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Interchangeable parts: The evolutionarily dynamic tRNA population in plant mitochondria

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ABSTRACT

Transfer RNAs (tRNAs) remain one of the very few classes of genes still encoded in the mitochondrial genome. These key components of the protein translation system must interact with a large enzymatic network of nuclear-encoded gene products to maintain mitochondrial function. Plants have an evolutionarily dynamic mitochondrial tRNA population, including ongoing tRNA gene loss and replacement by both horizontal gene transfer from diverse sources and import of nuclear-expressed tRNAs from the cytosol. Thus, plant mitochondria represent an excellent model for understanding how anciently divergent genes can act as "interchangeable parts" during the evolution of complex molecular systems. In particular, understanding the integration of the mitochondrial translation system with elements of the corresponding machinery used in cytosolic protein synthesis is a key area for eukaryotic cellular evolution. Here, we review the increasingly detailed phylogenetic data about the evolutionary history of mitochondrial tRNA gene loss, transfer, and functional replacement that has created extreme variation in mitochondrial tRNA populations across plant species. We describe emerging tRNA-seq methods with promise for refining our understanding of the expression and subcellular localization of tRNAs. Finally, we summarize current evidence and identify open questions related to coevolutionary changes in nuclear-encoded enzymes that have accompanied turnover in mitochondrial tRNA populations.

1. Introduction: Decoding the mitochondrial genome

The endosymbiotic origin of mitochondria has partitioned the eukaryotic cell into multiple genomic compartments, each with its own distinct information storage and processing pathways. Separate replication, transcription and translational machinery is required for the maintenance and expression of the mitochondrial genome (mitogenome) and is largely distinct from the machinery required for nuclear function. During the course of the approximately 2 billion years since the intracellular integration of the alphaproteobacterial progenitor of mitochondria and its host cell, the mitogenome has experienced extensive gene loss, with extant mitogenomes encoding only a small fraction of genes typically found in bacterial genomes (Sloan et al., 2018). This reduction in coding content has resulted in almost all components required for DNA replication, repair, and transcription being lost outright or functionally replaced by gene products encoded in the nuclear genome and imported into the mitochondrial matrix (Huynen et al., 2013). Yet despite this massive loss, all mitogenomes still retain at least some genes involved in protein synthesis (Roger et al., 2017). These stubbornly retained translation genes, in combination with those that have been functionally transferred to the nuclear genome or recruited from existing eukaryotic genes to function in the mitochondria, have created an enzymatic mosaic where mitochondrial protein synthesis depends on the orchestrated expression and assembly of gene products from both the nuclear and mitochondrial genomes.

Mitochondrial translation, like its counterpart in the cytosol, is the fundamental process of decoding messenger RNA (mRNA) molecules into polymers of amino acids. The site of protein synthesis is the ribosome, a massive enzyme complex composed of both RNAs (rRNAs) and proteins (Petrov et al., 2015). Amino acids are brought to the ribosome by transfer RNAs (tRNAs) where they are covalently bonded to form growing polypeptide chains. As the adapter molecules of translation, tRNAs are approximately 80 nt in length with a distinctive secondary "cloverleaf" structure composed of base-paired stems and unpaired loops, and a tertiary L-shaped configuration that must interact with the ribosome, mRNA, and amino acid (Rodnina et al., 2005; Zhang and Ferre-D'Amare, 2016). Translation is achieved based on a 3-nt anticodon sequence in each tRNA that is used to recognize appropriate codons with the mRNA. Amino acids are attached to their corresponding tRNAs by a group of specialized enzymes called aminoacyl tRNA synthetases (aaRSs) (O'Donoghue and Luthey-Schulten, 2003). In addition to their function in decoding, tRNAs can play a number of diverse biosynthetic and regulatory roles, including antibiotic biosynthesis, cell wall remodeling, translation control and even

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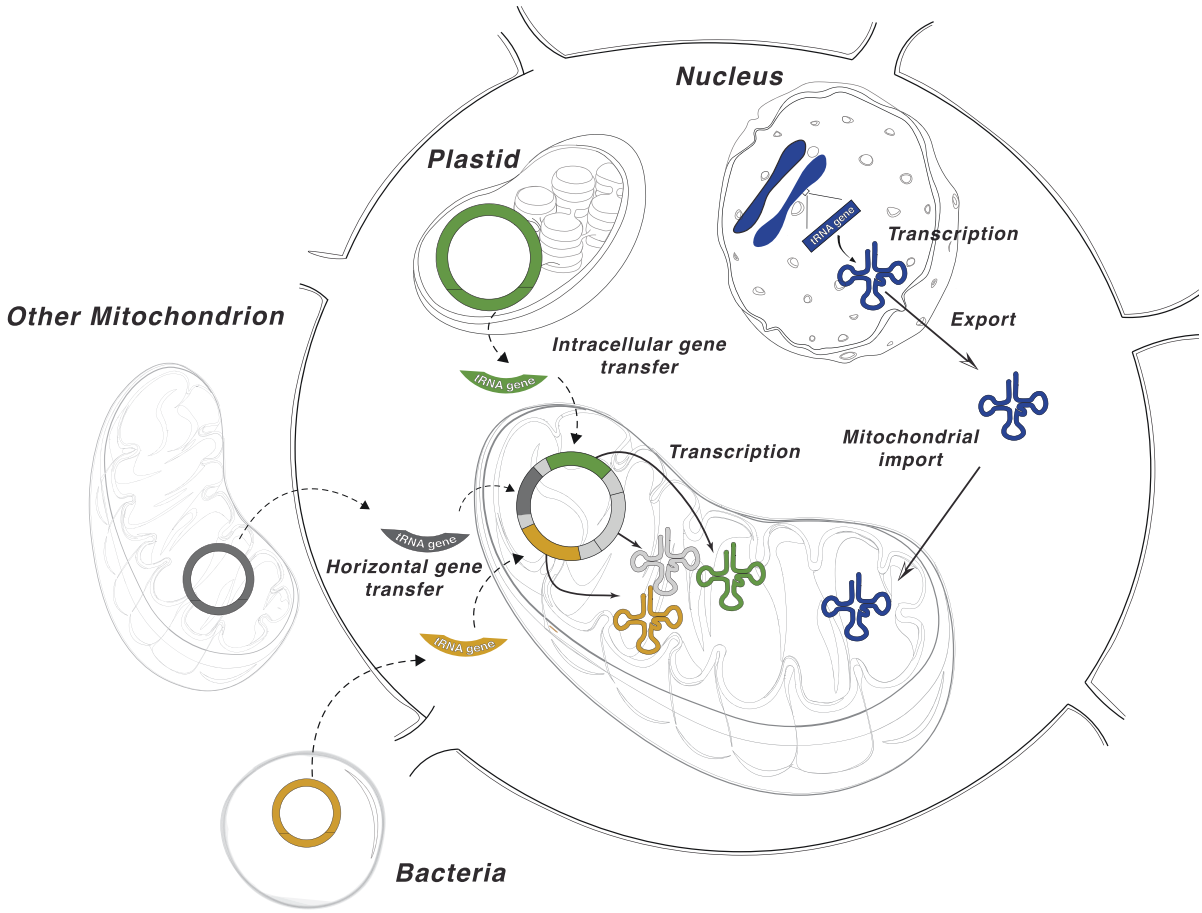


Fig. 1. The heterogeneous nature of the plant mitochondrial tRNA populations. The pool of tRNAs in the mitochondrial matrix of plants can contain a mix of tRNAs transcribed from native mitochondrial genes (light gray), intracellularly transferred genes from plastids (green), and horizontally transferred genes from other species (gold and dark gray), as well tRNAs expressed from nuclear genes and imported from the cytosol (blue). The light gray mt-tRNAs represent the native mitochondrial tRNAs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cellular signaling (Banerjee et al., 2010; Kirchner and Ignatova, 2015; Phizicky and Hopper, 2010; Raina and Ibba, 2014). tRNA-derived RNA fragments (tRFs) generated from both nuclear and mitochondrial tRNAs have also come under increased scrutiny for potential functions (Cognat et al., 2017). Finally, protein synthesis also requires a suite of additional enzymes involved in the maturation and processing of tRNAs and rRNAs as well as numerous initiation, elongation, and termination factors (Betat et al., 2014; Motorin and Helm, 2010).

The retention of certain translation genes in mitogenomes is clearly nonrandom, with the large and small subunit mitochondrial rRNA genes being universally retained, whereas genes such as aaRSs are only encoded in the nuclear genome (Duchêne et al., 2009; Ott et al., 2016). The presence and absence of other genes related to protein synthesis is much more lineage-specific. The number and identity of tRNAs encoded in the mitogenome is one of the greatest sources of variation. At one extreme, some eukaryotes have a sufficient set of mitochondrial tRNA (mt-tRNA) genes to decode all codons, but others lack any mitochondrial-encoded tRNAs whatsoever (Grosjean and Westhof, 2016; Salinas-Giegé et al., 2015; Schneider, 2011). Yet, despite this variability in mt-tRNA gene content across the eukaryotic tree of life, tRNA complements have stabilized for long periods of time in some clades. For example, the vast majority of bilaterian animals have the same 22 mt-tRNA genes (Lavrov and Pett, 2016).

This stability is in stark contrast to vascular plants, which have exceptionally dynamic and varied mitogenome tRNA content. In some species, the frequent insertion of foreign DNA into plant mitogenomes has inflated the number of mt-tRNA genes. Some of these insertions have functionally replaced the native mt-tRNA genes inherited from the ancestor of mitochondria (Duchêne and Maréchal-Drouard, 2001; Joyce and Gray, 1989; Kitazaki et al., 2011; Marchfelder et al., 1990). As tRNAs are likely the only class of mitochondrial genes to be functionally acquired from multiple foreign sources, they represent a fascinating system to study mitochondrial transcription and RNA processing pathways. Conversely, other plants have had their mt-tRNA gene content dwindle down to none (Hecht et al., 2011), with translation likely being maintained entirely by the import of nuclear-encoded tRNA counterparts (Murcha et al., 2016). In other lineages, this loss processes has not gone to completion, but instead appears to be rapid and ongoing (Petersen et al., 2015; Sloan et al., 2012a). As much of the enzymatic machinery required for mitochondrial tRNA maturation and function is distinct from cytosolic tRNA machinery, the evolution of cytosolic tRNA import raises numerous questions about the effect on the enzymatic network involved in mitochondrial translation.

This variation in tRNA metabolism makes plants one of the best systems to study the coevolutionary dynamics necessary for the replacement of one of the last remaining classes of mitochondrial genes. This review will focus on the origins of the heterogeneous nature of plant mt-tRNA genes and how the ongoing replacement of the bacterial-like mt-tRNAs with imported nuclear tRNAs can perturb the network of enzymatic interactions essential for translation. Additionally, we provide a short discussion on how recent advances in tRNA sequencing technologies make this an exciting time to test longstanding hypotheses about the expression and intermediate states necessary for functional mt-tRNA gene loss.

2. Plant mitochondrial tRNA populations: Rapid shifts and heterogeneous origins

The early sequencing of plant mitogenomes and the characterization of their mt-tRNA populations revealed a strikingly heterogeneous mixture of tRNAs (Dietrich et al., 1992; Knoop, 2004; Maréchal-Drouard et al., 1993). It became evident that the ancestral tRNA gene content within plant mitogenomes had been reshaped by a complex history of gene transfer events from different sources including plastids, other plants, algae, bacteria and possibly other distantly related lineages (Fig. 1). As the availability of plant mitogenome sequences has steadily increased, a more detailed view of donors and recipients in this dynamic process has come into focus. Moreover, many tRNAs that function in mitochondrial translation are encoded by nuclear genes and imported from the cytosol. The extent of this cytosolic replacement of native mt-tRNA genes also varies dramatically among plant lineages. Recent work involving instances of closely related plant species with radically different complements of mt-tRNA genes suggest that the reshaping of imported tRNA pools can happen remarkably fast. The following sections will outline how the propensity for gene transfer and the ongoing evolution of tRNA import have resulted in plant mitochondria having some of the most complex tRNA populations and tRNA metabolisms in the tree of life.

2.1. The ancestral set of mitochondrial tRNA genes

The variable number of tRNA genes encoded in extant mitogenomes raises questions about the ancestral set of tRNAs present in the last eukaryotic common ancestor (LECA) and those that were subsequently retained in the lineage leading to plants. Reconstructing the ancestral gene content of mitogenomes has been aided by sequencing efforts in diverse lineages of protists from across the eukaryotic tree of life that have unusually gene-rich mitogenomes, including jakobids, malawimonads, *Ancoracysta*, and *Diphylleia* (Burger et al., 2013; Janouškovec et al., 2017; Kamikawa et al., 2016; Valach et al., 2014). However, far more attention has been paid to proteins than tRNAs in inferring the ancestral mitogenome content (Janouškovec et al., 2017; Johnston and Williams, 2016; Lang et al., 1999; Roger et al., 2017). This bias may reflect some of the inherent challenges in studying tRNA evolution. The short length of tRNA genes (< 100 bp) limits statistical power in applying standard sequence-alignment techniques to infer gene histories and relationships. Furthermore, because of substitutions in anticodons as well as the fact that mitogenomes are famous for evolving non-standard genetic codes, some tRNAs genes are now associated with different decoding functions than the genes from which they evolved (Noutahi et al., 2019; Rogers and Griffiths-Jones, 2014; Yona et al., 2013).

Despite the challenges and limitations described above, a relatively clear picture about tRNA gene content in the LECA mitogenome emerges by comparing presence/absence patterns across eukaryotes (Fig. 2). At least 27 of the 31 tRNA genes listed as being of mitochondrial origin in Fig. 2 are likely to have been present in LECA (shown in bold), representing a sufficient set of tRNAs capable of decoding all amino acids. The tRNA-Arg(UCG) and tRNA-Leu(CAA) genes appear to frequently arise via duplication and anticodon mutation of other genes within those isoacceptor families (i.e., tRNAs with the same amino acid but different anticodons) (Turmel et al., 2013). As such, there is not necessarily compelling evidence for these two gene being in the LECA mitogenome despite their presence in the mitogenome of numerous extant eukaryotes. The tRNA-Ser(GGA) and tRNA-Val(GAC) genes are extremely rare in mitogenomes, making it difficult to confidently infer their origins, but it is possible that these two genes were also present in LECA based on the fact that there are found in the jakobid *Andalucia godoyi*, which harbors arguably the most ancestral-like mitogenome of any eukaryote studied to date (Burger et al., 2013).

This review will focus on the dynamic nature of mt-tRNA evolution within land plants, with an emphasis on angiosperm mt-tRNA populations. The complement of ancestral mt-tRNA genes appears to have remained largely stable for many hundreds of millions of years after LECA in the lineage that eventually gave rise to land plants. Even though there exists a large amount of diversity of mt-tRNA gene content and lineages with extreme tRNA gene loss in green algae (e.g., the widely used model system *Chlamydomonas reinhardtii* has only three tRNA genes in its mitogenome; Vinogradova et al., 2009), many green algae, including the hypothesized sister lineage to land plants (Zygnematales), have relatively complete, ancestral-like gene sets (Fig. 2) (One Thousand Plant Transcriptomes, 2019; Turmel et al., 2013). Similarly, non-vascular land plants (mosses, liverworts, and hornworts) also retained a majority of these ancestral tRNA genes, but with some clade-specific losses such as a suite of tRNA-Gln, tRNA-Arg, and tRNA-Ser genes being absent in hornwort mitochondria (Xue et al., 2010). Within vascular plants, there has been far more heterogeneity in mt-tRNA trajectories, including lineage-specific increases in the frequency of foreign DNA insertions and loss/replacement of native mt-tRNA genes.

2.2. Intracellular transfer of tRNA genes from plastids

While there are very few, if any, instances of bryophyte or algal mitogenomes having plastid-derived sequence, DNA of plastid origin is frequently inserted into the mitogenomes of vascular plants (Grewe et al., 2009; Liu et al., 2011; Sloan and Wu, 2014; Wang et al., 2007; Wang et al., 2018). Known as mitochondrial plastid DNAs (MTPTs), these intracellular transferred sequences are sparsely distributed in lycophytes and ferns (Grewe et al., 2009; Guo et al., 2017), but are ubiquitous in gymnosperms and angiosperms (Chaw et al., 2008; Guo et al., 2016; Jackman et al., 2019; Kan et al., 2020; Wang et al., 2018), with sequenced mitogenomes containing anywhere from 0.1 to 11.5% plastid-derived DNA (Alverson et al., 2010; Gandini and Sanchez-Puerta, 2017). In extreme examples, almost 90% of the corresponding plastid genome can be represented in the cumulative MTPT content within a mitogenome (Alverson et al., 2010; Rice et al., 2013; Sloan and Wu, 2014). Although there have been some instances of MTPTs contributing to the sequence evolution or regulation of mitochondrial protein and rRNA genes (Hao and Palmer, 2009; Nakazono et al., 1996; Sloan et al., 2010; Wang et al., 2012), the vast majority of these insertions are assumed to be nonfunctional. The identity and amount of plastid-derived sequence can vary dramatically, with MTPTs being gained and lost rapidly sometimes even varying in presence within a species (Alverson et al., 2010; Cummings et al., 2003; Sloan et al., 2012b). However, plastid-derived tRNA genes have proven to be a fascinating exception to this general trend of MTPT "insert and decay," with cases of MTPT tRNAs being retained for over hundreds of millions of years (Richardson et al., 2013; Wang et al., 2007). There is direct evidence that some of these plastid-derived sequences are transcribed into mature tRNAs, functionally replacing native mt-tRNA counterparts in mitochondrial translation (Duchêne and Maréchal-Drouard, 2001; Fey et al., 1997; Maréchal-Drouard et al., 1990; Miyata et al., 1998).

Some of the earliest functional replacements of native mt-tRNA genes appear to predate the divergence of gymnosperms and angiosperms, as all sequenced mitogenomes from both groups lack a native mitochondrial gene for tRNA-His and tRNA-Met (Fig. 2). Instead, a MTPT counterpart is encoded in the mitogenome of the majority of species (Richardson et al., 2013). Additional functional replacements of native mt-tRNAs with MTPTs occurred early in angiosperm evolution, including the loss of the native tRNA-Asn and tRNA-Trp genes, which phylogenetically corresponds to the gain of plastid-derived counterparts (Rice et al., 2013; Richardson et al., 2013). Interestingly, following the initial MTPT transfer, the tRNA-Trp gene was secondarily transferred to a linear plasmid in maize (*Zea*) (Leon et al., 1989). Other plastid-derived tRNA insertions are much more recent, suggesting an ongoing and dynamic process of mt-tRNA replacement in some lineages (Fig. 2). For example, in grasses, the expression of MTPT tRNA-Cys and tRNA-Phe genes appears to compensate for native mt-tRNA gene losses (Joyce and Gray, 1989). This dynamic situation complicates cases of

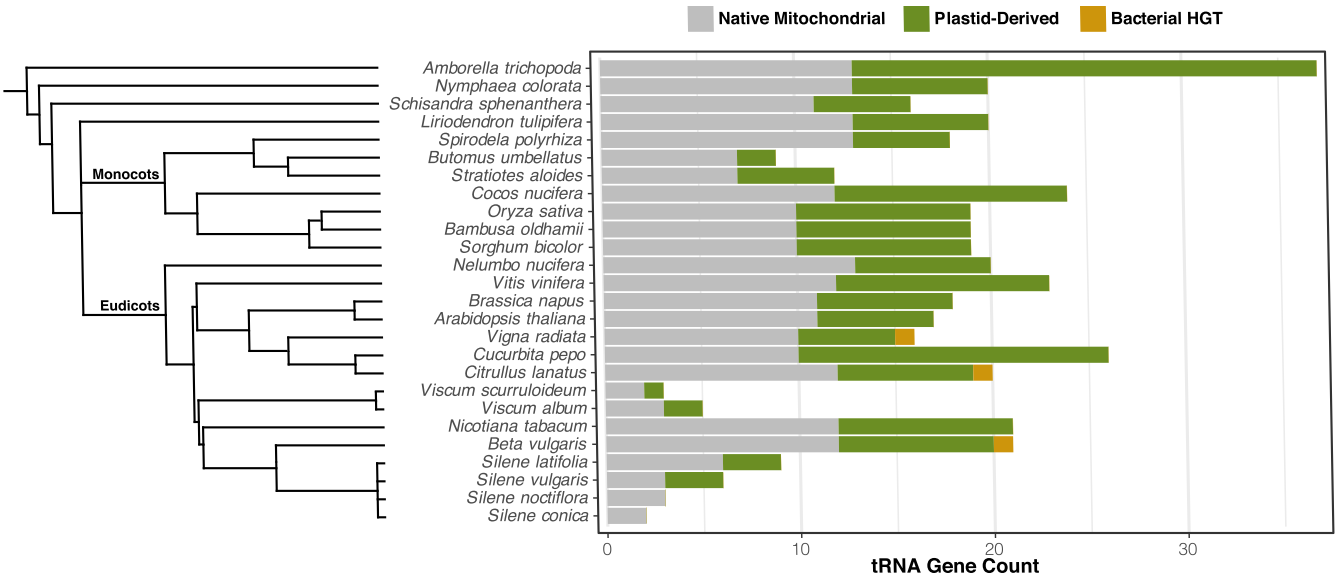


Fig. 2. Summary of tRNA gene content in mitogenomes from a sample of diverse streptophytic green algae and land plants. Filled squares indicate the presence of an intact gene sequence, with genes named based on a single-letter amino-acid abbreviation and with anticodon indicated parenthetically. For example, *trnA(ugc)* corresponds to tRNA-Ala with a UGC anticodon. Formyl-methionine is abbreviated fM. Genes shown in bold text are inferred to have been present in LECA (see main text). Disrupted or incomplete sequences inferred to be pseudogenes are not included. In some cases, gene classification is based on observed or inferred C-to-U RNA editing in the anticodon sequence (Grewe et al. 2009, Guo et al. 2017). Taxon abbreviations are defined as follows. Angio: angiosperms; Gymno: gymnosperms; F: Ferns; Lyco: lycophytes; Bryo: bryophytes; Charo: charophytes (i.e., streptophytic algae); Gene-Rich: other eukaryotes from lineages with unusually high retention of ancestral gene content in their mitogenomes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

very recent MTPT insertions, as it becomes difficult to assess whether associated tRNA genes are functional in the mitochondria or simply destined for loss. The frequent insertion of plastid-derived sequence also means that the same plastid tRNA gene can be transferred multiple times independently, complicating analyses of transfer age and homology.

2.3. Additional foreign sources of mt-tRNA genes

The propensity for plant mitogenomes to take up foreign sequence is not limited to MTPTs and intracellular gene transfer (IGT). There are also multiple instances in which other organisms have served as donors, creating an even more heterogeneous complement of mt-tRNA genes. Functional horizontal gene transfer (HGT) involving tRNA genes from bacterial sources has been documented in vascular plant mitogenomes. The first identified example was a tRNA-Cys gene in the sugar beet (*Beta vulgaris*) mitogenome that had little sequence homology to any known native plant or plastid tRNA genes (Kubo et al., 2000). Found to be most similar in sequence to bacterial tRNAs, this gene has now been identified in numerous angiosperm mitogenomes (Kitazaki et al., 2011). The bacterial-like tRNA-Cys gene was shown to be transcribed and aminoacylated in *Beta vulgaris*, suggesting that it is functional in mitochondrial translation (Kitazaki et al., 2011). In other angiosperm species, this tRNA-Cys gene is absent, but a plastid-derived tRNA-Cys copy is present, demonstrating how tRNA counterparts with radically different origins can fill the same role. More recently, five additional bacterial-like tRNA genes (tRNA-Arg, tRNA-Asp, tRNA-Lys, and two tRNA-Ser genes) were found in many vascular plant mitogenomes, including those of some lycophytes, ferns and gymnosperms (Fig. 2) (Guo et al., 2017; Knie et al., 2015). These genes exhibit apparent sequence homology to counterparts in Chlamydiae, a clade of intracellular bacteria. The conservation of some of these tRNA genes across multiple plant lineages suggests that they were gained early in vascular plant history and are likely functional. Notably, after the early replacement of the native tRNA-Asp by a chlamydial-like tRNA in vascular plants, it was then replaced yet again in angiosperms, this time by a plastid-derived counterpart (Knie et al., 2015).

The extent of tRNA HGT from bacteria has not been thoroughly explored across the larger green plant lineage, but there appear to be at least some instances of such acquisitions outside of land plants. For example, the tRNA-Thr(UGU) has been identified as non-native (Turmel et al., 2013), and it exhibits perfect or near-perfect sequence identity with many bacterial homologs. In addition, the tRNA-Thr (GGU) gene is another likely example of bacterial HGT. The most similar sequences to this gene outside of green plants are found in bacteria, suggesting that it may have been acquired by HGT in a common ancestor of extant green plants.

One of the largest sources of foreign DNA in some plant mitogenomes is HGT from the mitogenomes of other plant species (Bergthorsson et al., 2003; Park et al., 2015; Rice et al., 2013; Sanchez-Puerta et al., 2019). Plant mitochondria are complex organelles that undergo frequent fusion and homologous recombination (Arimura et al., 2004), resulting in populations of mitochondria with varied mitogenome content and structure (Arrieta-Montiel and Mackenzie, 2011). Possibly due to this tendency to fuse, many plant mitogenomes have horizontally acquired mitochondrial sequence, including tRNA genes, from other plant species. An extreme example is the angiosperm *Amborella trichopoda,* which has an unusually large mitogenome, containing foreign mitochondrial sequence from other angiosperms, mosses and green algae. Remarkably, some of these insertions appear to represent entire mitogenomes from moss and algal donors (Rice et al., 2013; Taylor et al., 2015). In a few cases, HGT from other green plant mitogenomes was inferred to functionally replace the corresponding ancestral mt-tRNA gene in *Amborella trichopoda*, but expression of the foreign genes has not been directly tested. This form of horizontal transfer also serves as a mechanism by which plastid-derived sequences in mitogenomes can be moved from species to species (Gandini and Sanchez-Puerta, 2017).

More recently, the mitogenomes of orchids (one of the largest families of angiosperms) were shown to contain HGTs of fungal mitochondrial sequence, including three tRNA genes (Sinn and Barrett, 2019). There is no evidence that the fungal-derived tRNA genes function in mitochondrial translation within orchids. Nevertheless, it is intriguing that this cluster of tRNA genes was anciently transferred in the ancestor of orchids and is still retained in many extant lineages and even appears to have been replaced by gene conversion during a

subsequent fungal transfer event in one major lineage.

In light of the numerous sources of foreign tRNA genes in plant mitogenomes, the lack of evidence for acquisition of tRNA genes from the nuclear genome is conspicuous. The nucleus is another common source of DNA insertions into plant mitogenomes (Goremykin et al., 2012; Notsu et al., 2002), but to our knowledge, there has never been a reported instance of a mitogenome with a functional nuclear-derived tRNA gene. This contrast may reflect simple probabilistic factors such as the low gene density of plant nuclear genomes, which makes it less likely that any random nuclear insert would contain a tRNA gene. In addition, unlike in plastids, which share some of the same transcriptional machinery with mitochondria (Kühn et al., 2009), expression of nuclear genes is driven by entirely distinct regulatory systems (Hummel et al., 2019) such that nuclear tRNA genes may be more likely to be transcriptionally inactive ("dead on arrival") when inserted into the mitogenome.

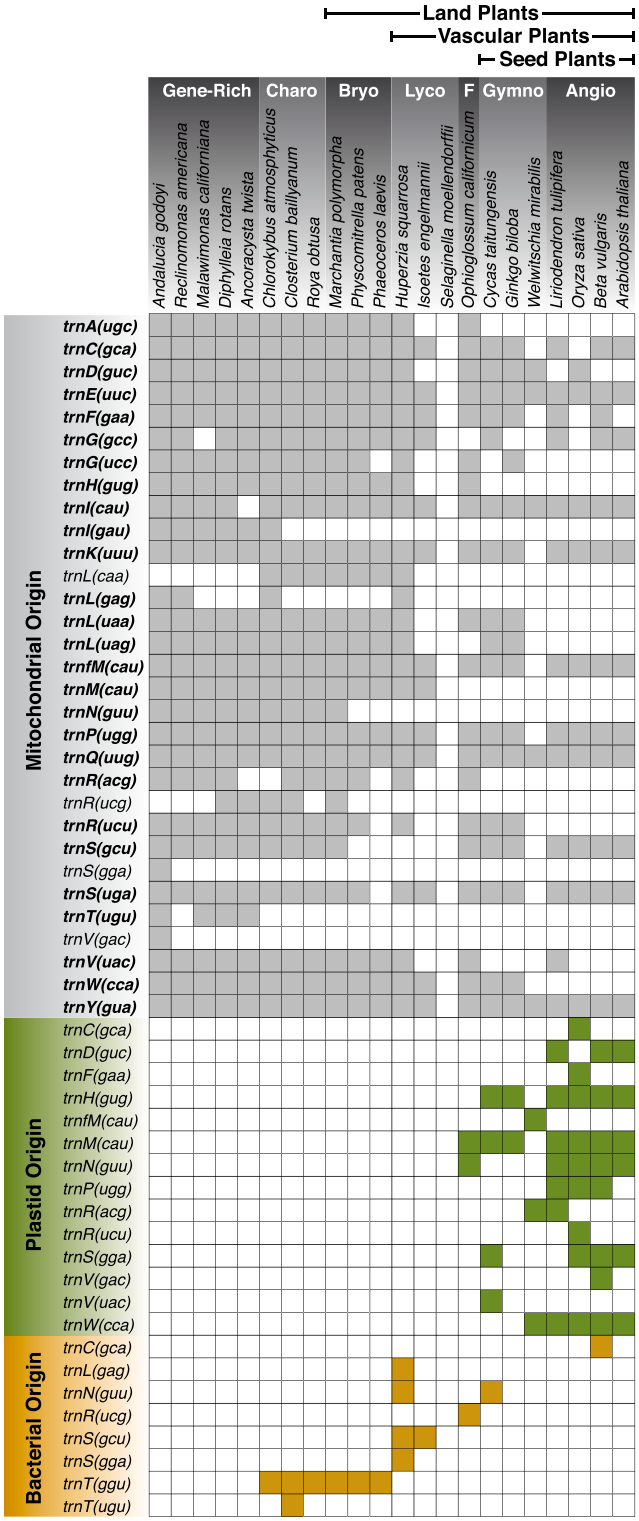


Fig. 3. Extensive variation in tRNA gene counts within the mitogenomes of a diverse sampling of angiosperms. Counts are based on intact gene sequences, excluding duplicate copies of the same gene. Genes are categorized based on their evolutionary origin, as indicated by color code. The tree on the left indicates evolutionary relationships among species, with the topology roughly following the Angiosperm Phylogeny Website v14 (http://www.mobot.org/MOBOT/research/APweb/). For *Amborella trichopoda*, which has been the recipient of extensive HGT from other plant species, the count for native mitochondrial tRNAs is limited to those inferred to be functional by Rice et al. (2013). These include cases of apparent vertical inheritance of the ancestral mitochondrial tRNA genes, as well as inferred cases of functional replacement by HGT of an orthologous gene copy from the mitogenome of another plant species. In contrast, HGTs that would have augmented the set of mitochondrial-derived tRNAs were assumed to be non-functional by Rice et al. (2013).

The frequent insertion of foreign tRNA genes complicates the "head count" of functional mt-tRNA genes. For example, the large mitogenome of *Amborella trichopoda* contains in excess of 150 individual tRNA gene copies, but the majority of these sequences originate from the insertion of foreign DNA, including mitogenome-scale HGT from other plant and algal species, as well as MTPTs that cumulatively represent almost an entire plastid genome. It is likely that most of these foreign inserts are non-functional. Similarly, 11.5% of the mitogenome of *Curcurbita pepo* (zucchini) is derived from MTPTs, including 16 of its 26 unique tRNA genes (Alverson et al., 2010), demonstrating how gene insertion events can greatly inflate mt-tRNA gene counts. For comparison, a more typical angiosperm mitogenome from the model system *Arabidopsis thaliana* has 11 unique native tRNA genes and six plastid-derived genes. Previous northern analysis only detected the expression of four of the six plastid-derived sequences, with no detection of tRNA-Met and tRNA-Trp (Duchêne and Maréchal-Drouard, 2001). However, recent high-throughput tRNA sequencing (tRNA-seq) analysis in *Arabidopsis* did find the plastid-derived tRNA-Met gene to be expressed and post transcriptionally modified with a CCA tail (Warren et al., 2019), implying that five (but probably not all six) of the MTPT tRNA genes in *Arabidopsis thaliana* are functional. Thus, commonly reported presence/absence matrices like the one shown in in Fig. 2 are unlikely to fully reflect functionality or homology because of the fluid nature of mt-tRNA gene sets. And although some work has been done to test for the transcription and aminoacylation for tRNA genes derived from MTPTs and HGTs (Duchêne and Maréchal-Drouard, 2001; Joyce and Gray, 1989; Kitazaki et al., 2011; Marchfelder et al., 1990), the majority of functional inference is based on mitogenome sequence alone. Researchers are often limited to using MTPT length and sequence divergence as "circumstantial" evidence for tRNA functionality in more recent transfers (Alverson et al., 2010; Richardson et al., 2013). Thus, despite tRNA genes being subject to extensive IGT and HGT, there is much work to do to explore the full scope of these phenomena and the mechanisms by which foreign tRNA genes can serve as interchangeable machinery in mitochondrial translation.

2.4. tRNA import from the cytosol complements missing mt-tRNA genes

Despite their many native and foreign tRNA genes, angiosperm mitogenomes still lack a minimally sufficient set of tRNA genes needed to carry out translation (Murcha et al., 2016). Instead, like other eukaryotes, plants depend on the import of expressed tRNAs from yet another source: the nuclear genome. Once thought to be uncommon, the import of nuclear-encoded tRNAs from the cytosol into the mitochondrial matrix has since been demonstrated to be the rule rather than the exception (Salinas-Giegé et al., 2015). All imported tRNAs are likely of eukaryotic origin, as we are

not aware of a documented case of a mt-tRNA gene being functionally transferred to the nuclear genome and targeted back to the mitochondria (Schneider, 2011). This is in sharp contrast to the numerous protein genes that were functionally transferred to the nuclear genome in the evolutionary history of mitochondria (Adams and Palmer, 2003; Timmis et al., 2004), a process that is still highly active in plants (Adams et al., 2002). The degree of cytosolic tRNA import varies across eukaryotes but appears to be minimal in many animal and fungal lineages as their mitogenomes have a mt-tRNA gene set capable of decoding all amino acids (Grosjean and Westhof, 2016). However, even in taxa with a sufficient set of mt-tRNA genes, import of cytosolic tRNAs has been observed (Mercer et al., 2011; Rinehart et al., 2005; Rubio et al., 2008). The function of imported tRNAs in such systems is not always clear, but in some cases these redundant tRNAs are important to maintain effective mitochondrial translation in stress conditions (Kamenski et al., 2007; Martin et al., 1979). Overall, however, the import of cytosolic tRNAs should generally account for a small percentage of the mitochondrial tRNA pool in these organisms (Mercer et al., 2011), and the identity of imported tRNAs has probably been stable for very long periods of time in some lineages (Alfonzo and Söll, 2009).

The apparent stasis observed in some eukaryotic lineages does not apply to plants, where the number of imported tRNAs has been shown to vary widely among species (Kumar et al., 1996). Under the assumption that decoding deficiencies in mt-tRNAs are compensated for by cytosolic import of nuclear-encoded tRNAs, it is clear that multiple plant linages have evolved extensive tRNA import dependencies to maintain mitochondrial translation. The most extreme case in land plants is the spikemoss *Selaginella*, a lycophyte that lacks any mt-tRNA genes and presumably relies entirely on cytosolic import (Hecht et al., 2011). These losses appear to be lineage-specific because all other sequenced lycophytes, ferns, gymnosperms and angiosperms encode at least some tRNA genes in their mitogenome. The gymnosperm *Welwitschia* also likely depends more heavily on import of cytosolic tRNAs than most plants because its mitogenome retains only eight tRNA genes (Fig. 2) (Guo et al., 2016).

The large number of sequenced mitogenomes available for angiosperms have revealed incredibly diverse trajectories for history of loss amongst the inherited set of ancestral mt-tRNA genes (Fig. 3). Angiosperms are expected to import an average of ~ 30–50% of their mitochondrial tRNA pool (Esser et al., 2006). However, based on observed patterns of tRNA gene loss from mitogenomes, this number may be significantly higher in some species and exhibit dramatic variation even between close relatives (Fig. 3). The sequencing and assembly of multiple mitogenomes from the genus *Silene* (Caryophyllaceae) revealed a general reduction in mt-tRNA gene content within the genus and a clear history of recent and ongoing loss, with species having anywhere from nine down to only two tRNA genes remaining in the mitogenome (Sloan et al., 2012a). Similarly extreme levels of reduction in mt-tRNA gene content have been more recently observed in species of the parasitic plant mistletoe (*Viscum*, Santalaceae), with *Viscum album* and *Viscum scurruloideum* retaining only five and three distinct mt-tRNA genes, respectively (Petersen et al., 2015; Skippington et al., 2015).

Such cases of ongoing and rapid mt-tRNA gene loss and presumed replacement by cytosolic import are noteworthy because they point to import specificity evolving remarkably fast. There are many longstanding and still unresolved questions on tRNA import mechanisms (Salinas et al., 2008), but what little is known about tRNA import in plants suggests that it is largely "complementary" (i.e., compensating for tRNA genes absent from the mitogenome rather than providing redundant tRNAs) and, at least in algae, "static" (i.e., tRNA pools were unaffected by manipulated mitogenome codon usage) (Salinas et al., 2012; Salinas et al., 2008; Vinogradova et al., 2009). Both of these observations provide evidence that tRNA import is a coevolved process based on historical tRNA requirements and mitogenome content. As such, instances of rapid mt-tRNA loss and replacement should provide a valuable opportunity to investigate this coevolutionary process "in the act".

Characterizing the evolutionary mechanisms necessary for the *de novo* import of cytosolic tRNA has been hindered by incomplete investigations of mitochondrial tRNA pools, as there has never been an exhaustive study of which tRNAs are imported in plant mitochondria (Salinas-Giegé et al., 2015). One reason for the lack of data on plant tRNA populations at subcellular levels is related to technological limitations in applying conventional sequencing methods to tRNA transcripts. Thus, investigations have largely been limited to targeted, hybridization methods. However, recent advances in tRNA-seq are opening the door for fine-scale characterization of tRNA populations that may accelerate investigations of tRNA import evolution (See Box 1).

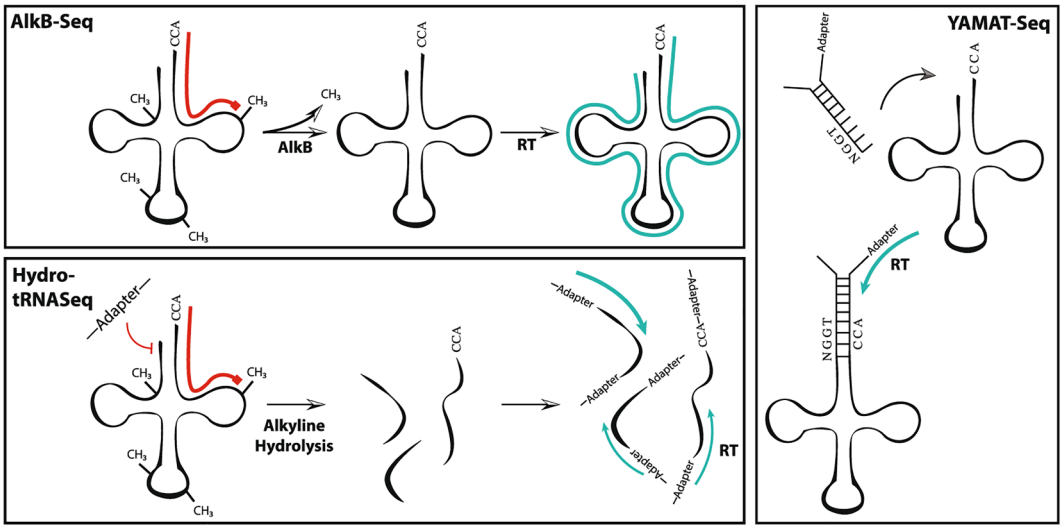
Box 1
Challenges and progress in high-throughput sequencing of tRNA populations.

It is ironic that in the half-century since a *Saccharomyces cerevisiae* tRNA became the very first complete nucleic acid to be sequenced (Holley et al., 1965), tRNAs have emerged as arguably the single most difficult class of molecules to analyze with modern sequencing technologies. One of the largest barriers to tRNA-seq is the extensive post-transcriptional modification of tRNA molecules (Wilusz, 2015), which carry over 100 different identified modifications (Motorin and Helm, 2010). Reverse transcription (RT) into cDNA is required for almost all current RNA sequencing methods, but many base modifications can cause stalling or dissociation of the RT enzyme due to interference with base pairing or steric hindrance (Motorin et al., 2007). As a result, tRNAs often yield truncated sequences or cDNA molecules that are entirely unsequenceable due to a lack of RT readthrough to necessary 5′ adapters. The ligation of sequencing adapters to the 5′- and 3′-end of tRNAs has also proven to be problematic. The compact secondary and tertiary structure of mature tRNAs makes the base-paired termini less accessible for ligation reactions (Lama et al., 2019). Because RT is normally primed off of the ligated 3′-adapter, molecules lacking these adapters are simply never sequenced.

Excitingly, the last five years has seen the introduction of multiple advances to circumvent these difficulties (see Figure). A breakthrough in tRNA-seq came from the utilization of the Escherichia coli dealkylating enzyme AlkB. Normally found in bacterial cells as a repair enzyme that removes aberrant DNA methylation (Mishina and He, 2006), wild type AlkB and engineered versions thereof were found to remove many RT-inhibiting modifications present on tRNAs (Cozen et al., 2015; Zheng et al., 2015). Treatment with AlkB prior to RT has resulted in detecting higher abundance and diversity of tRNA reads in numerous recent studies (Clark et al., 2016; Torres et al., 2019; Warren et al., 2019).

New methods have also been developed to increase the efficiency of adapter ligation and specifically target mature tRNA molecules within a heterogeneous RNA population. Y-shaped Adapter-ligated Mature tRNA sequencing, or YAMAT-seq, takes advantage of the unpaired discriminator base and CCA-tail found on all mature tRNAs by using adapters that complement this overhang (Shigematsu et al., 2017). Other methods have tried to simultaneously circumvent inhibitory modifications and secondary-structure issues by partial hydrolysis of tRNAs (Hydro-tRNASeq; Gogakos et al., 2017). The fragmentation of tRNAs appears to remove some modifications and free 3′- and 5′-ends for adapter ligation. This fragmentation, however, further complicates the mapping for what are already relatively short sequences, making it less suitable for certain biological questions that require distinguishing among genes from families of closely related sequences.

These protocols are very promising for tRNA biology; however, challenges remain for tRNA-seq. Namely, AlkB does not remove all modifications known to inhibit RT (Cozen et al., 2015; Zheng et al., 2015), and biases in adapter ligation persist (Lama et al., 2019), both of which lead to artefactual variation in tRNA-seq read abundance that may confound efforts to measure biological differences in tRNA expression levels. Nevertheless, the ability to generate full-length sequences from the tRNA populations of different cellular compartments represents a huge step forward in understanding the evolution of tRNA metabolism and key progress in investigating one of life's oldest and most fascinating molecules.



Box 1. Methods to increase the efficiency of reverse transcription (RT) and adapter ligation of tRNA molecules. Top left panel: RT-inhibiting modifications are removed with the enzyme AlkB prior to RT, allowing for production of full-sized cDNA molecules. Lower left panel: The base-paired 3′- and 5′-ends of tRNA molecules inhibit ligation reactions. The partial alkaline hydrolysis of tRNAs generates fragments which are more amenable for sequencing. Right panel: In order to specifically ligate adapters to mature tRNAs, Y-shaped adapters with an overhang that is complementary to the discriminator base and the CCA tail on mature tRNAs are utilized.

3. Coevolution and cytonuclear interactions in light of mt-tRNA gene loss and replacement

tRNAs are not standalone components in protein synthesis. Instead, they require large enzymatic networks for processing, editing, maturation and function (Table 1) (Phizicky and Hopper, 2010). Like nuclear-encoded tRNAs, mt-tRNAs undergo 5′- and 3′-end processing from longer primary transcripts as well as a suite of editing, modification, and functional maturation steps before fulfilling their role of delivering amino acids to the ribosome (Salinas-Giegé et al., 2015). With the exception of some components of the mitochondrial ribosome, all tRNA-interacting enzymes are encoded by the nuclear genome and imported into mitochondria. The incredibly dynamic nature plant of mt-tRNA gene loss and replacement raises questions about how the functional swapping of bacterial-like mt-tRNAs with anciently divergent counterparts perturbs the coevolved networks of interactions that are essential for mitochondrial translation.

3.1. The network of tRNA-interacting enzymes

The enzymes necessary for tRNA processing, maturation and function can be classified based on whether they function exclusively within one compartment (nucleus/cytosol, mitochondria, or plastids) or have activity in multiple compartments (Canino et al., 2009; Hopper and Nostramo, 2019; Rossmanith, 2012). In plants, the tRNA nucleotidyltransferase enzyme that adds the tail of CCA nucleotides found on the 3′ end of all mature tRNAs (CCAse) processes all cellular tRNAs regardless of where they are encoded or localized (von Braun et al., 2007).

Other tRNA-interacting enzymes can be organelle-specific. A partial division between nuclear and mitochondrial tRNA metabolic networks is apparent among aaRSs. Each aaRS interacts with cognate tRNAs based on amino-acid decoding (i.e., there would typically be one aaRS for each of the 20 different amino acids) and uses nucleotide identities at key positions for substrate recognition (Giegé et al., 1998). By and large, the bacterial-like tRNAs found in mitogenomes share little sequence similarity to eukaryotic tRNAs and are very poor substrates for eukaryotic aaRSs (Salinas-Giegé et al., 2015). Thus, eukaryotes must encode a largely separate set of aaRSs for mt-tRNA aminoacylation (Duchêne et al., 2009). Naively, we might expect that cytosolic and mitochondrial aaRSs would reflect the archaeal and alphaproteobacterial legacy of the respective host and endosymbiont lineages that gave rise to extant eukaryotes. However, aaRS evolutionary history appears far more complex and likely involves substantial HGT from other sources (Brindefalk et al., 2007; Doolittle and Handy, 1998). Despite these complex origins, early eukaryotic evolution does appear to have largely established separate sets of aaRSs that can be distinguished as either mitochondrial or cytosolic in function, although there are cases of aaRSs being shared between both compartments (Chien et al., 2014; Duchêne et al., 2001; Tolkunova et al., 2000).

The situation in plants is further complicated by the need to deliver aaRSs for a third translation system in the plastids. However, due to the widespread dual-functionality of aaRSs in both mitochondria and plastids, plants have fewer than the 60 aaRSs that would be expected if there were no overlap between the three translational systems. Possibly due to the bacterial-like nature of both plastid and mitochondrial tRNAs, many prokaryotic-like aaRSs are effective enzymatic partners for both classes of organellar tRNAs (Duchêne et al., 2005). In *Arabidopsis thaliana*, there are a total of 23 aaRSs thought to function in the mitochondria (Table 1), corresponding to 19 amino acids (there is no GlnRS in mitochondria because of the indirect pathway used to aminoacylate tRNA-Gln; see below). None of these 23 aaRSs seem to function exclusively in the mitochondria. A total of 17 *Arabidopsis* aaRSs have been identified as dual-targeted to the mitochondria and plastids but do not have cytosolic localization (Table 1). Only two of these enzymes (PheRS and SerRS) were assigned as the typical mitochondrial type inherited from the eukaryotic ancestor, implying an extensive history of horizonal gene transfer as most of the dual-targeted mitochondrial/plastid aaRSs were identified as having more recent bacterial origin. These include many aaRSs that can be traced to cyanobacteria and the presumed progenitor of plastids but also some that appear related to other bacterial lineages, implying additional sources of HGT and/or reflecting the limitations of phylogenetic resolution (Brandao and Silva-Filho, 2011; Brindefalk et al., 2007) concluded that all but two of the 17 dual-organellar aaRSs were of bacterial or mitochondrial origin, with HisRS and ProRS being the only exceptions. However, other phylogenetic analyses have placed this plant ProRS closest to bacterial enzymes, and the HisRS has exhibited ambiguous phylogenetic signal with association to a mix of both bacterial and archaeal enzymes (Brandao and Silva-Filho, 2011; Brindefalk et al., 2007). Therefore, it is possible that all 17 of the dual-organellar aaRSs

Table 1
mt-tRNA-interacting enzymes in *Arabidopsis thaliana*.

Enzyme	Arabidopsis ID	Function	Compartment[1]	Enzyme Origin[2]	Target Mitochondrial tRNAs[3]
AlaRS	At1g50200	aminoacylation	M|P|N	Eukaryotic: Cytosolic	trnA-cyto
AlaRS	At5g22800	aminoacylation	M|P	Cyanobacterial	trnA-cyto
ArgRS	At4g26300	aminoacylation	M|P|N[6]	Eukaryotic: Cytosolic	trnR-cyto
AsnRS	At4g17300	aminoacylation	M|P	Cyanobacterial[7]	trnN(guu)-cp
AspRS	At4g33760	aminoacylation	M|P	Cyanobacterial	trnD(guc)-cp
CysRS	At2g31170	aminoacylation	M|P	Other Bacterial	trnC(gca)-mt
GluRS	At5g64050	aminoacylation	M|P	Cyanobacterial[7]	trnE(uuc)-mt, trnQ(uug)-mt
GlyRS	At1g29880[5]	aminoacylation	M|N	Eukaryotic: Cytosolic	trnG(gcc)-mt, trnG-cyto
GlyRS	At3g48110	aminoacylation	M|P	Other Bacterial	trnG(gcc)-mt, trnG-cyto
HisRS	At3g46100	aminoacylation	M|P	Unclear[8]	trnH(gug)-cp
IleRS	At5g49030	aminoacylation	M|P	Cyanobacterial	trnI(cau)-mt, trnI-cyto
LeuRS	At1g09620	aminoacylation	M|N	Eukaryotic: Cytosolic	trnL-cyto
LysRS	At3g13490	aminoacylation	M|P	Cyanobacterial	trnK(uuu)-mt
MetRS	At3g55400	aminoacylation	M|P	Cyanobacterial	trnfM(cau)-mt, trnM(cau)-cp[10]
PheRS	At3g58140	aminoacylation	M|P	Eukaryotic: Mitochondrial	trnF-cyto
ProRS	At5g52520	aminoacylation	M|P	Other Bacterial[9]	trnP(ugg)-mt
SerRS	At1g11870	aminoacylation	M|P	Eukaryotic: Mitochondrial	trnS(gcu)-mt, trnS(gga)-cp, trnS(uga)-mt
ThrRS	At5g26830	aminoacylation	M|N	Eukaryotic: Cytosolic	trnT-cyto
ThrRS	At2g04842	aminoacylation	M|P	Cyanobacterial	trnT-cyto
TrpRS	At2g25840	aminoacylation	M|P	Cyanobacterial	trnW-cyto[11]
TyrRS	At3g02660	aminoacylation	M|P	Other Bacterial	trnY(gua)-mt
ValRS	At1g14610[5]	aminoacylation	M|N	Eukaryotic: Cytosolic	trnV-cyto
ValRS	At5g16715	aminoacylation	M|P	Other Bacterial	trnV-cyto
PRORP	At2g32230	5′-end processing	M|P	Eukaryotic	All
tRNase Z	At3g16260	3′-end processing	M	Eukaryotic	All
tRNase Z	At1g52160	3′-end processing	M|N	Eukaryotic	All
CCAse	At1g22660	CCA-tail addition	M|P|N	Eukaryotic	All
GatA	At3g25660	Glu/Gln amidation	M|P	Cyanobacterial	trnQ(uug)-mt
GatB	At1g48520	Glu/Gln amidation	M|P	Cyanobacterial	trnQ(uug)-mt
GatC	At4g32915	Glu/Gln amidation	M|P	Cyanobacterial	trnQ(uug)-mt
MTF[4]	At1g66520	met. formylation	M|P		trnfM(cau)-mt
TiLS[4]	At3g24560	trnI lysidine mod.	M|P		trnI(cau)-mt

[1] Compartment classifications (M: mitochondrial; P: plastid; N: nuclear/cytosolic) are based on targeting information from Duchêne et al. (2005), von Braun et al. (2007), Pujol et al. (2008), Canino et al. (2009), and Gobert et al. (2010).

[2] Enzyme origins are curated from Duchêne et al. (2005), Brindefalk et al. (2007), Pujol et al. (2008), Brandão et al. (2011), and Lechner et al. (2015). Enzymes identified as eukaryotic are inferred to have been present in LECA. For aaRSs, "mitochondrial" and "cytosolic" indicate the inferred functional role in LECA but do not necessarily imply deeper alphaproteobacterial or archaeal origins.

[3] In *Arabidopsis*, import of cytosolic tRNAs into the mitochondria has only been demonstrated for trnF and trnW. Other examples of cytosolic tRNAs are inferred to be imported based on the absence of corresponding genes in the mitogenome and documented import in other angiosperms (Salinas-Giegé et al. 2015).

[4] MTF and TiLS have not been characterized in plants, but the listed *Arabidopsis* genes represent candidates based on sequence similarity.

[5] Despite evidence of mitochondrial targeting, cytosolic-like GlyRS (At1g29880) and ValRS (At1g14610) may not be active in mitochondrial aminoacylation (Duchêne et al. 2009).

[6] Empirical evidence for plastid-targeting was obtained for ArgRS (At4g26300), but it is inferred to also function in the mitochondria and cytosol. See main text.

[7] Duchêne et al. (2005) identified AsnRS (At4g17300) and GluRS (At5g64050) as cyanobacterial in origin, but other studies have produced conflicting or uncertain results potentially pointing to other bacterial sources (Brindefalk et al. 2007, Brandão et al. 2011), so these classifications are tentative.

[8] Duchêne et al. (2005) identified HisRS (At3g46100) as archaeal in origin, but more recent studies have indicated an unresolved placement relative to bacteria and archaea (Brindefalk et al. 2007, Brandão et al. 2011).

[9] Duchêne et al. (2005) identified ProRS (At5g52520) as cytosolic in origin, but more recent studies have indicated a bacterial origin (Brindefalk et al. 2007, Brandão et al. 2011).

[10] Previous hybridization analysis of the plastid-derived trnM(cau) gene did not detect expression in *Arabidopsis* mitochondria (Duchêne and Maréchal-Drouard 2001), but we recently found evidence of mature tRNA transcripts from this gene using high-throughput sequencing (Warren et al. 2019).

[11] Previous hybridization analysis of the plastid-derived trnW(cca) gene did not detect expression in *Arabidopsis* mitochondria and instead detected import of a cytosolic counterpart (Duchêne and Maréchal-Drouard 2001).

in *Arabidopsis* have either bacterial or mitochondrial origin.

There are also six *Arabidopsis* aaRSs identified as having dual targeting to the mitochondria and cytosol, including two (AlaRS and ArgRS) that are also targeted to the plastids and thus appear to function in all three translation systems (Table 1). In the case of this ArgRS, there is only direct evidence of targeting to the plastid, but function in the mitochondria and cytosol is inferred based on the fact that disruption of the only other identified ArgRS gene in *Arabidopsis* does not affect viability (Berg et al., 2005; Duchêne et al., 2009). In contrast to the overwhelming bacterial/mitochondrial origin of the dual-organellar aaRSs described above, all six of the aaRSs with localization in both the mitochondria and cytosol appear to be ancestral cytosolic enzymes that are shared with other eukaryotes and have gained mitochondrial targeting. In four of these six cases (AlaRS, GlyRS, ThrRS, and ValRS), the presence of a cytosolic-like aaRS in the mitochondria creates apparent redundancy with the presence of a bacterial-like dual-organellar aaRS for the same amino acid. However, it cannot be assumed that both aaRSs are involved in aminoacylation in these examples of redundancy, as investigations of the cytosolic-like GlyRS and ValRS enzymes suggest that they are inactive within mitochondria and that only their bacterial-like counterparts function in mitochondrial aminoacylation (Duchêne et al., 2001; Duchêne et al., 2009). It is not clear why these enzymes might be targeted to the mitochondria yet not function in aminoacylation, but it has been hypothesized that it might reflect a role in chaperoned tRNA import from the cytosol, and aaRSs are known to take on alternative functions within the cell (Guo and Schimmel, 2013). Regardless, these examples of redundancy point to potential pathways for evolutionary transitions and loss/replacement of ancestral

mitochondrial enzymes.

Other enzymes involved in organelle protein synthesis are specific to bacterial-like translational machinery and have no equivalent functioning in the nucleus or cytosol. In most bacteria, archaea, and plastids, as well as plant mitochondria, the aminoacylation of tRNA-Gln is achieved through an indirect pathway, meaning it is not aminoacylated by a dedicated GlnRS. Instead, the mt-tRNA-Gln is first "incorrectly" charged with the amino acid Glu by a nondiscriminating GluRS and subsequently converted to Gln by a tRNA-dependent amidotransferase complex called GatCAB. In plants, this complex is dual targeted to mitochondria and plastids, and the genes that encode its protein subunits are cyanobacterial in origin, suggesting that they were acquired as part of plastid endosymbiosis (Pujol et al., 2008). Protein synthesis is also initiated differently in bacteria and organelles through the use of a formylmethionyl-tRNA, also known as tRNA-fMet (Coffin and Cossins, 1986; Takeuchi et al., 2001). The mt-tRNA-Met must first have a formyl group added to the amino group by an enzyme known as methionyl-tRNA formyltransferase (MTF) before it can be used to initiate translation (Ibba and Söll, 2004). Translation in plant mitochondria and plastids also relies on a bacterial-like modification of native tRNA-Ile, in which the cytidine in the CAU anticodon (which would typically decode AUG Met codons) is modified to lysidine by tRNA-Ile lysidine synthetase (TiLS), allowing it to decode AUA Ile codons (Suzuki and Miyauchi, 2010; Weber et al., 1990). The MTF and TiLS enzymes have not yet been described in plants, but obvious candidates exist based on sequence similarity (Table 1). Additional modifications are increasingly being recognized to play fundamental roles in mitochondrial translation, each with their own respective enzymatic machinery that must be imported into the mitochondrial matrix (Paris and Alfonzo, 2018).

Still other plant mt-tRNA metabolism machinery is seemingly derived in eukaryotes and not inherited from either the archaeal-like host or the alphaproteobacterial progenitor of mitochondria, including the RNase P enzyme utilized for the cleavage of 5′-leader sequences of mitochondrial-encoded tRNA precursors. It was once thought that all domains of life utilized an RNase P enzyme with a ribozyme catalytic domain (Altman, 2007); however, a more complex picture emerged with the discovery that the enzyme responsible for RNase P activity in human and plant organelles is composed entirely of protein, now known as the proteinaceous RNase P or PRORP (Gobert et al., 2010; Gutmann et al., 2012; Holzmann et al., 2008). Based on its distribution across diverse eukaryotic lineages, it appears that PRORP was likely present alongside the conventional RNase P ribozyme in LECA (Lechner et al., 2015). In addition to the originally described function in plant and animal mitochondria, some PRORPs have also been shown to have nuclear function, in some cases even serving as the only source of RNase P activity in the cell (Bonnard et al., 2016; Gutmann et al., 2012). The 3′-end processing of tRNAs relies on another type of enzyme known as tRNase Z. In *Arabidopsis thaliana*, there are four different tRNase Z genes with various targeting patterns (Canino et al., 2009), including two with mitochondrial localization (Table 1).

Overall, the many peculiarities of mt-tRNA metabolism highlight that, while translation is fundamentally similar across cellular compartments, organelle and cytosolic protein synthesis often require different, coevolved partners.

3.2. The coevolution of translational machinery across life and genomes

The genomic compartmentalization of mt-tRNAs and their nuclear-encoded enzymatic partners results in translational machinery being divided between two radically different genomes that differ in structure, mutation rates, inheritance and expression (Sloan et al., 2018). There is now clear evidence that this division of translational machinery between multiple genomes in eukaryotes has resulted in both mitonuclear epistasis and coevolution in mitochondrial tRNA metabolism. Some of the best described examples of these interactions involve aaRS and their mt-tRNA substrates, likely because aaRSs and their tRNA partners are under strong selection to maintain faithful decoding of transcripts into proteins (Salazar et al., 2003; Salinas-Giegé et al., 2015). In the fruit fly *Drosophila melanogaster,* an amino acid substitution in a mitochondrial-targeted TyrRS was found to be incompatible with a single-nucleotide mt-tRNA-Tyr polymorphism in a closely related species, *Drosophila simulans* (Meiklejohn et al., 2013). In separate genetic backgrounds, the nuclear-encoded aaRS amino acid substitution and the mitochondrial-encoded tRNA nucleotide substitution caused no fitness effects but, when present in the same organism, resulted in a significant decrease in development and fecundity. This epistatic effect demonstrates how single point mutations in tRNA networks can have large fitness effects on mt-tRNA metabolism. Similarly, mt-tRNA mutations disproportionally contribute to disease-causing phenotypes, with many of those pathologies resulting from perturbed aaRS interactions (Pearce et al., 2013). However, other studies have found this "coevolutionary crosstalk" between tRNAs and enzymatic partners to be more nuanced, inferring that a large portion of mt-aaRS amino acid substitutions at deep timescales are not driven by coevolutionary responses to mt-tRNA mutations (Adrion et al., 2016; Pett and Lavrov, 2015). Aside from aaRSs, other tRNA-interacting enzymes have adapted to mt-tRNA evolution, such as a modified EF-Tu enzyme which recognizes the unusual "armless" mt-tRNAs in nematodes (Arita et al., 2006).

While the above examples demonstrate possible coevolutionary responses to changes in mt-tRNA sequences, other work has focused on the effects of outright mt-tRNA gene loss and functional replacement. Certain lineages of non-bilaterian animals (sponges, placozoans, cnidarians, and ctenophores) have experienced extreme mt-tRNA loss. This loss of mt-tRNA genes appears to trigger the loss of nuclear-encoded enzymes formerly associated with the mt-tRNAs (Kohn et al., 2012; Pett and Lavrov, 2015). The absence of mt-tRNA genes generally corresponds to the parallel loss of the associated aaRS in the respective nuclear genomes of these animals (Haen et al., 2010). In species completely lacking mt-tRNAs genes, other nuclear tRNA-processing enzymes have been lost as well, including the genes encoding the GatCAB complex and the mitochondrial PRORP (Pett and Lavrov, 2015). The kinetoplastids present another case of extreme aaRS reduction precipitated by mt-tRNA gene loss in eukaryotes (Tan et al., 2002). The mitogenome of the kinetoplastid *Trypanosoma brucei* does not encode any mt-tRNAs (Schneider, 2001), which largely removes the requirement for two separate sets of aaRS for mitochondrial and cytosolic tRNA aminoacylation. In accordance with this, *Trypanosoma brucei* only encodes 23 aaRS genes (Charriere et al., 2006). However, surprisingly, even in this extreme example of merging cytosolic and mitochondrial tRNA metabolisms, separate eukaryotic aaRSs exist to aminoacylate the mitochondrial-imported tRNAs for Asp, Lys, and Trp, even though all tRNAs are encoded in the nuclear genome (Charriere et al., 2006; Charriere et al., 2009). The use of different aaRSs may be due to differences in post-transcriptional modification between tRNAs functioning in the cytosol vs. the mitochondria, which may make the two classes of tRNAs too dissimilar to be substrates for a single aaRS (Alfonzo et al., 1999; Charriere et al., 2009).

These studies point to broad effects of mt-tRNA gene loss, but the deep evolutionary timescales at which many of the gene loss/replacement events occurred can make it difficult to identify the evolutionary mechanisms and intermediate states that are required for the functional replacement of mt-tRNAs. These are significant questions as the functional replacement of native mt-tRNA genes with imported cytosolic counterparts represents swapping molecular parts after billions of years of evolutionary divergence.

3.3. Using plants to elucidate the evolutionary dynamics of mt-tRNA functional replacement

The ongoing replacement of mt-tRNA genes in plant lineages via the import of nuclear-encoded cytosolic counterparts poses questions about

which enzymatic partners are processing, modifying, and charging these tRNAs. Multiple scenarios could be envisioned, one being that that aaRSs and other nuclear-encoded tRNA-processing enzymes that were historically present only in the cytosol or nucleus have also gained import into the mitochondrial matrix and maintain their same interactions with newly imported cytosolic tRNA substrates. Mt-tRNA gene loss appears to precipitate this outcome at deep evolutionary timescales in groups with very few or no mt-tRNA genes remaining, such as the aforementioned Cnidaria and Ctenophora (Pett and Lavrov, 2015). These species largely lack mitochondrial-like aaRSs, which are assumed to be replaced by cytosolic versions of the enzymes. However, the import of nuclear tRNAs and cytosolic enzymes would seem to have required more or less simultaneous evolution because the import of either one without the other would have no functional effect. This “chicken and egg” problem of functional mt-tRNA replacement has been raised numerous times in mt-tRNA biology (Schneider, 2011; Small et al., 1999). Characterizing the subcellular localization of tRNAs and interacting enzymes in plant lineages with ongoing mt-tRNA loss represents a valuable opportunity to test hypotheses on the intermediate states and order of events necessary for functional mt-tRNA replacements, such as possible periods of redundant tRNA/enzyme import and the evolution of mitochondrial targeting peptides.

Conversely, a second evolutionary scenario could have arisen where mitochondrial aaRSs and other tRNA-interacting enzymes have been retained, adapting to recognize and process nuclear-encoded tRNAs. There is some evidence that this process has played a role in plant mt-tRNA metabolism as the organellar, bacterial-like GlyRS has gained substrate recognition for a cytosolically imported tRNA-Gly. Both a mitochondrial-encoded tRNA-Gly and an imported nuclear-encoded tRNA-Gly are necessary for mitochondrial translation in eudicot angiosperms because the mitochondrial-encoded tRNA-Gly(GCC) is unable to decode all four GGN Gly codons (Brubacher-Kauffmann et al., 1999). It was demonstrated that organellar GlyRSs from multiple angiosperms are able to aminoacylate both tRNAs (Duchêne et al., 2001; Salinas et al., 2005). This was surprising because prokaryotic and eukaryotic GlyRSs use a specific nucleotide identity at position 73 for activity, which is different between the two (nuclear and mitochondrial) tRNA-Gly genes (Giegé et al., 1998). This ability for the organellar, bacterial-like GlyRS to recognize cytosolic tRNA-Gly suggests the evolution of expanded substrate recognition based on comparisons with the GlyRS from *Escherichia coli*, which has limited activity on cytosolic tRNA substrates (Duchêne et al., 2001; Nameki et al., 1997).

More generally, data from *Arabidopsis thaliana* on tRNA gene content in the mitogenome and subcellular localization of aaRSs provide multiple examples supporting both of the evolutionary scenarios described above (Duchêne et al., 2009). The absence of tRNA-Arg and tRNA-Leu genes from the *Arabidopsis* mitogenome suggests cytosolic import of these tRNAs (which has been confirmed in other angiosperms; Salinas-Giegé et al., 2015), and the only mitochondrial-localized aaRSs for these tRNAs are also cytosolic in origin (Table 1). Therefore, these appear to be cases of matched retargeting of cytosolic tRNAs and interacting enzymes, as described in the first scenario. In contrast, for other examples of import of cytosolic tRNAs into *Arabidopsis* mitochondria that have been inferred (tRNA-Ile) or demonstrated (tRNA-Phe and tRNA-Trp; Duchêne and Maréchal-Drouard, 2001), the only corresponding aaRS localized to the mitochondria is bacterial-like, supporting the second evolutionary scenario, where existing mitochondrial machinery has adapted to function on cytosolic tRNAs. The cytosolic import of tRNA-Val and the aforementioned case of tRNA-Gly also fall in this category because only the bacterial-like aaRSs appear to be active in aminoacylation within the mitochondria even though a cytosolic-like aaRS is also localized to the mitochondria (Duchêne et al., 2009). The import of cytosolic tRNA-Ala and tRNA-Thr is less clear because bacterial-like and cytosolic-like aaRSs are both targeted to the mitochondria, and their relative contributions to aminoacylation are not known.

Although the adaptation of bacterial-like aaRSs to charge cytosolic tRNAs appears to be a viable evolutionary route in some cases, the dual-targeted nature of mitochondrial and plastid aaRSs could constrain enzymatic evolution, as very often the same enzyme must recognize both plastid and mitochondrial-encoded tRNAs (Table 1). It has yet to be determined if amino acid substitutions in organellar aaRSs and other tRNA-interacting proteins would have pleiotropic effects on plastid tRNA metabolism, possibility necessitating gene duplication and neofunctionalization events.

The two scenarios proposed above are not mutually exclusive, and it will be interesting to determine if certain evolutionary routes are taken repeatedly for lineages with independent mt-tRNA gene losses, or if tRNA-interacting networks are perturbed differently depending on the specific mt-tRNA gene. In addition, these two proposed coevolutionary responses to mt-tRNA loss do not represent the only possibilities. One fascinating example of an alternative mechanism to achieve functional mt-tRNA gene replacement comes from the apicomplexan *Toxoplasma gondii*, which depends entirely on cytosolic tRNA import for mitochondrial translation (Esseiva et al., 2004). What makes its mt-tRNA metabolism so exceptional is that no aaRSs have been found to be localized to the mitochondria in this species, meaning that tRNAs are imported already aminoacylated. This mechanism removes the evolutionary constraint of *de novo* aaRS import but raises questions about the recycling of tRNAs in these organisms. Because of the incredibly long half-life of tRNA molecules (up to multiple days; Hopper, 2013), a single tRNA can be assumed to be aminoacylated numerous times throughout its lifetime. Are the imported tRNAs in *Toxoplasma gondii* repeatedly transported back and forth across the mitochondrial membranes to be reloaded with amino acids? Alternatively, are imported tRNAs effectively “single-use” molecules that are degraded inside the mitochondrial matrix after unloading their amino acid? Both of these models seem energetically inefficient, but they represent the only obvious possibilities if all imported tRNAs are indeed aminoacylated in the cytosol. This apicomplexan aminoacylation mechanism is less likely to be ocurring in plants, as data on subcellular localization of aaRSs shows that plant mitochondria still have extensive aaRS import (Duchêne et al., 2005). But it is worth noting that other types of enzymatic interactions that need only occur once in the lifetime of a tRNA (e.g., end-processing, CCA-tailing, base modifications, etc.) may not suffer any inefficiency if the activity remains in the nucleus/cytosol followed by mitochondrial import of mature tRNAs. Regardless, the aminoacylation system in apicomplexans illustrates the diversity of mechanisms by which eukaryotic life maintains translation in the face of the mosaic origins of mitochondrial molecular biology.

4. Outlook: Catching functional mt-tRNA replacement in the act

It is a striking contrast that interactions between tRNAs and their enzyme partners are so coevolved that single nucleotide/amino-acid polymorphisms found in *Drosophila* species are sufficient to create highly deleterious incompatibilities (Meiklejohn et al., 2013) but, at the same time, there is such evolutionary interchangeability among anciently divergent tRNAs in plants and other lineages (Fig. 2). Reconciling this apparent paradox represents a key challenge for tRNA biology. The sequencing of mitogenomes from angiosperms such as *Silene* and *Viscum* (Petersen et al., 2015; Skippington et al., 2015; Sloan et al., 2012a) has revealed closely related species with very different complements of mt-tRNA genes, reflecting histories of gene loss that are both recent and extreme. These systems appear to have an even more active state of mt-tRNA gene replacement than what is already an incredibly dynamic plant mt-RNA metabolism. As such, they may offer new opportunities to describe the molecular coevolution and intermediate steps necessary for functional mt-tRNA replacement while it is actively occurring. Investigations into protein-RNA interactions, cellular trafficking, and genome evolution involved in mt-tRNA gene loss have the potential shed light on some of the most fundamental

questions in eukaryotic cell evolution, with the aid of advances in multiple fields of cell and molecular biology, including breakthroughs in tRNA-seq. And tracing the steps needed to replace such anciently divergence translational systems could have ripple effects in numerous fields including human health, synthetic biology, speciation, and the origin of eukaryotic life.

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