Elucidating Mechanisms that Direct Chloroplast Encoded Proteins to the Thylakoid Membrane

by

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Dr. Alice Barkan

The thylakoid membrane is the site of the light reactions of photosynthesis and is the destination for many integral membrane proteins that are involved in photosynthesis. My research aims to elucidate the mechanisms that target chloroplast-encoded proteins to the thylakoid membrane. Previous studies have revealed three separate pathways by which nuclear-encoded proteins are targeted to the thylakoid membrane, each of which evolved from secretion systems in the cyanobacterial ancestor. Plastid-encoded proteins are hypothesized to use these pathways to integrate into the thylakoid membrane as well. By profiling ribosomes in separated soluble and membrane fractions, the Barkan lab showed that many plastid mRNAs are translated on ribosomes that are anchored to the thylakoid membrane, and that the proteins encoded by these mRNAs are inserted into the thylakoid co-translationally. I conducted partitioned ribosome profiling experiments on maize mutants that lack each of the three thylakoid membrane translocon machineries to reveal which thylakoid targeted proteins are excluded from co-translational membrane integration in these specific mutants. I

found that the loss of certain translocon machinery had differential effects on the cotranslational targeting of plastid-encoded thylakoid membrane proteins.

Acknowledgements

I would like to thank Professor Alice Barkan for helping me to fully examine my research topic and consider various perspectives and contexts to interpret my results. I would also like to thank Professor Diane Hawley and Professor Roxann Prazniak for serving on my thesis committee. Thank you also to the members of the Barkan Lab for assisting me with many experiments and methodological questions.

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INTRODUCTION

The chloroplast

Plants and animals are composed of eukaryotic cells. Eukaryotic cells contain a membrane bound nucleus to house genetic material and membrane bound subcellular compartments, or “organelles” such as the mitochondrion and the chloroplast. The mitochondrion is the site of cellular respiration, where chemical energy called ATP is produced in the final step of sugar breakdown. Most eukaryotic cells contain mitochondria, but only plant cells contain chloroplasts. In a process known as photosynthesis, the chloroplast harnesses light energy from the sun to create chemical energy in the form of sugar.

A defining characteristic of chloroplasts and mitochondria is that they each contain their own genetic material in a small genome separate from the one housed in the cell nucleus. The presence of these organellar genomes provided the first clue that chloroplasts and mitochondria evolved from bacteria that had been engulfed by an ancient cell. Chloroplasts are derived from a photosynthetic bacterium (cyanobacterium) whereas mitochondria are derived from a non-photosynthetic bacterium (Raven and Allen, 2003). Bacteria are single-celled “prokaryotic” organisms and differ from eukaryotic cells by their lack of a membrane bound nucleus and lack of membrane bound organelles. The majority of the genes found in the bacterial ancestors of mitochondria and chloroplasts are no longer present in today’s organellar genomes. Depending on the species, each organellar genome retains approximately 20 to 100 genes from their bacterial ancestor. These organellar genes are necessary for the cell to

carry out either photosynthesis (in the case of chloroplasts) or respiration (in the case of mitochondria).

Gene expression

Genes consist of material called DNA, which makes up the genome of the cell. Genes must be expressed in order for them to fulfill their functions. The first step in gene expression is called transcription. Transcription involves making a single stranded RNA copy of the double-stranded genomic DNA. The single-stranded RNA is then modified in various ways and the finished product is known as a messenger RNA, or mRNA. After transcription and RNA processing, many finished mRNAs are then used as templates to create proteins, or "polypeptides", by a process known as translation. Proteins are the ultimate product of most genes, and it is the content of different proteins that are largely responsible for giving cells and organisms their particular properties.

Translation of protein coding mRNA is carried out by large structures called ribosomes. The ribosome 'reads' the genetic code provided by the mRNA transcript and constructs a polypeptide out of individual amino acids based on this genetic code. Ribosomes bind an mRNA and provide a structured site for polypeptide synthesis. Amino acids are transported to the ribosome by a special RNA called "transfer RNA" or tRNA. Each tRNA contains a nucleotide triplet that recognizes and binds to a complementary sequence on the mRNA. Successful recognition of an mRNA sequence by a tRNA results in the transfer of the appropriate amino acid to the ribosome. Within the ribosome, special RNA called 'ribosomal RNA' or rRNA catalyzes the linkage of the new amino acid to the previous amino acid. Translation along the length of the

whole mRNA transcript results in a polypeptide coded from the information stored in the DNA (Figure 1).

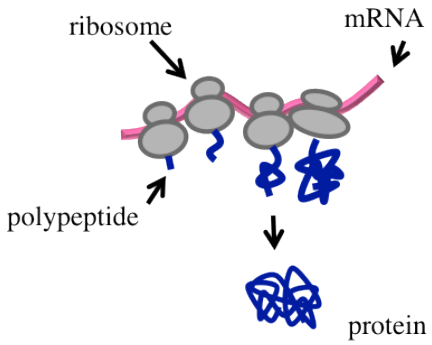

Figure 1. A polysome

Ribosomes translate along the length of an mRNA, resulting in a polypeptide that folds into a protein. This structure is also known as a polysome.

Membranes compartmentalize the cell

A defining characteristic of a protein is its behavior in an aqueous environment. Some are hydrophobic (water hating), some are hydrophilic (water loving), and some are amphipathic (a mixture of hydrophobic and hydrophilic). It is energetically unfavorable for a hydrophobic protein to linger in an aqueous environment, just as it is energetically unfavorable for a hydrophilic protein to linger in a non-aqueous environment. Cellular membranes are primarily made of amphipathic phospholipids, such that they self organize with their hydrophilic portions exposed to the aqueous environment and their hydrophobic portions associating with each other to form “lipid bilayers” (Figure 2).

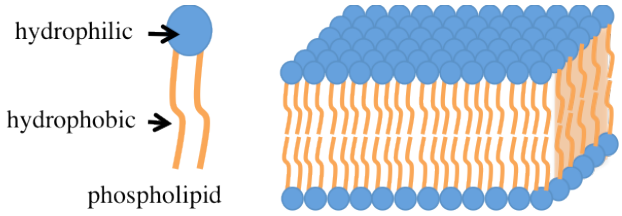

Figure 2. Phospholipid bilayer

A phospholipid has a hydrophilic head and a hydrophobic tail. These molecules can arrange themselves into a lipid bilayer and are the basis for cellular membranes.

These energy considerations make membranes able to compartmentalize regions in a cell by preventing the random passages of proteins between these regions. The strategic placement of membranes within the cell organizes where proteins go and how they function.

Newly synthesized proteins must be guided to the appropriate compartment in the cell before they can function. Some mRNAs encode proteins that are destined to be embedded in a membrane. These proteins must be 'integrated' into the membrane before they can function and are therefore called integral membrane proteins. Integration into the membrane may occur after the protein has been completely synthesized, in a process called post-translational integration. Alternatively, integration can take place co-translationally, while the ribosomes are still attached to the mRNA and still synthesizing the protein (Figure 3). There are certain signals within membrane proteins that target them to the correct membrane in the cell and dictate the particular arrangement that the protein will take in the membrane.

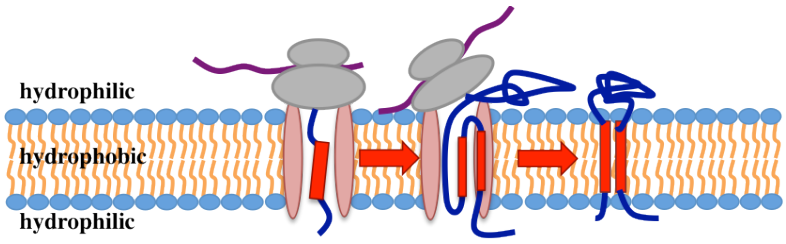

Figure 3. Co-translational integration of a membrane protein

The protein is inserted directly into the membrane from the translating ribosome.

The thylakoid membrane system

The chloroplast is divided into several sub-compartments. These compartments are the inner and outer envelope membranes, the inter-membrane space, the aqueous stroma interior, and the thylakoid membrane system (Figure 4).

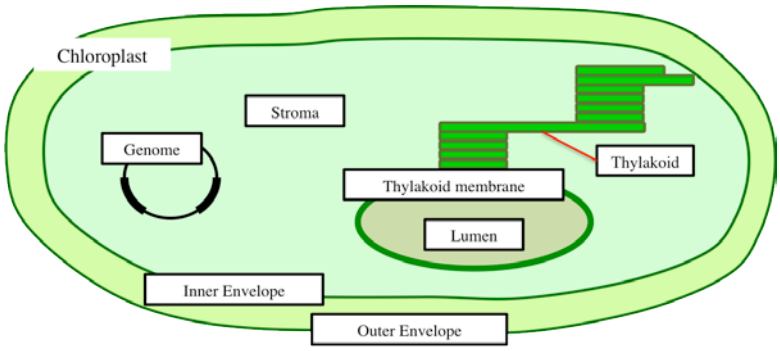

Figure 4. Anatomy of a chloroplast

The chloroplast is divided into several sub-compartments including the inner and outer envelope membranes, the aqueous stroma interior, and the thylakoid membrane system.

The thylakoid system itself is subdivided, consisting of a hydrophilic compartment called the lumen surrounded by a membrane. The thylakoid membrane is the site of the light reactions of photosynthesis and is very protein rich. The thylakoid membrane harbors five major multi-subunit photosynthetic protein complexes: Photosystem I

(PSI), the cytochrome b6f complex (cyt b6f), Photosystem II (PSII), ATP synthase, and NADH dehydrogenase. These complexes work in conjunction to complete the steps of photosynthesis by using light energy from the sun to synthesize sugars that can be used as food by the plant. The sugars synthesized through the process of photosynthesis fuel all life on earth.

Every subunit in a photosynthetic complex must be correctly inserted into the thylakoid membrane before they can assemble together into a functional complex. Protein complexes in the thylakoid membrane are comprised in similar proportions of subunits that are encoded by the nuclear genome and by the chloroplast genome (Zhang et al., 2001). The chloroplast genome of *Zea mays* contains about 100 genes, which encode machinery for photosynthesis and gene expression (Maier et al., 1995). In addition, the chloroplast requires about 3000 gene products from the nuclear genome to function; these include genes of cyanobacterial ancestry as well as genes that evolved after the chloroplast was incorporated into a primordial plant cell. Since photosynthetic complexes require subunits from two different genomic sources in the cell, careful regulation and timing of protein integration into the thylakoid membrane is essential to assemble functional photosystems.

Mechanisms of protein targeting to the thylakoid membrane

Nuclear-encoded proteins destined for integration into the thylakoid membrane are fully synthesized in the cytosolic compartment outside of the chloroplast. These proteins must then be imported into the chloroplast from the cytosol before insertion into the thylakoid membrane. The passage of proteins through cellular membranes from one compartment to another is mechanistically complicated because protein surfaces are

typically hydrophilic and will be repelled by the hydrophobic membrane as water is repelled by oil. Therefore, molecular machines called translocases have evolved. A translocase is a protein channel that allows the selective movement of certain proteins through a membrane.

Fully synthesized nuclear proteins in the cytosol are imported into the chloroplast through a translocon pathway in the envelope membrane of the chloroplast (Jarvis, 2008). The imported proteins remain in the aqueous interior (stroma) of the organelle unless they have a particular sorting signal, in which case they are directed to a different sub-compartment in the chloroplast. Nuclear-encoded and plastid-encoded proteins in the chloroplast stroma can be localized to the thylakoid lumen, the thylakoid membrane, or the inner envelope membrane (Celedon and Cline, 2013). Previous studies have found three ancestral translocases in the thylakoid membrane by which nuclear encoded proteins are integrated or imported. Each of these translocases evolved from secretion systems in the cyanobacterial ancestor of the chloroplast. Each translocase operates by a different mechanism and has different energy requirements for translocation. These ancestral translocases are the cpSec (chloroplast secretory), the cpSRP (chloroplast Signal Recognition Particle), and the cpTat (Twin arginine translocation) pathways (Albiniak et al., 2012).

The thylakoid membrane cpSec pathway

The cpSec pathway was the first thylakoid membrane translocon to be discovered in chloroplasts and is derived from the Sec system in bacteria (Laidler et al., 1995). The cpSec pathway translocates nuclear-encoded proteins across the thylakoid membrane using the energy of ATP hydrolysis. During cpSec transport, proteins are

unfolded and moved through a thylakoid membrane embedded translocase composed of the cpSecY and cpSecE subunits (Schuenemann et al., 1999). Proteins can also be integrated into the thylakoid membrane by this cpSecY/E translocase. The cpSecA subunit is another protein involved in the cpSec pathway. This protein binds N-terminal signal sequences, and hydrolyses ATP to feed the unfolded polypeptide through the protein channel into the thylakoid lumen (Albiniak et al., 2012).

Although some proteins use the cpSecA subunit to integrate via the cpSecY translocon, cpSecY is believed to have additional functions that are independent of cpSecA. Mutants that lack the cpSecA subunit (encoded by the *tha1* gene in maize) experienced a phenotype that was much milder than mutants lacking the cpSecY subunit (*csy1*) (Roy and Barkan, 1998; Voelker et al., 1997). This indicates that cpSecY is responsible for the integration of some membrane proteins that do not need cpSecA. Null cpSecY mutants also experience a global defect in chloroplast translation (Roy and Barkan, 1998), but the basis for this is unknown.

The thylakoid membrane cpSRP pathway

The cpSRP pathway integrates proteins with hydrophobic trans-membrane segments into the thylakoid membrane. The cpSRP pathway uses the freely moving signal recognition particle to recognize a signal motif on a protein in the stroma (Schünemann, 2004). The cpSRP guides a subset of proteins in the stroma to the membrane-bound GTPase cpFtsY. The protein is then directed to an adjacent membrane-embedded translocon called Alb3, which sometimes acts in conjunction with SecY/E to integrate the unfolded protein into the thylakoid membrane. Alb3 acts in conjunction with cpSRP54 and a chloroplast-specific component, cpSRP43 to post-

translationally integrate abundant nuclear-encoded light harvesting chlorophyll proteins into the thylakoid membrane (Wang and Dalbey, 2011). Although it is currently believed that chloroplast Alb3 requires cpSRP and cpSecY/E to function, the bacterial homolog of Alb3 (YidC) has been shown to act independently of these pathways to integrate some proteins into the bacterial cytoplasmic membrane (Serek et al., 2004).

The thylakoid membrane cpTat pathway

While proteins imported into the thylakoid through the SecY/E translocon must be unfolded, the cpTat pathway can transport completely folded proteins (Celedon and Cline, 2013). This capability is essential for the transport of nuclear-encoded proteins that bind a prosthetic group such as an Fe-S cluster or zinc atom in the stroma. The irreversible unfolding of these proteins in the chloroplast stroma would eliminate their ability to function, so a pathway that can transport folded proteins into the thylakoid is essential for chloroplast biogenesis and viability. Whereas the cpSec pathway requires ATP hydrolysis to provide energy for translocation, the cpTat pathway requires only the presence of a transmembrane electrochemical gradient for protein import (Cline and Mori, 2001). TAT stands for Twin Arginine Translocon, so named because its substrates have an N-terminal signal sequence that includes a "twin arginine" sequence. Two conserved subunits, cpTatC and Hcf106, form the cpTat receptor complex in the membrane (Cline and Mori, 2001). They are assisted by a third subunit, Tha4, to facilitate transport of the protein through the thylakoid membrane. It is still unknown how the cpTat pathway is able to import massive folded proteins into the thylakoid without jeopardizing the trans-thylakoid proton gradient that drives ATP synthesis.

A subset of plastid-encoded integral thylakoid membrane proteins are localized co-translationally

While a considerable amount is known about how nucleus-encoded proteins are targeted and imported through the thylakoid membrane, very little is known about how plastid-encoded proteins are localized to the thylakoid membrane. Approximately half of the proteins that make up the photosynthetic complexes in the thylakoid membrane are encoded by the chloroplast genome. To understand how photosynthetic complexes are assembled, it is crucial that we fully grasp the mechanisms by which plastid-encoded proteins are targeted to the thylakoid. Unlike nuclear-encoded proteins, plastid-encoded proteins can be targeted to the thylakoid membrane either during or after translation. Before the targeting mechanisms of plastid-encoded proteins can be examined, it must be determined whether chloroplast-encoded integral thylakoid membrane proteins are co-translationally or post-translationally localized to the thylakoid membrane.

Zoschke and Barkan developed an assay using ribosome profiling to determine which plastid-encoded proteins are translated on membrane-associated ribosomes and to learn about the signals that target them for co-translational integration (Zoschke & Barkan, 2015). They showed that ribosomes synthesizing co-translationally targeted proteins are not bound to the membrane at the start of translation, but transition to the membrane at a certain position inside the open reading frame. This is illustrated by the ribosome profiling data for *psbB*, a plastid mRNA that encodes a co-translationally inserted membrane protein (Figure 5). At a certain point in the wild-type *psbB* mRNA a signal prompts the transition from soluble ribosomes to membrane bound ribosomes. A

similar pattern is observed for mRNAs encoding all co-translationally targeted proteins. By analyzing the position at which the transition from the soluble to membrane phase occurs, Zoschke and Barkan concluded that membrane attachment is triggered by exposure of either a cpSecA-dependent N-terminal signal peptide or the first transmembrane sequence of the nascent peptide from the ribosome's exit channel. Integral membrane proteins whose first trans-membrane segment is so close to the stop codon that it is never exposed from the ribosome prior to translation termination are translated off the membrane and post-translationally integrated.

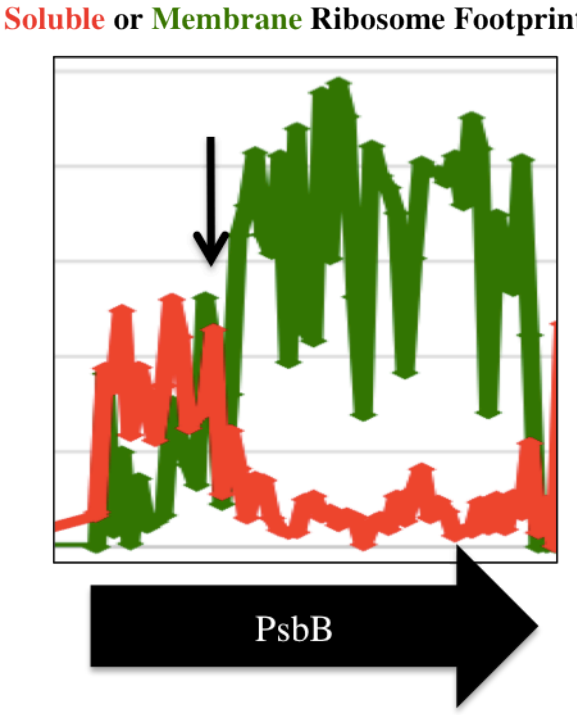

Figure 5. The co-translational integration of wild-type *psbB* mRNA is shown by a transition from soluble ribosomes to membrane bound ribosomes marked by the arrow.

My research: elucidating the mechanisms by which co-translationally integrated plastid-encoded proteins are localized to the thylakoid membrane

With this assay, Zoschke and Barkan identified 19 integral membrane proteins encoded by the chloroplast genome that are co-translationally integrated into the thylakoid membrane. My project builds on these findings by addressing which thylakoid targeting machineries participate in co-translational targeting of these 19 proteins. We hypothesized that chloroplast-encoded proteins are targeted to the thylakoid lumen and membrane using the same three pathways as nuclear-encoded proteins: cpSec, cpTat, and cpSRP. My research aimed to elucidate the mechanisms by which chloroplast-encoded proteins are co-translationally targeted to the thylakoid membrane.

To do this, I used maize mutants that lack components of each of the three thylakoid membrane translocon machineries. If the normal transition from soluble- to membrane-tethered ribosomes within mRNAs encoding co-translationally targeted proteins fails to occur in a particular mutant, I would conclude that the mutant lacks the machinery needed for co-translational targeting. Furthermore, this analysis may reveal a shared sequence or signal pattern by which chloroplast gene products are targeted to the thylakoid membrane by one or more of these three translocation pathways.

There are two important limitations of the ribosome profiling assay I used to address these questions. First, it reports the position and efficiency of only the initial engagement of the translating ribosomes with the thylakoid membrane; the integration of subsequent trans-membrane segments is not revealed in the data. In addition, only transcripts that are co-translationally integrated into the thylakoid membrane can be

studied by this assay. The assay cannot identify the machineries that participate in the post-translational integration of proteins into the thylakoid.

METHODS

Non-photosynthetic maize mutants

Maize (*Zea mays)* is a model organism in plant biology. The maize genome has been thoroughly sequenced and studied such that many mutations in the genome can be precisely mapped back to a particular gene. Many mutations in maize are caused by 'transposable elements,' which are repeated sequences of DNA that frequently change position in the genome. Transposable elements make up over half of the maize genome (Meyers et al., 2001). When a transposable element inserts into or near a gene, it sometimes disrupt the genes function and cause an observable change in the properties of the organism. Plants that appear normal are called 'wild-type (WT)' and plants that do not appear normal are said to have a 'mutant phenotype' (Figure 6).

Figure 6. Maize mutants showing a gradient of non-photosynthetic phenotypes compared to WT (left).

The Barkan Lab has a large collection of non-photosynthetic maize mutants that are caused by the transposable element Mutator (*Mu*). As *Mu* jumps around the maize genome, it can insert itself into genes that encode for essential photosynthetic components and inhibit their expression. These mutant non-photosynthetic plants will

only live as long as they can gain chemical energy from their seed; after the seed stores are depleted, which takes several weeks, the plant cannot photosynthesize and will die. We analyze the properties of these seedlings ~8 days after planting, at a time when even the wild-type seedlings have not yet transitioned to reliance on photosynthesis for their growth.

The Barkan lab previously identified mutants lacking components of the cpSec, cpSRP, and TAT thylakoid machineries (Asakura et al., 2004; Voelker and Barkan, 1995; Voelker et al., 1997). I used these mutants to examine how co-translational integration of chloroplast-encoded proteins is disrupted when a particular translocon is mutated. From the cpSec pathway, I used a mutant called *csy1* that lacks the cpSecY protein and a mutant called *tha1* that lacks the cpSecA protein. The *csy1* seedlings have an ivory phenotype and the *tha1* seedlings have a pale green phenotype compared to WT. From the cpSRP pathway, I used a mutant called *csr1* that lacks the cpFtsY membrane-bound SRP receptor. *csr1* seedlings have a pale yellow phenotype compared to WT. From the cpTat pathway, I used a mutant called *hcf106* that lacks the Hcf106 subunit.

Ribosome profiling

I used a genome-wide method called ribosome profiling in conjunction with the maize mutants described above to characterize the suborganellar localization of translation *in vivo.* Ribosome profiling gives a snapshot of what mRNAs are being actively translated and to what degree during a cell at the moment of harvest (Zoschke et al., 2013). This method is used to map the positions of small mRNA pieces that are protected from exogenous nucleases by bound ribosomes called “ribosome footprints”.

The original ribosome profiling method used deep sequencing to map ribosome footprints to specific mRNA locations (Ingolia et al., 2009). However, Zoschke and Barkan modified the approach by using high-resolution microarrays to map the ribosome footprints, to decrease the cost and time involved in each assay (Zoschke et al., 2013). They further modified the approach to apply it to separated membrane and soluble fractions to examine separately the suborganellar translation of transcripts on soluble and membrane-associated ribosomes (Zoschke & Barkan, 2015). This is the approach I used for my experiments.

Plant cells are lysed and treated with chloramphenicol to stall translation. The lysed cells are treated with micrococcal nuclease to cleave mRNA strands between ribosomes (Figure 7). The solution is then partitioned into 'membrane' and 'soluble' fractions by centrifugation. The membrane fraction contains the thylakoid membranes and all ribosomes that are tethered to the membrane. The soluble fraction contains contents of the thylakoid lumen and the chloroplast stroma and ribosomes translating nascent peptides that are not tethered to a membrane. Each translating ribosome protects a small (20-35 nucleotide) piece of mRNA from digestion with nuclease. To purify the ribosome footprints, the separated ribosomes (monosomes) are purified by centrifugation through a sucrose cushion, and the 'ribosome footprints' are then purified from the monosomes by denaturing polyacrylamide gel electrophoresis. The purified ribosome footprints from the soluble and membrane fraction are labeled with fluorescent red and green dye respectively and then combined and used to probe a high resolution microarray with overlapping 50-mer probes spanning all chloroplast genes.

The results allow me to determine the position of each ribosome footprint to a resolution of approximately 30 nucleotides.

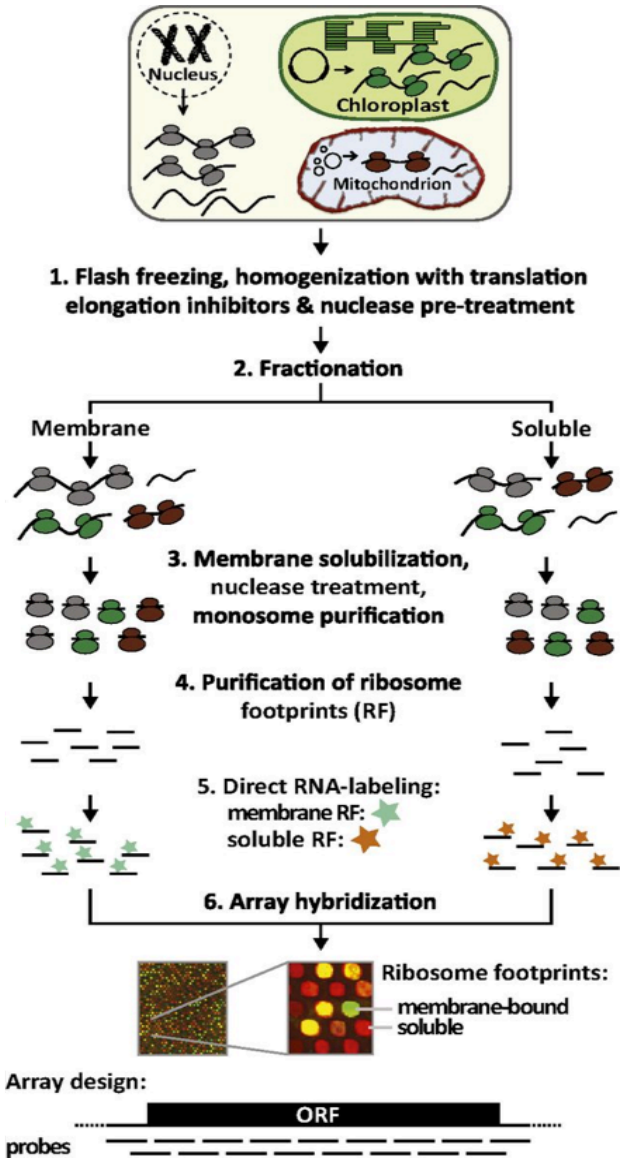

Figure 7. Method for profiling chloroplast ribosome positions in separated membrane and soluble fractions

Used with permission from Reimo Zoschke and Alice Barkan PNAS 2015; 112:E1678-E1687 (Zoschke & Barkan, 2015).

Microarray

A microarray is a collection of microscopic spots of DNAs of known sequence attached to a solid surface. The genomic position of each DNA sequence on the surface of the microarray is known. Microarrays can determine the abundance of RNA or DNAs with particular sequences in a complex mixture, by taking advantage of complementary base-pairing. DNA and RNA sequences are composed of four nucleotides: A, T/U, C, and G. Two strands of DNA or RNA can form a stable interaction by base-pairing; an A can pair with a T or a U and a C can pair with a G. Each spot on a microarray contains short pieces of DNA that have the exact same sequence. When RNA is 'hybridized' to the microarray, any RNA molecules that include a sequence that is complementary to a particular DNA on the microarray will form a stable base-paired interaction with that spot (Figure 8a).

When fluorescent-labeled mRNA pieces are 'hybridized' to a microarray, mRNA base-pairs with the complementary DNA sequence on the microarray and creates a detectable color on that particular spot. The fluorescent labeled RNA is detected by lasers to reveal a red:green color ratio for each spot on the array (Figure 8b). The ratio of red to green dye in each spot indicates the relative abundance of membrane or soluble ribosome footprints that hybridized to that particular spot. This indicates the amount of membrane-associated or soluble ribosomes that are translating that particular piece of the mRNA at the time of tissue harvest.

Zoschke and Barkan showed that 19 chloroplast-encoded proteins are co-translationally targeted to the membrane. I examined whether co-translational targeting of these 19 proteins is disrupted in each maize mutant described above. To do this, I

plotting separately the fluorescent signal from ribosome footprints isolated in the soluble and membrane-bound fractions (Zoschke & Barkan, 2015).

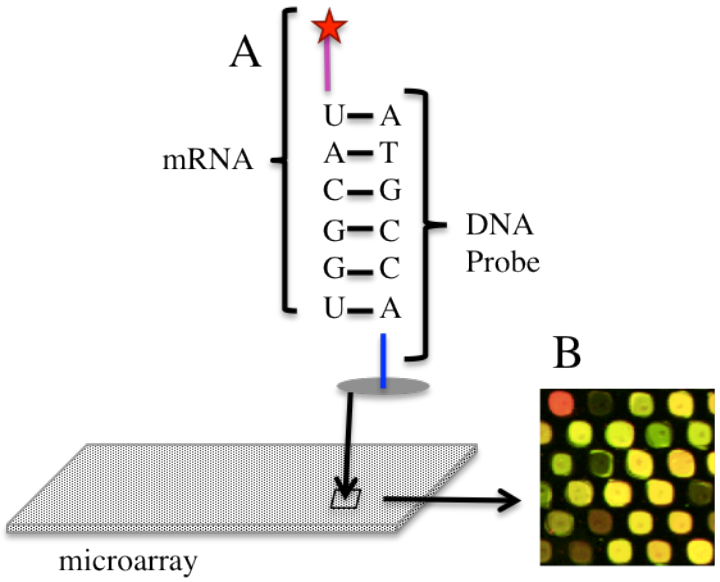

Figure 8. Diagram of a DNA microarray and mechanism of hybridization

(A) Hybridized mRNA will form a stable base-paired interaction with a specific DNA sequence on a microarray spot. (B) mRNA labeled with red or green dye can be detected by lasers to reveal a pattern that indicates the ratio of mRNA abundance.

Polysome gradient analysis

During the process of translation, ribosomes start at the beginning of the protein-coding region. As they move along, they make room for additional ribosomes to begin. Thus multiple ribosomes usually translate the same mRNA simultaneously, in a structure called a polysome (Figure 1). The relative mass of a polysome indicates the number of translating ribosomes on that mRNA transcript, which indicates the relative amount of translation that mRNA is experiencing.

To determine polysome size, cell extracts are sedimentated through a sucrose gradient. The rate of sedimentation of an mRNA increases with the number of translating ribosomes attached to it. Therefore, mRNAs that sediment more rapidly are attached to a higher number of translating ribosomes than mRNAs that sediment more slowly. I used this assay to determine whether the overall translation rates for several chloroplast mRNAs differed between wild-type and mutant samples.

RESULTS

Genome-wide analysis of ribosome profiling reveals distinctive differences in mutants lacking thylakoid translocon machinery

Profiling ribosomes in separated membrane and soluble fractions of WT and the *csr1*, *tha1,* and *csy1* mutants revealed genome-wide differences in both soluble and membrane-bound ribosomal abundance (Figure 9). We are still working out best practices for normalizing and comparing the data from different fractions and different mutants. Therefore, the conclusions I am able to make so far are somewhat limited. In the graphs below, the data were qualitatively normalized by equalizing the soluble signal of genes unaffected by the translocon mutants, such as those encoding tRNAs and rRNAs, between all four samples.

The genome-wide view reveals genome-wide differences in signal as well as different patterns in the translation of specific plastid-encoded genes in each mutant. The overall signal for the *csy1* mutant was extremely low, which is expected given the loss of thylakoid membranes and plastid ribosomes reported for this mutant (Roy and Barkan, 1998). The pleiotropic nature of these defects complicates the interpretation of this dataset, so the *csy1* data are discussed only for the one gene for which the data are informative. The abundance of soluble and membrane-associated ribosomes on specific plastid-encoded genes can be examined by looking at the membrane and soluble signal on an enlarged section of the plot. This magnified view is used to examine the specific targeting mechanisms of individual plastid-encoded mRNAs to the thylakoid membrane in the absence of certain translocon subunits.

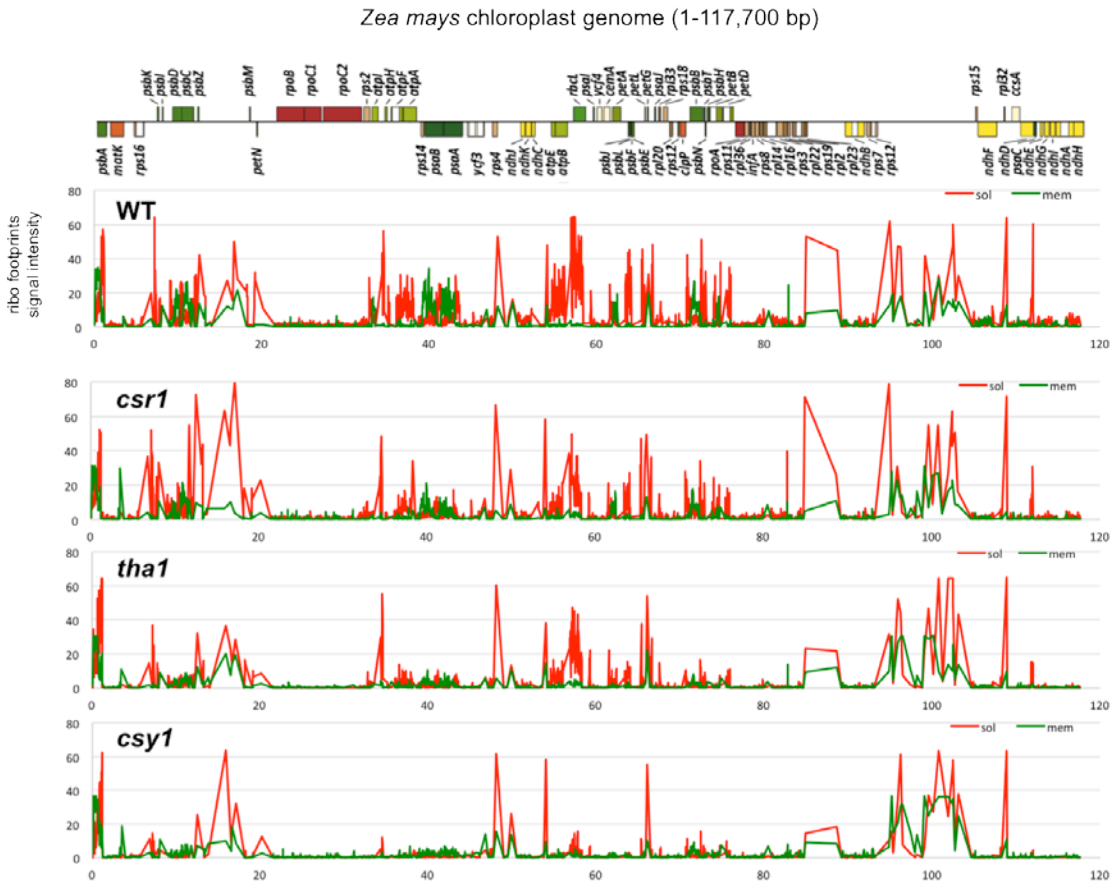

Figure 9. Overview of soluble and membrane-associate ribosome signal aligned with the chloroplast genome.

Whole-genome comparison of wild-type ribosome distribution to three mutants: *csr1* (cpFtsY), *tha1* (cpSecA), and *csy1* (cpSecY).

Differential effects of cpSecA on the cotranslational targeting of plastid-encoded membrane proteins

The petA gene encodes the *cytochrome f* subunit of the Cytochrome-b6f complex, and is the only chloroplast-encoded protein that is known to require cpSecA for its targeting to the thylakoid membrane (Figure 10). Cytochrome f is co-translationally localized to the thylakoid membrane by a 35 residue cleavable N-

terminal signal peptide specific for cpSecA (Röhl & Van Wijk, 2001; Zoschke & Barkan, 2015).

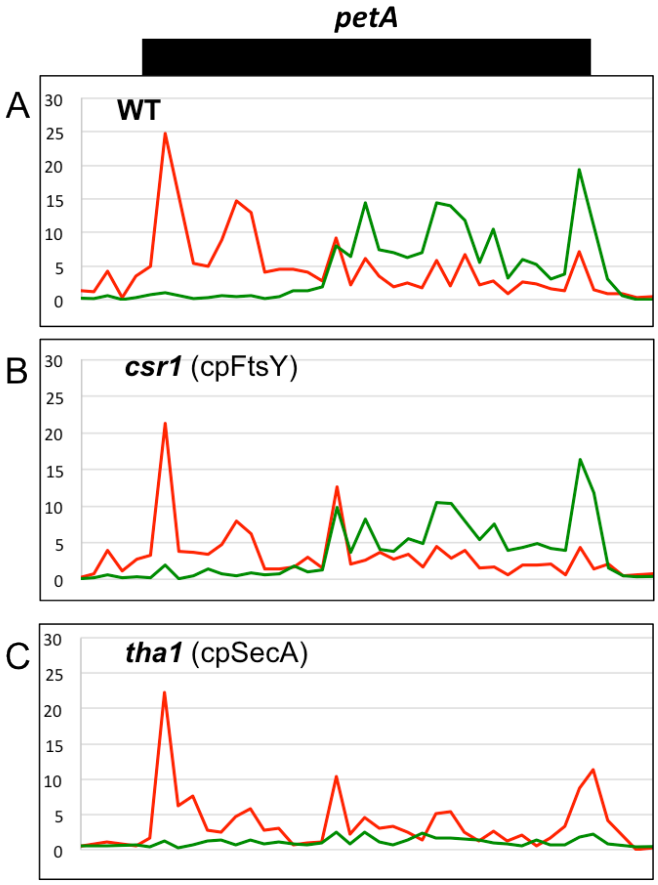

Figure 10. Relative signal from soluble and membrane-associated ribosomes translating the *petA* mRNA

(a) WT; (b) *csr1* mutant lacking cpFtsY; (c) *tha1* mutant lacking cpSecA

As anticipated, membrane-associated translation of the *petA* transcript is disrupted in the *tha1* mutant compared to WT. The loss of membrane-associated ribosomes in the *tha1* mutant seems to be accompanied by a normal amount of soluble translating ribosomes downstream of the position where ribosomes become attached to the membrane in WT. This is what would be expected if the role of cpSecA is limited to

engagement of the nascent protein with the membrane. Analysis of membrane-associated ribosomes on *petA* in the *csr1* mutant showed that initial engagement of the *cyt-f* nascent peptide with the thylakoid membrane is independent of cpFtsY. This result is not surprising, since FtsY and SecA act in separate pathways in bacteria (Serek et al., 2004).

The petB gene, also located in the chloroplast genome, encodes the *cytochrome-b6* subunit of the thylakoid Cytochrome-b6f complex (Figure 11). Cytochrome b6 is cotranslationally targeted to the thylakoid membrane (Zoschke & Barkan, 2015). My data shows that initial engagement of the *petB* transcript with the thylakoid membrane may be less efficient in the absence of cpFtsY. The abundance of soluble ribosomes translating *petB* is normal but membrane associated ribosomes appear to be mildly depleted in the *csr1* mutant compared to WT. However, a rise in the membrane signal occurs at the same position as in WT indicating that some degree of co-translational targeting of *petB* is retained in the *csr1* mutant. The partial depletion of ribosome abundance in the *csr1* mutant was a phenomenon observed for several transcripts and is discussed in detail below.

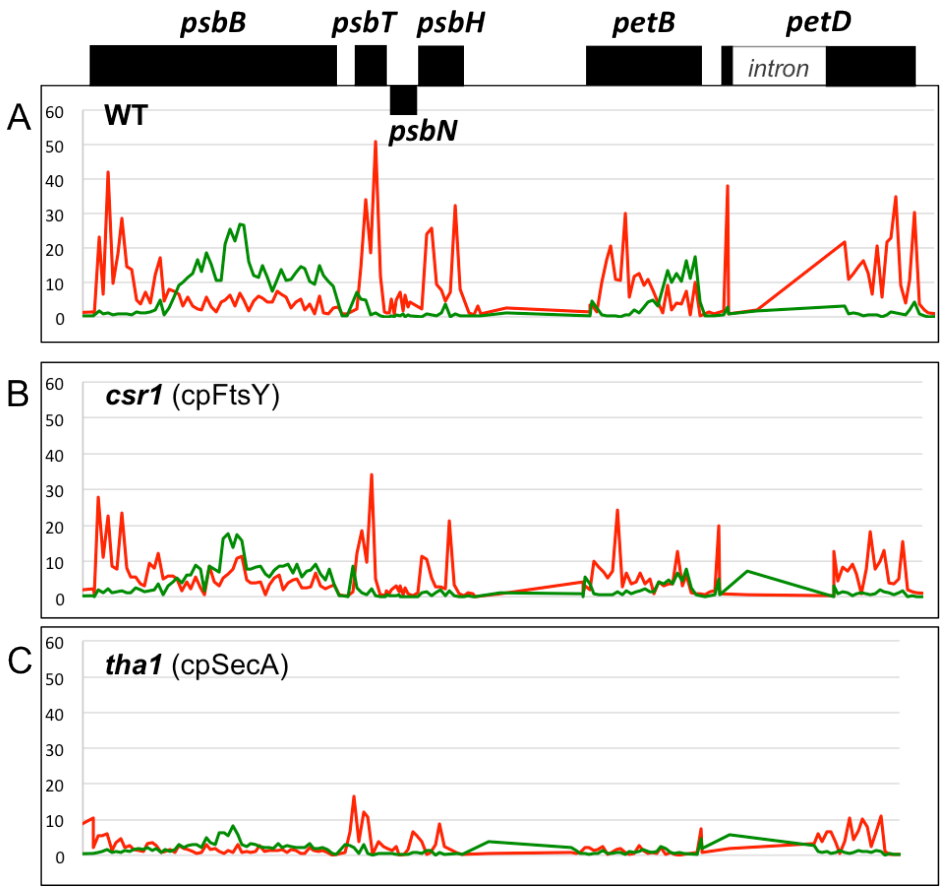

Figure 11. Relative abundance of soluble and membrane-associated ribosomes translating a polycistronic transcription unit including *psbB*, *petB*, and *petD*

(a) WT; (b) *csr1* mutant lacking cpFtsY; (c) *tha1* mutant lacking cpSecA

A subset of plastid-encoded proteins require cpFtsY for membrane-associated translation

I examined the results obtained from genome-wide ribosome profiling on separated membrane and soluble fractions isolated from *csr1* tissue to assay the role of the cpFtsY thylakoid targeting machinery in co-translational integration of other plastid-encoded membrane proteins. This analysis revealed that cpFtsY is required for the membrane-associated translation of a subset of proteins. The co-translational integration

of the membrane protein encoded by the *atpI* transcript is severely disrupted in the *csr1* mutant. Although there are an abundance of membrane-associated ribosomes on the *atpI* transcript in WT, these ribosomes are fully depleted in the *csr1* mutant (Figure 12). A severe decrease in membrane-associated ribosomes was also observed for the *atpF* and the *ndhF* transcripts in the *csr1* mutant (Figure 12, 13).

The loss of membrane-associated ribosomes on *atpI, atpF,* and *ndhF* was accompanied by an equivalent decrease in soluble ribosomes. There appear to be fewer translating ribosomes on these transcripts overall. Yet, ribosome abundance on nearby genes (such as atpH), seems to be unaltered (Figure 12). I considered several possible explanations for this phenomenon. When the membrane and soluble fractions are partitioned after treatment with nuclease, the membranes in the 'membrane fraction' are solubilized using non-ionic detergent. This releases ribosomes tethered to the thylakoid membrane so they can be purified through the sucrose gradient. No non-ionic detergent was added to the soluble fraction since it was assumed that all ribosomes would already be in solution. If ribosomes translating these particular transcripts are attached to a non-thylakoid low-density membrane, they will not be solubilized and therefore will not pellet through the sucrose gradient.

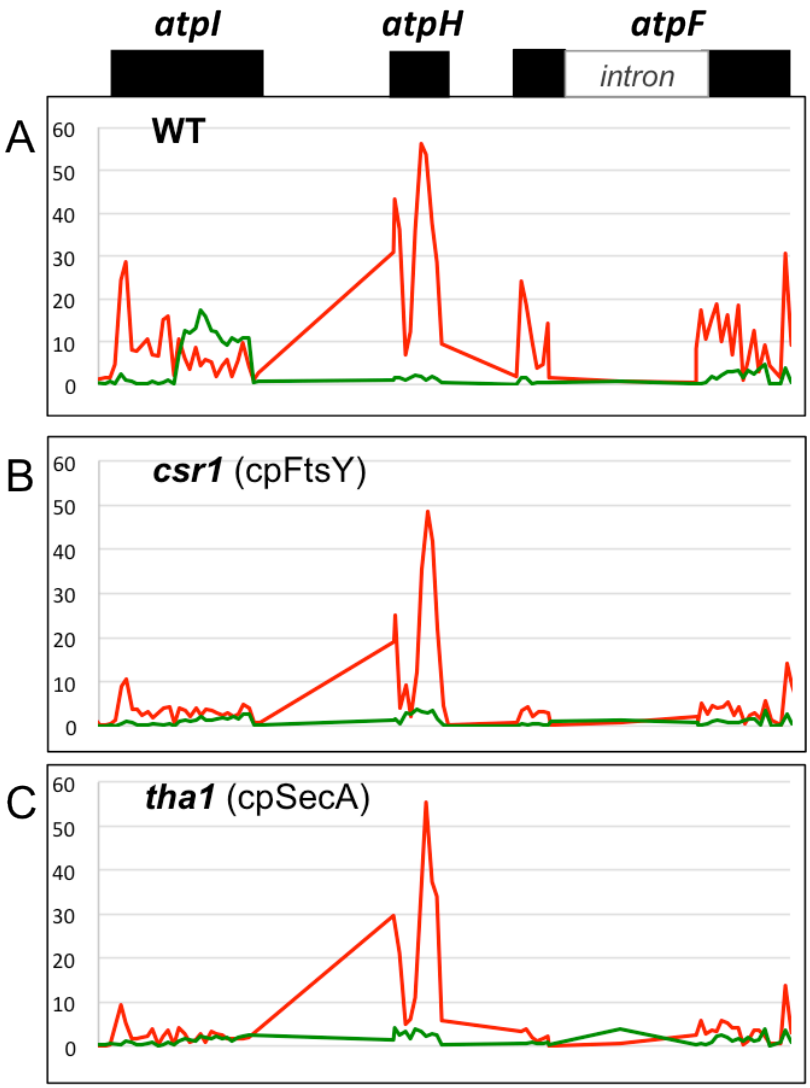

Figure 12. Relative abundance of soluble and membrane-associated ribosomes translating the polycistronic transcription unit including *atpI*, *atpH*, and *atpF*

(a) WT; (b) *csr1* mutant lacking cpFtsY; (c) *tha1* mutant lacking cpSecA

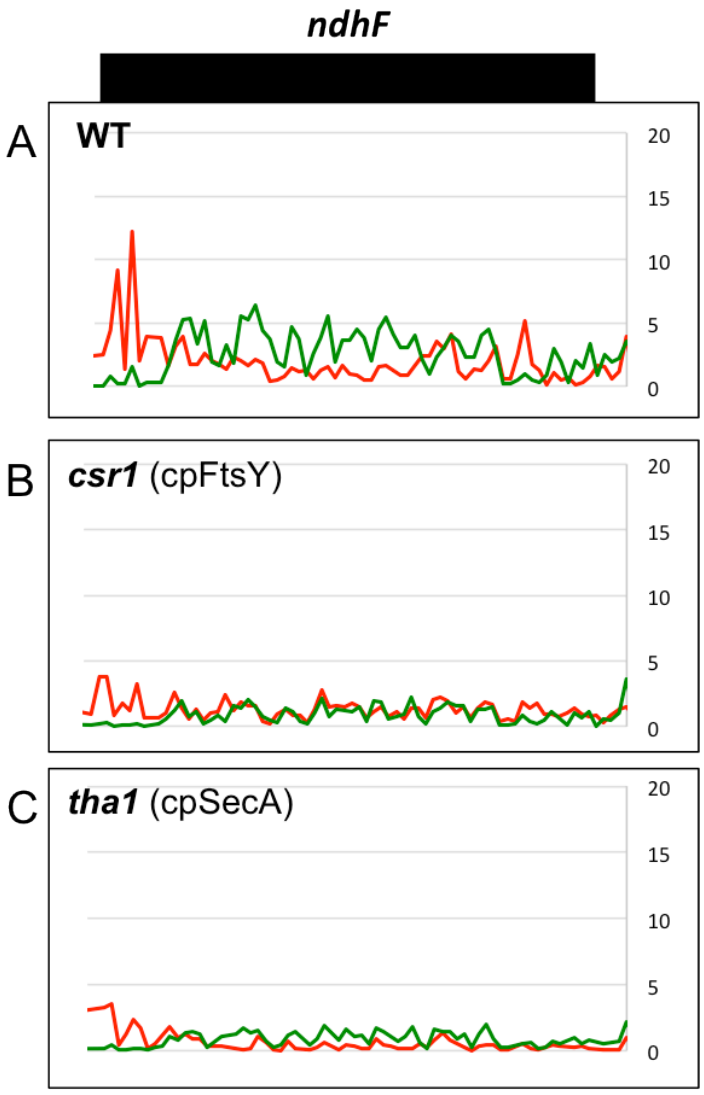

Figure 13. Relative abundance of soluble and membrane-associated ribosomes translating *ndhF* mRNA

(a) WT; (b) *csr1* mutant lacking cpFtsY; (c) *tha1* mutant lacking cpSecA

However, additional ribosome profiling experiments were performed in *csr1* tissue in which non-ionic detergents were added to the soluble fraction in an attempt to

resolve this issue. When looking at the genome-wide differences between *csr1* with no detergent added and *csr1* with detergent added to the soluble fraction, there appears to be an overall increase in the abundance of soluble signal in csr1 (+) detergent (Figure 14). However, zooming in on a co-translationally integrated membrane protein such as atpI reveals that the increase in soluble signal appears to be non-specific and is still less than WT level (Figure 15). The transition from soluble to membrane translating ribosomes is lost in *csr1* (+) detergent, indicating that adding detergent to the soluble fraction does not specifically recover the soluble ribosomes lost in the *csr1* mutant. This negates the hypothesis that these soluble ribosomes are being lost on a low-density membrane.

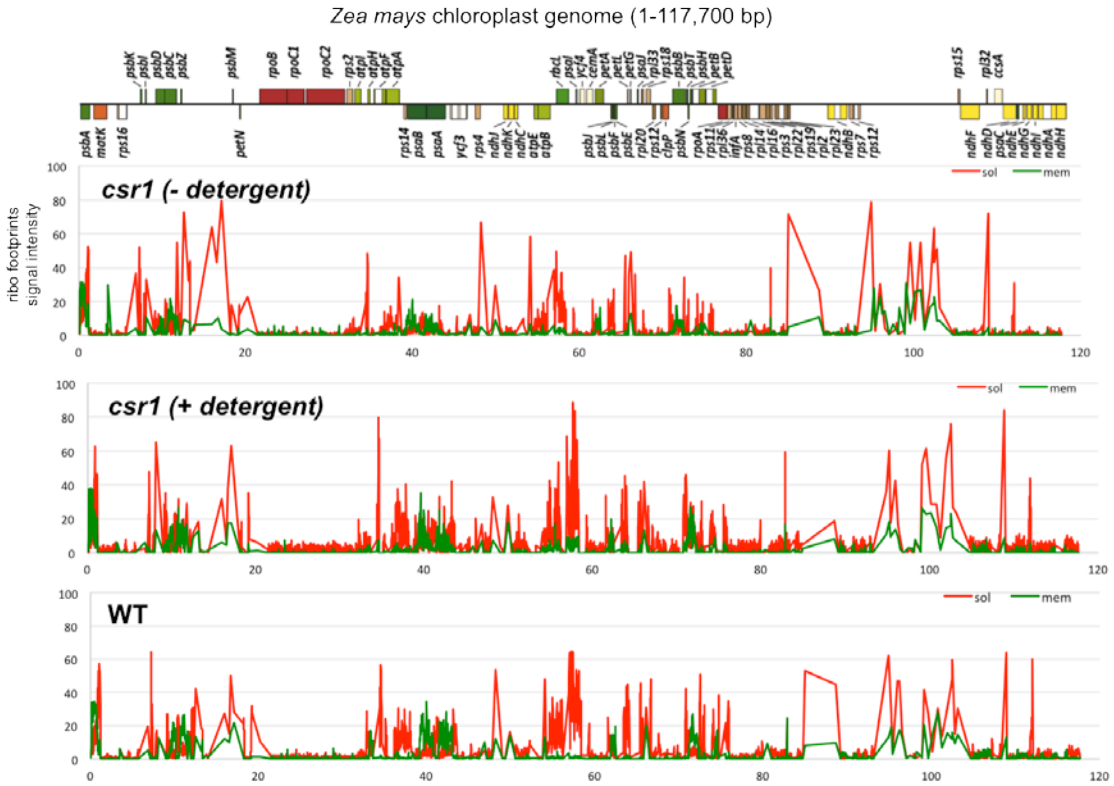

Figure 14. Overview of soluble and membrane-associated ribosome signal in the *csr1* mutant in the presence or absence of detergent in the soluble fraction.

Whole-genome comparison between wild-type ribosome distribution, the *csr1* (cpFtsY) mutant (-) detergent, and *csr1* (+) detergent.

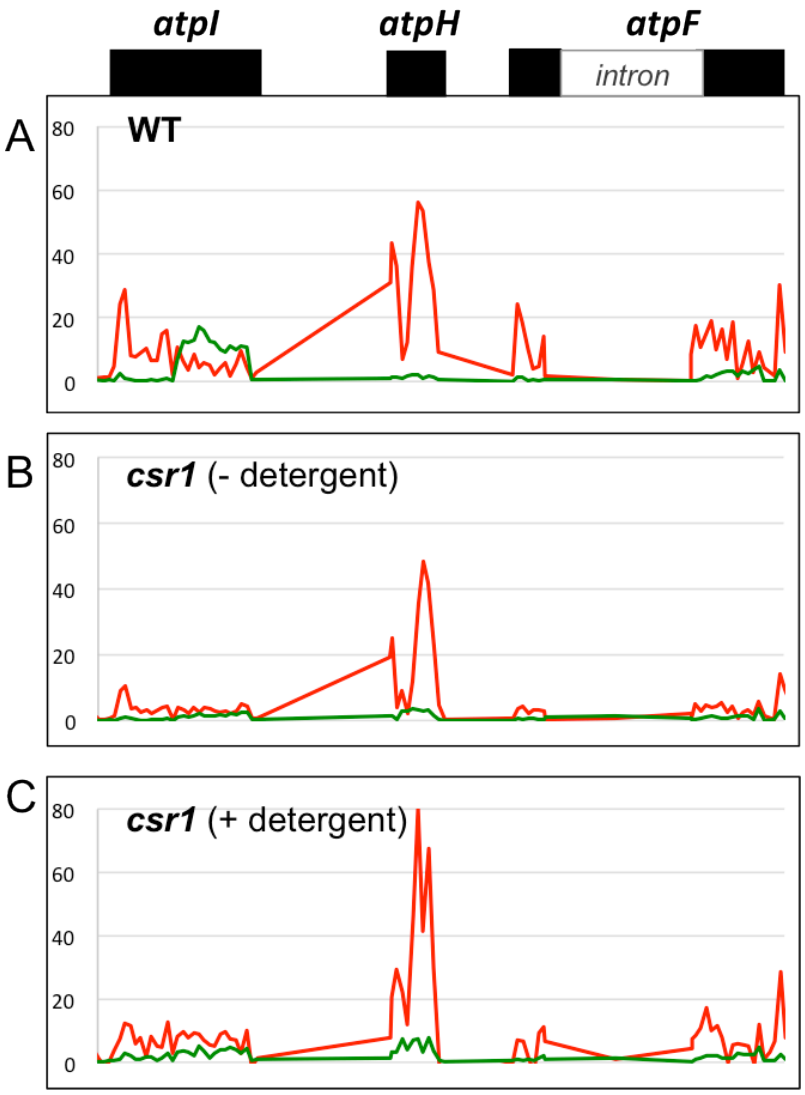

Figure 15. Comparison of relative abundance of soluble ribosomes translating integral membrane proteins in the presence or absence of detergent in the soluble fraction

(a) WT; (b) *csr1* mutant with no added non-ionic detergent to the soluble fraction; (c) *csr1* mutant with non-ionic detergent added to the soluble fraction.

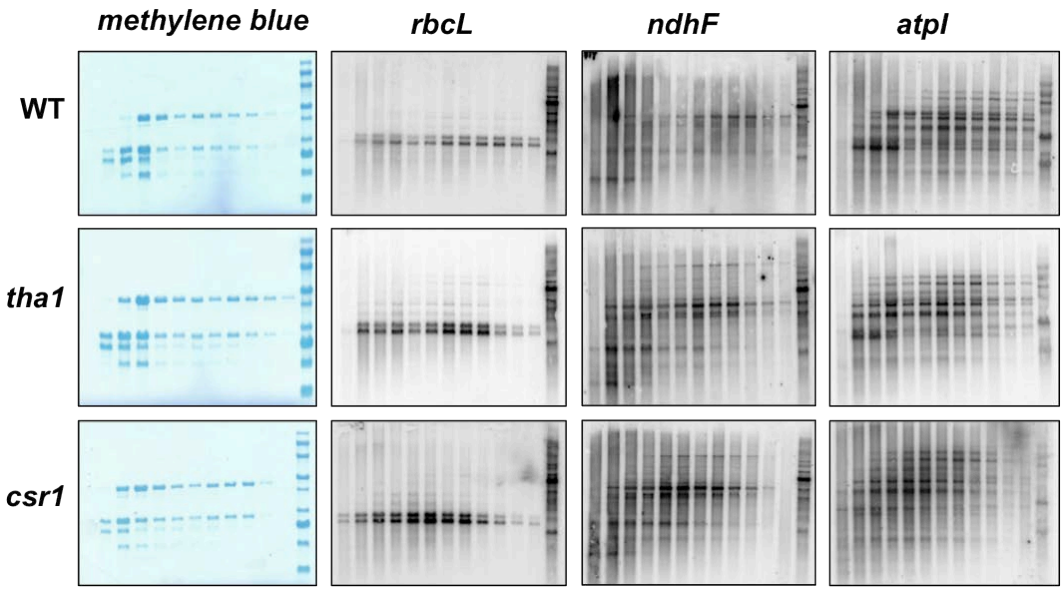

Figure 16. Polysome gradient analysis

WT, *tha1*, and *csr1* polysome gradients stained with methylene blue to show position of ribosomal RNAs and probed for *rbcL*, *atpI*, and *ndhF* mRNA.

It is possible that there are overall fewer translating ribosomes on these transcripts in the absence of cpFtsY. To examine this possibility, I performed polysome gradient analysis on the *csr1* and *tha1* mutants to look at the overall levels of translation of *atpI* and *ndhF* transcripts (Figure 16). I compared the distribution of ribosome amount on these transcripts in the mutants to wildtype and *rbcL*, a soluble plastid-encoded mRNA used as a control for global loss of translation. I found that there is an overall modest decrease in translating ribosomes on *ndhF*, *atpI*, and *rbcL* compared to wildtype in the *csr1* mutant. However, this is unlikely to account for the complete absence of ribosome footprint signal on *ndhF* and *atpI* that I saw in my microarray assays, since translation of *rbcL* was still detected robustly in the *csr1* mutant.

Another possibility is that the *atpI*, *atpF*, and *ndhF* translating ribosomes were not detected by the microarray assay because the footprint size changes in the absence

of cpFtsY. It was recently reported that cytosolic ribosomes in certain stages of the elongation cycle leave very short footprints (22 nt) (Lareau et al., 2014). Footprints of this length would probably not be detected with our microarray assay due to their low stability. We are currently addressing the possibility that we failed to detect a population of ribosome footprints in these mutants by using deep sequencing instead of microarrays.

Pleiotropic defects in *csr1* reduce the translation of a subset of plastid-encoded proteins that localize into the thylakoid membrane independently of cpFtsY

The *atpI*, *atpF*, and *ndhF* transcripts experience a significant defect in membrane-associated and soluble translating ribosomes in the *csr1* mutant. However, an additional subset of proteins experienced a mild reduction of both membrane and soluble translating ribosomes in the absence of cpFtsY. This subset includes petB, psbB, ndhA, ndhB, psaB, psaA, psbC, and psbD (Figures 11, 17 – 20). These eight transcripts experience a slight reduction of both soluble and membrane associated ribosomes in the *csr1* mutant compared to WT. However, these transcripts experience a rise in the membrane signal at the same position as in WT, indicating that co-translational localization is retained. The successful transition from soluble to membrane-associated ribosomes indicates that membrane targeting of these proteins is independent of cpFtsY.

The loss of cpFtsY confers pleiotropic defects in the localization of many plastid-encoded proteins to the thylakoid membrane, including those that it does not directly integrate. Asakura et al. first discovered pleiotropic defects in the biogenesis of the thylakoid membrane in the absence of cpFtsY. The thylakoid membrane is

unstacked and reduced in abundance in the *csr1* mutant compared to WT (Asakura et al., 2004). The abundance of subunits in all five thylakoid bound photosystems is depleted in the *csr1* mutant, but it is unlikely that cpFtsY is involved in the localization of all of these proteins to the membrane. In the *csr1* mutant, the abundance of cpSecY, Alb3, and cpTatC are increased perhaps as a compensatory mechanism for the lack of cpFtsY (Asakura et al., 2004). This finding rules out the possibility that cpFtsY is required for the insertion of other translocon subunits, thereby indirectly reducing the accumulation of other thylakoid membrane proteins. The pleiotropic defects caused by the loss of cpFtsY will be discussed in more detail below.

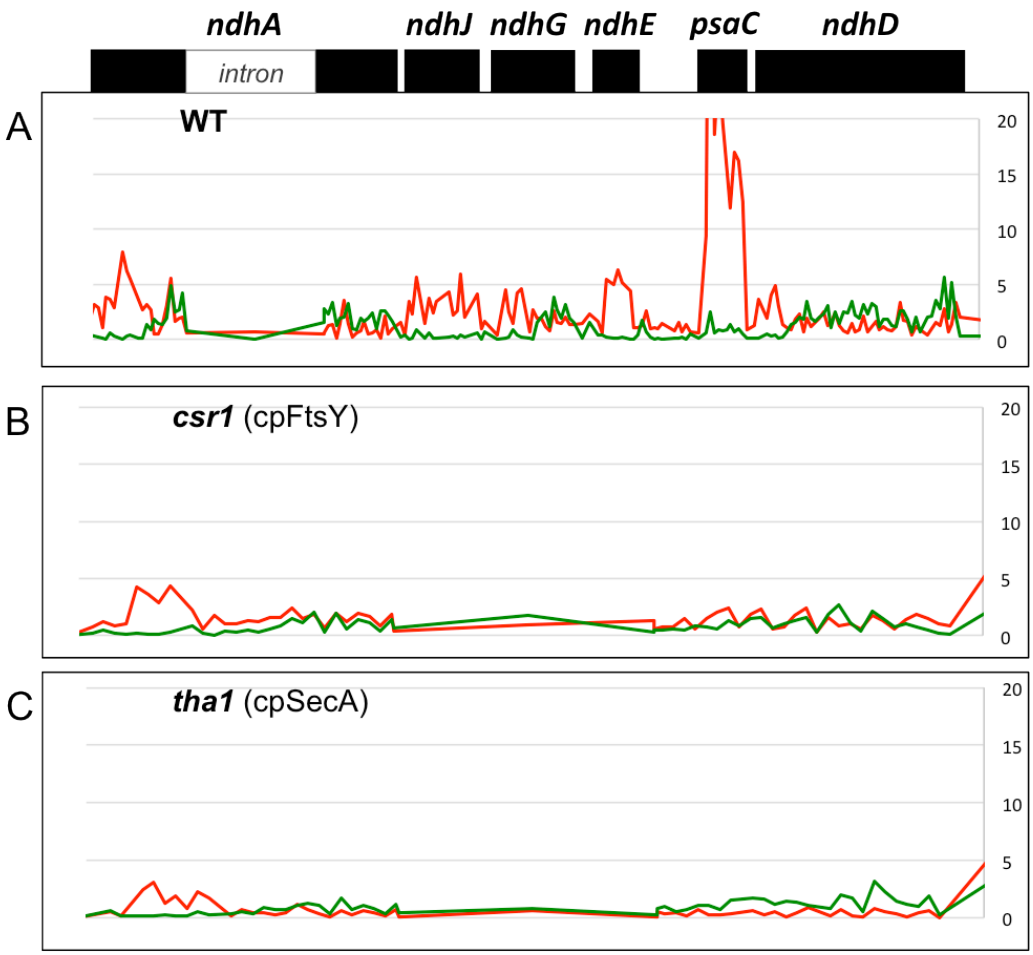

Figure 17. Relative abundance of soluble and membrane-associated ribosomes translating a polycistronic transcription unit including *ndhA*.

(a) WT; (b) *csr1* mutant lacking cpFtsY; (c) *tha1* mutant lacking cpSecA.

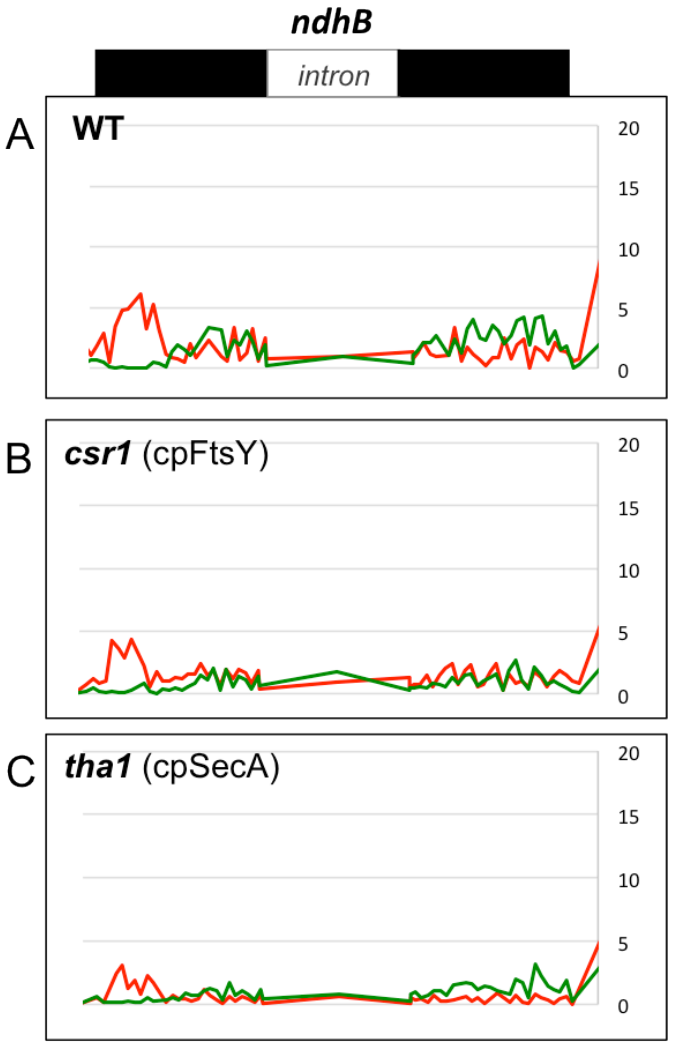

Figure 18. Relative abundance of soluble and membrane-associated ribosomes translating *ndhB* mRNA.

(a) WT; (b) *csr1* mutant lacking cpFtsY; (c) *tha1* mutant lacking cpSecA

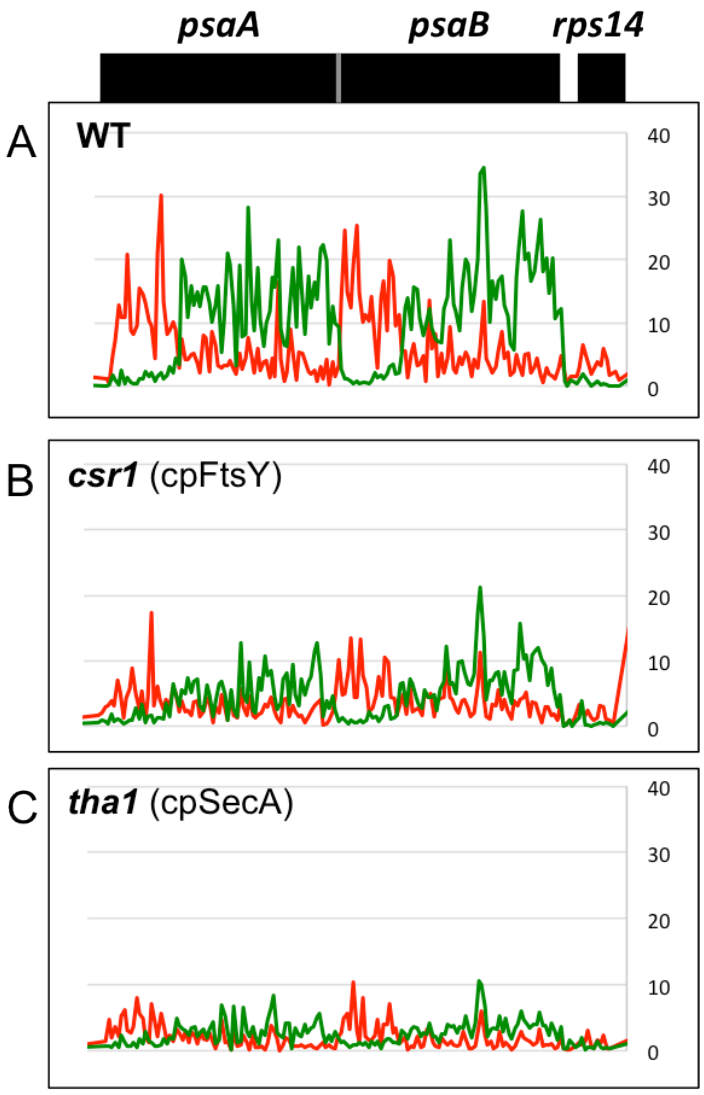

Figure 19. Relative abundance of soluble and membrane-associated ribosomes translating a polycistronic transcription unit including *psaA* and *psaB*

(a) WT; (b) *csr1* mutant lacking cpFtsY; (c) *tha1* mutant lacking cpSecA

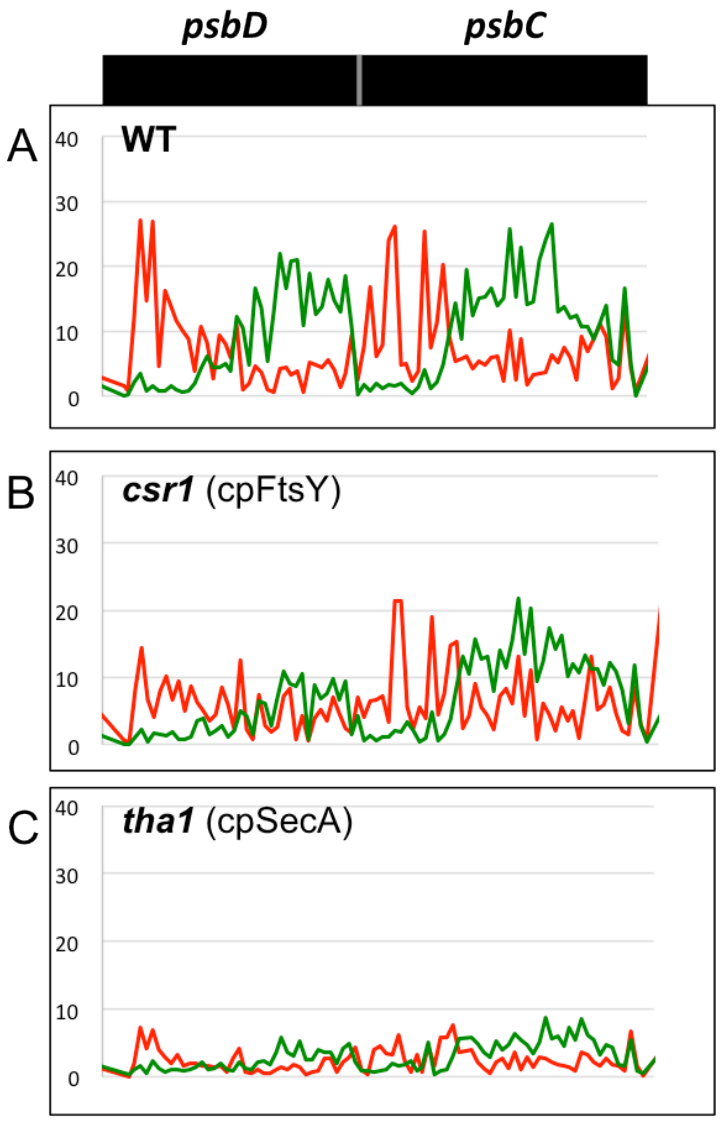

Figure 20. Relative abundance of soluble and membrane-associated ribosomes translating a polycistronic transcription unit including *psbC* and *psbD*

(a) WT; (b) *csr1* mutant lacking cpFtsY; (c) *tha1* mutant lacking cpSecA

Loss of cpSecY results in a global chloroplast translation defect

The pleiotropic defect in membrane protein localization caused by the lack of cpFtsY mildly reduces the translation of many chloroplast membrane proteins. In contrast, the loss of cpSecY results in the complete loss of translating ribosomes on all transcripts investigated except for *psbA*, which is a special case that will be addressed below. It is tempting to believe that all chloroplast-encoded membrane proteins require the cpSecY/E translocon pathway to integrate into the thylakoid membrane. This hypothesis is supported by homologous experiments performed in bacteria that found that the majority of membrane proteins are inserted into the ER by the Sec pathway (Klostermann et al., 2002).

However, another possibility is that not all of these proteins are localized to the membrane by the cpSecY/E translocon exclusively, but are instead affected by a severe pleiotropic defect caused by the loss of a major protein insertion pathway. Thylakoid membranes in the cpSecY null mutant are severely depleted (Roy and Barkan, 1998). The cpSecY/E translocon is known to integrate many proteins into the thylakoid membrane that are crucial to the assembly of each photosynthetic complex. As described previously, the loss of a single subunit from a thylakoid photosynthetic complex may reduce the accumulation of that complex (Voelker and Barkan, 1995). This could explain why the membrane-associated translation of so many plastid-encoded membrane proteins is disrupted.

Another pleiotropic defect observed in the *csy1* mutant is a global defect in chloroplast translation (Roy and Barkan, 1998). This phenomenon can be illustrated by comparing ribosome abundance on mRNAs encoding the membrane protein petB to the

soluble protein petD, both of which have depleted ribosome accumulation in the *csy1* mutant (Figure 11). PetD is a soluble protein that is never localized to the thylakoid membrane, and is translated entirely on soluble ribosomes. In the *cys1* mutant, the abundance of ribosomes in *petD* is depleted. This provides *in vivo* evidence of a pleiotropic defect in global chloroplast translation caused by the loss of cpSecY.

The D1 nascent peptide engages with the thylakoid membrane independently of cpFtsY, cpSecA, and cpSecY

The D1 protein is the reaction center of Photosystem II and is encoded by the psbA gene in the chloroplast genome. D1 is damaged by photon bombardment during photosynthesis and must be newly synthesized and replaced often (Rintamäki et al., 1996). This requires the D1 protein to have a high turnover rate, characterized by both rapid protein synthesis and rapid localization to the thylakoid membrane. Soluble and membrane-associated ribosomes translating the *psbA* transcript are consistently at WT levels in the *csr1*, *csy1* and *tha1* mutants (Figure 21). In fact, *psbA* is unique in this way. This indicates that the initial engagement of the D1 nascent peptide with the thylakoid membrane is not exclusively dependent on the cpFtsY, cpSecY, or cpSecA subunits. The translation of D1 appears to be at least somewhat independent of the pleiotropic defects that may be caused by a loss of these subunits, although its final integration and function in the membrane cannot be observed by this assay. It is possible that the D1 protein does not require these subunits for initial engagement with the membrane, but it may require one or more of them for subsequent integration steps.

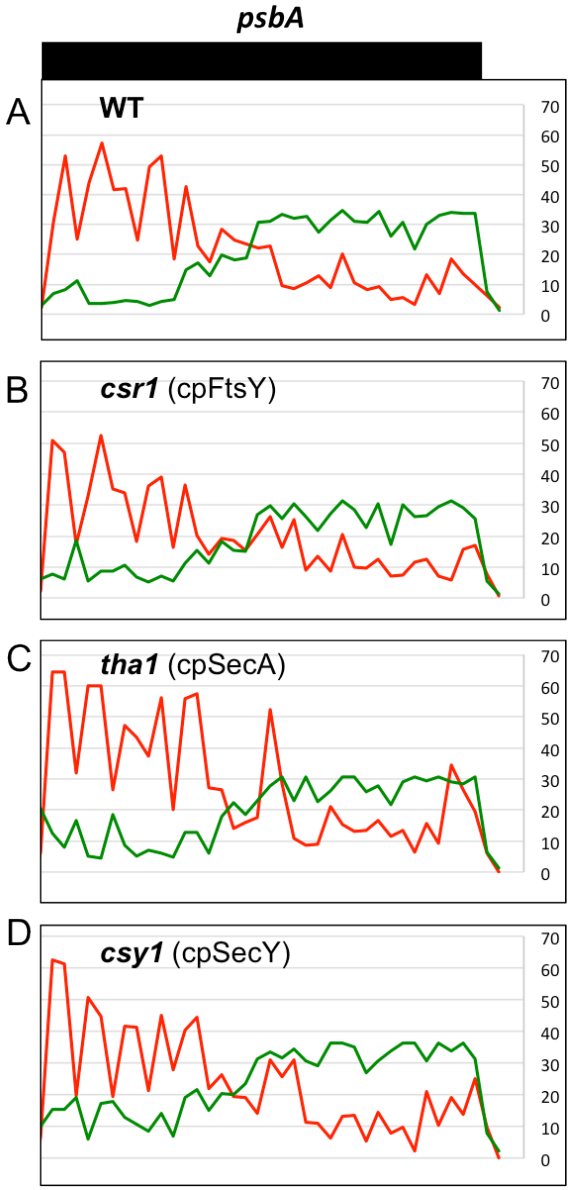

Figure 21. Relative abundance of soluble and membrane-associated ribosomes translating *psbA* mRNA

(a) WT; (b) *csr1* mutant lacking cpFtsY; (c) *tha1* mutant lacking cpSecA; (d) *csy1* mutant lacking cpSecY

In the absence of cpFtsY, *psbA* mRNA accumulates to normal levels and steady state levels of D1 protein are reduced but entirely not depleted (Asakura et al., 2004). The reduction of D1 protein could be explained by the pleiotropic, non-specific effect that the loss of cpFtsY has on the assembly of all photosystem complexes. D1 protein was found to be successfully integrated into the thylakoid membrane in the absence of cpFtsY (Asakura et al., 2004). However, in vitro crosslinking experiments have shown that cpSRP54 interacts strongly, although transiently, with the D1 nascent peptide (Nilsson and Van Wijk, 2002). This suggests that D1 may be able to integrate into the thylakoid membrane by multiple mechanisms, including but not limited to the cpSRP/cpFtsY translocon pathway.

The D1 nascent peptide's successful engagement with the thylakoid membrane in the absence of cpSecY was surprising. Current research supports a model in which D1 integrates into the thylakoid co-translationally via the cpSecY/E translocon pathway (Zhang et al., 2001; Walter et al., 2015). In vitro crosslinking experiments have shown that D1 elongation intermediates are in close proximity to cpSecY (Zhang et al., 2001). Polysome gradient analysis of the *psbA* transcript in *csy1* mutant tissue revealed that the number of ribosomes associated with the *psbA* transcript was dramatically decreased in the absence of cpSecY (Roy and Barkan, 1998). Previous studies have also shown that in the absence of cpSecY, accumulation of the D1 protein is reduced <10% of WT (Roy and Barkan, 1998). Since the ribosome profiling assay cannot detect integration steps subsequent to initial engagement, it is possible that the D1 nascent peptide is able to engage with the membrane but is unable to integrate. After translation, the aggregated D1 protein tethered to the membrane would be degraded by proteases in the stroma,

resulting in depletion of the D1 protein as detected by immunoassay. These possibilities will be discussed below.

Examining targeting behavior of plastid-encoded membrane proteins in the absence of *hcf106*

I examined ribosome behavior in a mutant lacking the hcf106 protein from the cpTat thylakoid membrane integration pathway. The cpTat pathway is specialized for the import of pre-folded proteins through the thylakoid membrane. Proteins that can be integrated co-translationally are able to use the cpSec or cpSRP pathways, which require proteins to be unfolded prior to transport. There are not any chloroplast-encoded proteins that are known to bind prosthetic groups in the stroma, but analysis of ribosome profiling done on this mutant will provide a whole-genome view of the effects of losing *hcf106* in the chloroplast (Celedon and Cline, 2013). I have performed this ribosome profiling experiment but I am still in the process of analyzing the data.

DISCUSSION

Maize mutant lacking cpFtsY exhibits pleiotropic defects on initial engagement of plastid-encoded nascent peptides with the thylakoid membrane

All thylakoid-bound photosystem complexes are reduced in the *csr1* maize mutant (Asakura et al., 2004). The loss of cpFtsY appears to result in a pleiotropic defect in which the assembly and accumulation of photosynthetic complexes in the thylakoid membrane is severely reduced. This possibility is supported by ribosome profiling analysis on the *csr1* mutant. atpI, atpF, and ndhF appear to be the only three plastid-encoded thylakoid membrane proteins investigated that absolutely require cpFtsY for co-translational targeting to the thylakoid membrane. The overall abundance of ribosomes translating eight other plastid encoded membrane proteins (petB, psbB, ndhA, ndhB, psaB, psaA, psbC, and psbD) is mildly reduced in the *csr1* mutant but not entirely depleted, and the position at which ribosomes transition from the soluble to membrane phase is similar to WT.

An interesting commonality between atpI, atpF, and ndhF is that they share an unusual topology in the thylakoid membrane. They are the only three co-translationally inserted plastid-encoded thylakoid membrane proteins known to have their N-terminus in the lumen of the thylakoid (Zoschke & Barkan, 2015). Other plastid encoded membrane proteins have either a stromal N-terminus or an unknown topology. It is possible that cpFtsY is required for the co-translational integration of these proteins into the thylakoid membrane for topological reasons. Perhaps cpFtsY is required to correctly orient the incoming nascent peptide, and without this subunit these proteins are unable to co-translationally engage the thylakoid membrane.

cpFtsY co-precipitates from the thylakoid membrane in a stable complex with the cpSecY and Alb3 subunits, suggesting that cpFtsY, cpSecY, and Alb3 interact and function independently of co-translational integration of proteins into the membrane (Walter et al., 2015). The stable association between translocon subunits in the membrane could explain the pleiotropic effects observed *in vivo* in the absence of cpFtsY. The lack of cpFtsY reduces accumulation of ribosomes on many transcripts that may not actually require cpFtsY for integration into the membrane. The loss of cpFtsY may destabilize other translocon subunits (such as Alb3 or cpSecY) that are required for the integration of many plastid-encoded proteins into the membrane.

Proposing a cpSec-independent integration mechanism for PsbA similar to the bacterial YidC membrane integration pathway

Alb3 is a chloroplast homolog of the bacterial translocon YidC. YidC integrates proteins recognized by the SecY/E translocon into the ER membrane in bacteria. YidC works by facilitating the lateral release, folding, and assembly of the transmembrane domains of Sec-dependent proteins from the SecE/Y translocase into the lipid bilayer (Scotti et al., 2000). However, YidC has also been shown to integrate membrane proteins independently of the Sec translocon, a trait which is shared by the chloroplast Alb3 homolog (Klostermann et al., 2002). In chloroplasts, Alb3 exists in the thylakoid membrane as part of a translocon complex including the cpSecY subunit and the cpFtsY subunit (Klostermann et al., 2002; Moore et al., 2003). Despite being located in the same translocon complex, Alb3 can function as an insertase independent of its other components. Nuclear-encoded light-harvesting chlorophyll proteins integrate into the thylakoid membrane using cpSRP54 and Alb3, but independently of cpSecY

(Klostermann et al., 2002). This observation supports the hypothesis that Alb3 may function independently of cpSecY in chloroplasts in the integration of plastid-encoded proteins as well.

Alb3 plays a role in the co-translational integration of the D1 protein into the thylakoid membrane, although the exact mechanism of this localization and integration is unknown (Walter et al., 2015). A model in which cpFtsY and Alb3 form a complex together to insert thylakoid membrane proteins independently of cpSecY has been proposed (Moore et al., 2003). This model is supported by Asakura et al.'s observation that in the *csr1* mutant there is a reduction of D1 protein and in the *alb3* mutant there is a total loss of D1 protein (Asakura et al., 2008). Ribosomes translating the D1 protein in the *csr1* mutant successfully tether to the membrane at the same position on the *psbA* transcript as in WT (Figure 21). This indicates that in the *csr1* mutant the initial engagement of the D1 nascent peptide with the thylakoid membrane is successful, so cpFtsY is not explicitly required for this targeting step. Since the D1 nascent peptide associates with cpSRP54, it is possible that in a normal chloroplast D1 is able to localize to the thylakoid membrane by cpFtsY in addition to other mechanisms.

The D1 nascent peptide may have the ability to localize to the membrane independently of cpFtsY and cpSecY via Alb3, where initial engagement of the nascent peptide with the Alb3 subunit is successful in the *csr1*, *csy1*, and *tha1* mutants. However, subsequent integration events may require the use of cpSecY for lateral movement or assembly of the D1 protein from the Alb3 translocon into the lipid bilayer. This is the exact opposite of the bacterial homolog mechanism, in which YidC is necessary to integrate proteins initially recognized by the SecY/E translocon (Scotti et

al., 2000). Alb3, cpFtsY, and cpSecY are known to form a stable complex in the thylakoid membrane, which suggests an interactive function between the subunits (Walter et al., 2015). The D1 protein's high turnover rate necessitates its ability to localize to the thylakoid membrane quickly and efficiently. The best way to achieve rapid insertion is to allow the D1 nascent peptide to integrate into the thylakoid membrane by a variety of simultaneous mechanisms. The D1 protein may therefore also be able to localize and integrate into the thylakoid membrane by an undiscovered translocon.

Lack of ribosomes translating stromal precursors when membrane-associated ribosomes are depleted

A decrease in the abundance of a membrane protein should theoretically be coupled with an increase in abundance of its mRNA precursor in the stroma (Voelker et al., 1997). This observation is not reflected in the ribosome profiling data, in which case depletion of membrane-associated ribosomes is coupled with an equivalent depletion of soluble translating ribosomes (with the exception of *petA*). These soluble ribosomes should theoretically be translating the stromal precursor, before the first transmembrane sequence or N-terminal signal peptide is revealed and the nascent peptide localizes to the membrane. Further investigations must be conducted to understand why a global defect in translation occurs in the absence of a membrane-bound receptor.

BIBLIOGRAPHY

Albiniak, A.M., Baglieri, J., and Robinson, C. (2012). Targeting of lumenal proteins across the thylakoid membrane. J. Exp. Bot. *63*, 1689–1698.

Asakura, Y., Hirohashi, T., Kikuchi, S., Belcher, S., Osborne, E., Yano, S., Terashima, I., Barkan, A., and Nakai, M. (2004). Maize mutants lacking chloroplast FtsY exhibit pleiotropic defects in the biogenesis of thylakoid membranes. Plant Cell *16*, 201–214.

Asakura, Y., Kikuchi, S., and Nakai, M. (2008). Non-identical contributions of two membrane-bound cpSRP components, cpFtsY and Alb3, to thylakoid biogenesis. Plant J. *56*, 1007–1017.

Celedon, J.M., and Cline, K. (2013). Intra-plastid protein trafficking: How plant cells adapted prokaryotic mechanisms to the eukaryotic condition. Biochim. Biophys. Acta - Mol. Cell Res. *1833*, 341–351.

Cline, K., and Mori, H. (2001). Thylakoid ΔpH-dependent precursor proteins bind to a cpTatC-Hcf106 complex before Tha4-dependent transport. J. Cell Biol. *154*, 719–729.

Ingolia, N.T., Ghaemmaghami, S., Newman, J.R.S., and Weissman, J.S. (2009). Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science *324*, 218–223.

Jarvis, P. (2008). Targeting of nucleus-encoded proteins to chloroplasts in plants. New Phytol. *179*, 257–285.

Klostermann, E., Droste Gen Helling, I., Carde, J.-P., and Schünemann, D. (2002). The thylakoid membrane protein ALB3 associates with the cpSecY-translocase in Arabidopsis thaliana. Biochem. J. *368*, 777–781.

Laidler, V., Chaddock, A.M., Knott, T.G., Walker, D., and Robinson, C. (1995). A SecY homolog in Arabidopsis thaliana: Sequence of a full-length cDNA clone and import of the precursor protein into chloroplasts. J. Biol. Chem. *270*, 17664–17667.

Lareau, L.F., Hite, D.H., Hogan, G.J., and Brown, P.O. (2014). Distinct stages of the translation elongation cycle revealed by sequencing ribosome-protected mRNA fragments. Elife *2014*.

Maier, R.M., Neckermann, K., Igloi, G.L., and Kössel, H. (1995). Complete sequence of the maize chloroplast genome: gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. J. Mol. Biol. *251*, 614–628.

Meyers, B.C., Tingey, S. V., and Morgante, M. (2001). Abundance, distribution, and transcriptional activity of repetitive elements in the maize genome. Genome Res. *11*, 1660–1676.

Nilsson, R., and Van Wijk, K.J. (2002). Transient interaction of cpSRP54 with elongating nascent chains of the chloroplast-encoded D1 protein; "cpSRP54 caught in the act."FEBS Lett. *524*, 127–133.

Raven, J.A., and Allen, J.F. (2003). Genomics and chloroplast evolution: what did cyanobacteria do for plants? Genome Biol. *4*, 209.

Rintamäki, E., Kettunen, R., and Aro, E.M. (1996). Differential Dl dephosphorylation in functional and photodamaged photosystem II Centers: Dephosphorylation is a prerequisite for degradation of damaged D1. J. Biol. Chem. *271*, 14870–14875.

Röhl, T., and Van Wijk, K.J. (2001). In Vitro Reconstitution of Insertion and Processing of Cytochrome in a Homologous Chloroplast Translation System. J. Biol. Chem. *276*, 35465–35472.

Roy, L.M., and Barkan, A. (1998). A SecY homologue is required for the elaboration of the chloroplast thylakoid membrane and for normal chloroplast gene expression. J. Cell Biol. *141*, 385–395.

Schuenemann, D., Amin, P., Hartmann, E., and Hoffman, N.E. (1999). Chloroplast secY is complexed to secE and involved in the translocation of the 33-kDa but not the 23-kDa subunit of the oxygen-evolving complex. J. Biol. Chem. *274*, 12177–12182.

Schünemann, D. (2004). Structure and function of the chloroplast signal recognition particle. Curr. Genet. *44*, 295–304.

Scotti, P.A., Urbanus, M.L., Brunner, J., de Gier, J.W., von Heijne, G., van der Does, C., Driessen, A.J., Oudega, B., and Luirink, J. (2000). YidC, the Escherichia coli homologue of mitochondrial Oxa1p, is a component of the Sec translocase. EMBO J. *19*, 542–549.

Serek, J., Bauer-Manz, G., Struhalla, G., van den Berg, L., Kiefer, D., Dalbey, R., and Kuhn, A. (2004). Escherichia coli YidC is a membrane insertase for Sec-independent proteins. EMBO J. *23*, 294–301.

Voelker, R., and Barkan, A. (1995). Two nuclear mutations disrupt distinct pathways for targeting proteins to the chloroplast thylakoid. EMBO J. *14*, 3905–3914.

Voelker, R., Mendel-Hartvig, J., and Barkan, A. (1997). Transposon-disruption of a maize nuclear gene, tha1, encoding a chloroplast SecA homologue: In vivo role of cp-SecA in thylakoid protein targeting. Genetics *145*, 467–478.

Wang, P., and Dalbey, R.E. (2011). Inserting membrane proteins: The YidC/Oxa1/Alb3 machinery in bacteria, mitochondria, and chloroplasts. Biochim. Biophys. Acta - Biomembr. *1808*, 866–875.

Zhang, L., Paakkarinen, V., Suorsa, M., and Aro, E.M. (2001). A SecY Homologue is Involved in Chloroplast-encoded D1 Protein Biogenesis. J. Biol. Chem. *276*, 37809–37814.

Zoschke, R., Watkins, K.P., and Barkan, A. (2013). A rapid ribosome profiling method elucidates chloroplast ribosome behavior in vivo. Plant Cell *25*, 2265–2275.

Zoschke, R., and Barkan, A. (2015). Genome-wide analysis of thylakoid-bound ribosomes in maize reveals principles of cotranslational targeting to the thylakoid membrane. Proc. Natl. Acad. Sci. USA. *112*, 1678-1687.