novo* biosynthesis of cytokinins is the most common pathway, the prenylation of tRNAs by the tRNA isopentenyltransferases followed by their degradation also allows the release of cytokinins and represents a minor pathway for cytokinins biosynthesis (Miyawaki *et al.*, 2006) (Kurakawa *et al.*, 2007) (Kieber and Schaller, 2014).

These two examples perfectly illustrate how tRNAs or aa-tRNAs can be hijacked from the translation process to produce metabolites. Although so far restricted to a very few examples in plants, it is tempting to speculate that other alternative functions may exist. In particular, diverting aminoacyl-tRNAs to aminoacylate membrane lipids is a well-known process in bacteria (Katz *et al.*, 2016) and has been recently described in fungi (Yakovov *et al.*, 2020). A similar role in plants remains speculative but worth to decipher.

### *tRNAs as primers for initiation of reverse transcription in plant pararetroviruses*

Surprisingly, there is an astonishing relationship between tRNAs and viruses. The two following sub-sections report on various aspects of this relation. The first one relates on the use of specific host tRNAs to prime reverse transcription of viral RNAs (**Fig. 1b**), the second one is linked to tRNA mimicry with the finding of tRNA-like structures (TLS) on viral genomes (**Fig. 1c**).

In 1970, the discovery of an RNA-dependent DNA polymerase (thus called reverse transcriptase) in two retroviruses, murine leukemia virus (MuLV) (Baltimore, 1970) and Rous

sarcoma virus (RSV) (Temin and Mizutani, 1970), demonstrated that genetic information can be transferred from RNA to DNA. Since then, numerous reverse transcriptases have been described in retroviruses. These reverse transcriptases require specific tRNAs, such as tRNA<sup>Trp</sup> for MuLV or tRNA<sup>Trp</sup> for RSV as primers to initiate DNA synthesis (Marquet *et al.*, 1995). To note, the reverse transcription of mobile elements, the retrotransposons, also requires tRNAs as primers. In plants, some double-stranded DNA viruses were found to replicate via an RNA intermediate and were named pararetroviruses. The most well-known representative is the Cauliflower Mosaic Virus (CaMV), a virus infecting cruciferae. Forty years ago, the involvement of a reverse transcription step in the replication of the virus was demonstrated, and the implication of a tRNA strongly suspected (Pfeiffer and Hohn, 1983). Indeed, as for retroviruses and retrotransposons, the host initiator tRNA<sup>Meti</sup> initiates the replication (Turner and Covey, 1984), (Hull *et al.*, 1987), (**Fig. 1b**). Retroviruses, retrotransposons and plant pararetroviruses share another feature: the specificity of the reverse transcription priming is ensured by a complementarity between the 3' extremity of the tRNA and a sequence called PBS for primer binding site. For instance, in the CaMV, there is a 14 nt sequence of perfect homology between the 3' extremity of tRNA<sup>Meti</sup> and the PBS so that a strict initiation of reverse transcription occurs.

As a whole, primer tRNA appears essential for the life cycle of plant pararetroviruses. More generally, a small set of specific tRNA species serve as primers of reverse transcription even in distantly related retrotransposons, retroviruses and pararetroviruses and we can wonder why tRNAs were selected rather than other abundant RNA species. Is that related to a close evolutionary origin between viruses and tRNAs, and/or to the need to highly regulate the reverse transcription process? These questions remain unanswered to date.

#### *Roles of tRNA-like structures (TLS) of viral RNA genomes*

A number of positive strand viral RNA genomes lacks a poly(A) tail at their 3'-extremities, an important feature for their translation and stability. To cope with this lack, many of them possess a tRNA-like structure (TLS) at their 3'-termini (**Fig. 1c**). As explained in detailed reviews (Wu *et al.*, 2021) (Dreher, 2009), a TLS structure presents some criteria of a true tRNA molecule: for instance, folding in a cloverleaf secondary structure, recognition by tRNA-specific enzymes (e.g. RNases P or Z, tRNA-nucleotidyl transferase, aminoacyl-tRNA synthetases), formation of a tertiary complex with GTP and an elongation factor. The first TLS to be identified was in the RNA genome of the turnip yellow mosaic virus (TYMV), (Yot *et al.*, 1970). Since then, various TLSs have been found in many plant viral genomes. With the exception of tobnaviruses, plant viral TLSs perfectly mimic tRNAs and are aminoacylated by either valine, histidine, or tyrosine. Several important, but widely variable, functions have been attributed to viral TLSs. For instance, valylation of TYMV TLS is essential to support infectivity while the TLS of tomato aspermy cucumovirus is not even aminoacylated. TLSs are indeed also implicated in the replication step of viruses, in particular as minus strand initiation start. In some cases, translation enhancement by TLSs has been observed in a manner that resembles a poly(A) tail (Miras *et al.*, 2017). In the brome mosaic virus (BMV), by being implicated in RNA recombination, the presence of TLSs could be an advantage for the virus. In addition, in this virus, TLSs are involved in the encapsidation of the tripartite viral genomes. It has also been suggested that some plant viral RNAs use TLSs as carriers to be transported in phloem sieve tubes (Tolstyko *et al.*, 2020). Finally, the role of TLSs in the stabilization of viral genomic RNAs is also well known. As a matter of fact, the integrity of

the CCA triplet (thus repaired by the host tRNA-nucleotidyl transferase) present at the 3'-end of TLS sequences is important.

#### *tRNA or TLS as stabilizing structures*

In the same vein, the presence of tRNA at the 3' extremity of organellar mRNAs also appears to be an ancestral event. Recent work showed that mitochondrial-encoded tRNAs from the freshwater alga, *Cyanophora paradoxa*, have two functions within the organelle: on the one hand, as expected, they are key players of mitochondrial translation and, on the other hand, they are involved in a peculiar punctuation processing mechanism leading to mRNA-tRNA hybrids with a CCA triplet at their 3'-extremities (**Fig. 1d**), (Salinas-Giege *et al.*, 2021). The presence of highly folded tRNA structures at the 3'-ends of mRNAs likely impede exonuclease activities and thus stabilize the mRNAs until they reach the mitochondrial ribosomes to be translated. *C. paradoxa*, a glaucophyte representing the most basal group of Archeplastida, is considered as a living fossil among photosynthetic organisms. The existence in this organism of mitochondrial tRNA genes, located just downstream of protein-coding genes, serving as stabilizing factors is reminiscent to the presence of numerous tRNA pseudogenes or TLSs (also called t-elements) located close to RNA extremities in the mitochondria of higher plants such as Arabidopsis (Forner *et al.*, 2007) or potato (Varre *et al.*, 2019). It is tempting to speculate that such "t-elements" derive from true tRNA genes; they progressively became pseudogenes and lost their ability to be correctly processed and active in translation in the mitochondria of higher plants but still kept another essential function in mRNA stabilization.

#### *Transport of tRNA:mRNA hybrids*

Similar to viral RNAs and organellar mRNAs, the presence of tRNA or TLS as part of plant cytosolic mRNAs represents an additional example of their key role in gene expression regulation and signaling. Indeed, mobility of mRNAs represents an important aspect of cell-to-cell communication. The mobility of RNAs and their importance as signaling molecules has been described in detail (Maizel *et al.*, 2020). Using grafting experiments in Arabidopsis, Zhang *et al.* demonstrated that mRNA:TLS transcripts can move bidirectionally in phloem sieve elements and that the TLS seems to be determinant for long distance transport of mRNAs between tissues (**Fig. 1e**), (Zhang *et al.*, 2016a). These hybrids appear enriched in the population of graft-mobile mRNAs. Interestingly, the authors also showed that once transported, these mRNAs can be translated. The presence of 19 dicistronic tRNA:mRNA transcripts was also demonstrated in grapevine (Fabres *et al.*, 2021). The authors suggest that the use of TLS as mobile signals for mRNA transcripts to move over long distances is linked to the appearance of plant vasculature. The functional role of these mobile mRNAs in distant tissues and the mechanism allowing the transport of mRNA:TLS transcripts remain to be further addressed.

Collectively, it is striking to note the large number of cases in which TLSs are found associated with either viral RNAs or mRNAs, and the number of functions in which they may be implicated (e.g. replication, stabilization, transport, translation). Is this reminiscent of the world of RNA and why is it so well conserved during evolution? These intriguing questions could be related to the origin of life.

### *Short interspersed nuclear elements (SINEs)*

Finally, the last example illustrating the importance of tRNAs, apart their role in protein synthesis, is their implication in the emergence of nonautonomous retrotransposons, the SINEs. Again, this strongly suggests that primordial ancestral tRNAs had the potentiality to be drivers of evolution likely already in the “RNA world”.

Short interspersed nuclear elements (SINEs) are short (100 to 600 bp) non-autonomous retroelements transcribed by RNA polymerase III (pol III), (Kramerov and Vassetzky, 2011). They are found in a wide variety of eukaryotic genomes and rely on long interspersed elements (LINEs) for their propagation. The best-known family is the Alu family. In most cases, SINEs are ancestrally derived from tRNA genes. SINEs derived from tRNAs have a composite structure (**Fig. 1f**). They comprise a TLS at their 5'-extremity, with the A and B boxes characteristic of pol III internal promoters, followed by a tRNA-unrelated portion and a poly(A) rich sequence at the 3'- end. Since the discovery of the first SINEs in plants, found in the Waxy gene of *Oryza sativa* (Umeda *et al.*, 1991), many other SINEs have been identified in photosynthetic organisms (Seibt *et al.*, 2016). For example, various families have been identified in *A. thaliana*, in *Brassica* species (Pelissier *et al.*, 2004), and in other plants (Gadzalski and Sakowicz, 2011). A family of 35 SINE members has been found in *Populus trichocarpa* (Michaud *et al.*, 2011) and more than 200 copies of such elements have been identified in the unicellular green alga, *Chlamydomonas reinhardtii* (Cognat *et al.*, 2008). Remarkably, while in most cases, the typical tRNA cloverleaf secondary structure is difficult to retrieve, both in *C. reinhardtii* and in *P. trichocarpa*, the SINE tRNA-related sequences have retained mostly perfect cloverleaf structures. Compared to human where SINEs represent more than 10% of the genome, they correspond to usually less than 0.3% of plant genomes. While the roles of SINEs have been well investigated in human (Zhang *et al.*, 2021), only limited information is available about the functions of plant SINEs. In Solanaceae, Seibt and colleagues demonstrated that SINE retrotransposons are important contributors to gene and genome evolution: SINEs were shown to be involved in an increased length of UTRs and introns, to be responsible for the formation of splice sites, exons, start and stop codons or to form tandem-like structures (Seibt *et al.*, 2016). The genome-wide impact of the *Au* SINE family was extensively studied in wheat species (Ben-David *et al.*, 2013). This work supports the existence of an *Au* SINE activity throughout the evolution of wheat and its polyploidization, followed by an epigenetic regulation via cytosine methylation. The *Au* Sine family has thus likely contributed to the diversification of wheat species by inducing genetic and epigenetic changes. As a whole, tRNA-related SINE retroelements are likely important players of the evolution and the organization of the plant nuclear genome, but overall, this remains largely poorly understood and undoubtedly, other regulatory functions of plant SINEs remain to be deciphered.

### **The emerging world of tRNA-derived RNAs**

High-throughput small RNA sequencing technologies have greatly expanded the repertoire of small non-coding RNAs. Among them, RNA fragments derived from tRNAs have grown considerably in recent years. Produced through enzymatic cleavage of tRNAs, they have been described in all domains of life, including plants, as novel actors in the regulation of genomes expression (Megel *et al.*, 2015) (Kumar *et al.*, 2016) (Magee and Rigoutsos, 2020) (Alves and Nogueira, 2021). Many names and acronyms have been assigned to the small RNAs derived from tRNAs. Among them, we can cite tRFs, tsRNAs, tiRNAs, tRNA halves

among others. Recently, a consensus nomenclature has been proposed with the name tDR for tRNA-derived RNAs (Holmes *et al.*, 2022). A bioinformatic tool, tDRnamer (<http://trna.ucsc.edu/tDRnamer/index.html>), allows its standardization: the name takes into account the size of the tDR, its position relative to its corresponding tRNA, the nature of the tRNA. A suffix indicates if the RNA sequence can correspond to several tRNA transcripts and their number as well as the possible modifications of the tRNA carried by the tDR. Here, we adopted this novel acronym tDR and we present our current knowledge of the tDRs population, biogenesis, and functions in photosynthetic organisms.

### *The population of tDRs*

To characterize tDR populations, classical high-throughput small RNA sequencing methods are commonly used. They usually require 5'-monophosphate (5'-P) and 3'-hydroxyl (3'-OH) for the ligation of adapter molecules, but recent data show that other types of extremities (such as 5'-OH, 5'-ppp, 3'-p, 2'3'-cyclic phosphate or 3'-aminoacyl), not compatible with such classical ligation procedure, may exist [see for instance (Wang *et al.*, 2021)]. In addition, nucleotide modifications, such as methylation, strongly impede the reverse transcription step and result in stops or misincorporations. Thus, it is important to note that, due to these sequencing biases (*e.g.* blocked extremities, modifications) and limitation in the length of the analyzed RNAs, part of the tDR population is likely missing in current analyzes. Novel approaches such as ARM-seq (Cozen *et al.*, 2015), PANDORA-seq (Shi *et al.*, 2021), CPA-seq (Wang *et al.*, 2021), or RTcB sRNA-seq (Gu *et al.*, 2022) represent important tools to enlarge the tDRs repertoire.

Nevertheless, the data obtained so far already provided interesting insights regarding tDR pools. tDRs can be divided into two groups according to the region of the tRNA they derive and according to their size (**Fig. 2a**). Short tDRs (*circa* 15-28 nt) originate from the 5'-extremity of mature tRNA after cleavage in the D region (tDR-5D) or from the 3'-extremity after cleavage in the T region (tDR-3T). Long tDRs (*circa* 30-40 nt) correspond to mature tRNA halves generated through cleavage in the anticodon region (tDR-5A, tDR-3A).

In plants studied so far, tDRs originating from mature tRNAs represent more than 99.8% of the tDRs population. tDRs derived from precursor tRNAs (in particular from the 3' trailer sequences released after cleavage by RNase Z) are an exception (Cognat *et al.*, 2017) (Alves *et al.*, 2017), as compared to mammals where they are more abundant (Liao *et al.*, 2010). Interestingly, the population of tDRs is derived from only a part of different tRNA species. For instance, only 12 of 46 nuclear and 10 of 30 plastidial isoacceptor tRNAs are predominantly cleaved to form tDR-5D (Cognat *et al.*, 2017). Among the short tDRs, those of 19 and 20 nt are the most abundant. The tDRs-5D result mostly from cleavages in the D-loop and to a lesser extent in the D-arm. The opposite is true for tDR-3T where cleavages are more abundant in the T-arm than in the T-loop. Zahra and collaborators analyzed small RNA libraries from 6 angiosperms (*A. thaliana*, *Solanum lycopersicum*, *Cicer arietinum*, *Medicago truncatula*, *Oryza sativa* and *Zea mays*) and obtained similar results (Zahra *et al.*, 2021). A few internal tDRs were also retrieved.

In Arabidopsis, tDRs from nuclear, plastidial, and mitochondrial tRNAs were found. Three-quarters of the identified tDRs are from nuclear tRNAs, about one-quarter are of plastidial origin and less than 1% are from mitochondrial tRNAs (Cognat *et al.*, 2017). The presence of the different classes of plastidial tDRs were also found in *Brassica rapa* (Wang *et al.*, 2011). Within different tissues (seedlings, leaves, and flowers), the tDR population slightly varies. In contrast, the population in roots shows notable differences. It is not only due to changes in

the plastidial tDRs population (because of the absence of chloroplasts in roots compared to photosynthetic tissues), but also to fluctuations in the nuclear tDR population (Cognat *et al.*, 2017) (Wang *et al.*, 2011) (Alves *et al.*, 2017). Globally, few tDR-3T are found in roots. Concerning tDR-5D, four of them represent more than 75% of the tDR-5D population in roots (Arg (ACG), Arg (TCG), Cys (GCA), and Gly (TCC)). An accumulation of tDR-5D has also been observed in mature pollen grains (Martinez *et al.*, 2017).

Importantly, it is now well described that the accumulation of several tDRs is induced by various stresses (**Fig. 2b**). For instance, oxidative stress triggers the accumulation of a variety of short and long tDRs (Thompson *et al.*, 2008) (Alves *et al.*, 2017). Variations in tDRs population were also observed in different plants, Arabidopsis, wheat, rice, turnip, under various abiotic stresses (e.g. salt, drought, cold, heat, uv), (Loss-Morais *et al.*, 2013) (Alves *et al.*, 2017) (Cognat *et al.*, 2017). Both up- or down-regulation of specific tDRs were observed and the variations are not always the same depending on the plant species. Whether this reflects different biological functions or biases due to the various approaches used remains to be studied in more details. In addition, Byeon and collaborators have demonstrated a transgenerational response to heat stress implicating a differential accumulation of tDRs in *Brassica rapa*. Upon heat stress, they first observed a down-regulation of glutamyl tDR-5D and an up-regulation of aspartyl tDR-5D in different organs. Then they showed that in the progeny of the stressed plants, there was still an accumulation of aspartyl tDR-5D, while other tDRs (Ala, Arg, Tyr) were reduced (Byeon *et al.*, 2017, 2019). The authors propose that tDRs could contribute to the improvement of plant responses to future stress *via* the regulation of several molecular pathways but this remains to be established. Accumulation of specific tDRs (in particular Gly and Asp tDRs) was also found during phosphate starvation in Arabidopsis (Hsieh *et al.*, 2010; Hsieh *et al.*, 2009) (Megel *et al.*, 2019), changes in tDR abundance depending on phosphorous was also observed in barley (Hackenberg *et al.*, 2013), and a global decrease in tDRs was obtained following a treatment of tomato leaves with the phytohormone ABA (Abscisic Acid), (Luan *et al.*, 2020). Finally biotic stresses were also shown to influence the population of tDRs. For example, in wheat, tDR deriving from tRNA Glu (CTC), Lys (CTT), and Thr (CGU) are highly expressed during *Fusarium graminearum* infection (Sun *et al.*, 2022).

#### *The biogenesis of tDRs*

Production of tDRs implies cleavage of tRNA molecules by endoribonucleases. From bacteria to eukaryotes, many enzymes have been identified during the last decade (e.g. (Megel *et al.*, 2015) (Chen *et al.*, 2021)). Non exhaustively, we can cite Colicin in *E. coli*, the RNases T2 in *Saccharomyces cerevisiae* or in *Tetrahymena thermophila* and a multitude of nucleases in mammals such as ELAC2, RNase L, the RNase A Angiogenin, the RNase III Dicer, or nucleases of the Schlafen family (Yang *et al.*, 2018). Most of these nucleases cleave tRNAs in the anticodon region to generate long tDRs and very few data are available concerning the biogenesis of short RNAs after cleavage in the D- or T- regions. This complexity shows the difficulty to define precisely which enzyme does which job, and much remains to be done to address this question. This is particularly true in plants (**Fig. 2c and d**). While the implication of Dicer-like 1 (DCL1) to generate specific tDRs in pollen grains has been proposed (Martinez *et al.*, 2017), other data where plant DCLs are not involved in tDR biogenesis were presented by two teams (Alves *et al.*, 2017) (Megel *et al.*, 2019). Rather, both teams demonstrate that the biogenesis of both short and long tDRs in leaves, flowers, seed coats, and endosperms, relies on the activity of endoribonucleases belonging to the RNase T2 family named RNS.

Indeed, in *Arabidopsis* grown under phosphate starvation conditions, the induction of RNS1 is correlated with the accumulation of specific tDRs previously observed by Hsieh and collaborators (Hsieh *et al.*, 2010). How can we explain such a discrepancy? Each *Arabidopsis* RNS has a specific expression profile but together all the members of the family cover the whole plant, including flowers and pollen, thus we expect a rather broad and constitutive activity of tRNA cleavage by this family of endoribonucleases. DCLs, also highly expressed in pollen, may be responsible for only a very limited, but essential, number of specific tRNA cleavages, especially to produce specific tDRs such as the ones of 19 nt. To generate tDR-5D of 19 nt in length, tRNA cleavage occurs in the D- loop. As DCL enzymes usually cleave double stranded RNAs, what also remains to be elucidated is the mode of action of these enzymes to produce tDRs. How DCLs but also RNSs interact with tRNAs remains unknown and await structure/function analysis.

Another essential parameter to take into account for the biogenesis of tDRs is the presence of numerous modifications on eukaryotic tRNAs (Suzuki, 2021). These modifications, by either promoting or inhibiting tRNA cleavages, will affect the abundance of tDRs (Lyons *et al.*, 2018) (Chen *et al.*, 2021). For instance, in *Drosophila melanogaster*, under heat stress, the presence of a methylated cytosine at position 38 ( $m^5C$ ) of tRNA<sup>Asp</sup>, tRNA<sup>Gly</sup>, and tRNA<sup>Val</sup> reduces the production of tDR-5A (Durdevic *et al.*, 2013). By contrast, in humans, decreased methylation of cytidines 48 and 49 ( $m^5C$ ) increases tDR5A production. This is due to an increased affinity between Angiogenin and unmodified tRNAs (Blanco *et al.*, 2014). In plants, tRNAs are also modified and these modifications may vary under stress conditions (Wang *et al.*, 2017a) or during plant development (Pfitzinger *et al.*, 1990). However, yet, no data are available on the role played by tRNA modifications in the production of tDRs in these organisms. It is tempting to speculate, that as for the other organisms studied so far, their presence or absence will impact tRNA cleavage in plants. This is still an open question that could be addressed by analyzing *Arabidopsis* knock-down mutant lines of modifying enzymes.

### *The roles of tDRs*

In all organisms, the tDRs population appears complex and comprises thousands of small non-coding RNAs. Many of these tRNA-derived RNAs are likely to be degradation by-products related to tRNA turnover or tRNA quality control (Megel *et al.*, 2015). It is also an efficient way to recycle nutrients and deliver phosphate and nitrogen to the cells. Nevertheless, there is now growing evidence that tDRs play additional functions that have important biological consequences (e.g. in cancer, neurological diseases, viral infection, epigenetic inheritance), and several reviews listing the multiple roles to which they have been attributed so far as well as the underlying molecular mechanisms exist (Schimmel, 2018) (Magee and Rigoutsos, 2020) (Su *et al.*, 2020) (Chen *et al.*, 2021). In plants, the functions of tDRs are still poorly understood (**Fig. 3**).

Specific tDRs were found associated with argonaute (AGO) proteins (Loss-Morais *et al.*, 2013) (Alves *et al.*, 2017) (Cognat *et al.*, 2017) (Ren *et al.*, 2019) (Trolet *et al.*, 2019) (Gu *et al.*, 2022), thus suggesting their implication in the regulation of gene expression *via* the RNA-induced silencing complex RISC, similar to a microRNA-like pathway. Indeed, Martinez and collaborators observed an accumulation of tDR-5D of 19 nt in *Arabidopsis* pollen grains, accumulation also found in the reproductive organs of rice, maize, and the moss *Physcomitrium patens* (Martinez *et al.*, 2017). The *Arabidopsis* 19 nt long tDRs were found incorporated onto AGO1. Bioinformatics analysis to search for target mRNAs with sequence

complementarity with the tDRs showed that they could target mostly protein-coding mRNAs but also a smaller proportion of transposable elements (TEs). Finally, they demonstrated that a tDR-5D derived from initiator tRNA<sup>Met</sup> is able to target the Athila6A transcript, a member of the Gypsy family of retroelements, and to induce its cleavage (**Fig. 3a**). As germinative cells lose heterochromatin during epigenetic reprogramming, the use of a tDR as a microRNA to target and degrade TEs, maybe a way to avoid their reactivation and maintain the stability of the genome. More recently, it was proposed that a tDR-5D derived from tRNA<sup>Ala</sup> associates with AGO1 and targets the mRNA coding for the cytochrome 450 71A13 involved in the biosynthesis of camalexin, a toxin for pathogens. Upon infection of Arabidopsis with *Botrytis cinera*, the abundance of the tDR would be reduced and the production of camalexin increased to de-repress the anti-fungal defense (Gu *et al.*, 2022). In wheat, upon infection with *Fusarium graminearum*, there is a very high accumulation of tDRs, in particular, three deriving from tRNA<sup>Lys</sup>(CTT). Potential targets including genes implicated in replication, translation, protein degradation, and resistance to pathogens were suggested. Yet, there is no experimental evidence demonstrating a role of these tDRs in the regulation of gene expression via RNA silencing, but this could represent another interesting example. Finally, a very interesting discovery comes from a study focusing on the interaction between the rhizobial bacterium *Bradyrhizobium japonicum* and the soybean *Glycine max*, during root nodule formation (**Fig. 3b**). Ren and collaborators showed the presence in the nodules of bacterial tDRs-3T with a size range between 18 to 24 nt. More specifically, they demonstrated that three tDRs (from bacterial tRNA<sup>Val</sup>(CAC), tRNA<sup>Gly</sup>(TCC), and tRNA<sup>Gln</sup>(CTG)), found loaded in AGO1, target three genes, GmRHD3, GmHAM4 and GmLRX5, associated with nodule initiation and development. The authors then demonstrated that, by their ability to interact with AGO1, tDRs target and repress genes that prevent symbiosis. Here, by acting as positive regulators of nodulation, the rhizobial tDRs represents a nice example of cross-kingdom regulation between bacteria and plants.

While a few tDRs were found associated with AGO proteins, many of them are not. A few studies have shown that RNAs deriving from tRNAs are able to inhibit translation independently of any RISC complex (**Fig. 3c**). In pumpkin, tDRs, among other small non-coding RNAs, accumulate in the phloem sap. The authors demonstrated that this complex population of small RNAs triggers translation inhibition *in vitro* and suggested that tDRs were the best candidates to play this role and act as long-distance signaling molecules (Zhang *et al.*, 2009). To address the question of whether plant tDRs can act as regulators of protein synthesis, *in vitro* experiments were performed with Arabidopsis tDRs. Both long (from tRNA<sup>Ala</sup>, tRNA<sup>Leu</sup> or tRNA<sup>Cys</sup>) and short tDRs (from tRNA<sup>Ala</sup> or tRNA<sup>Asn</sup>) are capable of inhibiting translation *in vitro* (Nowacka *et al.*, 2013) (Lalande *et al.*, 2020). In mammals, translation repression by tDR-5A derived from tRNA<sup>Ala</sup> was also demonstrated and shown to be dependent on the presence of the 4 guanine residues located at its 5'-extremity that enable G-quadruplex formation (Ivanov *et al.*, 2014). By contrast, Arabidopsis alanine tDR-5D also contains 4 Gs at the 5'-extremity but this is not a pre-requisite for translation inhibition. Analysis of Arabidopsis ribosomal fractions showed that alanine tDR can associate with active polyribosomes and modulate translation but how this is achieved remains to be elucidated (Lalande *et al.*, 2020). So far inhibition of protein synthesis by tDRs was only provided *in vitro*. It becomes essential to provide evidence of their involvement in the regulation of translation *in vivo* and to characterize the biological processes impacted, such as stress response or plant development.

## Concluding remarks

tRNA biology appears much more complex than first envisaged. With the discovery of the genetic code and the indispensability of tRNAs to transform genetic information into proteins during the translation process, it seemed that the circle was closed. This loop was reopened with the discovery of novel functions attributed to these macromolecules. We have just presented some of them in this review dedicated to the plant kingdom but this is likely only the tip of the iceberg. During evolution, nuclear tRNA genes have been duplicated/multiplied/dispersed in eukaryotic genomes such as those of the plant world. Point mutations have progressively generated tRNA isoacceptors (*i.e.* different anticodons for the same amino acid) and tRNA isodecoders (*i.e.* same anticodon but with sequence differences). Moreover, the transition from the prokaryotic to the eukaryotic world is correlated with a significant increase in both the number and the complexity of base modifications found on tRNAs (Boccaletto *et al.*, 2022). Altogether, the result is an immense and complex reservoir of tRNAs that opens the door to the emergence, in the course of evolution, of various and unanticipated roles that for the most part have yet to be determined. Identifying these different functions, related or not to translation, remains a challenge. The development of new high throughput technologies should facilitate this work. The study of tRNA epitranscriptome variation during cell development or during response or adaptation to stresses could lead to the characterization of certain tRNAs involved in specific processes. This is particularly true for plants, which, as sessile organisms, must quickly and continuously adapt to the environment. In human and yeast, variation in tRNA modification levels were observed during cell cycle and in response to environmental stresses (e.g. (Chan *et al.*, 2010) (Huber *et al.*, 2019) (Chen *et al.*, 2021)). In plants, such changes were also shown in different stress conditions such as drought, salt or cold (Wang *et al.*, 2017b) but variations during cell development have not been studied yet. Many tRNA modifications are essential for decoding efficiently the corresponding codons, and dynamic changes in tRNA modification levels in response to stress conditions can favor the selective translation of subpopulations of mRNAs (Ignatova *et al.*, 2020) (Lin *et al.*, 2018). Variations in tRNA modification levels can also affect the stability of tRNAs (Huang and Hopper, 2016) (Torrent *et al.*, 2018). In frame of the canonical role of tRNAs, protein synthesis efficiency will then be affected. In addition, as described above, the biogenesis of tDRs can be impacted by tRNA modification changes, thereby potentially influencing the regulatory functions of tDRs. Importantly, it is worth to mention here that, in addition to affect tDR biogenesis, altered modification status may also influence the function of resulting tDRs (Zhang *et al.*, 2016b). The importance of the many tRNA modifications represents a large biological field to be discovered. tRNA epitranscriptomic comparative studies could bring new clues on which modifications and which tRNA isoacceptors and/or isodecoders are differentially affected during cell development or stress response, a first step towards the understanding of their various roles in central biological processes.

The number of tRNA genes but also their localization on the genome is an aspect to be taken into consideration regarding their potential functions. Very few studies in this field exist. It is known that environment and three-dimensional structure of chromatin can affect the transcription of tRNA genes by RNA pol III (see *e.g.* (Thompson *et al.*, 2003) in *S. cerevisiae*). Conversely, the spatial organization of tRNA genes on a genome could influence the 3D structure of the chromatin. Indeed, in *S. cerevisiae*, Hamdani and collaborators have shown that tRNA genes and RNA Pol III transcription elements contribute to chromatin organization and local domain formation (Hamdani *et al.*, 2019). Whether this is a

widespread phenomenon and whether it occurs in photosynthetic organisms remains to be elucidated. The annotation of plant tRNA genes (<http://plantrna.ibmp.cnrs.fr/>)(<http://14.139.61.8/PtRNAdb/index.php>) and the use of recent technologies including high throughput chromatin conformation capture and derivatives allowing local- and long-distance chromatin interactions (Domb *et al.*, 2022) could help demonstrating the role of tRNA genes in shaping genome structure.

Finally, the discovery of tRNA-derived RNAs with multiple functions, in particular as regulators of genome expression, completely changed the paradigm. Indeed, tRNA-derived RNAs are no longer considered to be simple degradation products, in fact, they have greatly expanded the tRNA world, even though they remain quite enigmatic. Many unanswered questions remain: which ribonuclease for which tDR biogenesis? Among the vast repertoire of RNA fragments, which tDR for which biological process? Which molecular processes and which tDR interactors for which biological process? In plants, the situation is even more complex as tDRs derived from organellar (plastidial and mitochondrial) tRNAs have been observed. Plant organellar tDRs are not generated within the organelles (Cognat *et al.*, 2017). Do these organellar tDRs play a role in retrograde signaling is another open question. Finally, among the most outstanding questions, it is still debated how, through the process of evolution, the present-day tRNA structure arose (Schimmel, 2018). Among the various hypotheses, it has been suggested that a combination of tRNA halves is at the origin of modern tRNAs (Zuo *et al.*, 2013). This is a “chicken and egg” issue, and both possibilities are not exclusive: emergence of modern tRNAs via the fusion of smaller RNAs at the beginning of life to ensure protein synthesis and biogenesis of a diversity of small non-coding RNAs through the cleavage of tRNAs over evolution to acquire new ways of regulating gene expression. At this point, it is worth recalling that tDRs are present in the three domains of life, Archaea, Bacteria and Eukarya, positioning them among the oldest classes of small non-coding RNAs. Similarly, tRNAs are also considered among the oldest and most conserved non-coding RNAs and their complex relation with viruses, as described above, is intriguing from an evolutionary point of view. As suggested by (Chen *et al.*, 2021), while tRNAs are, nowadays, considered as major players of protein translation, another primary ancestral role may have been to be drivers of early life by providing the possibility, through fragmentation, to produce a large set of tDRs, opening up the potential to acquire new regulatory functions. This attractive hypothesis will need to be addressed in the future.

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**Funding**

This work was supported by the Interdisciplinary Thematic Institute IMCBio, as part of the ITI 2021-2028 program of the University of Strasbourg, CNRS and Inserm, supported by IdEx Unistra (ANR-10-IDEX-0002), and by SFRI-STRAT'US project (ANR 20-SFRI-0012) and EUR IMCBio (ANR-17-EURE-0023) under the framework of the French Investments for the Future Program."

**Author contribution**

Both authors, M.C. and L.D., contributed to the writing of the manuscripts and to the drawing of the figures.

**Conflict of interest**

No potential conflict of interest was reported by the authors.

Accepted Manuscript

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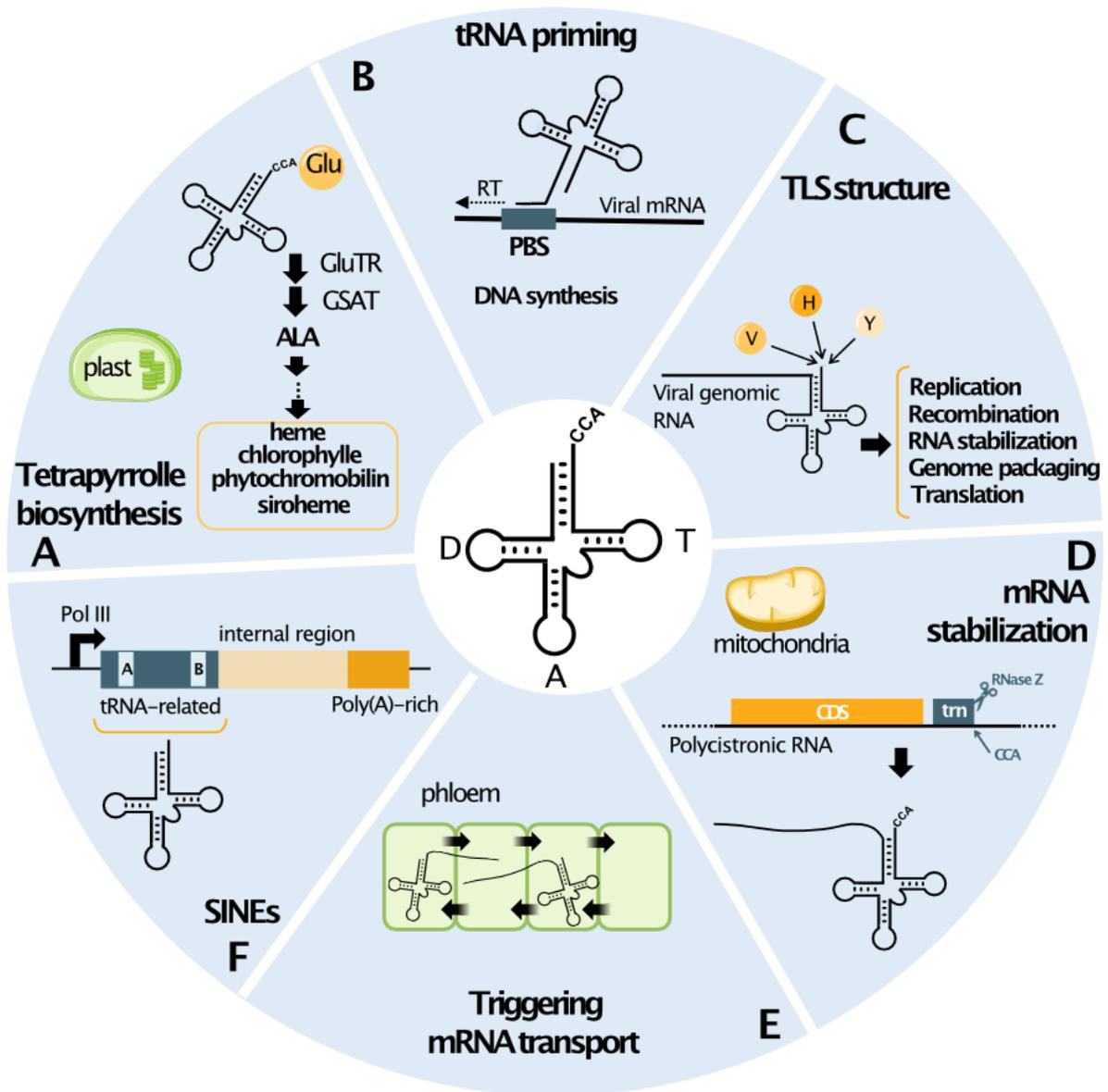
## Figure legends

**Fig. 1.** Overview of additional functions, besides protein synthesis, of tRNAs in the plant cell. The typical cloverleaf secondary structure of a tRNA, with its D- T- and A- regions, is depicted in the middle. **(a)** Plastidial tRNA<sup>Glu</sup> is required for the biosynthesis of 5-aminolevulinic acid (ALA), the precursor of tetrapyrroles. **(b)** A tRNA is used as a primer for reverse transcription (RT) of RNA of pararetroviruses. The 3' extremity of the tRNA sequence is complementary to the primer binding site (PBS) located at the 5' extremity of the viral RNA. **(c)** The different roles of tRNA-like structures (TLS) found at the 3' extremity of various plant viral RNA genomes. TLS can be charged with Valine (V), Histidine (H) or Tyrosine (Y). **(d)** In mitochondria, tRNAs (as illustrated here in *Cyanophora paradoxa*) or "t-elements" deriving from tRNAs, present at the 3'- end of mRNAs can prevent their degradation from exonucleases. **(e)** Discistronic mRNA:tRNA transcripts circulate at long distance *via* the phloem vasculature. **(f)** Short interspersed nuclear elements (SINEs) are ancestrally derived from tRNA genes and transcribed by polymerase III thanks to the A and B boxes internal promoters. In a few cases, the classical tRNA cloverleaf structure is conserved.

**Fig.2.** Overview of tRNA-derived RNAs (tDR) biogenesis. **(a)** The major classes of short (tDR-5D, tDR-3T) and long (tDR-5A, tDR-3A) tDRs originate from nucleus (N)-encoded, plastid (P)-encoded and mitochondrion (M)-encoded tRNAs. **(b)** tDR populations fluctuate under stress conditions (*e.g.* phosphate deprivation, UV, heat) or depending on the tissue or developmental stage (*e.g.* roots, leaves, flowers, siliques). **(c)** In pollen, the Dicer-Like ribonuclease DCL1 was shown to be involved in the production of short tDRs. **(d)** The RNases T2 (RNS1 to 4) are the major actors of short and long tDRs biogenesis in various tissues.

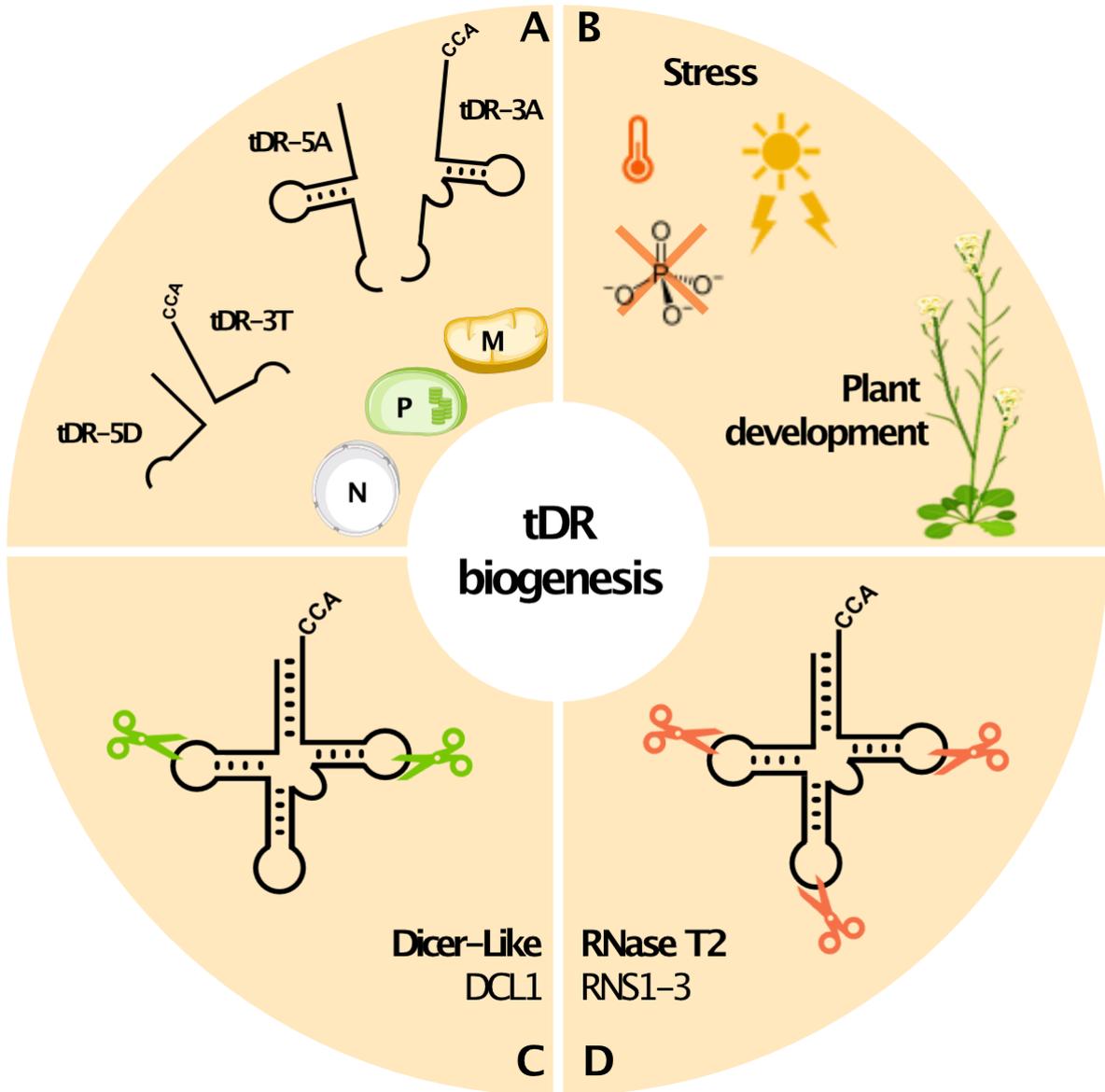
**Fig.3.** Overview of the functions of plant tDRs. **(a)** tDRs can associate with the argonaute protein AGO1 in the RISC complex to target and cleave RNA such as transposable elements (TE). **(b)** Through their ability to interact with AGO1, bacterial tDRs target and repress genes that prevent symbiosis in Fabaceae. **(c)** tDRs can modulate the translation process by having the capability to be associated with polyribosomes.

Figure 1



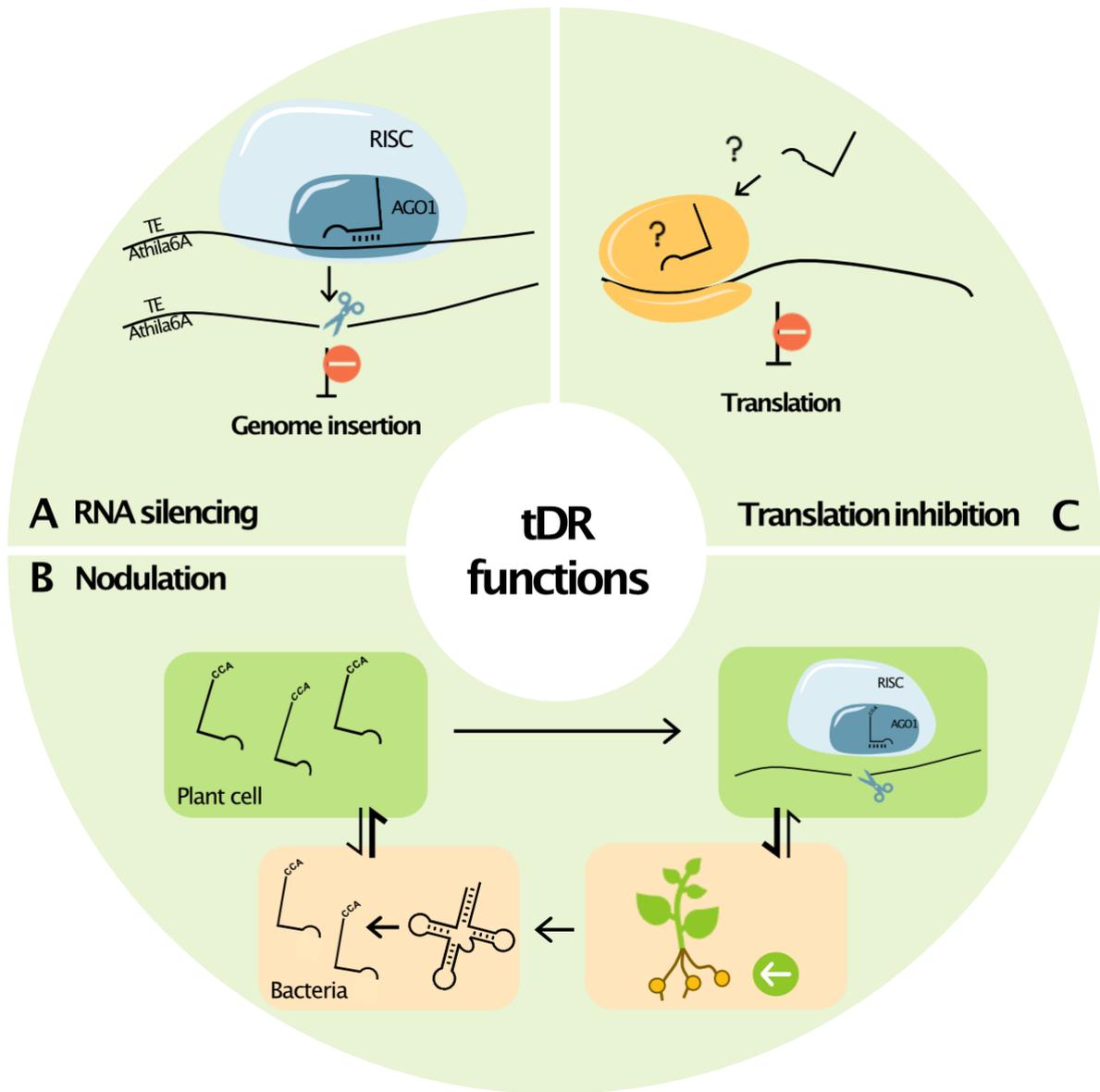
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Figure 2



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Figure 3



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