Extensive retargeting of plant aminoacyl tRNA synthetases correlates with mitochondrial tRNA gene loss

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Abstract

The number of tRNAs encoded in plant mitogenomes varies considerably, with some lineages exhibiting rapid reductions. Loss of these bacterial-like mitochondrial tRNAs necessitates the import of nuclear-encoded counterparts that share little sequence similarity, raising questions about the identity and trafficking of enzymes necessary for the maturation and function of these newly imported tRNAs. In particular, the aminoacyl tRNA synthetases (aaRSs) that charge tRNAs are usually divided into distinct classes that specialize on either organellar or cytosolic tRNAs. Here, we investigated the evolution of aaRS subcellular localization in five species from a plant lineage (*Sileneae*) that has experienced extensive and rapid mitochondrial tRNA loss. By analyzing full-length mRNA transcripts (PacBio Iso-Seq), we found predicted retargeting of many ancestrally cytosolic aaRSs to the mitochondrion as well as scenarios where enzyme localization does not appear to change despite functional tRNA replacement. In most cases of transit peptide acquisition, the cytosolic enzyme gained a coding extension prior to the loss of the cognate mitochondrial tRNAs – suggesting a transitional state where organellar and cytosolic aaRSs colocalize to mitochondria. The ongoing functional replacement of *Sileneae* mitochondrial tRNAs and subsequent evolution of tRNA-interacting enzymes present an opportunity to study the coevolutionary dynamics of plant cytonuclear genetics.

Introduction

Mitochondrial protein synthesis requires the expression and interaction of gene products encoded in two separate genomes. Even though most genes involved in mitochondrial translation are encoded in the nuclear genome, all mitochondrial genomes (mitogenomes) encode some translational machinery (Roger et al. 2017). For example, mitogenomes typically encode at least some transfer RNAs (tRNAs) but lack the genes that encode the enzymes necessary for tRNA maturation, modification, and aminoacylation. This partitioning of translational proteins and RNAs across distinct genomes has resulted in chimeric enzymatic networks, with gene products being produced in separate subcellular locations by disparate genetic systems (mitochondrial and nuclear) (Sloan et al. 2018). Vascular plants have even more complex translational systems because of the presence of a second endosymbiotic partner, the plastid. Plastids are additional sites for protein synthesis and exploit some of the same machinery utilized in mitochondrial translation (Duchêne et al. 2005; Pujol et al. 2008). Therefore, the plant mitochondrial translation apparatus provides an opportunity to study the coevolutionary processes necessary to maintain protein synthesis in lineages with tripartite genetic systems.

One of the most fundamental enzymatic processes in translation is the interaction between tRNAs and their cognate aminoacyl-tRNA synthetases (aaRSs or RSs). Before tRNAs carry amino acids to the ribosome for polypeptide synthesis, aaRSs must first ligate the correct amino acid to their 3′-ends. There are a total of 23 described aaRSs: one for each of the twenty proteinogenic amino acids (with the exception of lysine, which has two), and two additional aaRSs for the rare amino acids pyrrolysine and phosphoserine (Rubio Gomez and Ibba 2020). In vascular plants, all aaRS genes are encoded by the nuclear genome (Duchêne et al. 2009). Therefore, aaRSs that function in organellar protein synthesis must be translated by cytosolic ribosomes, targeted to the correct organelle, and translocated across multiple membranes (Duchêne et al. 2009; Ghifari et al. 2018). These subcellularly trafficked aaRSs are generally bacterial-like and distinct from their cytosolic counterparts—largely originating from intracellular gene transfers (plastid and mitochondrial transfers to the nuclear genome) or horizontal gene

transfers from other bacterial sources (Doolittle and Handy 1998; Duchêne et al. 2005; Brandao and Silva-Filho 2011; Rubio Gomez and Ibba 2020).

Somewhat surprisingly, analyses of aaRS genes in *Arabidopsis thaliana* did not find the expected 20 aaRS genes for each subcellular compartment (cytosol, mitochondria, and plastid) (Small et al. 1999; Duchêne et al. 2005). Instead, most aaRSs imported into the mitochondria are also trafficked to plastids — reducing the number of aaRSs in *A. thaliana* to only 45 (Duchêne et al. 2005). These dual-targeted aaRSs must then interact with both mitochondrial tRNAs (mt-tRNAs) and plastid tRNAs to enable translation in these bacterial-like systems.

The import of nuclear-encoded aaRSs into plant organelles involves N-terminal peptides that are recognized by translocase proteins on outer organelle membranes (Berglund et al. 2009; Ge et al. 2014; Ghifari et al. 2018). These transit peptides are cleaved after translocation and can vary considerably in length from fewer than 20 amino acids to over 100 (averaging around 42-50 residues) (Huang et al. 2009; Ge et al. 2014; Murcha et al. 2014). Mitochondrial transit peptides often form amphipathic alpha helices with many hydrophobic and positively charged amino acids (Huang et al. 2009; Schmidt et al. 2010). Plant mitochondrial transit peptides are also particularly rich in Ser residues, and many have a loosely conserved motif containing an Arg residue near the peptide cleavage site (Huang et al. 2009; Ge et al. 2014). Despite these general structural features, there is almost no primary amino acid sequence conservation in transit peptides (Lee et al. 2008; Kunze and Berger 2015), and these domains are considered some of the fastest evolving (non-neutral) sites (Williams et al. 2000; Christian et al. 2020).

Dual-targeted aaRSs that function in both mitochondria and plastids contain an ambiguous N-terminal transit peptide that is recognized by each of the organelle outer membranes (Peeters and Small 2001; Duchêne et al. 2005). How the nonhomologous protein translocation machinery on plastid and mitochondrial outer membranes can interact with the same transit peptide is still not entirely clear (Yogev and Pines 2011; Carrie and Small 2013). While plastid-specific transit peptide sequences generally lack the helical structure found on mitochondrial transit peptides, both organelle transit peptides have very similar amino acid compositions with many hydrophobic and positively charged residues (Bruce 2001;

Ge et al. 2014; Christian et al. 2020). Not surprisingly, dual-targeted transit peptides often exhibit intermediate properties between plastid and mitochondrial-specific transit peptides (Pujol et al. 2007; Berglund et al. 2009).

Even though most of the aaRSs imported into plant organelles are dual-targeted and bacterial-like, there are also multiple exceptions. In *A. thaliana*, there are five eukaryotic-like (cytosolic) aaRSs that are dual-localized to mitochondria and the cytosol (Mireau et al. 1996; Duchêne et al. 2005). The import of these eukaryotic-like aaRSs contributes to the complex nature of mitochondrial tRNA metabolism in plants, where the import of some nuclear-encoded tRNAs is also necessary because the mitogenome contains an incomplete set of tRNAs (Michaud et al. 2011). The five aaRS enzymes shared between the cytosol and mitochondria in *A. thaliana* correspond to tRNAs that are also imported from the cytosol – thereby maintaining phylogenetic congruence between the imported tRNA and interacting enzyme (Duchêne et al. 2005). This coevolutionary pairing of tRNA and aaRS is likely necessary due to the highly discriminating nature of aaRSs (Rubio Gomez and Ibba 2020). The correct attachment of the corresponding amino acid to RNA is essential for the faithful decoding of the genome, and tRNA aminoacylation is a highly accurate process whereby aaRS enzymes use certain nucleotide positions (identity elements) on the tRNA for substrate recognition (Giegé et al. 1998). As eukaryotic tRNAs have little sequence similarity with the bacterial-like mitochondrial and plastid tRNAs, they would be expected to make poor substrates for bacterial-like aaRSs (Salinas-Giegé et al. 2015). However, there are cases of an aaRS and tRNA that functionally interact despite originating from different domains of life (Duchêne et al. 2005; Warren and Sloan 2020). For example, a eukaryotic ProRS appears to have replaced all bacterial counterparts in the genome of *A. thaliana*, despite the species retaining a bacterial-like mitochondrial tRNA-Pro. Therefore, mitochondrial tRNA-Pro must then be aminoacylated by a eukaryotic enzyme. Notably, two eukaryotic ProRSs exist in the *A. thaliana* genome, and only one of those genes contains an organelle transit peptide – suggesting that some enzymatic differentiation may be necessary for bacterial-like tRNA recognition (Duchêne et al. 2005).

Despite a few aaRS/tRNA phylogenetic incongruencies, there exists a general rule of tRNAs encoded in the mitogenome being charged by enzymes that are bacterial in nature. Questions then arise as to the trafficking of aaRSs in plants that have undergone recent and extensive mt-tRNA loss. For example, mitogenomes from the angiosperm tribe *Sileneae* have experienced extensive losses of mt-tRNA genes (Fig. 1) (Sloan et al. 2012a), and recent analysis suggests that these mt-tRNAs are being functionally replaced by import of nuclear-encoded counterparts (Warren et al. 2021).

The almost complete loss and replacement of native mt-tRNAs with nuclear-encoded tRNAs in *Sileneae* species raises multiple alternative scenarios as to the identity of the aaRSs that aminoacylate these newly imported tRNAs. It is possible that the ancestral organellar aaRSs retain mitochondrial localization and have adapted to recognize a novel substrate (a eukaryotic tRNA) or that these aaRSs were already less discriminating enzymes that can effectively charge substantially divergent tRNA molecules. Alternatively, cytosolic aaRSs may have gained targeting to the mitochondria and act on the newly imported tRNA – effectively replacing both partners in the mitochondrial tRNA/aaRS system with eukaryotic counterparts (Haen et al. 2010; Pett and Lavrov 2015).

Here, using genomic and transcriptomic approaches, we test for the evolution of mitochondrial transit peptides, gene duplication events, and changes in transit peptide targeting in four different species from the genus *Silene* as well as *Agrostemma githago*, a close outgroup in the tribe *Sileneae*. We find that functional replacement of mt-tRNAs can precipitate rapid evolution in predicted subcellular targeting of aaRSs, but also that "one solution does not fit all", as responses differ depending on the species and aaRS.

Results

Identification and characterization of Sileneae aaRS gene content

To test for gain of mitochondrial import of cytosolic aaRSs in response to the import of nuclear-encoded tRNAs, we used PacBio Iso-Seq technology (Zhao et al. 2019) to sequence full-length mRNA transcripts from five *Sileneae* species: *A. githago, S. conica*, *S. latifolia, S. noctiflora*, and *S. vulgaris* to search for mitochondrial transit peptides. In addition, previously generated genome assemblies from the same

species (Krasovec et al. 2018; Williams et al. 2020; Warren et al. 2021) were searched for genes or putative transit peptides that may not have been detected by Iso-Seq because of lower expression levels.

Transcripts identified as homologous to annotated *A. thaliana* aaRSs were aligned, and maximum likelihood trees were generated to infer the history of each gene family. To investigate the possible acquisition of mitochondrial transit peptides, the subcellular targeting of each aaRS was predicted using multiple software packages: TargetP v.2.0 (Almagro Armenteros et al. 2019), LOCALIZER v.1.0.4 (Sperschneider et al. 2017), and Predotar v.1.04 (Small et al. 2004).

This analysis successfully identified transcripts from each *Sileneae* species corresponding to known *A. thaliana* organellar and cytosolic aaRSs for each amino acid (Supp. Table 1). As expected, *Sileneae* aaRSs that were homologous to organelle-targeted aaRSs in *A. thaliana* had very high predicted probabilities of being localized to mitochondria, plastids, or both (Supp. Figs. 1-20).

Frequent and rapid acquisition of N-terminal extensions in cytosolic aaRSs genes associated with mt-tRNA loss in Sileneae

In *Sileneae*, mt-tRNA genes decoding 13 amino acids have been lost in one or more species compared to *A. thaliana*, and a 14th (mt-tRNA-Phe) was lost independently in *A. thaliana* and some *Sileneae* species (Fig. 1). These losses raise the question as to which aaRSs are charging the newly imported tRNA replacements. In six of these 13 cases (GlnRS, LysRS, MetRS, ProRS, TrpRS, and TyrRS), we identified an N-terminal extension (Fig. 2) and predicted mitochondrial targeting of the cytosolic enzyme in multiple *Sileneae* species (Fig. 3). In these cases, the corresponding *A. thaliana* enzyme is not mitochondrial-targeted, implying evolutionary gain(s) of transit peptides and targeting in *Sileneae* (Fig. 3). There is also some evidence supporting gains of mitochondrial targeting for three additional cytosolic aaRSs (AspRS, GluRS, and GlyRS), but interpreting these cases is more complex (see below). These examples of retargeting of cytosolic aaRSs imply that ancestral pairings between aaRSs and tRNAs are maintained and have simply expanded their function to include mitochondrial translation.

Many aaRS genes existed as multicopy gene families, and there were multiple cases where an N-terminal extension was only present on a specific clade of genes within an aaRS family: GlnRS, TyrRS, MetRS, and ProRS (Figs. 4 and 5). In these cases, it appears that mitochondrial localization happened following a gene duplication event. The age of these duplications varied considerably, as the two groups of cytosolic MetRS enzymes predate the divergence of *A. thaliana* and *Sileneae* (Fig. 5), whereas the duplication of GlnRS, ProRS, and TyrRS were specific to the lineage leading to *Sileneae* (Fig. 4). There were also multiple LysRS genes with and without coding extensions in *A. githago*, *S. latifolia* and *S. vulgaris*; however, these genes did not form clearly distinct clades (Fig. 4). TrpRS was the only cytosolic aaRS enzyme predicted to gain a mitochondrial transit peptide that was clearly present as a single copy in *Silene* (Fig. 4).

For aaRS genes predicted to be mitochondrially localized, alternative transcription start sites commonly resulted in the expression of two isoforms – producing transcripts with and without coding extensions. For MetRS, GlnRS, LysRS, and TrpRS, an isoform that lacked an N-terminal extension (but was otherwise identical or nearly identical to the extension-containing transcripts) generally exhibited much higher expression levels (inferred from Iso-Seq read counts) than transcripts with coding extensions.

The identified N-terminal extensions were generally found on orthologs from multiple species within the *Sileneae* clade (Fig. 4-5), suggesting a single origin preceding the divergence of these species. A handful of exceptions to this pattern did exist where the presence of an N-terminal extension was unique to a single species. For example, we found a duplicate cytosolic AspRS gene in *S. vulgaris* that was strongly predicted to be mitochondrially targeted, but no other *Sileneae* species appear to have gained mitochondrial targeting for AspRS (Supp. Fig. 4). Additionally, N-terminal extensions on a cytosolic ProRS in *A. githago* (Fig. 5) and a cytosolic TyrRS in *S. vulgaris* (Fig. 4) appear to be nonhomologous to the extensions found in other species because no significant similarity was found with a blastn comparison at an e-value threshold of 0.1. Therefore, these cases likely represent independent gains of transit peptides.

In the majority of cases where a cytosolic enzyme was predicted to have gained mitochondrial targeting, an N-terminal extension was obvious in alignments and was contiguous with the downstream coding sequence in the respective genome assembly (i.e., not separated by an intron). However, alternative splicing for GluRS in *A. githago* (low expression; two Iso-Seq reads) and *S. conica* (moderate expression; 50 Iso-Seq reads) resulted in isoforms that were predicted to be mitochondrially-targeted (Supp. Fig. 7). These alternative isoforms were shorter than the more typical GluRS isoforms. In each case, a 5′ portion of the open reading frame (ORF) was replaced by a shorter, novel exon (the alternative *S. conica* and *A. githago* isoforms were 65 and 21 amino acids shorter, respectively). Therefore, despite the predicted gain of mitochondrial targeting, the truncated length of the core ORFs makes it unclear whether these alternatively spliced transcripts produce aaRSs that are functional in aminoacylation.

Functional replacement of mt-tRNAs is not always associated with retargeting of cytosolic aaRSs

Although evolution of N-terminal transit peptides appears to have occurred repeatedly in *Sileneae* aaRSs (Figs. 2-5), there were also numerous examples where a cytosolic enzyme did not experience an obvious change in N-terminal sequence despite the loss of the cognate mt-tRNA gene and functional replacement via import of the cytosolic tRNA (Fig. 1) (Warren et al. 2021). For AsnRS, CysRS, HisRS, PheRS, and SerRS, organelle localization was not predicted by any of the software programs (Supp. Figs. 3, 5, 14, 16), and the length of the enzymes did not differ substantially from the corresponding *A. thaliana* ortholog(s) in alignments. The organellar aaRSs for Asn (Supp. Fig. 3), Cys (Supp. Fig. 5), His (Supp. Fig. 9), and Phe (Supp. Fig. 14) did retain predicted transit peptides for mitochondrial localization, suggesting that these organellar aaRSs might now be charging the newly imported cytosolic tRNAs. SerRS presented a unique situation because neither the cytosolic nor organellar genes in *Sileneae* are predicted to be mitochondrially targeted (Supp. Fig. 16). Instead, the organellar SerRS is predicted to be plastid-specific (Supp. Fig. 16), making the identity of the aaRS that charges mitochondrially imported tRNA-Ser unclear.

Some substantially truncated transcripts originating from HisRS and AspRS resulted in ORFs that began in the middle or the C-terminal end of the gene body and were predicted to have a high probability of being mitochondrially targeted. These lowly expressed transcripts could represent transcript degradation, and we did not consider them to be strong evidence of aaRS function in the mitochondria due to the lack of an intact full-length protein typically required for aminoacylation. Some aaRS protein domains are known to have functions outside of aminoacylation (Ibba and Francklyn 2004; Pang et al. 2014), so it is possible that a truncated aaRS gene product is organelle localized and has novel functions, but we did not include these truncated sequences in this analysis.

Although *A. thaliana* lacks mt-tRNA-Phe, the loss of this mt-tRNA gene in *Sileneae* appears to be an independent event, as multiple relatives (including *S. chalcedonica*) still retain a mt-tRNA-Phe in the mitogenome (Fig. 1). In *A. thaliana,* only a single organellar PheRS was found to localize to both plastids and mitochondria and is therefore expected to charge both native plastid tRNAs as well as imported tRNA-Phe in mitochondria. However, the enzymatic coevolutionary response to losing this mt-tRNA may be sustainably different in *Sileneae* as there has been a gene duplication event in the organellar PheRS gene family (Fig. 6). One of the PheRS paralogs has a stronger prediction for mitochondrial targeting than plastid targeting, whereas the inverse is true for the other paralog (Fig. 6). This history of gene duplication and N-terminal sequence evolution may point to specialization of these organellar-targeted genes and the adaptation of one paralog for recognition of imported cytosolic tRNAs in the mitochondria. Regardless, tRNA-Phe is the only mt-tRNA lost three times independently in this angiosperm dataset, yet there is no evidence of cytosolic PheRS gaining mitochondrial import in any of these lineages. This may be due to unique evolutionary constraints for the import of a functional cytosolic PheRS. Cytosolic PheRS is a complex aaRS composed of two heterodimers with α- and β-subunits, with both subunits being essential for tRNA-Phe aminoacylation (Safro et al. 2013). The import of both subunits and successful assembly of the dimer is presumably essential for aminoacylation inside the mitochondrial matrix, thus requiring the almost simultaneous *de novo* acquisition of a targeting peptide on

both subunits for functional replacement. This import requirement may have made it unusually difficult to functionally replace the organellar PheRS with its multi-subunit cytosolic counterpart.

The evolution of putative aaRS transit peptides can occur prior to the loss of mt-tRNAs

Phylogenetically, the acquisition of putative transit peptides by cytosolic aaRSs in *Sileneae* rarely matched the precise timing of the loss of the cognate mt-tRNA (Fig. 3). Only GlnRS and potentially TrpRS showed a perfect correlation between mt-tRNA loss and predicted cytosolic aaRS retargeting. Extensions were detected on some cytosolic TrpRS genes in species that still retain a mt-tRNA-Trp gene, but they fell below the targeting prediction cutoff for strong likelihood of mitochondrial localization. For the cytosolic enzymes GluRS, LysRS, TyrRS, and ProRS, an N-terminal extension was present in species that still retained a mt-tRNA (Figs. 3-4). For example, a predicted targeting peptide was present on the cytosolic LysRS transcripts from *A. githago* despite that species retaining both a mt-tRNA-Lys gene and an organellar LysRS with predicted mitochondrial targeting. Similarly, mitochondrial localization was also predicted for cytosolic TrpRS and TyrRS in *S. vulgaris*, yet both cognate bacterial-like tRNAs are still encoded in the *S. vulgaris* mitogenome. If these targeting predictions are correct, then the colocalization to the mitochondria of two aaRSs (both an organellar and cytosolic type) would be a widespread phenomenon in *Sileneae* (Fig. 2), raising questions about the enzymatic function and substrates of both aaRSs.

Retargeting of cytosolic aaRSs to mitochondria may precipitate loss of organellar aaRS dual-targeting

Predicting organelle-specific versus dual-targeted enzymes with *in silico* methods is difficult due to the shared characteristics of mitochondrial, plastid, and dual transit peptides. Nevertheless, the gain of mitochondrial localization of a cytosolic aaRS enzyme leads to multiple hypothesis about the fate of the organelle-targeted counterpart. The replacement of bacterial-like tRNAs and the cognate aaRS partners in mitochondrial translation is unlikely to lead to the outright loss of organellar aaRS because plant cells also contain plastids, which retain a full complement of bacterial-like tRNA genes that require an organellar

aaRS for aminoacylation. This dual functionality of many plant aaRSs likely constrains outright aaRS gene loss because of the pleiotropic effects on plastid translation. However, one testable prediction is that ancestrally dual-targeted aaRSs that localize to both organelles will lose or reduce mitochondrial targeting and specialize on plastid function when a cytosolic counterpart gains mitochondrial localization. In agreement with this, we observed a decreased probability of mitochondrial targeting for multiple organellar aaRS genes when a cytosolic enzyme was predicted to be retargeted to the mitochondria.

The targeting of GlyRS enzymes presents an interesting situation in *A. thaliana* where both a cytosolic enzyme and a dual-targeted organellar enzyme are localized to the mitochondria (Figs. 2 and Supp. Fig. 8). In *Sileneae*, a putative transit peptide on the cytosolic GlyRS is also present, but it is possible that it was gained independently. Unlike in *A. thaliana*, *Sileneae* species have lost the mt-tRNA-Gly gene, suggesting a complete replacement of the bacterial Gly decoding system in *Sileneae* mitochondria (Fig. 2). This functional replacement of tRNA/aaRS corresponds with a decrease in the predicted probability of mitochondrial localization (Supp. Fig. 8).

Retargeting of cytosolic MetRS is also associated with changes in dual-targeting predictions for the organellar aaRSs. Although multiple organellar MetRS genes in *Sileneae* experienced only a marginal decrease in mitochondrial targeting prediction compared to *A. thaliana*, *S. vulgaris* had virtually no signal of mitochondrial localization (Supp. Fig. 13) and is the only species in the lineage that has lost both tRNA-Met genes (elongator Met and initiator fMet, Fig. 1). This observation raises the possibly that the loss of both tRNA genes has obviated the need for an organellar MetRS in *S. vulgaris* mitochondria.

In addition, there was a correspondence between the strength of the prediction for mitochondrial targeting of a cytosolic TrpRS and the reduction of a mitochondrial-targeting signal in the organellar TrpRS in *Silene* (Supp. Fig, 18). This association was strongest in the species that have lost the cognate mt-tRNA gene, as *S. noctiflora* and *S. conica* had the greatest reduction in mitochondrial targeting probability for the organellar enzymes. *Silene vulgaris* shows some indication of mitochondrial targeting of the cytosolic TrpRS but still retains the mt-tRNA-Trp gene. Interestingly, this species has two different

copies of the organellar TrpRS, one of which is predicted to be dual targeted, while the other is predicted to be exclusively plastid localized (Fig. 4).

Discussion

The rapid functional replacement of *Sileneae* mt-tRNA genes with nuclear-encoded counterparts raises questions about the identity of the aaRSs that aminoacylate the newly imported tRNAs. Here, we used predictive subcellular localization software in conjunction with full-length cDNA sequencing and nuclear genome assembly data to test for hypothesized changes in aaRS targeting and import in association with tRNA replacement.

One longstanding question related to mt-tRNA replacement in plants is whether tRNA or aaRS import happens first, as it has been assumed that the import of one without the other would be nonfunctional in translation (Small et al. 1999). The data presented here provide evidence for two different scenarios that could facilitate the loss of mt-tRNAs – the first being that a permissive organellar aaRS enzyme is capable of charging newly imported, nuclear-encoded tRNAs. Evidence for this intermediate state arises from roughly half of mt-tRNA gene losses, which are not associated with a discernable gain of mitochondrial targeting peptides on cognate cytosolic enzymes, leaving the organellar aaRS as the only functional enzyme to charge imported tRNAs. Furthermore, recent work to detect tRNA import in *Sileneae* (Warren et al. 2021) found cases of redundant tRNA import (import of a cytosolic tRNA prior to the loss of the mt-tRNA gene) for tRNA-Asn, tRNA-Glu, and tRNA-His. The results from the present study suggest that organellar aaRSs are capable of charging all three of these cytosolic tRNAs (Supp. Fig. 3, Supp. Fig. 7, Supp. Fig. 9). This type of mismatch between a bacterial-like aaRS and a eukaryotic tRNA is not unprecedented (Salinas-Giegé et al. 2015). Aminoacylation studies on aaRSs in plants and other taxa have also found organellar aaRSs to be less discriminating than bacterial or eukaryotic counterparts (Salinas-Giegé et al. 2015).

The second transition scenario is more complex and involves the *de novo* evolution of mitochondrial import of a cytosolic aaRS enzyme. If the aaRS targeting predictions in this study are

accurate (see below for discussion of some of the limitations of *in silico* predictions), a cognate cytosolic aaRS has also gained mitochondrial import in the remaining cases where an mt-tRNA gene is lost. It is possible that these imported synthetases have a function other than aminoacylation, thereby predisposing the system to mt-tRNA replacement once the partnered cytosolic tRNA gains import. Alternatively, an intermediate state of mitochondrial translation may exist whereby both the organellar system (a bacterial-like tRNA/aaRS) and a cytosolic system (eukaryotic-like tRNA/aaRS) are cofunctional in mitochondria. Evidence for the latter scenario comes from *Sileneae* species predicted to have both a cytosolic and organellar aaRS localized to the mitochondria for the same amino acids (Lys, Pro, and Tyr). A similar situation exists in *A. thaliana* where both imported tRNA-Gly and mt-tRNA-Gly are necessary for translation (Salinas et al. 2005). The presence of both imported and native tRNAs that decode the same amino acid (but different codons) is mirrored by the import of both an organellar and cytosolic GlyRS (Fig. 3) (Duchêne et al. 2005). The bacterial-like GlyRS was found to effectively aminoacylate both tRNA counterparts, whereas the cytosolic GlyRS had poor actively with a mt-tRNA-Gly substrate (Duchêne et al. 2001). The cytosolic GlyRS was suggested to be inactive in the mitochondrial matrix in *A. thaliana* because the enzymatic extracts from mitochondria were hydrophobic and appeared to be inactive when fractionated by chromatography (Duchêne et al. 2001). Nevertheless, it would be interesting to determine whether similar scenarios exist in *Sileneae* where an organellar or cytosolic aaRS has cross-functionality and is able to effectively charge both tRNA counterparts. Alternatively, there may exist a functional partitioning of matched aaRSs and tRNAs based on phylogenetic origins. Once both aaRSs are functional within the mitochondria, it becomes easy to envision a scenario where an inactivating mutation in the mt-tRNA gene makes the system wholly dependent on the cytosolic tRNA/aaRS pair.

The acquisition of mitochondrial transit peptides appears to evolve rapidly, as there were multiple instances where novel transit peptides were only detected in a single species. There were even instances where a class of aaRS genes gained transit peptides independently multiple times (e.g., ProRS [Fig. 5], and TyrRS [Fig. 4]). The likelihood and rate of proteins gaining organelle import have been experimentally explored by testing the localization of bacterial-derived sequences, *in silico* methods, and

the predictive localization of randomized sequences (Baker and Schatz 1987; Christensen et al. 2005; Xu et al. 2013; Christian et al. 2020; Costello et al. 2020). These studies have suggested that protein retargeting is relatively common. The fact that the most frequently occurring residues in transit peptides are represented by the codons in the largest codon families in the genetic code may explain the high probability of *de novo* evolution (Mackenzie 2005).

Although the localization of aaRSs to plant mitochondria is largely mediated by N-terminal presequences, there are two cases in *A. thaliana* (LeuRS and ArgRS) where a cytosolic enzyme is localized to mitochondria but lacks an identifiable N-terminal transit peptide (Duchêne et al. 2005). For cytosolic LeuRS, western blots and *in vivo* uptake studies have confirmed its presence in *A. thaliana* mitochondria, and recent work has shown that the import is dependent on the cognate tRNA-Leu (Reinbothe et al. 2021). The cytosolic ArgRS in *A. thaliana* also lacks a transit peptide, but the ArgRS enzyme with an N-terminal extension was shown to exclusively localize to plastids (Duchêne et al. 2005), suggesting that the cytosolic ArgRS is targeted to the mitochondria by an unknown mechanism. These examples highlight the need for experimental investigation into the localization of aaRS, particularly in systems like *Sileneae* that are undergoing rapid evolution in mt-tRNA gene content. The curious example of SerRS localization in *Sileneae*, where neither the cytosolic nor organellar enzymes were confidently predicted to be mitochondrially localized, may point to either a failure to recognize a mitochondrial transit peptide or an import mechanism that is not dependent on an N-terminal sequence. Interestingly, cytosolic SerRS enzymes have undergone a duplication in *Sileneae*, something seen in other cytosolic aaRS classes that have gained mitochondrial localization.

However, if the targeting predictions presented here are correct, there appears to be widespread replacement of organellar aaRSs with cytosolic counterparts, possibly rendering the organellar enzyme nonfunctional or redundant in mitochondria. It could therefore be expected that these bacterial-like enzymes would be lost due to lack of selective constraint. This trend of organellar aaRS gene loss correlating with mt-tRNA gene loss has been described in multiple non-bilaterian animal systems, including cnidarians, comb jellies, and sponges (Haen et al. 2010; Pett and Lavrov 2015). In the case of

plants, the presence of the plastid necessitates the retention of organellar aaRSs. Accordingly, we saw a trend towards loss of predicted mitochondrial targeting (i.e., plastid specificity) for multiple organellar aaRSs (GlyRS, MetRS, TrpRS) following mt-tRNA loss. The loss of dual targeting is infrequent in plants, but it does occur and can be associated with gene duplication and neofunctionalization, resulting in two organelle-specific proteins (Xu et al. 2013). The apparent large scale loss of mitochondrial targeting in response to cytosolic aaRS retargeting in *Sileneae* represents a complex chain of coevolutionary events involving three genomic compartments.

Finally, the existence of what appears to be two very different routes to the replacement of the bacterial aaRS/tRNA system in plant mitochondria (permissive aaRSs and redundant aaRS import) may offer insight into each enzyme's activity and address a striking contrast encountered in aaRS evolution. On one hand, aaRSs have successfully undergone horizontal gene transfer across some of the deepest splits in tree of life without disrupting their function (Doolittle and Handy 1998; Brindefalk et al. 2007). On the other hand, aaRSs are also highly discriminating enzymes. Even within mitochondrial translation systems, there are multiple examples of single nucleotide substitutions in mt-tRNAs resulting in severe reductions in aminoacylation (Yarham et al. 2010; Liu and Chen 2020). In one described case of aaRS/tRNA incompatibility in *Drosophila*, a single amino acid polymorphism in the mitochondrial TyrRS negatively interacted with a nucleotide polymorphism in mt-tRNA-Tyr to produce a diseased phenotype of delayed development and reduced fecundity (Meiklejohn et al. 2013). The replacement of a mt-tRNA with a cytosolic tRNA represents a far more radical change in substrates. The retargeting of only certain cytosolic aaRSs may offer clues as to which organellar aaRS have maintained substrate specificity that requires the import of a phylogenetically congruent (cytosolic) aaRS for functional mt-tRNA gene replacement.

Materials and Methods

Tissue generation and growth conditions

Tissue generation, RNA extraction, and library construction for *S. noctiflora* was done in a previously described study (Williams et al. 2020), while data for the other four *Sileneae* species were newly generated for this study. The following seed collections or accessions were used: *A. githago* Kew Gardens Millennium Seed Bank (0053084), *S. vulgaris* S9L (Sloan et al. 2012b), *S. latifolia* UK2600 (from the line originally used for mitogenome sequencing in Sloan et al. (2010)), and *S. conica* ABR (Sloan et al. 2012a). Seeds were germinated in small plastic pots with Plantorium Greenhouse brand potting soil in a growth chamber at 23 °C with a light setting of 8-hour light/16-hour dark at 100 μE m^{-1} s^{-1}. One week after germination, chamber settings were modified to promote flowering ("long-day" conditions) with 16-hour light/8-hour dark.

RNA extraction and Iso-Seq library construction

RNA was extracted from *A. githago* (hermaphrodite), *S. conica* (hermaphrodite), *S. latifolia* (male), and *S. vulgaris* (male-fertile hermaphrodite) with a Qiagen RNeasy Plant Mini Kit, using RLT buffer with 10 μl beta-mercaptoethanol. RNA was DNase treated with a Qiagen RNase-Free DNase Set. Separate RNA extractions were performed on leaf tissue and an immature flower sample (~5 days post flower development) for *A. githago*, *S. vulgaris*, and *S. latifolia*. Two different tissues were used to increase detection of diverse transcripts, but the two RNA samples were pooled equally by mass for each species prior to library construction, so individual reads cannot be assigned to leaf or floral tissues. Only leaf tissue was used for *S. conica* as the individual had not yet begun flowering at the time of RNA extraction. Both tissue types were harvested at 4 weeks post-germination, and RNA integrity and purity were checked on a TapeStation 2200 and a Nanodrop 2000.

Iso-Seq library construction and sequencing was performed at the Arizona Genomics Institute. Library construction was done using PacBio's SMRTbell Express Template Prep Kit 2.0. The four libraries were barcoded and pooled. The multiplexed pool was sequenced with a PacBio Sequel II platform on two SMRT Cells using a Sequencing Primer V4, Sequel II Bind Kit 2.0, Internal Control 1.0,

and Sequel II Sequencing Kit 2.0. Raw movie files were processed to generate circular consensus sequences (CCSs) using PacBio's SMRT Link v9.0.0.92188 software (Pacific Biosciences 2020). Demultiplexing was performed with lima v2.0.0 and the --isoseq option. Full-length non-chimeric (FLNC) sequences were generated with the refine command and the --require_polya option in the IsoSeq3 (v3.4.0) pipeline. Clustering of FLNCs into isoforms was then performed with the cluster command in IsoSeq3 with the --use-qvs option. The two SMRT Cells produced similar outputs with 5.8M and 5.9M raw reads, which resulted in 3.9M CCSs for each cell (3.5M and 3.4M retained after demultiplexing). The results of demultiplexing, FLNC filtering, and clustering are shown in Supp. Table 2.

Extraction of aaRS transcript sequences

Arabidopsis aaRS genes were identified from published sources (Duchêne et al. 2005; Warren and Sloan 2020) and the corresponding protein sequences were obtained from the Araport11 genome annotation (201606 release). Homologs from the high-quality (HQ) clustered isoforms from each species were identified with a custom Perl script (iso-seq_blast_pipeline.pl available at GitHub: https://github.com/warrenjessica/Iso-Seq_scripts) that performed a tBLASTn search with each *Arabidopsis* aaRS sequence, requiring a minimum sequence identity of 50% and a minimum query length coverage of 50%. All HQ clusters that satisfied these criteria were retained by setting the --min_read parameter to 2 (the IsoSeq3 clustering step already excludes singleton transcripts).

Transcript processing and targeting prediction

The longest ORF was extracted from each aaRS transcript using the EMBOSS v. 6.6.0 (Rice et al. 2000) getorf program with the options: -minsize 75 -find 1. Many Iso-Seq transcripts differed in length by only by a few nucleotides in the UTR region but resulted in identical ORFs. Therefore, all identical ORFs were collapsed for downstream targeting and phylogenetic analysis. Collapsed ORFs were translated into protein coding sequences for localization analysis. TargetP v.2.0 (Almagro Armenteros et al. 2019), LOCALIZER v.1.0.4 (Sperschneider et al. 2017), and Predotar v.1.04 (Small et al. 2004) were each used to predict targeting probabilities of each coding sequence. All programs were run with the plant option.

Determination of gene copy number and genome assembly scanning for undetected genes

Very similar transcripts can be the product of different genes, alleles, or sequencing errors. In order to infer the number of unique genes for each related set of transcripts in a species, CD-HIT-EST v. 4.8.1 (Fu et al. 2012) was used to further cluster transcripts into groups. For this clustering step, sequences were first aligned with MAFFT v. 7.245 (Katoh and Standley 2013) with default settings and trimmed by eye to remove terminal sequence ends with gaps and N-terminal extensions that were not present on all sequences. Any two sequences in which the coding region shared greater than 98% sequence similarity were collapsed into a single gene cluster (CD-HIT-EST options -c 0.98 -n 5 -d 0). Each cluster of transcripts was considered a single gene, and the transcript with the highest expression and longest length was retained as the representative sequence for the gene.

To check for the possibility that a cytosolic gene had gained a transit peptide but was undetected in Iso-Seq data (due to low expression or representation in the sequencing library), all cytosolic genes that appeared to lack transit peptides were checked for immediately upstream start codons in the corresponding nuclear genome assembly (Warren et al. 2021) . Representative transcripts from each gene cluster were translated and BLASTed (tblastn) against the nuclear assembly, and scaffolds with a hit to the first exon of the protein were extracted and analyzed with the ExPASy Translate tool (Artimo et al. 2012). The ORF found in the genome assembly was then compared to the ORF generated from the transcript and inspected for length differences. If an upstream Met was present, the upstream sequence was appended to the rest of the gene and re-run through the targeting prediction software described above.

Occasionally, when BLASTing cytosolic aaRS proteins to nuclear assemblies, additional genes were discovered that were entirely absent from the Iso-Seq data (genes marked with ** in Supp. Figs. 3,4,13 and 19). In these cases, the region that aligned to the first exon of the expressed paralog was used for phylogenetic and targeting analysis.

Sequence alignment and maximum likelihood phylogenetic analysis

After clustering transcripts by sequence similarity (see above), the coding region of the longest transcript for each gene was retained for phylogenetic analysis. If two or more transcripts were tied for the longest length, the one with higher expression level was used. Retained sequences for each aaRS gene family were aligned using MAFFT v. 7.245 (Katoh and Standley 2013) with default settings. Sequences were trimmed by eye to remove poorly aligned regions, and maximum likelihood trees were produced using RAxML v.8.2.12 (Stamatakis 2014) with a GTRGAMMA model and rapid bootstrap analysis with a 100 replicates.

Data Availability

The CCSs from each Iso-Seq library are available via the NCBI Sequence Read Archive (SRA) under BioProject PRJNA799780. Trimmed and untrimmed alignments for final aaRS sequences can be found on Dryad at https://doi.org/10.5061/dryad.0k6djhb20.

Acknowledgements

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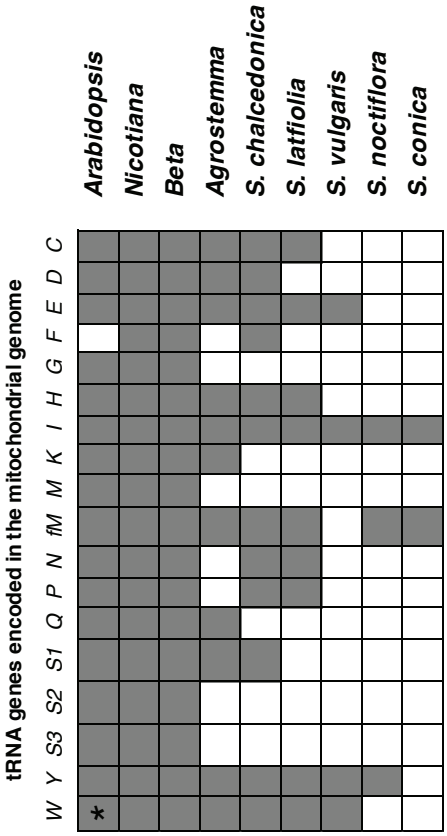

Figure 1| The loss of mt-tRNA genes in the mitogenomes of multiple *Sileneae* species. Singe-letter codes are used for amino acid abbreviations. Gray filled squares indicate the presence of the corresponding tRNA gene in the mitogenome. *Arabidopsis* tRNA-Trp (W) is marked with an * because multiple studies have failed to detect its expression and the import of a nuclear-encoded tRNA-Trp has been demonstrated (Duchêne and Marechal-Drouard 2001; Warren et al. 2020)

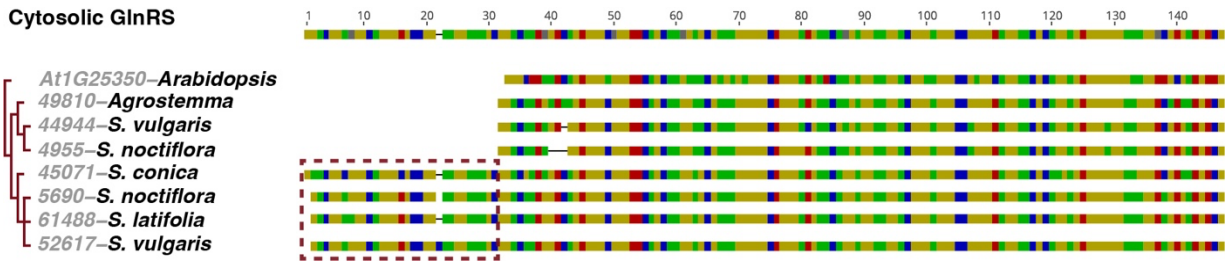

Figure 2| Alignment of cytosolic GlnRS genes with predicted mitochondrial transit peptides indicated. Top bar indicates the consensus amino acid sequence and position numbering. Colors represent polarity with red – acidic, blue – basic, green – polar, yellow – nonpolar. Transit peptides (or N-terminal extensions) were predicted using TargetP v.2.0 (Almagro Armenteros et al. 2019) and are outlined in the dashed, red box. Numbers with the taxa names indicate unique ID numbers for each gene. No upstream coding sequence for the genes without predicted transit peptide could be found in transcripts or genome assemblies. Figure generated with Geneious Prime software (v. 2022.0.1).

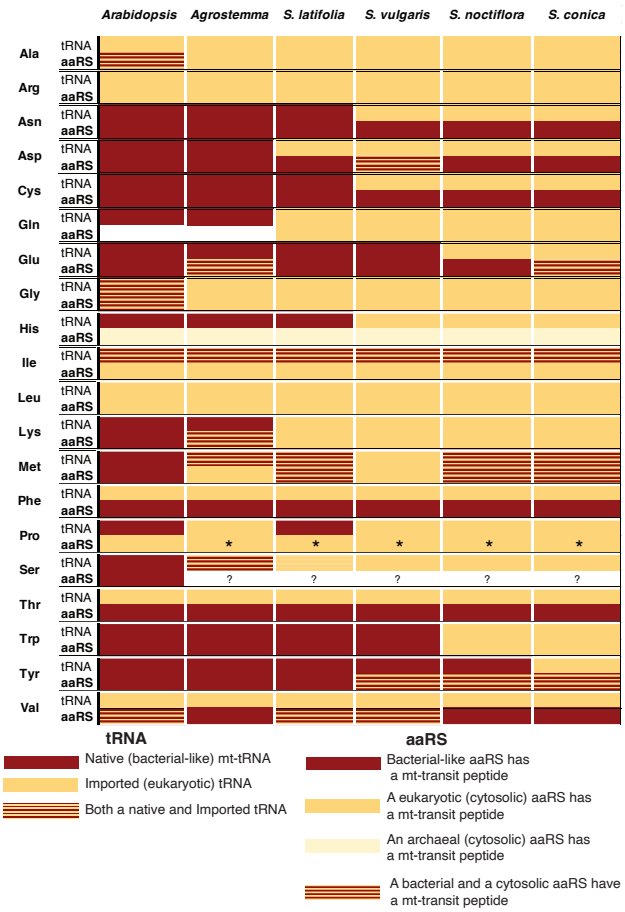

Figure 3| The origin of mitochondrial tRNAs and the cognate mitochondrially-localized aaRSs in *A. thaliana* and multiple *Sileneae* species. The mitochondrial localization of aaRSs has been experimentally demonstrated in *A. thaliana* (see Supp. Table 1, (Duchêne et al. 2005)) and has been assigned for *Silenae* species based on localization prediction software. Mt-tRNAs and aaRSs have complex gene histories. Thus, tRNAs and aaRSs classified as "bacterial-like" include those derived from the mitochondria (alphaproteobacterial-like), the plastids (cyanobacterial-like), or other bacterial origins. All bacterial-like tRNAs are still encoded in the mitogenomes, whereas all eukaryotic mitochondrial tRNAs are imported from the cytosol. Solid colors represent the presence of only a single phylogenetic class of a tRNA or aaRS, while striped boxes indicate the presence of multiple aaRSs/tRNAs with differing phylogenetic origins. The cytosolic TyrRS in angiosperms is archaeal in origin, and it appears to have been retargeted to the mitochondria in some *Silene* species. For this figure, an aaRS was classified as being predicted to be mitochondrially-targeted if the enzyme had 50 or more percentage points of targeting likelihood to the organelle (cumulatively between the three targeting prediction software programs). See Supp. Figs 1-20 for more detailed targeting data for each gene family. An organellar GlnRS does not exist in most plant mitochondria (including *Arabidopsis*) as tRNA-Gln is aminoacylated by an indirect transamidation pathway with a nondiscriminating GluRS (Salinas-Giegé et al. 2015). This ancestral state appears to be retained in *Agrostemma*. All ProRS enzymes have eukaryotic origins, but organelle and cytosolic genes are distinct. ProRS enzymes marked with an * indicate that both organellar and cytosolic copies have a coding extension predicted to function as a transit peptide. No SerRS enzymes in *Sileneae* were strongly predicted to be targeted to the mitochondria.

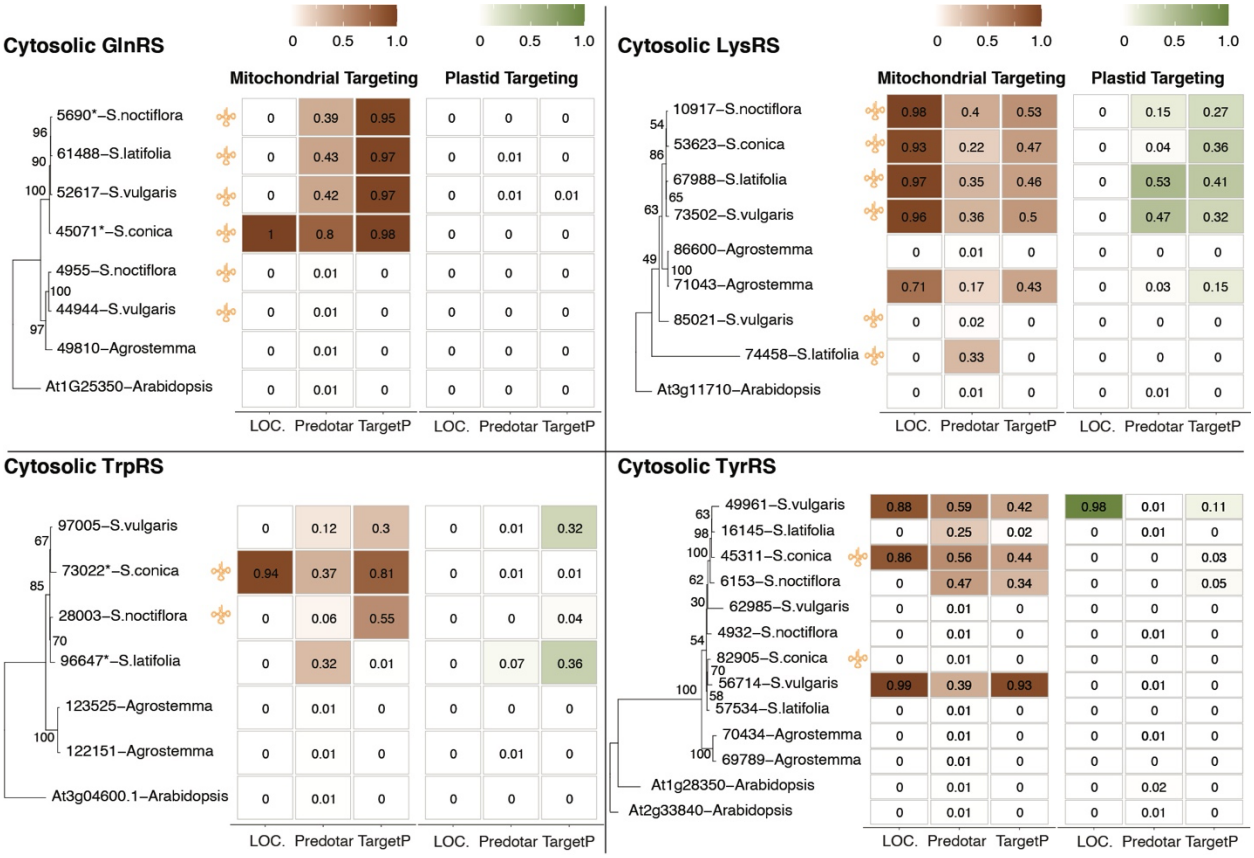

Figure 4| Maximum likelihood trees and targeting probabilities of cytosolic aaRSs in *A. thaliana* and *Sileneae* that have gained an N-terminal extension predicted to function as a mitochondrial transit peptide. Shown are the aaRSs in which a corresponding mt-tRNA gene has been lost in one or more *Silene* species (after the divergence of the *Silene* and *Agrostemma*). The orange tRNA symbol indicates that the species has lost the mt-tRNA, and therefore requires the import of a nuclear-encoded tRNA. Genes marked with an * indicate that this gene was detected in Iso-Seq data but without the 5´ extension present in the nuclear assembly.

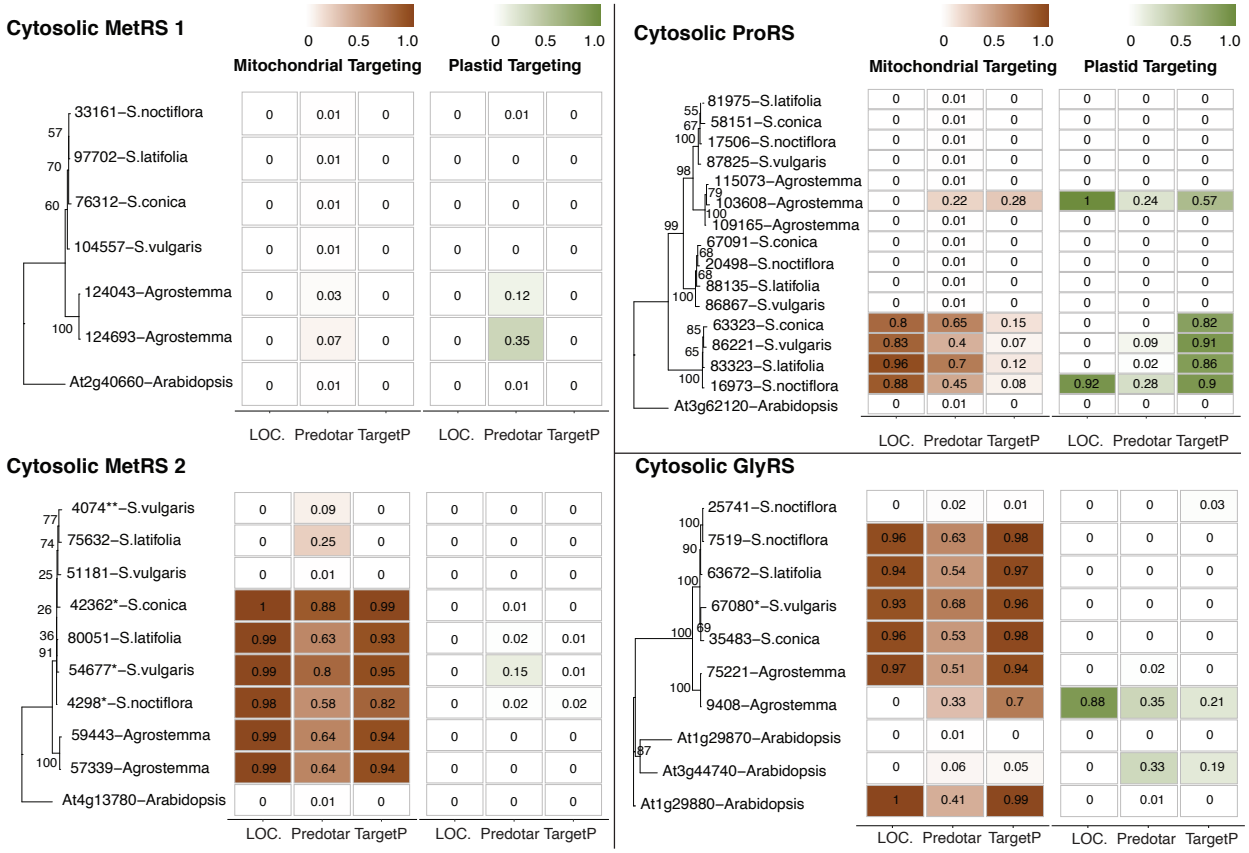

Figure 5| Maximum likelihood trees and targeting probabilities of cytosolic aaRS in *A. thaliana* and *Sileneae* that have gained an N-terminal extension predicted to function as a mitochondrial transit peptide. Shown are the aaRSs in which all *Silene* and *Agrostemma* species have lost the corresponding mt-tRNA. Genes marked with an * indicate that this gene was detected in Iso-Seq data but without the 5´ extension present in the nuclear assembly. Genes marked with ** indicate that this gene was undetected by Iso-Seq data but was identified in the nuclear assembly.

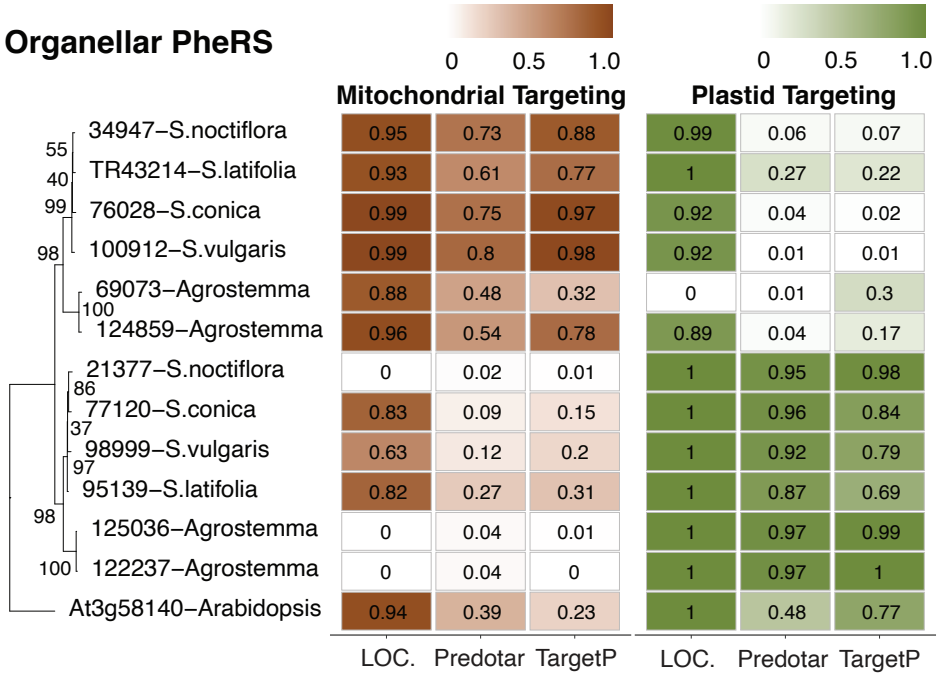

Figure 6| Maximum likelihood tree and targeting probabilities of organellar PheRS genes in *A. thaliana* and *Sileneae*. Mt-tRNA-Phe has been lost in *A. thaliana* as well as in all *Silene* and *Agrostemma* species.

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