

BIOGENESIS OF CHLOROPLAST THYLAKOID TRANSLOCASE SUBUNIT CPTATC

By

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To my family and teachers

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LIST OF ABBREVIATIONS

-2TC	Tandem tetra-Cysteine Protein tag for Chromophore Assisted Light Inactivation
ADP	Adenosine diphosphate
Alb3	Albino 3. A membrane-integrated subunit of the cpSRP protein translocase.
Alb3.1	Alb3 of <i>Chlamydomonas reinhardtii</i>
Alb3.2	Alb3-like protein of <i>Chlamydomonas reinhardtii</i>
Alb4	Albino4. Putative thylakoid protein translocase that is homologous to Alb3
Anti-	Antibodies to-
Arf	ADP ribosylation factor. Small GTPase involved in endomembrane vesicle trafficking
at-	<i>Arabidopsis thaliana</i> antibodies
ATP	Adenosine triphosphate
AVI	Anthocyanic vesicle inclusion
Az	Sodium azide
B1-	A specific Invitrogen Gateway recombinase site
B2-	A specific Invitrogen Gateway recombinase site
B5-	A specific Invitrogen Gateway recombinase site
B5r-	A specific Invitrogen Gateway recombinase site
BLAST	Basic local alignment search tool
BN-	Blue Native
CALI	Chromophore Assisted Light Inactivation
CAO	Chlorophyllide a Oxygenase
Chl	Chlorophyll
cm	Centimeters of distance

-CO ₃	Carbonate
Col-0	Arabidopsis Columbia ecotype
CF ₀ II	Membrane-integrated subunit of the mitochondrial ATPase protein complex
Cp	Chloroplast
cpOxa1p	Chloroplast homolog of bacterial YidC, which is similar to mitochondrial Oxa1p. Chloroplast Oxa1p is also known as Alb3.
cpSecA	Subunit A of the cpSec protein translocase
cpSecA2	Subunit A of the cpSec2 protein translocase
cpSecE	Subunit E of the cpSec protein translocase
cpSecE2	Hypothetical cpSec2 protein translocase subunit 'E.'
cpSecY	Subunit Y of the cpSec protein translocase
cpSecY2	Subunit Y of the cpSec2 protein translocase
cpSRP43	The 43 kilodalton subunit of the cpSRP protein translocase
cpSRP54	The 54 kilodalton subunit of the cpSRP protein translocase
cpTat	Twin arginine protein translocase of chloroplast thylakoids
cpTatC	Subunit C of the cpTat protein translocase
CTAB	Centrimonium bromide
Cys	Cysteine
Δ	Deletion mutation
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
DTT	Dithiothreitol
E	Envelope membrane fraction
EDT ₂	Ethane dithiol
ER	Endoplasmic reticulum

FtsY	Filamentous thermosensitive Y. A thylakoid membrane associated subunit of the cpSRP protein translocase. Also known as the cpSRP receptor
G/C	Guanine/Cytosine content (in percent) found within a given stretch of DNA sequence
GFP	Green fluorescent protein
GspB	286 kilodalton cell wall-anchored protein of <i>Streptococcus gordonii</i>
GTP	Guanosine triphosphate
GV3101	A strain of <i>Agrobacterium tumefaciens</i> that is used to transform plants
Hcf106	High chlorophyll fluorescence 106. A membrane-integrated subunit of the cpTat translocase
HK	HEPES-KOH pH 8. A buffer used to hypotonically lyse isolated chloroplasts <i>in vitro</i> .
Hsp70	The 70 kilodalton heat shock protein of chloroplasts
Hsp93	The 93 kilodalton heat shock protein of chloroplasts
i-	Intermediate processed form of a translocated chloroplast protein
IB	Import buffer
IP	Immunoprecipitation
kD	Kilodalton
LHCP	Light-harvesting chlorophyll-binding protein
M	Chloroplast membrane fraction
-M	Molar concentration
m-	Mature processed form of a translocated chloroplast protein
µg	Micrograms of mass
min	Minutes on time
mL	Milliliter of volume
µL	Microliter of volume

mm	Millimeters of distance
mM	Millimolar concentration
μM	Micromolar concentration
Mr	Molecular weight markers
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog medium
mW	Milliwatts of power
NC	The non-conserved peptide region of cpTatC
ng	Nanograms of mass
nm	Nanometers of distance
N/V	Nigericin Valinomycin
NigVal	Nigericin Valinomycin
OE-	Oxygen evolving complex subunit-
OP	Orthophenanthroline
Oxa1p	Oxidase Assembly 1. Mitochondrial inner membrane protein integrase that is homologous to YidC in bacteria and Alb3 of chloroplasts.
Oxa2	Oxidase Assembly 2. Similar to Oxa1p
P	Pellet
p-	Precursor form of a translocated chloroplast protein
P1-	A specific Invitrogen Gateway recombinase site
P2-	A specific Invitrogen Gateway recombinase site
P5-	A specific Invitrogen Gateway recombinase site
P5r-	A specific Invitrogen Gateway recombinase site
P36	Chloroplast envelope phosphate translocator
-PAGE	Polyacrylimide gel electrophoresis
PC	Plastocyanin

PCR	Polymerase chain reaction
pET-14b	A member of the bacterial pET expression vector series
PG	Post-germination
Plsp1	Chloroplast signal peptidase
pMDC7	Arabidopsis binary vector that contains the estrogen inducible system
PMF	Proton Motive Force
PMSF	Phenylmethanesulfonyl fluoride
Pre-	Precursor form of protein in question
PS	Photosystem
ps-	<i>Pisum sativum</i> antibodies
Psb-	Photosystem II subunit-
Psa-	Photosystem I subunit-
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Rab	Sub-family of small GTPases that are involved in in endomembrane trafficking
RB47	An mRNA binding protein of <i>Chlamydomonas reinhardtii</i> that is involved in chloroplast protein translation
RNAi	RNA interference
RT-	Reverse transcription
RUBISCO	Ribulose bisphosphate carboxylase oxygenase
S	Chloroplast soluble fraction
S	Supernatant
SDS-	Sodium dodecyl sulfate, protein denaturing detergent.
Sec	Secretory protein translocase of chloroplast thylakoids
SOE	Splicing by overlap extension

SP6	Phage DNA dependent RNA polymerase that is used to synthesize mRNA <i>in vitro</i>
SRP	Signal recognition particle protein translocase of chloroplast thylakoids
SSU	RUBISCO small subunit
+T	Chloroplast membrane fraction that has been treated with thermolysin protease
T1	The first segregating generation produced from self-pollinating transformed Arabidopsis
T2	The second segregating generation produced from self-pollinating YidC2
TBS	Tris-buffered saline solution
TCA	Trichloroacetic acid
TEM	Transmission Electron Microscopy
T-DNA	Transfer DNA
Th	Thermolysin protease
Tha4	Thylakoid Assembly 4. A membrane-integrated subunit of the cpTat protein translocase
Thy	Thylakoids
TIC	Translocon at the inner envelope membrane of plant chloroplasts
TIC40	40 kilodalton subunit of the translocon at the inner envelope membrane of plant chloroplasts
TIC110	110 kilodalton subunit of the translocon at the inner envelope membrane of plant chloroplasts
TIGR	The Institute for Genomics Research
TLCK	N- α -Tosyl-L-Lysiny-Chloromethylketone
TM	Transmembrane domain
TOC	Translocon at the outer envelope membrane of plant chloroplasts
TOC75	75 kilodalton subunit of the translocon at the outer envelope membrane of plant chloroplasts

TOC132/120	132 and 120 kilodalton subunits of the translocon at the outer envelope membrane of plant chloroplasts
TOC159	159 kilodalton subunit of the translocon at the outer envelope membrane of plant chloroplasts
Tp	Translation product
tp-	Transit peptide
Tr	Trypsin protease
UTR	Untranslated region
W	Watts of power
Ws	Wassilewskija ecotype of Arabidopsis
VIPP1	Vesicle inducing protein in plastids 1
YidC	Bacterial membrane protein integrase that is similar to Alb3 of chloroplasts and Oxa1p of mitochondria
YidC2	Bacterial membrane protein that is similar to YidC

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Thylakoid membranes have a unique complement of proteins, most of which are synthesized in the cytosol, imported into the stroma, and translocated into thylakoid membranes by specific thylakoid translocases. Known thylakoid translocases contain a core multi-spanning, membrane-integrated subunit which is also nuclear-encoded and imported into chloroplasts before being integrated into thylakoid membranes. Thylakoid translocases play a central role in determining the composition of thylakoids, yet the manner by which the core translocase subunits are integrated into the membrane is not known. We used biochemical and genetic approaches to investigate integration of the core subunit of the chloroplast Tat translocase, cpTatC, into thylakoid membranes. *In vitro* import assays show that cpTatC correctly localizes to thylakoids if imported into intact chloroplasts, but it does not integrate into isolated thylakoids. *In vitro* transit peptide processing and chimeric precursor import experiments suggest that cpTatC possesses a stroma-targeting transit peptide. Import time course and chase assays confirmed that cpTatC targets to thylakoids via a stromal intermediate, suggesting that it might integrate through one of the known thylakoid translocation pathways. However, chemical inhibitors to the cpSecA-cpSecY and cpTat pathways did not impede cpTatC localization to

thylakoids when used in import assays. Analysis of membranes isolated from *Arabidopsis thaliana* mutants lacking cpSecY or Alb3 showed that neither is necessary for cpTatC membrane integration or assembly into the cpTat receptor complex. These data suggest the existence of another translocase, possibly one dedicated to the integration of chloroplast translocases.

Alb4 and Sec2 are recently discovered putative chloroplast protein translocases. Results from *in vitro* import experiments suggested that cpSecY2 and SecA2 both localize to chloroplasts. Inducible RNAi enabled a CPSECY2 transcript reduction to ~40% of wild type levels in *Arabidopsis* seedlings. Reductions to CPSECY2 transcripts resulted in bleached cotyledons and plastids that mostly lack thylakoids, similar to phenotypes that have been observed in cpTatC, Alb3, and cpSecY knockout mutants. Reduction of CPSECY2 transcripts gave way to reductions of cpTatC transcript and protein levels. TIC40, cpSecY, and TIC110 protein levels were also reduced in cpSecY2 RNAi seedlings, suggesting that cpSec2 may be involved in the biogenesis or stability of bacterially-conserved core thylakoid translocase subunits, and novel eukaryotic-derived subunits of the chloroplast import apparatus.

CHAPTER 1 MECHANISMS OF THYLAKOID BIOGENESIS

Summary

Pigments, lipids, and proteins are the materials that constitute chloroplast thylakoids. The arrangement of lipids and pigments and the organization of macromolecular complexes of proteins in thylakoid membranes enable oxygenic photosynthesis, which supports Earth's biosphere by transforming electromagnetic radiation into chemical energy. The expression and activity of chloroplast envelope- and endoplasmic reticulum membrane-localized lipid synthesis and transport proteins determines chloroplast lipid content. Inner envelope membranes are internalized to become thylakoid membranes by two hypothetical processes: inner envelope membrane invagination and/or vesicle transport. Photosynthetic proteins reach thylakoids through the help of specific chloroplast envelope and thylakoid protein translocases. Here, I review several aspects of thylakoid membrane internalization and protein transport, and discuss how each contributes to thylakoid biogenesis.

Introduction

Plant chloroplasts are derived from an ancient endosymbiotic event in which a photosynthetic cyanobacterium took up residence within a mitochondria-containing eukaryote (Dyall et al., 2004). The subsequent loss or transfer of most endosymbiont genes to the host genome created a complex genetic system that coordinates nuclear anterograde and chloroplast retrograde signaling with a reduced endosymbiont genome and nuclear dominance over chloroplast gene expression (Woodson and Chory, 2008). Nuclear dominance allowed plant cells to coordinate plastid development with the developmental priorities of the plant, relegating the endosymbiont to organelle status. The plastid is distinguished from its bacterial relative by an ability to differentiate among several plastid types, each with distinctive structure,

composition, and chemistry. Proplastids are progenitor plastids present in meristematic cells; they are small semi-spherical organelles that mostly lack internal membranes (Figure 1-1) (Buchanan et al., 2000). Etioplasts arise from proplastids that have received some, but not all, of the environmental cues that are needed to trigger chloroplast development. Etioplasts contain a large paracrystalline mass of internal lipids called the prolamellar body. Prolamellar bodies contain chlorophyll biosynthetic proteins and other pre-chloroplast proteins that can be used for rapid differentiation into thylakoids (Figure 1-1) (Blomqvist et al., 2008). Etioplasts accumulate in plant tissues until light-induced genes direct inner membrane re-differentiation and thylakoid biogenesis.

Similar to cyanobacteria, chloroplasts possess internal photosynthetic thylakoid membranes made up of densely packed protein complexes that are embedded in membrane lamellae. Chloroplast thylakoid lamellae are either exposed to the stroma or arranged as stacks called grana. The stroma is an aqueous compartment that is analogous to the cytoplasm of the original endosymbiont. The stroma is contained within a bilayered inner envelope membrane (Figure 1-1). The outer envelope is a second membrane that falls outside the inner envelope. The envelope inter-membrane space and the thylakoid lumen are aqueous compartments of chloroplasts that are topologically analogous to the periplasm of the original endosymbiont.

Studies of chloroplast biogenesis have led to interesting discoveries and developmental questions regarding when and how plastid inner membranes accumulate the lipids and proteins that define thylakoid identity. Such research has identified proteins that synthesize, modify, and internalize plastid inner membrane lipids. Other studies describe protein translocases that target nuclear- and plastid-encoded proteins to thylakoid membranes. The following review discusses how chloroplast lipid internalization and protein translocation contribute to thylakoid biogenesis.

Thylakoid Lipids are Imported from the Envelope Membrane

Thylakoid lipids are synthesized through the dual activity of chloroplast envelope- and endoplasmic reticulum-derived prokaryotic and eukaryotic synthesis pathways respectively, but the final steps of thylakoid lipid synthesis occur in the envelope (Benning, 2008). The mechanisms of envelope to thylakoid lipid transport are poorly understood, but some evidence suggests thylakoids acquire lipids through vesicle transport from, and/or direct continuity with, the inner envelope membrane.

Vesicle transport from the inner envelope to thylakoid membranes

Rapidly developing chloroplasts contain stromal vesicles that are thought to traffic lipids from the inner envelope to thylakoid membranes. Vesicles and stroma-facing buds of the inner envelope membranes have been observed in thin sections of mature chloroplasts in *Arabidopsis* and pea (Westphal et al., 2001b; Kroll et al., 2001; Morre et al., 1991). Increased numbers of vesicles appear to accumulate from inner envelope buds in chloroplasts within leaves that are incubated at decreasing temperatures (Morre et al., 1991). In cold temperatures, chloroplast vesicle accumulation is correlated with slow transport of radiolabeled lipids from the envelope to thylakoids in an *in vitro* lipid synthesis and transport assay (Andersson et al., 2001). Cold temperature is also known to reduce vesicle transport in the endomembrane system. For instance, cold temperature caused increased transitional endoplasmic reticulum (ER) vesicle accumulation and reduced vesicle fusion with the cis-Golgi in animal cells (Morre et al., 1989).

Biochemical studies have suggested that proteins similar to those used for cytosolic vesicle trafficking mediate chloroplast vesicle transport. Cytosolic vesicle trafficking is facilitated by a number of small guanosine triphosphate (GTP) binding proteins such as Arf proteins, which initiate ER vesicle budding and coat protein recruitment, and Rab protein family members, which regulate vesicle docking to, and fusion with, endomembranes (Gillingham and Munro,

2007; Haucke, 2003). Stromal vesicles did not accumulate in chloroplasts that were incubated in the cold with non-hydrolysable forms of GTP (Westphal et al., 2001b). The dependence of stromal vesicle formation on GTP was put in context when stromal vesicles accumulated in chloroplasts that were incubated with inhibitors to known cytosolic vesicle fusion proteins such as protein phosphatase 1 and calmodulin, which are involved homotypic membrane fusion during yeast vacuole formation (Westphal et al., 2001b; Mayer et al., 1996; Mayer, 1999).

Aside from observations of chloroplast ultrastructure and the results of biochemical experiments, other evidence for envelope to thylakoid vesicle transport has come from studies of Vesicle Inducing Protein in Plastids 1 (*VIPPI*). *VIPPI* got its name from mutants in plants and cyanobacteria that exhibit a lack of stromal vesicles and an inability to maintain thylakoid membranes (Kroll et al., 2001; Westphal et al., 2001a). *VIPPI* function appears to be involved in thylakoid maintenance because *Arabidopsis vipp1* mutants germinate and initially develop as wild type seedlings. It isn't until several weeks after germination that *vipp1* mutants lose their thylakoids (Kroll et al., 2001). In cold temperatures, reduced lipid trafficking between the chloroplast envelope and thylakoids is correlated with an increase in stromal vesicles, but cold induced stromal vesicles are missing in *vipp1* mutants (Andersson et al., 2001; Kroll et al., 2001). Together, these studies suggest that *VIPPI* is involved in a chloroplast vesicle transport system that moves lipids between the envelope and thylakoid membranes.

However, the hypothesis that *VIPPI* is involved in vesicle transport was challenged by genetic studies in cyanobacteria. Complete loss of *VIPPI* reduced the abundance of photosystem I complexes, and inducible depletion of *VIPPI* reduced photosynthetic activity in cyanobacteria without resulting in thylakoid membrane loss (Fuhrmann et al., 2009; Gao and Xu, 2009). Further, *VIPPI* depletion reduced cell viability before any disruption to thylakoid membrane

structure. The above findings have been met by a hypothesis that *VIPP1* function in cyanobacteria is different than the function of *VIPP1* in plant thylakoids. A study in *Arabidopsis* showed that decreased *VIPP1* protein levels did not affect the assembly of thylakoid protein complexes, and instead, affected thylakoid membrane formation (Aseeva et al., 2007). The differences between *VIPP1* function in cyanobacteria and plant chloroplasts might be expected if vesicular transport from the inner envelope to thylakoids arose after endosymbiosis, as a function that was inherited from the eukaryotic cell (Vothknecht and Soll, 2005). Indeed, chloroplast vesicles have only been observed in embryophytes and not cyanobacteria or other photosynthetic eukaryotes such as charophytes, chlorophytes, glaucocystophytes, or rhodophytes (Westphal et al., 2003).

A second possibility for membrane internalization during thylakoid biogenesis: inner envelope invagination

The view that chloroplast inner envelope invaginations represent early developing thylakoid membranes mostly stems from structural studies that revealed proplastid and chloroplast envelope invaginations that emanate into the stroma (Morre et al., 1991; Carde et al., 1982). Similar to conditions that stimulate stromal vesicle accumulation, cold temperatures also brought on inner envelope invaginations in mature chloroplasts from tobacco and pea (Morre et al., 1991). These studies relied on electron microscopy, which kills the specimen during sample preparation, preventing one from following the potential development of inner envelope invaginations into thylakoid lamellae. Leech et al. tried to follow thylakoid biogenesis by observing membrane structure in developing chloroplasts along the length of greening maize leaves (Leech et al., 1973). In between proplastids at the leaf base and mature chloroplasts at the leaf tip, were plastids that contained elongated pre-granal thylakoids that extended across the pre-chloroplast. The pre-granal thylakoids appeared to converge at either end of the plastid, but

it was unclear if the thylakoid lamellae were continuous with the inner envelope membrane. The process was easier to observe in *Chlamydomonas reinhardtii*, in which chloroplast greening produced envelope invaginations that appeared to take on thylakoid structure, ultimately resulting in thylakoids that were attached to the envelope membranes (Hooper et al., 1991).

Modern cryo-fixation methods and three dimensional electron tomography (3D EM) have presented chloroplast internal membrane structure with better detail and preservation than was previously possible from using thin sections of chemically fixed specimens (Staehein, 2003). Interestingly, 3D EM micrographs revealed attachment sites between thylakoid membranes and the inner envelope or cell membranes in plant chloroplasts or cyanobacteria, respectively (van de Meene et al., 2006; Shimoni et al., 2005).

Vesicles or invaginations?

Existing evidence for chloroplast vesicle transport needs to be substantiated by studies that characterize mechanisms of vesicle budding from the inner envelope, transport through the stroma, and fusion with thylakoid membranes. The results of chemical inhibitor experiments suggest that proteins similar to those involved in endomembrane vesicle transport mediate vesicle budding and transport. Bioinformatics studies have identified several predicted chloroplast proteins that bear homology to vesicle budding GTPases (Andersson and Sandelius, 2004), but evidence to confirm the chloroplast localization or specific biochemical function of any candidate protein in a chloroplast vesicle transport pathway has not been reported. Vesicle transport persists as a potential mode of chloroplast lipid internalization, but until chloroplast vesicle transport mechanisms and their associated proteins are better characterized, the process will remain hypothetical.

Ultrastructural studies of chloroplasts in plants and *C. reinhardtii* have revealed inner envelope membrane invaginations and thylakoid biogenesis from inner envelope invaginations,

respectively. These studies and the discovery of envelope-thylakoid membrane contact sites in cyanobacteria and plant and algal chloroplasts support the hypothesis that inner envelope invaginations give rise to thylakoids. However, more functional characterization is needed to understand the mechanisms that invaginate the inner envelope membrane.

Thylakoid Protein Translocases Determine Thylakoid Protein Composition

The plastid genome encodes roughly 50 thylakoid proteins, whereas about half of thylakoid membrane proteins and all thylakoid lumen proteins are encoded in the nucleus (Kieselbach and Schroder, 2003; Peltier et al., 2002; Race et al., 1999). Much of what is known about the localization of nuclear-encoded thylakoid protein translocases has come from biochemical studies of isolated mature chloroplasts. Nuclear-encoded thylakoid proteins are synthesized in the cytosol and are imported into the chloroplast by novel translocases of the outer (TOC) and inner (TIC) chloroplast envelope membranes (Inaba and Schnell, 2008). TOC and TIC specifically bind chloroplast precursor proteins at N-terminal chloroplast transit peptides, which are cleaved by a transit peptidase as chloroplast precursors are imported into the stroma (Figure 1-2). For thylakoid precursor proteins that possess bipartite transit peptides, cleavage of the chloroplast transit peptide reveals a second targeting peptide that is cleaved by a thylakoid lumen peptidase upon protein transport into the thylakoid (Schunemann, 2007). Other thylakoid proteins lack cleavable thylakoid targeting transit peptides and instead contain non-cleaved internal hydrophobic targeting peptides. In either case, cleavage of the chloroplast transit peptides reveals sequences that target proteins for integration into or transport across thylakoid membranes by one of several bacterially conserved thylakoid protein translocation pathways: the Twin Arginine (cpTat), chloroplast Signal Recognition Particle (cpSRP), chloroplast Secretory (cpSec), or the spontaneous pathway (Figure 1-2) (Cline and Dabney-Smith, 2008).

The cpTat translocase is known to utilize the trans-thylakoid membrane proton motive force to transport folded proteins across chloroplast thylakoid membranes (Cline and Theg, 2007). Nuclear-encoded Light Harvesting Chlorophyll Binding Proteins (LHCPs) are multi-spanning membrane proteins that are targeted to photosynthetic thylakoid membranes post-translationally through the interaction of stromal intermediate LHCP with cpSRP (Reed et al., 1990; Payan and Cline, 1991; Yuan et al., 1993; Li et al., 1995). LHCPs are inserted into thylakoid membranes by a membrane-integrated translocase called Alb3 (Moore et al., 2000), which is homologous to a known bacterial membrane protein integrase, YidC. Chloroplast Sec translocates partially unfolded soluble proteins and hydrophobic proteins across or into thylakoid membranes, respectively (Schunemann, 2007). During reconstituted translation of plastid-encoded photosystem II reaction center protein D1, an emerging hydrophobic stretch of D1 was observed to cross-link with cpSRP, implicating cpSRP in co-translational protein targeting in chloroplasts (Nilsson and van Wijk, 2002). Soluble subunit of the cpSRP translocase target D1 to the cpSec translocase (Zhang et al., 2001). Plastid-encoded cytochrome f is also co-translationally inserted into thylakoids by the cpSec translocase, but cytochrome f requires cpSecA for insertion (Nohara et al., 1996; Rohl and van Wijk, 2001). Chloroplast SecA is related to bacterial SecA, an ATPase motor that assists hydrophilic domains of Sec substrates through the Sec channel protein, SecY (Dalbey and Chen, 2004). Chloroplast Sec substrates are also known to translocate via cpSec without a need for cpSecA (van Wijk et al., 1995).

Several proteins integrate into thylakoid membranes *in vitro* in the absence of ATP or a proton motive force (PMF); integration is thought to occur spontaneously because it proceeds despite pre-treating thylakoids with proteases that degrade thylakoid translocases. Among proteins known to integrate spontaneously are single spanning proteins such as the ATPase CF₀II

subunit, and photosystem II (Psb-) subunits X, Y, and W (Robinson et al., 1996; Michl et al., 1994; Lorkovic et al., 1995; Kim et al., 1996). A few polytopic thylakoid membrane proteins are also known to be inserted spontaneously: the early light inducible protein 2, PsbS, and photosystem I (Psa-) subunits G, K, and S (Kim et al., 1998; Kim et al., 1999; Mant et al., 2001; Woolhead et al., 2001).

Genetic studies suggest that wild type thylakoid membrane structure is sustained by translocase-assisted insertion of photosynthetic proteins into thylakoid membranes. When translocases are missing, thylakoid membranes lose their structure or vanish completely, which has been observed when loss of function mutations arise in genes that encode thylakoid translocases such as Alb3, cpTatC, cpSecY, cpSecA, FtsY, or double mutants of the 54- and 43-kD subunits of cpSRP (Roy and Barkan, 1998; Skalizky, 2006; Sundberg et al., 1997; Hutin et al., 2002; Asakura et al., 2004; Asakura et al., 2008; Motohashi et al., 2001). Pleiotropic effects that come from mutating genes that encode thylakoid protein translocases preclude making specific conclusions about translocase function during thylakoid biogenesis; however, genetic studies show that translocases are necessary for proper thylakoid biogenesis and maintenance of thylakoid structure.

The localization and function of thylakoid protein translocases has mostly been studied in mature isolated chloroplasts. The same studies have not been performed with differentiating proplastids because isolating significant numbers of proplastids is not feasible. As a result, little is known about when photosynthetic proteins are inserted into developing thylakoid membranes. Proteins could be inserted before lipids are internalized, on the envelope membrane itself, or on budded vesicles or invaginated lamellae. Experiments are needed to understand when and where photosynthetic proteins are inserted during thylakoid biogenesis. Learning when thylakoid

protein translocases are expressed and where they localize in developing chloroplasts will define when and where chloroplast inner membranes become thylakoids.

Biogenesis of the photosynthetic apparatus during thylakoid biogenesis. The timing and location(s) at which photosynthetic proteins are inserted into early developing thylakoids are not known, but indirect evidence suggests that photosynthetic proteins can integrate into inner envelope membranes. *Chlamydomonas reinhardtii* RB47 and several other mRNA binding proteins, that help translate plastid-encoded photosynthetic proteins, co-fractionated with low-density chloroplast membranes whose lipid composition is identical to that of inner envelope membranes (Zerges and Rochaix, 1998). Density gradient centrifugation separated the mRNA binding proteins and low-density membranes into two different fractions. The first fraction contained isolated inner envelope membranes, and the second contained thylakoid-associated inner envelope membranes. Zerges and Rochaix interpreted their data to suggest that thylakoid-associated inner envelope membranes could exist as vesicles or part of continuous attachments between envelope and thylakoid membranes.

Associations between chloroplast mRNA binding proteins and the inner envelope membrane are particularly interesting in light of studies that found that chlorophyll is synthesized and assembled with LHCPs at inner envelope membranes in greening *Chlamydomonas reinhardtii*. Dark grown wild type and mutant *y-1* both accumulate mRNA that encode thylakoid membrane proteins, but unlike wild type, *y-1* does not synthesize thylakoid membrane proteins or chlorophyll in the dark (Hooper and Stegeman, 1976). During *y-1* chloroplast greening, light elicits chlorophyll synthesis, which in turn elicits Photosystem (PS) I and II biogenesis and LHCP assembly into light harvesting complexes at the inner envelope membrane (Hooper et al., 1982; Maloney et al., 1989; Hooper et al., 1991). PSI and PSII seem

to localize in close proximity to one another in the membrane because their activities couple shortly after greening is initiated (White and Hooper, 1994). Chlorophyllide a Oxygenase (CAO), which plays a role in chlorophyll synthesis, is also localized to the inner envelope membranes of greening *Chlamydomonas* and the inner envelope and thylakoid membranes in both *Arabidopsis* and pea chloroplasts (Eggink et al., 2004; Reinbothe et al., 2006).

The results of recent studies into the function and localization of chloroplast signal peptidase, Plsp1, indirectly suggest that the photosynthetic apparatus is assembled at the inner envelope membranes during thylakoid biogenesis. The 75 kD subunit (TOC75) of the TOC translocase is not processed in *Arabidopsis* that harbor a homozygous knockout mutation in the gene that encodes Plsp1 (Inoue et al., 2005). Interestingly, the thylakoid lumen targeting transit peptides of soluble plastocyanin and the 23- and 33-kD subunit of the oxygen-evolving complex (OE33) are also not processed in *Arabidopsis pls1p* mutants (Shipman-Roston et al., 2010; Inoue et al., 2005). The ability to process both envelope and thylakoid proteins is thought to be related to a dual localization of Pls1p to the inner envelope and pro-thylakoids in proplastids and to thylakoids in mature chloroplasts, respectively (Shipman and Inoue, 2009). Although the role that Pls1p plays during thylakoid biogenesis is still unclear, Pls1p localization studies have drawn a connection between the inner envelope and thylakoid membranes before and after thylakoid biogenesis.

Conclusions and Perspectives

Until the mechanisms for chloroplast vesicle transport and inner envelope membrane invagination are clarified, both remain possible modes for chloroplast membrane internalization. Existing data points to inner envelope invaginations as the initial site of thylakoid biogenesis, and vesicle transport as a means to maintain thylakoids (Figure 1-3). On the other hand, vesicle

transport might not be necessary if thylakoid membranes and inner envelope membranes were continuous, because membrane continuity would allow for lateral lipid transfer.

The membrane continuity hypothesis implies that inner envelope and thylakoid membranes are different domains of a chloroplast inner membrane system. Such a relationship would not be unique to chloroplast inner membranes, as several organelles possess membranes with multi-domain structure. The endoplasmic reticulum has lamellar, tubular, and nuclear envelope domains (English et al., 2009). The mitochondria inner membrane is organized into tubular cristae that span the organelle and peripheral stretches that are located near the outer mitochondria membrane (Mannella, 2006). Recent use of 3D EM tomography has described the multi-domain architecture of thylakoid membranes, which are organized into stromal and stacked grana lamellae (Shimoni et al., 2005; Garab and Mannella, 2008; Mustardy et al., 2008).

Membrane domains can be defined by differences in membrane structure and protein composition. Similarly, both the inner envelope and thylakoid membranes share identical lipid composition and bilayer leaflet distribution (Rawyler et al., 1995; Rawyler et al., 1992; Benning, 2008), but differ in membrane structure and protein composition.

Thylakoid protein translocase expression, localization, and activity determine when and where most photosynthetic proteins integrate into chloroplast inner membranes. Findings that show the photosynthetic apparatus is assembled at inner envelope membranes, and that translocases are needed to insert photosynthetic proteins, together suggest that biogenesis of the photosynthetic apparatus requires protein translocases to be in place at the inner envelope to facilitate thylakoid biogenesis.

The cpSec translocase integrates plastid-encoded thylakoid membrane proteins such as those that are bound by inner envelope membrane-associated mRNA binding proteins, and

cpSRP and Alb3 target and integrate LHCPs into thylakoid membranes in pea chloroplasts (Zhang et al., 2001; Li et al., 1995; Moore et al., 2000). Associations between these translocated substrates and the inner envelope imply that Alb3 and cpSec protein translocases need to be present at inner envelope membranes in advance of thylakoid biogenesis. Interestingly, cpSecY localizes to both thylakoids and inner envelope membranes or cell membranes in cyanelles or cyanobacteria (Yusa et al., 2008; Nakai et al., 1993), and indirect evidence suggests that TatC exists at the cell membrane in cyanobacteria (Spence et al., 2003). The cpTat translocase is comprised of subunits cpTatC, Tha4, and Hcf106, which are all detected in the inner envelope and thylakoid membranes of pea chloroplasts (Fincher et al., 2003). Verifying the localization of thylakoid protein translocases in the envelope membranes of early developing chloroplasts could support the available data that suggest thylakoid biogenesis occurs at the inner envelope. Furthermore, learning how protein translocases are integrated into chloroplast inner membranes will clarify early developmental steps in chloroplasts that precede thylakoid biogenesis.

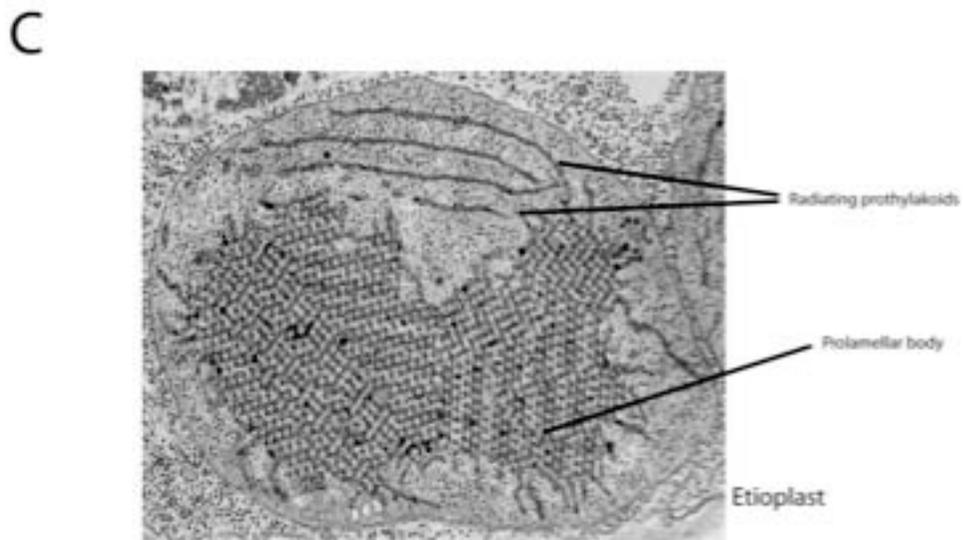
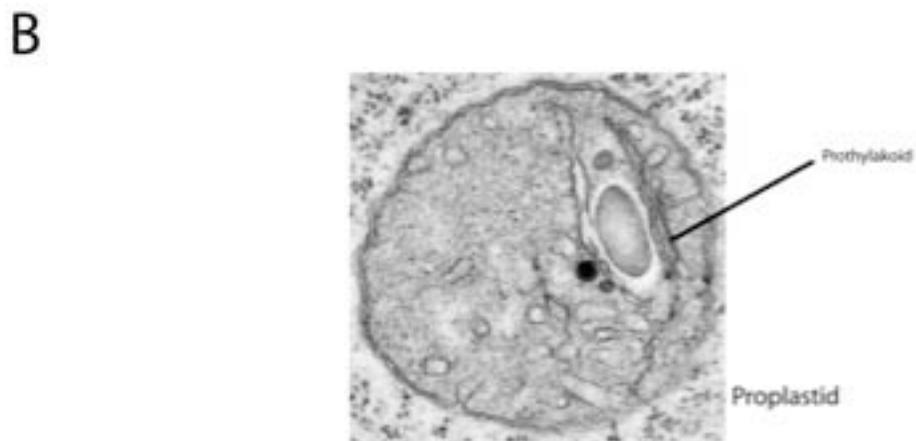
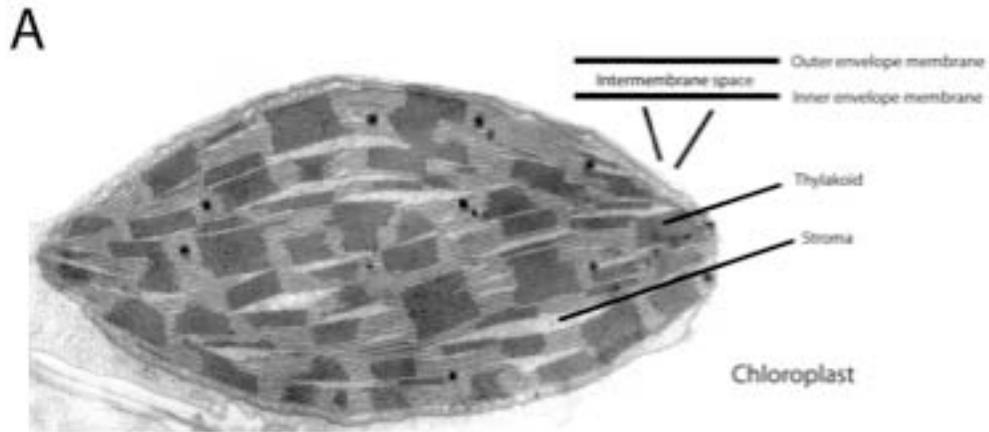


Figure 1-1. Transmission electron micrographs of a chloroplast A), proplastid B), and an etioplast C). Plastid compartments are labeled. The edge of the chloroplast is expanded to illustrate the relative arrangement of the envelope compartments. Images were provided by Dr. Brian Gunning (Gunning, 2003).

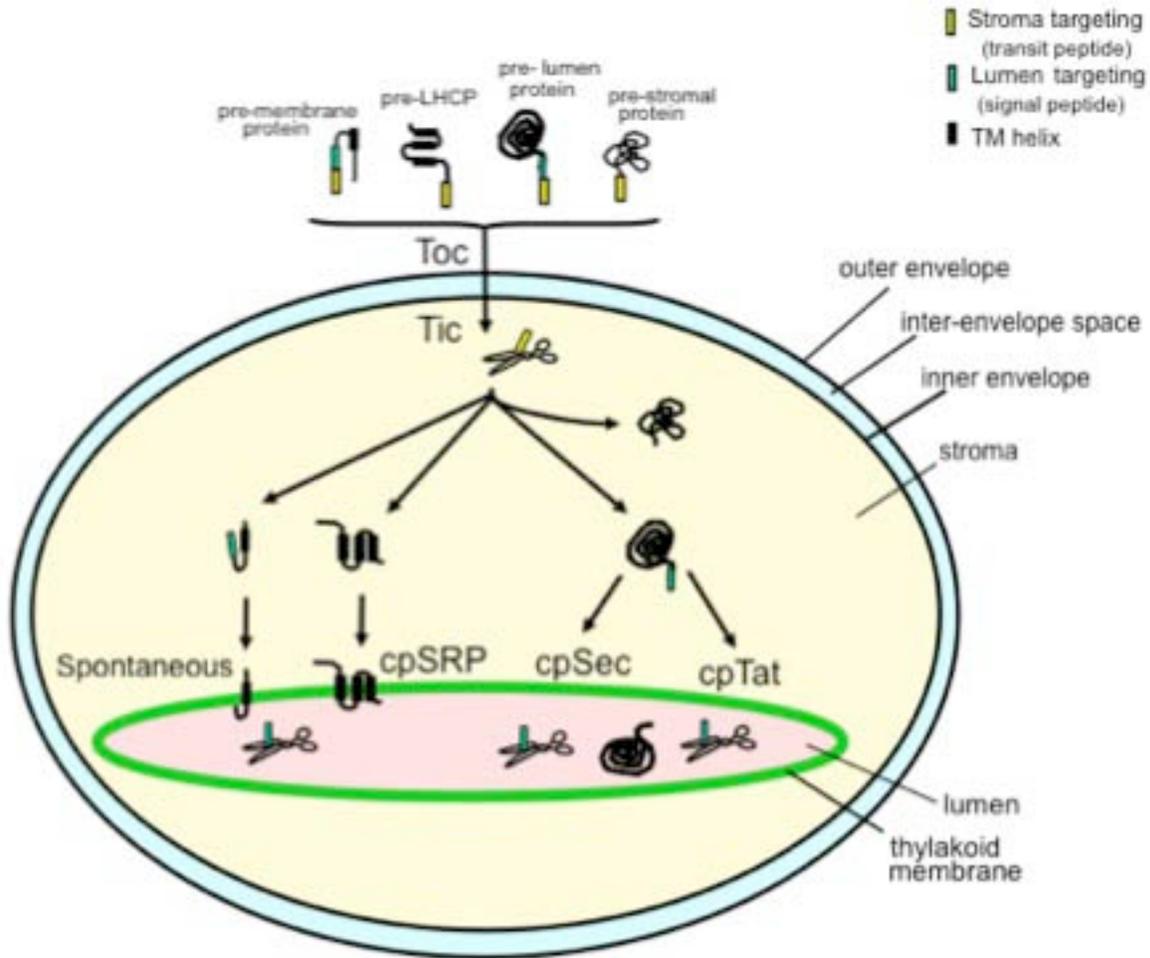


Figure 1-2. Nuclear-encoded thylakoid proteins are targeted to thylakoids by chloroplast protein translocases (model adapted from (Cline and Dabney-Smith, 2008)). Thylakoid precursor proteins are imported into chloroplasts by the envelope-localized TOC/TIC translocases. Once inside the chloroplast, a stromal transit peptidase (scissors in stroma) cleaves the stromal targeting transit peptide, revealing targeting sequences that direct thylakoid precursor proteins to one of several bacterially-conserved protein translocase pathways. Once inserted into thylakoid membranes, or transported into the thylakoid lumen, a lumen-localized peptidase (scissors in lumen) cleaves thylakoid-targeting peptides from thylakoid-targeted proteins.

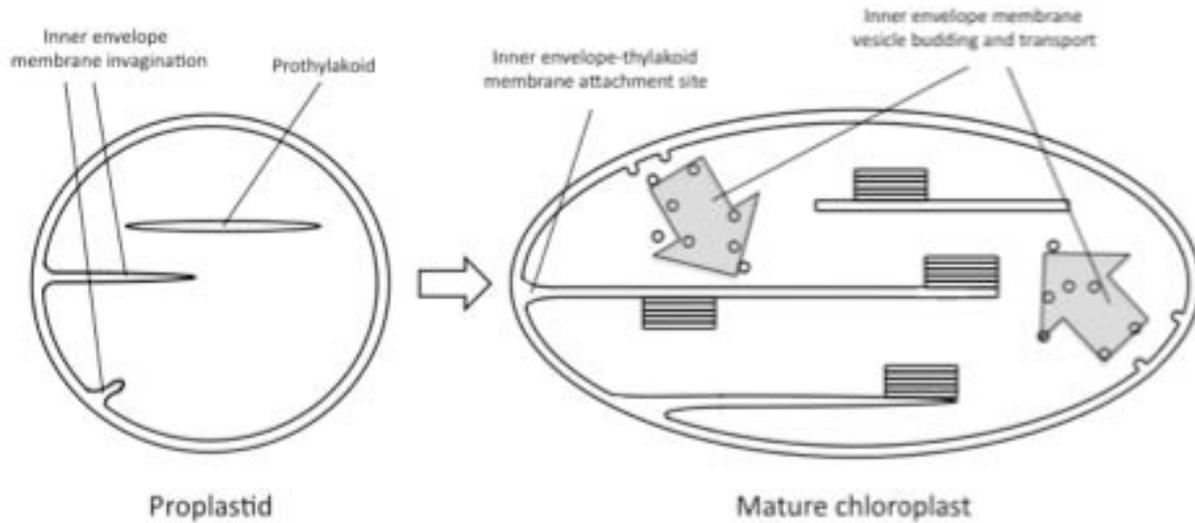


Figure 1-3. Chloroplast inner membrane internalization. The thylakoid membranes of mature chloroplasts are thought to arise through inner envelope membrane invagination and/or vesicle transport between the inner envelope and internal membranes of pre-chloroplasts such as proplastids.

CHAPTER 2
LOCALIZATION AND INTEGRATION OF THYLAKOID PROTEIN TRANSLOCASE
SUBUNIT CPTATC

Introduction

Most of what is known about thylakoid protein translocation comes from studies of developed chloroplasts. Following import into the plastid, all nuclear-encoded thylakoid proteins studied to date are inserted into thylakoids from the plastid stroma by one of four conserved translocation pathways, all of which are evolutionarily derived from a bacterial endosymbiont (Schunemann, 2007). Some membrane proteins integrate into thylakoids by an unassisted or ‘spontaneous’ mechanism. The remaining known membrane proteins and luminal proteins are inserted by translocases. The cpSRP-FtsY-Alb3 translocase integrates a class of light harvesting membrane proteins, the cpSecA-cpSecYE translocase transports globular luminal proteins and integrates some membrane proteins, and the cpTat translocase transports folded luminal proteins and integrates a very limited number of membrane proteins (Schunemann, 2007). The cpSecYE and SRP subunits also appear to co-translationally integrate at least one plastid-encoded membrane protein (Zhang et al., 2001; Nilsson and van Wijk, 2002). Little is known about how membrane-bound translocase subunits integrate into thylakoid membranes. Some translocase subunits, e.g. cpSecE and the cpTat subunits Hcf106 and Tha4, ‘spontaneously’ integrate into the membrane (Steiner et al., 2002; Fincher et al., 2003). In fact, Hcf106 and Tha4 are found in the envelope as well as the thylakoids, as might be expected of proteins that integrate spontaneously. However, the mechanism of integration of the multi-spanning core subunits cpSecY, Alb3, and cpTatC, is completely unknown.

We have begun to examine the targeting pathway of the core subunits. The present work focuses on cpTatC because we have an efficient biochemical assay for its localization. When incubated with intact chloroplasts, pcpTatC is imported, integrated into thylakoids, and

assembled into the cpTat receptor complex (Fincher et al., 2003). In contrast to spontaneously integrating membrane proteins, membrane integration of cpTatC appears to require a translocase because neither the precursor nor the mature form of cpTatC integrates into isolated thylakoids under any experimental conditions examined (Fincher et al., 2003). Here, we present evidence that cpTatC targets to thylakoid membranes via a stromal intermediate, and that cpTatC membrane integration is not altered by competition with precursors of the cpSec and cpTat pathways. Furthermore, cpTatC was found integrated and assembled into the cpTat receptor complex in membranes isolated from Arabidopsis seedlings that carry loss of function mutations in genes encoding Alb3 and cpSecY. These data suggest cpTatC integrates via a translocase other than those already known to exist at thylakoid membranes. The possible involvement of two new candidate translocases will be discussed.

Results

Chloroplast TatC Precursors Possess a Stroma-targeting Transit Peptide

cpTatC is synthesized as a precursor (pcpTatC) with a 50-residue amino-terminal targeting peptide that is cleaved upon import into chloroplasts (Mori et al., 2001). Two experiments were conducted to determine if pcpTatC possesses a stroma-targeting transit peptide. First, radiolabeled pcpTatC was incubated with isolated stroma to determine if its transit peptide is cleaved by the stromal transit peptidase (Richter and Lamppa, 1999). Isolated stroma cleaved pcpTatC to a protein the size of mature cpTatC (mcpTatC), similar to cleavage of the precursor to the small subunit of ribulose biphosphate carboxylase oxygenase (pSSU) to mature size SSU (mSSU) (Figure 2-1A). Reduction in size was due to amino-terminal processing because stroma had no effect on *in vitro* translated mcpTatC. Processing was conducted by the stromal peptidase because ortho-phenanthroline, an inhibitor of the stromal transit peptidase, prevented processing of pcpTatC as well as pSSU (Figure 2-1A).

In a second experiment, the transit peptides and small portions of the mature domain of pSSU and pcpTatC were swapped (Figure 2-1B, 2-2B), and the chimeric precursor proteins were assayed for localization in a chloroplast import assay. As shown in Figure 2-1C, pcpTatC is imported into chloroplasts, processed to mature size, and localized primarily to the membranes. Integration into the membranes is shown by the appearance of characteristic degradation products upon treatment of the membranes with thermolysin (Mori et al., 2001). The same import and localization pattern was obtained for SSUtpcpTatC, which was directed by the known stroma-targeting SSU transit peptide, except that import was more efficient. Similarly, the cpTatC transit peptide directed cpTatCtpSSU import into the stroma, albeit much less efficiently than the homologous transit peptide. These results demonstrate that cpTatC possesses a stroma-targeting transit peptide.

The cpTatC Non-conserved Stromal Domain is not Necessary for Targeting to Thylakoid Membranes or Assembly into the 700 kD cpTat Receptor Complex

Precursors that possess stroma-targeting transit peptides do not necessarily reach the chloroplast stroma. For example, the outer envelope translocase subunit TOC75 also has a cleavable stroma-targeting transit peptide, but a second peptide sequence stops transfer before TOC75 reaches the stroma (Inoue and Keegstra, 2003). The possibility that cpTatC possesses a similar membrane retention domain was examined by looking for sequence elements that might serve as localization signals. Targeting signals are frequently extra peptides that have reduced sequence conservation among orthologous proteins as they do not constitute functional domains of the protein (Bruce, 2001). Mature cpTatC proteins possess long hydrophilic amino-terminal extensions before the first transmembrane domain (Mori et al., 2001), whereas bacterial TatC proteins have short hydrophilic amino termini (Buchanan et al., 2002). Alignment of cpTatC proteins from a variety of plant species revealed a conserved region constituting transmembrane

domains, soluble loops, and 29 residues amino-proximal to the first transmembrane domain. Most sequence divergence was found in regions that represented transit peptides and the first ~50 residues of the amino terminus of the mature protein (Figure 2-2A). To assess its potential role in targeting, the non-conserved region (NC) was deleted from SSUtpcpTatC, producing SSUtp Δ NCcpTatC (Figure 2-2A, 2-2B). This precursor imported into chloroplasts, localized to thylakoids, and produced the same degradation products as wild type pcpTatC (Figure 2-3B). In thylakoids, cpTatC is present in a ~700 kD receptor complex that can be detected by Blue Native PAGE (BN-PAGE) (Cline and Mori, 2001). Imported Δ NCcpTatC also assembled into a large receptor complex, but with a reduced molecular weight (Figure 2-3B). The lower molecular weight would be expected if all of the cpTatC subunits in the complex were Δ NCcpTatC, although that remains to be determined. Treating the digitonin-solubilized membrane extract with Hcf106 antibodies before BN-PAGE caused a band shift to higher molecular weight, verifying that Δ NCcpTatC is assembled with Hcf106 in the cpTat receptor complex (data not shown). Thus, the non-conserved stromal domain of cpTatC is not necessary for targeting of cpTatC to thylakoid membranes or assembly into the 700 kD cpTat receptor complex.

Chloroplast TatC Precursor Import and Protease Accessibility Assays Reveal a Stromal mcpTatC

A small percentage of imported cpTatC is always found in the soluble fraction of chloroplasts recovered from import assays (Figure 2-3B, lane 3). A protease accessibility assay was performed to test whether the soluble cpTatC was stromal or located in the inter-envelope membrane space (Jackson et al., 1998). When incubated with intact chloroplasts, thermolysin digests exposed outer envelope proteins, but it cannot penetrate the outer envelope membrane (Figure 2-4B). Trypsin can cross the outer envelope to access the intermembrane space, allowing digestion of TOC75, but it cannot cross the inner envelope membrane, preventing

digestion of TIC110 (Figure 2-4B). Radiolabeled pcpTatC was imported into chloroplasts and the chloroplasts were then treated with buffer, trypsin, or thermolysin. After treatment, repurified chloroplasts were fractionated into soluble and membrane fractions. The percentage of soluble cpTatC from the trypsin treated chloroplasts was essentially the same as that from the thermolysin treated chloroplasts, indicating that the soluble mcpTatC is located in the stroma rather than the inter-envelope membrane space (Figure 2-4A).

Chloroplast TatC Targets to Thylakoid Membranes Via a Stromal Intermediate

In order to determine whether the stromal mcpTatC is a stromal intermediate or a dead-end import product, a rapid stopping time course of a protein import assay was conducted (Reed et al., 1990). During the first five minutes of import, mcpTatC accumulated in the stroma at a rate similar to import into chloroplasts (Figure 2-5A), after which its accumulation slowed and then declined. Initially, membrane-associated mcpTatC accumulated more slowly, but it continued to accumulate after stromal mcpTatC accumulation leveled off and declined. Similar accumulation kinetics were obtained from three independent experiments (Figure 2-5B). These import kinetics are similar to those exhibited by thylakoid proteins that are known to target via stromal intermediates (Reed et al., 1990; Cline et al., 1992).

An import-chase assay was conducted to determine if stromal cpTatC is the direct precursor to thylakoid-integrated cpTatC (Reed et al., 1990; Li and Schnell, 2006). After a brief period of pSSUtpcpTatC import, all remaining precursor was removed from the chloroplast surface by treatment with thermolysin. Chloroplasts were returned to import reaction conditions, and samples were removed at subsequent time points to follow the sorting of imported protein between the internal chloroplast compartments. pSSUtpcpTatC was used in this experiment instead of pcpTatC, because the SSU transit peptide imparts increased import efficiency upon cpTatC (Figure 2-1C), and produces proportionally more stromal mcpTatC at early time points.

The data show that cpTatC chases from stroma to thylakoids (Figure 2-6A). Similar results were obtained from three independent chase experiments (Figure 2-6B). Taken together, the results of the time course and chase experiments indicate that cpTatC targets to thylakoid membranes via a stromal intermediate.

Chloroplast TatC is Neither Integrated by the cpTat nor the cpSecA/cpSecYE Pathways

Targeting of cpTatC to thylakoid membranes via a stromal intermediate suggests that a conserved thylakoid translocase might serve as the cpTatC integrase (See Discussion). We used *in organello* competition to determine if cpTatC is integrated either by the cpSec or cpTat pathways (Cline et al., 1993). *In organello* competition involves pre-importing chemical quantities of either a cpSec or cpTat pathway precursor into chloroplasts before adding radiolabeled test precursor to the assay. The TOC-TIC import apparatus imports chloroplast precursor proteins much faster than the cpSec or cpTat apparatus can transport them from the stroma, which results in amounts of stromal intermediate that saturates the thylakoid translocases. Radiolabeled stromal intermediate accumulates if both stromal species use the same thylakoid translocase. As shown in Figure 2-7, pre-import of the cpTat precursor pOE23 or the cpSec pathway precursor pOE33 neither caused accumulation of stromal cpTatC nor a decrease in membrane associated cpTatC. The efficacy and specificity of the competition is shown in Figure 2-7B and 2-7C for control proteins: radiolabeled pOE23, which accumulates as intermediate only when competing with pre-imported pOE23, and radiolabeled pOE33, which accumulates as intermediate only after pre-import of pOE33.

Other inhibitors were applied to import assays: 1) azide to inhibit the ATPase domain of cpSecA and 2) ionophores to dissipate the proton gradient, which is required by the cpTat and which stimulates the cpSecA/cpSecYE and SRP/Alb3 pathways. Neither treatment had a significant effect on cpTatC targeting to thylakoids (Figures 2-8, and 2-9).

Neither cpSecY nor Alb3 are Necessary for cpTatC Integration

In bacteria, SecYE and YidC, the bacterial orthologs to cpSecYE and Alb3, operate with other translocase subunits in a modular fashion, e.g. SRP can target precursors to SecYE or YidC, and SecA is not always required to integrate membrane proteins through SecYE (Fandl et al., 1988; Valent et al., 1998; Scotti et al., 1999; Bloois et al., 2004). To more directly determine if Alb3 or cpSecY are necessary to integrate cpTatC, membranes were isolated from Arabidopsis seedlings carrying loss of function mutations in genes that encode either Alb3 or cpSecY (Skalitzky, 2006; Sundberg et al., 1997). Chloroplasts could not be analyzed from mutant seedlings, which contain grossly deformed plastids with few internal membranes. The isolated membranes were tested for the presence, membrane integration, and assembly status of cpTatC. Thylakoids isolated from wild type Arabidopsis were also analyzed to help identify translocase-specific and non-specific bands. As shown in Figure 2-10A, cpTatC was detected in membranes from both mutants. Carbonate did not extract cpTatC from mutant membranes (Figure 2-10C) (Mori et al., 2001), indicating that cpTatC is integrally associated with the membranes. Moreover, cpTatC was found assembled into the 700 kD Tat receptor complex in membranes from mutant seedlings (Figure 2-10B). Samples of membranes from mutant seedlings were also queried using antibodies that detect core translocase subunits Alb3, cpSecY, or Alb4 (Figure 2-10A and 2-10C). Neither Alb3 nor cpSecY were detected in membranes isolated from each respective mutant (Figure 2-10A). The minor band migrating slightly below the Alb3 band in the *alb3* membranes is a non-specific band that is extracted by alkaline carbonate (Figure 2-10C). Interestingly Alb4 and cpSecY were present in the *alb3* membranes and Alb3 and Alb4 were present in the *secy* membranes (Figure 2-10A). Antibodies to Pisum Alb3 were used to detect Alb4 in isolated Arabidopsis membranes because Pisum Alb3 antibodies were found to cross-react with Arabidopsis Alb4 (Figure 2-11). Alkaline carbonate extraction (Figure 2-10C)

verified that Alb3, cpSecY, and Alb4 were integrally associated with the wild-type and mutant membranes, although Alb3 was frequently low in abundance in alkaline extraction experiments with *secy* membranes. We occasionally observed a double banding pattern when detecting cpSecY (Figure 2-10A); the lower band may result from some partial degradation during isolation. In addition to showing that cpTatC does not require either cpSecY or Alb3 for integration, these findings raise important questions regarding the integration machinery for other translocase subunits Alb3, Alb4, and cpSecY.

Discussion

Cavalier-Smith classified cell membranes into two categories: ‘genetic’ and ‘derived’ (Cavalier-Smith, 2000). Genetic membranes always arise by growth and division, and they are continuously maintained through the germ line from cell to daughter cell. Genetic membranes acquire proteins through the action of specific translocases and frequently synthesize their own polar lipids. Derived membranes obtain lipids and proteins from genetic membranes by membrane flow, e.g. vesicles. The endoplasmic membrane and plastid inner envelope membrane are examples of genetic membranes, whereas the tonoplast and Golgi membranes are derived. The thylakoid membrane of plant chloroplasts has features of both genetic and derived membranes. In mature chloroplasts, thylakoids take up their unique complement of proteins through a set of specific translocases (Cline and Theg, 2007; Schunemann, 2007) and thylakoids are passed to daughter chloroplasts by division (Cran and Possingham, 1972). Conversely, during chloroplast biogenesis, thylakoids are thought to arise from the inner envelope by membrane invagination and fission (Carde et al., 1982; Benning et al., 2006; Kobayashi et al., 2007). This poses the question. “At what point do inner plastid envelope invaginations become thylakoids?” The key may lie in determining when, where, and how thylakoid translocases are

inserted into the membranes because they play a central role in determining the protein composition of the thylakoid membrane and lumen.

Chloroplast TatC, SecY, and Alb3 are thylakoid translocase proteins derived from the cyanobacterial progenitor of chloroplasts (Dalbey and Kuhn, 2000). In bacteria, the orthologous proteins are co-translationally integrated into the cytoplasmic or thylakoid membranes by SRP and SecY/E/G and/or YidC, similar to other multi-spanning membrane proteins. Co-translational insertion insures that highly hydrophobic proteins are not exposed to the aqueous environment where they would aggregate. As the chloroplast evolved, genes encoding thylakoid translocases were relocated to the nucleus, precluding a co-translational mode of integration and necessitating alternate strategies to integrate these proteins into the membrane. One strategy might integrate cpTatC into the inner envelope during plastid import by a novel insertion mechanism. Such a strategy is used by a class of mitochondrial inner membrane proteins and at least two chloroplast inner envelope proteins (Brink et al., 1995; Knight and Gray, 1995; Sirrenberg et al., 1996). Membrane flow might relocate cpTatC to thylakoids. Another possibility would be to import cpTatC to the stroma allowing it to enter a conserved, but altered, translocation pathway. The localization of LHC apoproteins is an example of this latter strategy. preLHC apoproteins are imported into the plastid stroma where they bind cpSRP, a modified SRP adapted to post-translational insertion, which targets them to a conserved FtsY receptor before subsequent integration via Alb3.

Our studies show that cpTatC possesses a stromal-targeting transit peptide, appears to lack an envelope retention domain, and proceeds to thylakoids via a stromal intermediate. This suggests that cpTatC is integrated by a conserved translocase, either directly into the thylakoid membrane or, alternatively, into the inner envelope membrane. In that regard, a small amount of

envelope-associated cpTatC was generally detected in protein import assays (Fincher et al., 2003), although it was not technically feasible to determine if envelope-associated cpTatC is an intermediate or an off-pathway product.. At the time of this study, three conserved translocases were known: cpSecA/cpSecY/E, cpSRP/cpFtsY/Alb3, and cpTat, all of which are thylakoid localized in mature chloroplasts (Fincher et al., 2003). Chloroplast SecA is homologous to SecA, an ATP-powered translocation motor required to move large hydrophilic domains across the membrane through a SecYE channel (Andersson and Heijne, 1993). Chloroplast SRP and cpFtsY are involved in targeting precursors to Alb3 (Moore et al., 2000). A number of genetic studies have ruled out certain accessory subunits from being involved in cpTatC integration. The cpTat substrate OE23 was correctly localized to the thylakoid lumen in maize seedlings that lack cpSecA (Voelker et al., 1997). Mature size OE23 was also detected in membranes isolated from Arabidopsis plants that carry loss of function mutations in SRP subunits cpSRP54 and SRP43 (Hutin et al., 2002). The chloroplast Tat pathway was indirectly ruled out by the fact that *in vitro*-integrated Tha4 complemented cpTat transport function of maize thylakoids from a Tha4 null mutant line (Fincher et al., 2003). Our biochemical studies verify that cpTatC does not employ the cpSec and cpTat translocases in the known configurations.

Asakura *et al.* previously detected cpSecY and cpTatC in protein extracts from maize seedlings harboring a loss of function mutation in the gene encoding FtsY; and more recently, cpTatC and cpSecY were detected in protein extracts from Arabidopsis *alb3* and *ftsY* mutants (Asakura et al., 2004; Asakura et al., 2008). We extend these findings by showing that cpTatC is integrated into membranes and assembled into the cpTat receptor complex in the absence of Alb3 or cpSecY. These findings are particularly relevant because in *E. coli*, indirect evidence suggests that TatC is integrated in a SecYE-dependent manner (Yi et al., 2003).

We also found that cpSecY and Alb4 were integrated in membranes from *alb3* mutant seedlings and Alb3 and Alb4 were integrated in membranes from *cpsecy* mutant seedlings. This is contrary to evidence from *E. coli* that suggests the Alb3 homolog, YidC, is integrated in a Sec and SRP dependent manner (Koch et al., 2002). We cannot rule out the possibility that Alb3 alone integrates Alb3 and that cpSecY/E integrates cpSecY; however, it is clear from our results that cpTatC is not integrated by any of the known translocases, implying the existence of an uncharacterized plastid translocase. Although the translocase may be novel, it is more likely to be conserved because cpTatC is derived from the bacterial endosymbiont, which originally integrated this multi-spanning protein from the topologically equivalent (i.e. to stroma) cytoplasm.

Two new conserved candidate cpTatC integrases were recently discovered: Alb4 and SecY2 (Gerdes et al., 2006; Skalitzky, 2006). Alb4 is homologous to Alb3 and is located in the thylakoid membranes in chloroplasts. SecY2 possesses a predicted transit peptide, and when incubated with intact chloroplasts, is imported, processed to a smaller protein, and is recovered with the membrane fraction (unpublished results of the authors). The general importance of Alb4 is unclear as anti-sense RNA and T-DNA insertion lines failed to show a striking phenotype (Gerdes et al., 2006). On the other hand, Arabidopsis plants that are homozygous for loss of function mutations in SecY2 are embryo lethal. In addition, promoter swapping experiments between cpSecY and SecY2 showed that these two proteins perform non-redundant functions (Skalitzky, 2006). Certainly this second plastid SecY is a candidate for insertion/translocation of proteins not accommodated by the known translocases. This arrangement is demonstrated by certain gram positive bacteria which possess an additional SecY that translocates a specific subset of proteins (Siboo et al., 2008). Considering the essential

nature of the plastid SecY2, deciphering its function will require a conditional mutation or an inducible silencing approach.

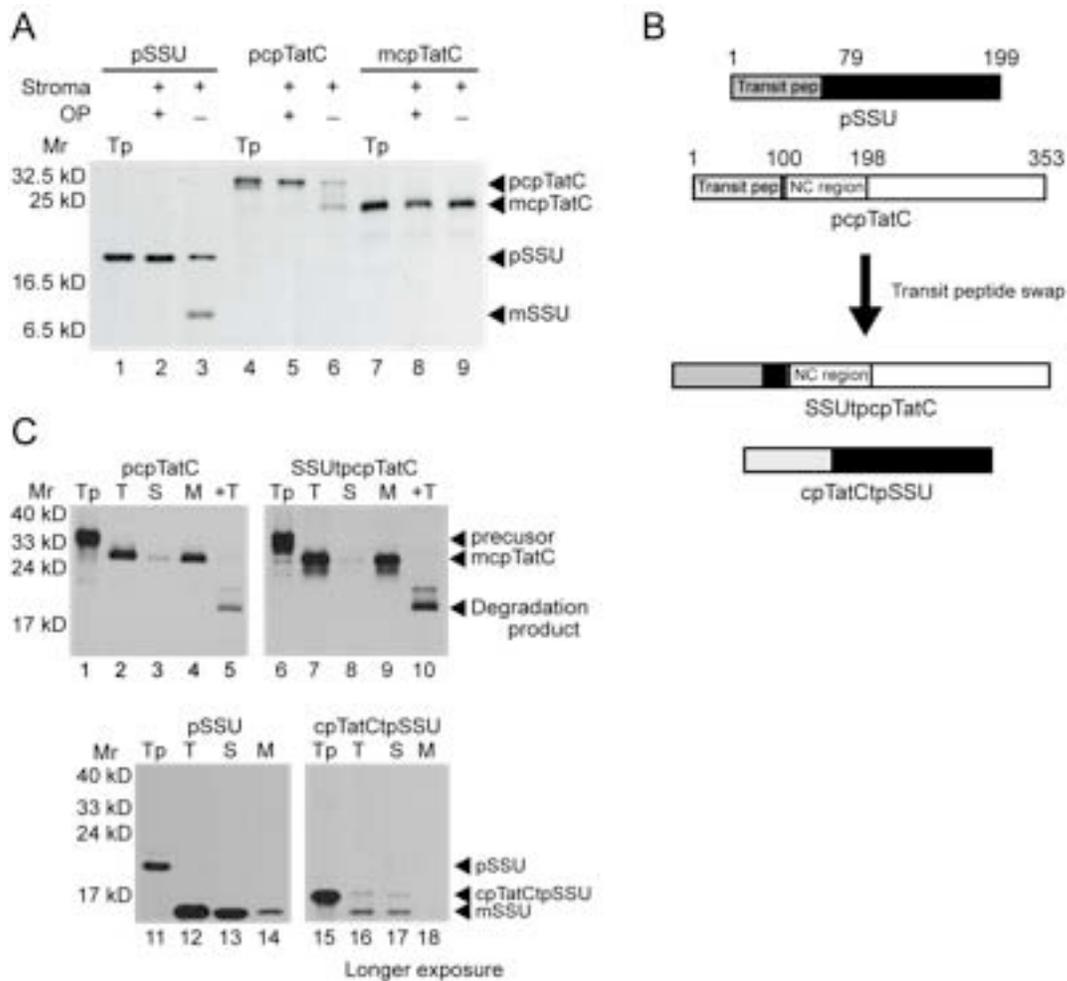


Figure 2-1. Precursor to cpTatC possesses a stromal targeting transit peptide. A) Radiolabeled pSSU, pcpTatC, and mature cpTatC (mcpTatC) were each incubated with isolated stroma and 3 mM PMSF with or without 10 mM 1,10-phenanthroline (OP), as shown above the panel, at 25°C, for 30 minutes. Samples were analyzed using SDS-PAGE and fluorography. B) Scheme illustrating swapping of the pSSU and pcpTatC transit peptides. Numbers above constructs represent the number of amino acids from the translation start site. C) Radiolabeled pcpTatC, SSUtpcpTatC, pSSU, and cpTatCtpSSU were each incubated with chloroplasts in a protein import assay (Experimental Procedures, Appendix) for 20 minutes. Chloroplasts were treated with 100 µg/mL thermolysin and then re-purified, washed, lysed, and fractionated. Translation product (Tp) equivalent to 5% of each assay, chloroplasts (Cp), stroma (S), membranes (M), and thermolysin-treated membranes (+T) were analyzed using SDS-PAGE and fluorography. The positions of molecular weight markers are shown at left.

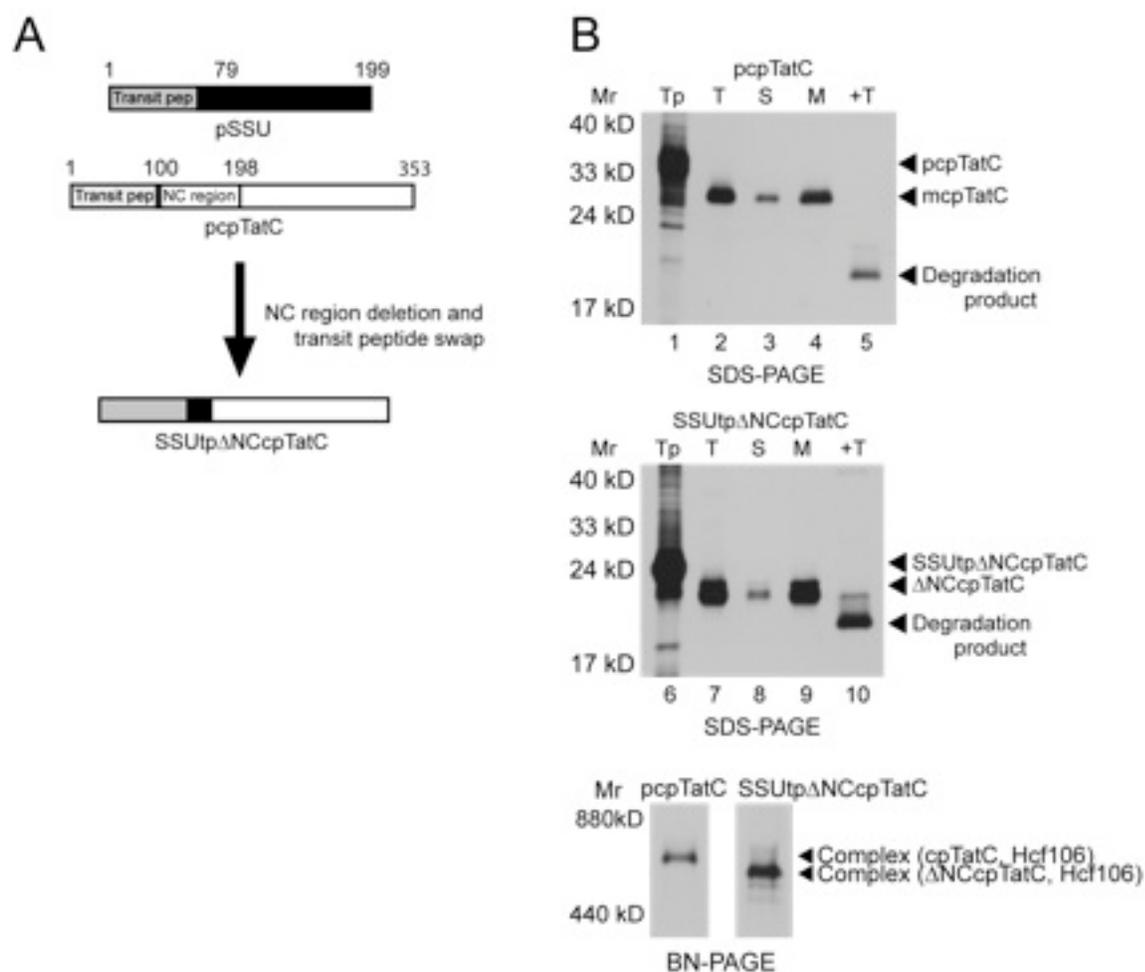


Figure 2-3. The cpTatC non-conserved stromal domain is not necessary for cpTatC localization. A) Scheme illustrating deletion of the cpTatC non-conserved region (NC region). Numbers above constructs represent the number of amino acids from the translation start site. B) Radiolabeled pcpTatC and SSUtpΔNCcpTatC were incubated with chloroplasts in a protein import assay ('Experimental Procedures,' Appendix) for 20 minutes. Chloroplasts were treated with 100 μg/mL thermolysin, re-purified, washed, lysed, and fractionated into stroma and membranes. Translation product (Tp) equivalent to 5% of each assay, chloroplasts (Cp), stroma (S), membranes (M), and thermolysin-treated membranes (+T) were analyzed using SDS-PAGE and fluorography. Membranes were also dissolved in 1% digitonin and analyzed using Blue Native PAGE and fluorography. The positions of molecular weight markers (Mr), or monomeric (440 kD) and dimeric (880 kD) ferritin for BN-PAGE, are shown at left.

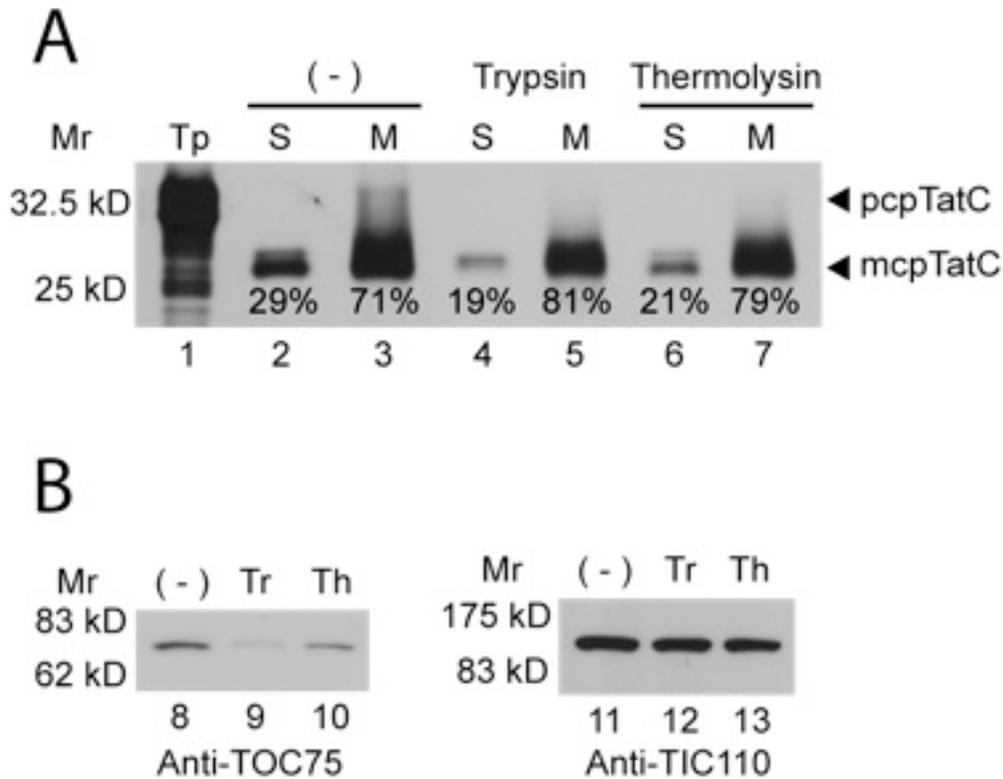


Figure 2-4. Soluble cpTatC is located in the stroma, not the envelope intermembrane space. A) Radiolabeled pcpTatC was incubated with chloroplasts in a protein import assay (Experimental Procedures, Appendix) for 10 minutes. Recovered chloroplasts were treated with IB (-), trypsin, or thermolysin. Proteases were inhibited before chloroplast re-isolation, lysis, and fractionation. Translation product (Tp) equivalent to 5% of each assay, stroma (S), and membrane (M) fractions were analyzed using SDS-PAGE and fluorography. Gel band extraction was used to quantify radiolabeled cpTatC in each chloroplast fraction for each treatment. Numbers below bands represent the relative percentages of cpTatC found in each fraction. B) Chloroplasts from IB- (-), trypsin- (Tr), and thermolysin-treated (Th) samples were also analyzed by SDS-PAGE and immunoblotting with antibodies to TOC75 (applied at 1:2500) or TIC110 (applied at 1:5000). The positions of molecular weight markers (Mr) are shown at left.

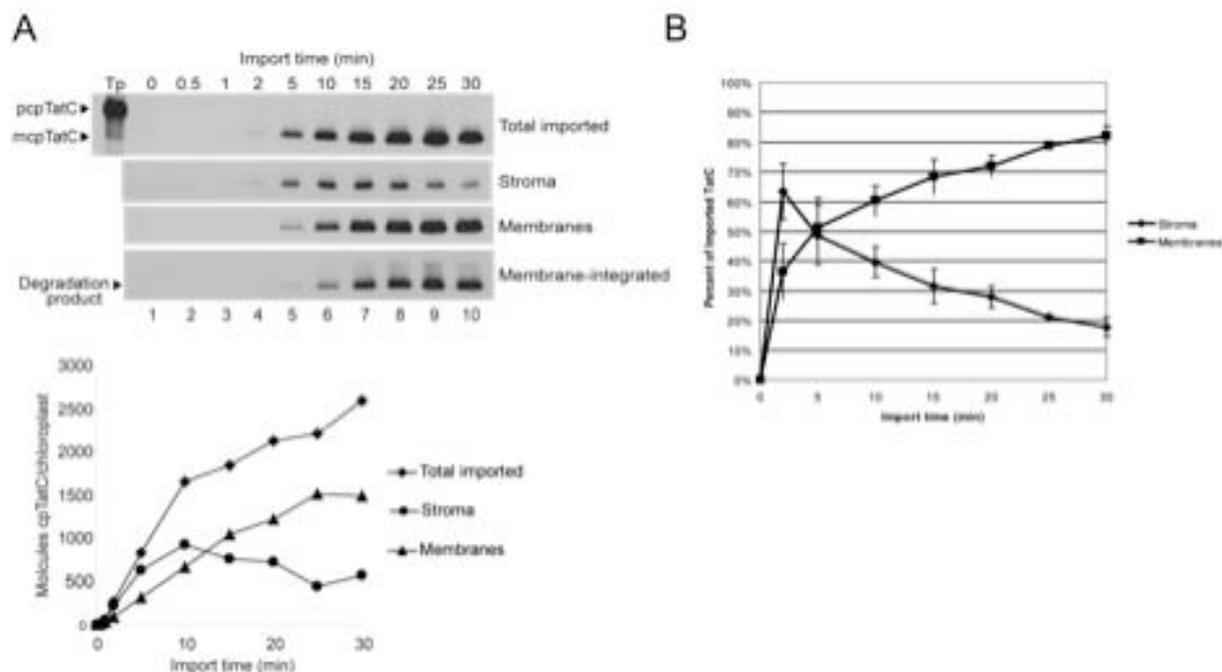


Figure 2-5. Stromal cpTatC behaves like a stromal targeting intermediate. A) Radiolabeled pcpTatC was incubated with chloroplasts in an import time course assay (Experimental Procedures, Appendix). At the times indicated, samples of the import reaction were transferred to 3.3 mM HgCl₂ to terminate import and localization. Chloroplasts in each sample were treated with 100 μg/mL thermolysin, repurified, brought to equal chlorophyll concentration, lysed, and fractionated into membranes and stroma. Translation product (Tp) equivalent to 5% of each assay, chloroplasts (Total imported), stroma, membranes, and thermolysin-treated membranes (Membrane-integrated) were analyzed using SDS-PAGE and fluorography. Gel band extraction was used to quantify radiolabeled cpTatC in each chloroplast fraction at each time point. Molecules of cpTatC per chloroplast estimated from DPMs in bands and chlorophyll content of each sample are plotted for each fraction and time point. B) Three import time course experiments were conducted (see ‘Experimental Procedures,’ Appendix). Radiolabeled cpTatC in each chloroplast fraction was quantified. The average percentages of stroma- and membrane-associated cpTatC relative to total imported cpTatC are plotted for each time point, across three replicate cpTatC import time course experiments.

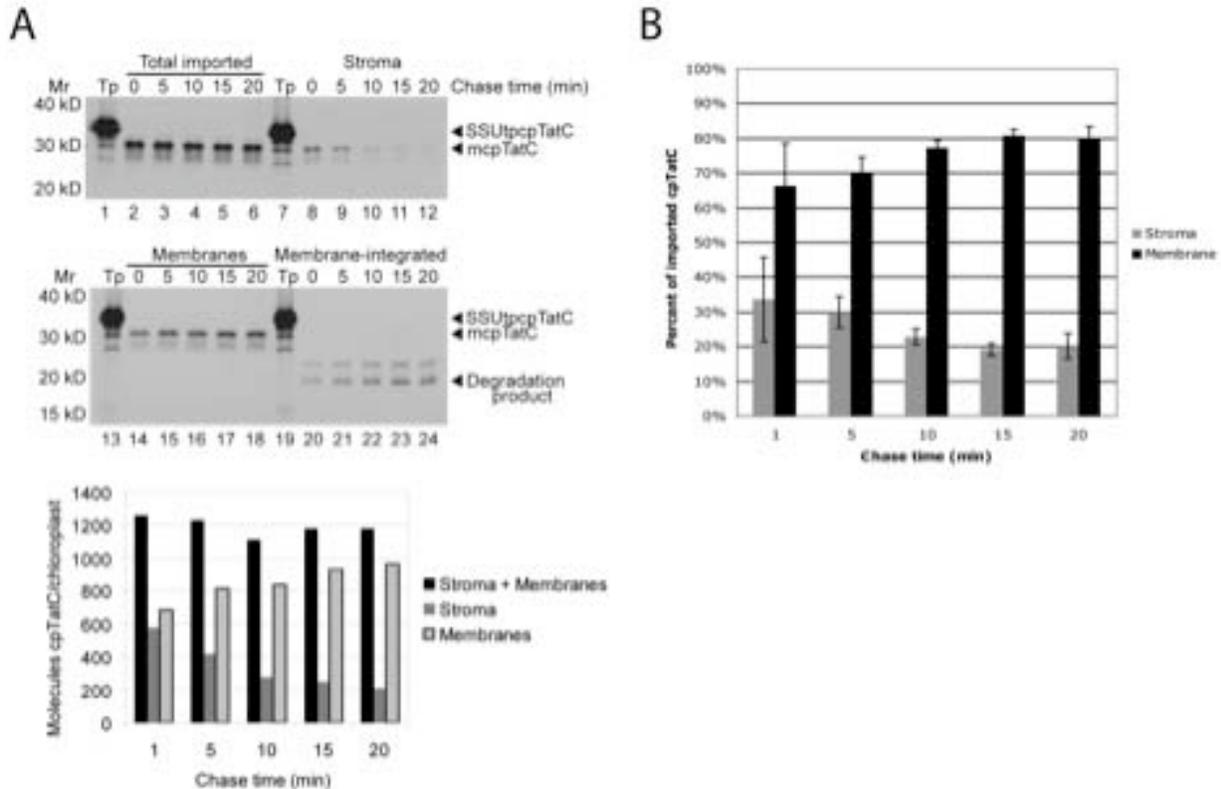


Figure 2-6. Stromal intermediate cpTatC is a direct precursor to thylakoid-integrated cpTatC. A) Chloroplasts and radiolabeled SSUtpcpTatC (Tp) were incubated in an import chase time course assay (Experimental Procedures, Appendix). After 5 minutes of import, chloroplasts were diluted, treated with thermolysin, repurified, resuspended to the original import reaction volume, and placed back in import reaction conditions. At time points, samples of chloroplasts were re-isolated, washed, lysed, and fractionated. Chloroplasts ('Total imported'), stroma, membranes, and thermolysin-treated membranes ('Membrane-integrated') were analyzed using SDS-PAGE and fluorography. The positions of molecular weight markers (Mr) are shown at left. Radiolabeled cpTatC in each chloroplast fraction was quantified from DPMs in bands and chlorophyll content of each sample. The molecules of cpTatC per chloroplast of stroma, membrane, and sum of stroma and membrane cpTatC were plotted for each time point. B) Three import chase time course experiments were conducted (see 'Experimental Procedures,' Appendix). Radiolabeled cpTatC in each chloroplast fraction was quantified. Plotted are the average percentages of stroma- and membrane-associated cpTatC relative to total imported cpTatC at each time point, across three replicate cpTatC import chase time course experiments.

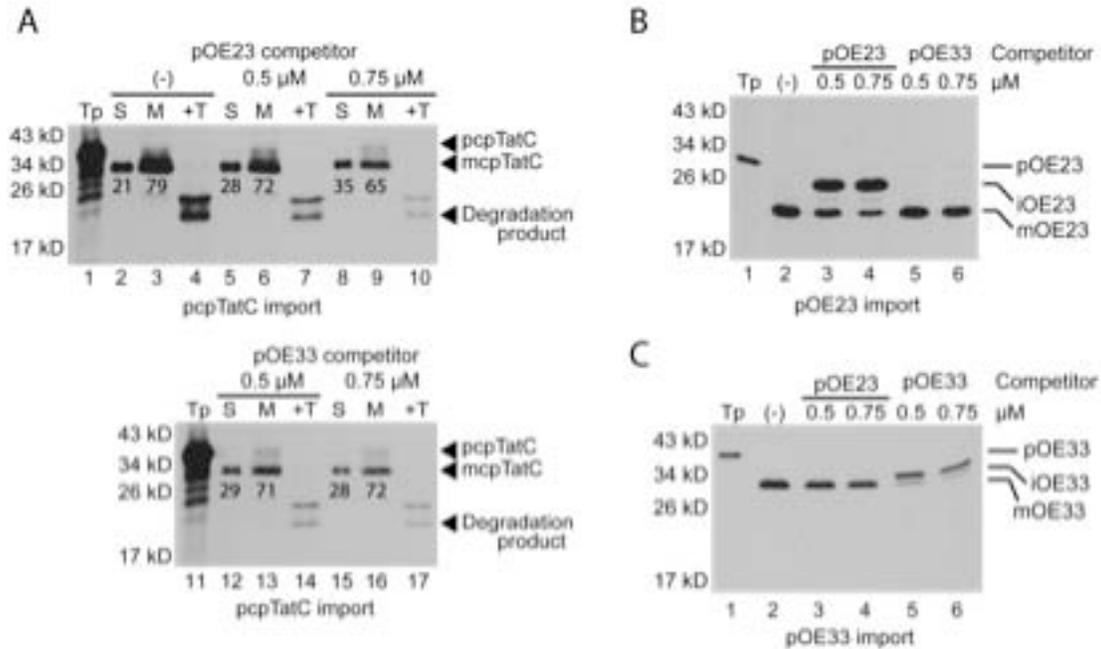


Figure 2-7. *In organello* competition indicates that cpTatC integration does not proceed via the cpTat or cpSec pathways. Chloroplasts were incubated with 5 mM ATP, 1.5 mM DTT, and either 0.3 M urea (-) or unlabeled bacterially expressed pOE23 or pOE33 (competitor) at the final concentrations depicted above the panels for 7 min in the light. Radiolabeled pcpTatC A), pOE23 B), or pOE33 C) was added to the reaction mixtures and the import reaction allowed to continue for an additional 15 minutes. Chloroplasts from pcpTatC reactions were recovered, lysed, and fractionated into stroma (S) and membranes (M). An aliquot of the membrane fraction was treated with thermolysin (+T). Samples were analyzed by SDS-PAGE and fluorography. Chloroplasts from import reactions with radiolabeled pOE23 or pOE33 were recovered and directly analyzed. Gel band extraction was performed to quantify the relative percentages of stromal and membrane-associated cpTatC, which are represented by numbers below the bands in panel A). The positions of molecular weight markers (Mr) are shown at left.

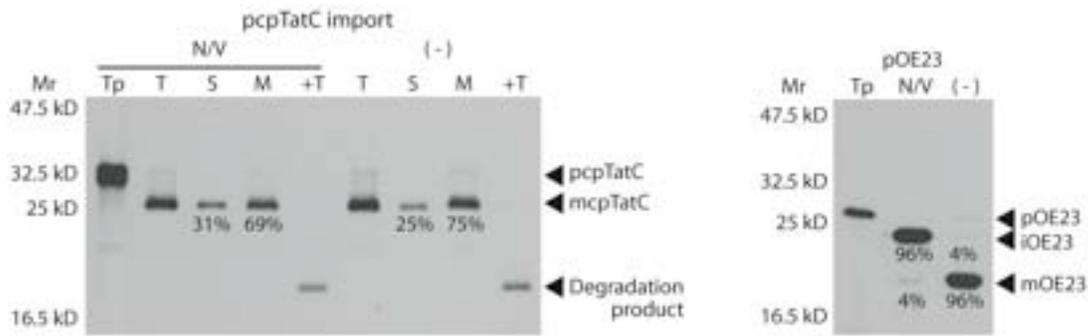


Figure 2-8. pcpTatC targeting is unaffected by protonophores. Radiolabeled pOE23 or pcpTatC was incubated with chloroplasts, that were pretreated with (Nig/Val) or without (-) 0.5 μ M nigericin and 1 μ M valinomycin, in a protein import assay (Supplemental Procedures, Appendix) for 20 minutes. Chloroplasts were treated with 100 μ g/mL thermolysin and repurified. Chloroplasts from pcpTatC import reactions were lysed, and fractionated. Chloroplasts from pOE23 import reactions and total imported cpTatC (T), stroma (S), membrane (M), and thermolysin-treated membrane fractions (+T) were analyzed using SDS-PAGE and fluorography. Gel band extraction was used to quantify radiolabeled intermediate OE23 (iOE23), and mature OE23 (mOE23) and cpTatC (mcpTatC) in each chloroplast fraction for each treatment. Numbers below bands represent the relative percentages of cpTatC or OE23 found in each fraction. Translation products (Tp). The positions of molecular weight markers (Mr) are shown at left.

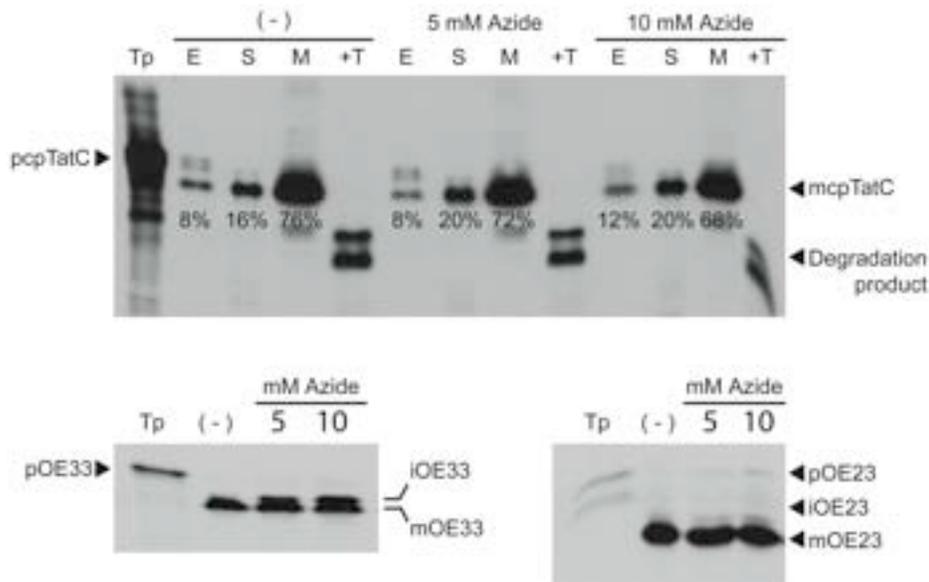


Figure 2-9. pcpTatC targeting is unaffected by azide. Radiolabeled pcpTatC, pOE33, or pOE23 was incubated with chloroplasts, that were pretreated without (-) or with 5 mM or 10 mM sodium azide, in a protein import assay (Experimental Procedures, Appendix) for 15 minutes. Chloroplasts from pcpTatC import reactions were repurified, lysed, and fractionated as described in Experimental Procedures. Chloroplasts from pOE23 and pOE33 import reactions and total imported cpTatC (T), stroma (S), membrane (M), and thermolysin-treated membrane fractions (+T) were analyzed using SDS-PAGE and fluorography. Gel band extraction was used to quantify radiolabeled mature cpTatC (mcpTatC) in each chloroplast fraction for each treatment. Numbers below bands represent the relative percentages of cpTatC found in each fraction. Translation products (Tp).

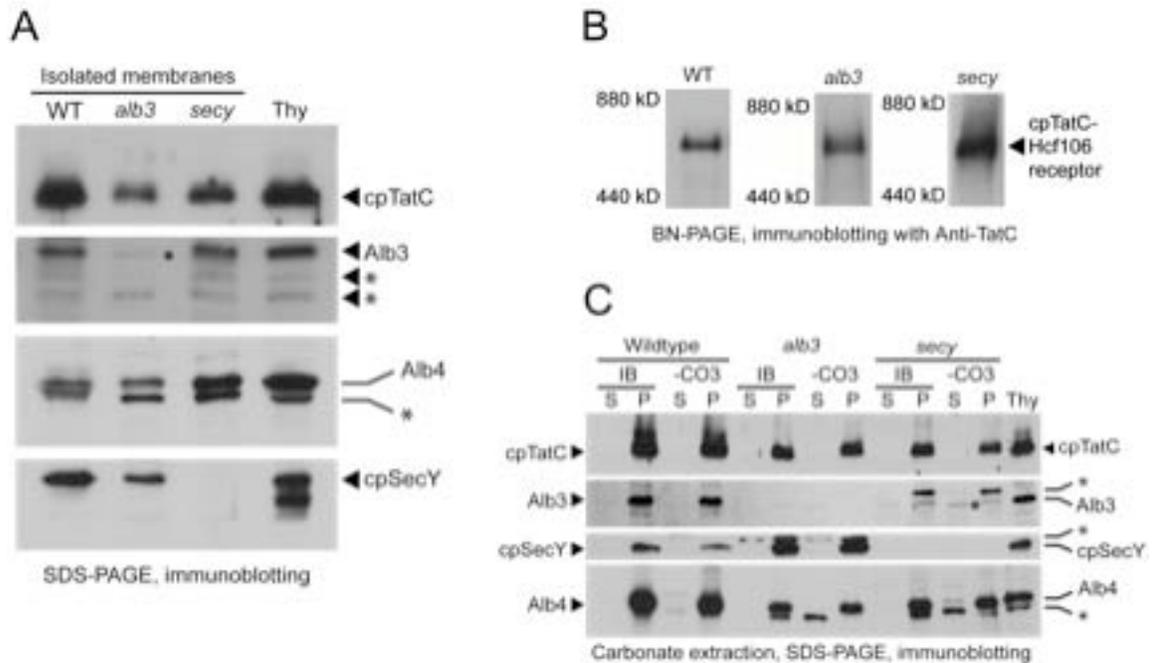


Figure 2-10. Neither Alb3 nor SecY are necessary for cpTatC integration. A) Arabidopsis seedlings were germinated on MS media, grown, and harvested as described (Experimental Procedures, Appendix). Membranes were isolated from seedlings and samples of equal tissue mass were analyzed by SDS-PAGE and immunoblotting. B) Isolated membranes were also dissolved in 2% digitonin and analyzed by BN-PAGE and immunoblotting using antibodies to atcpTatC. C) Membranes were subjected to alkaline carbonate (-CO₃) or mock extraction (IB). Supernatants (S) and membrane pellets (P) from each extraction were analyzed using SDS-PAGE and immunoblotting. Thylakoids (Thy) prepared from isolated Arabidopsis chloroplasts were analyzed in the far right lanes of each SDS-PAGE gel to provide reference bands for the other lanes. (*) Non-specific bands (refer to page 36). All antibodies were applied at 1:5000.

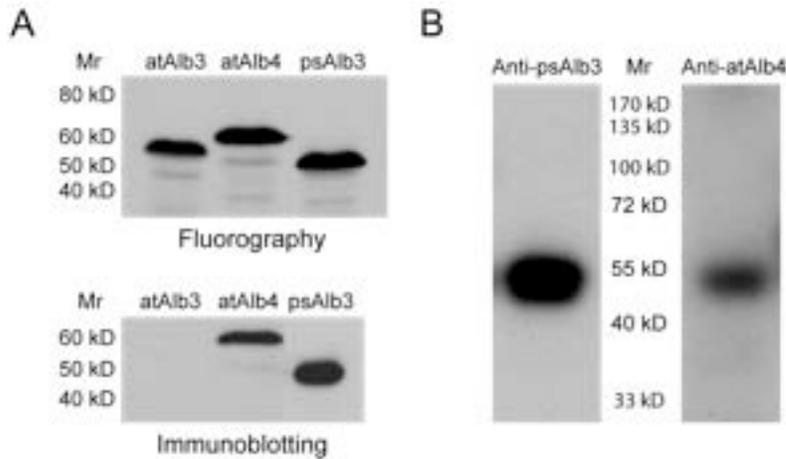


Figure 2-11. Antibodies that detect psAlb3 protein, cross-react with atAlb4, but not atAlb3. A) Radiolabeled precursors to atAlb3, atAlb4, and psAlb3 were translated *in vitro* and analyzed by SDS-PAGE and fluorography or immunoblotting with antibodies to psAlb3 (applied at 1:40000). The positions of molecular weight markers (Mr) are shown at left. B) Isolated Arabidopsis thylakoids were analyzed by SDS-PAGE and immunoblotting with antibodies to psAlb3 (applied at 1:5000) and atAlb4 (applied at 1:5000). The positions of molecular weight markers (Mr) are shown between lanes.

CHAPTER 3 CHLOROPLAST SEC2

Introduction

Conservative sorting is said to occur when bacterially derived chloroplast precursor proteins reach their final localization through mechanisms that evolved from the bacterial endosymbiont (Cline and Henry, 1996). Targeting of cpTatC via a stromal intermediate implies that cpTatC integrates into thylakoid membranes through a conserved thylakoid translocase. Alb3 and cpSecY are conserved thylakoid translocases that integrate membrane proteins. Loss of function mutants for either translocase exhibit the same seedling lethal phenotype as cpTatC mutants, but neither Alb3 nor cpSecY are required for cpTatC integration (Martin et al., 2009). It is possible that cpTatC integrates into thylakoid membranes via some alternative pathway. Alb3, Alb4, and cpSecY might also rely on such a pathway because Alb4 integration does not require Alb3 or cpSecY and neither Alb3 nor cpSecY is required to integrate cpSecY or Alb3, respectively (Martin et al., 2009). It is possible that stromal intermediate cpTatC integrates into thylakoids through one of two recently discovered putative translocases, Alb4 or SecY2 (Accession number NM_128710) (Gerdes et al., 2006; Skalitzky, 2006).

Alb4 was originally reported to be an Alb3-like domain of a larger more complicated chloroplast inner envelope protein called Artemis (Fulgosi et al., 2002). A later study of the Alb3-like domain revealed that it was not a domain of Artemis, but a separate integral thylakoid membrane protein (Gerdes et al., 2006). The authors named the protein Alb4 by virtue of its high protein sequence homology to the thylakoid translocase Alb3. Alb4 and Alb3 are members of a family of membrane protein integrases that includes Oxa1p and YidC from mitochondria and bacteria (Luirink et al., 2001). Similar to chloroplast Alb proteins, two Oxa proteins exist within the mitochondria of plants, fungi, and animals (Funes et al., 2004). Experiments in fungi

suggest that Oxa1p integrates membrane proteins co-translationally, whereas Oxa2 integrates membrane proteins post-translationally (Preuss et al., 2005). Some bacteria also possess two YidC proteins; YidC2 was recently characterized in *Streptococcus mutans* as having a partially redundant function to YidC1 (Hasona et al., 2005; Dong et al., 2008; Funes et al., 2009).

Two Alb proteins are also present in *Chlamydomonas reinhardtii*. The first paralog, Alb3.1, is involved in light-harvesting complex assembly, but it is not required for cell viability. Alb3.2 is necessary for cell viability and is involved in photosystem assembly and/or stability (Gohre et al., 2006). Plant Alb3 is necessary for integration of light harvesting chlorophyll binding proteins into thylakoid membranes, but no Alb4 substrates are currently known (Moore et al., 2000). As is the case for a loss of function mutant of Alb3.2, a plant Alb3 loss of function mutation produces a seedling lethal phenotype (Sundberg et al., 1997). An Alb4 loss of function mutant has not been identified; however, knocking down Arabidopsis Alb4 protein expression to 10% of wild type levels resulted in normal looking plants that exhibited slightly de-stacked thylakoid grana within larger and more spherical chloroplasts (Gerdes et al., 2006). Because homozygous cpTatC mutation produces an albino seedling lethal phenotype, Alb4 appears an unlikely cpTatC integrase (Motohashi et al., 2001). Nevertheless, until an Alb4 knockout mutant is available, Alb4 must be considered a potential cpTatC integrase.

A predicted open reading frame found in the Arabidopsis genome was initially named SECY2 based on sequence homology to CPSECY (Mori and Cline, 2001). Similar to Arabidopsis cpSecY, Arabidopsis SecY2 is predicted to contain ten transmembrane domains and a 34 amino acid chloroplast transit peptide (Hofmann and Stoffel, 1993; Emanuelsson et al., 2000). Screens for embryo lethal mutations in Arabidopsis independently identified putative SECY2 (Skalitzky, 2006). Arabidopsis that harbor a homozygous knockout mutation in SECY2

are embryo lethal due to a developmental arrest at the globular stage of embryo development (Skalitzky, 2006). SecY2 appears to have a function distinguishable from cpSecY because promoter swap, complementation experiments demonstrated that CPSECY and SECY2 are not functionally redundant (Skalitzky, 2006).

The Arabidopsis genome also contains a predicted chloroplast protein that bears 47% identity and 62% similarity to chloroplast SecA (cpSecA). Chloroplast SecA is a soluble ATP powered motor that aids the transport of cpSec substrates through the cpSecYE channel (Mori and Cline, 2001). Similar to cpSecA, SecA2 (Accession number NM_102014) is predicted to possess a 58 amino acid plastid targeting transit peptide (Emanuelsson et al., 2000). Disruption of the SecA2 gene results in an embryo lethal phenotype (Donna Fernandez, personal communication). Arabidopsis SecY2 and SecA2 may represent subunits of a second chloroplast Sec system, cpSec2.

Most of what is known about Sec2 translocases comes from genetic and biochemical studies in gram-positive bacteria. Sec2 translocates large glycosylated cell surface adhesins that serve as virulence factors for several pathogenic bacteria (Bensing et al., 2004; Wu et al., 2007; Mistou et al., 2009; Bensing and Sullam, 2002). Interestingly, a particular cell surface adhesin, GspB of *Streptococcus gordonii*, is translocated by Sec2, but does not possess a signal peptide (Bensing et al., 2004). The lack of a signal peptide or signal anchor for the export of a Sec substrate is unusual (Mori and Ito, 2001) and might signify a mode of translocation that is unique to Sec2. Also interesting are the hypotheses about how Sec2 translocases arose in gram-positive bacteria. In one instance, Wu et al. (2007) observed low G/C content and a putative transposase gene amidst the genes that encode the Sec2 subunits in *Streptococcus parasanguis*. Wu et al.

(2007) interpret that Sec2 arose in *S. parasanguis* through horizontal gene transfer as a part of a gene island from another bacterium.

Similar to bacterial Sec translocases, the Sec translocase of chloroplasts includes Y, E, and A subunits (Mori and Ito, 2001). Unfolded thylakoid Sec substrates proceed through a membrane integrated cpSecYE channel with assistance from a soluble cpSecA subunit (Schunemann, 2007). The essential nature of Arabidopsis (*at-*) SecY2 and SecA2 for embryo development makes them candidate cpTatC integrases and possibly responsible for other essential plastid functions. However, first it was important to obtain experimental evidence for their localization to plastids. Here, I present biochemical evidence for plastid localization of both SecY2 and SecA2 from Arabidopsis.

Results

Arabidopsis SecY2 is a Membrane Integrated Plastid Protein that can be Found in Envelope Membranes

To determine if SecY2 is capable of localizing to plastids, *in vitro* translated radiolabeled precursor to Arabidopsis SecY2 (pcpSecY2) was incubated with isolated pea chloroplasts in an *in vitro* import assay. Radiolabeled pcpSecY2 was imported into isolated pea chloroplasts, processed to a mature 48.3 kD size, and localized to membranes (Figure 3-1A, lanes 2 and 4). Carbonate extracted membranes retained cpSecY2, indicating that cpSecY2 is integrated into the membrane (Figure 3-1B). Protease treating membranes produced two degradation products that measured 24.1 kD and 18.2 kD in size (Figure 3-1A, +T long exposure). Degradation products are expected from protease treatment of membrane-integrated proteins that possess both exposed and membrane protected regions (Mori et al., 2001). Together, these results represent one piece of evidence that shows Arabidopsis SecY2 is a membrane-integrated plastid protein.

In lanes 2 and 4 of Figure 3-1, and later in Figure 3-2 (compare lanes 2 and 6 to lane 8), total imported and envelope cpSecY2 migrates as smaller proteins than does thylakoid cpSecY2. Rubisco is a highly abundant 49 kD protein that could interfere with the migration of a less abundant 48.5 kD protein such as cpSecY2. The differences in cpSecY2 migration seen in chloroplasts and stroma, versus what is observed in membranes, might be due to the fact that chloroplasts and stroma both contain Rubisco whereas membranes are mostly free of Rubisco.

Although cpSecY localizes to membranes in plant plastids, SecY is found in both the cell and thylakoid membranes of cyanobacteria, and in the envelope and thylakoid membranes of cyanelles (Nakai et al., 1993; Laidler et al., 1995; Yusa et al., 2008). As such, cpSecY2 localization was studied to address the possibility that cpSecY2 associates with envelope membranes. Chloroplast SecY2 localization was compared to that of a radiolabeled inner envelope phosphate translocator (P36), the precursor of which was co-imported with pcpSecY2 (Block et al., 1983; Flugge and Heldt, 1979) (Figure 3-2). Fractionation quality was judged by the relative amounts of envelope and thylakoid marker proteins, TIC110 and cpTatC, that could be found in each respective fraction. The envelope fraction was free of thylakoid contamination because cpTatC was only found in the thylakoid fraction. However, fractionation was not complete, because a small amount of TIC110 was found in the thylakoid fraction (Figure 3-2, lower panels). Conversely, P36 was found in the thylakoid fraction, indicating that the thylakoid fraction was partly contaminated with envelope membranes (Figure 3-2, upper panel). If cpSecY2 were localized to thylakoids only, I would have expected a fractionation pattern similar to that of cpTatC. The results of this experiment suggest cpSecY2 that at least some cpSecY2 is localized to envelope membranes.

Arabidopsis SecA2 is a Plastid Protein

In vitro translated radiolabeled precursor to Arabidopsis SecA2 was incubated with isolated pea chloroplasts in an *in vitro* import reaction in order to test if SecA2 is capable of plastid localization. SecA2 was imported into chloroplasts and processed to a mature 116 kD size protein that localized to the stroma (Figure 3-3). Two smaller translation products were also imported into chloroplasts and localized to the stroma (Figure 3-3, 'Truncated mcpSecA2'). These precursors likely arose from premature termination of pcpSecA2 translation because they possessed enough of the pcpSecA2 N-terminus to be imported into chloroplasts.

Discussion

The above experiments provide biochemical evidence that Arabidopsis SecY2 and SecA2 are plastid proteins because *in vitro* chloroplast import assays were previously shown to verify *in vivo* plastid localization (Chua and Schmidt, 1978). However, the results do not rule out the possibility that Arabidopsis cpSecA2 or cpSecY2 are also targeted to mitochondria (Carrie et al., 2009). In future studies, an *in vitro* dual localization assay could address possible mitochondrial localization (Pavlov et al., 2007). Potential localization of Arabidopsis SecY2 to mitochondria might alternatively be tested *in vivo* by isolated Arabidopsis mitochondria.

My data do not indicate whether or not cpSecY2 or cpSecA2 are present in chloroplasts, i.e. as opposed to other types of plastids. Transcription profiling data available from Genevestigator show that cpSecY2 is transcribed predominantly in the shoot tissue, and less so in the roots, whereas cpSecA2 is expressed at low levels in all tissues (Hruz et al., 2008). Thus the expression data suggest cpSecY2 and cpSecA2 are present in chloroplasts.

The membrane and stroma localizations of *in vitro* imported cpSecY2 and cpSecA2 match the localizations of the corresponding cpSec subunits, cpSecY and cpSecA. It is interesting that imported cpSecY2 is found in the envelope membranes. One could interpret that the envelope

membrane is an intermediate location for cpSecY2 that is on pathway for ultimate targeting to thylakoid membranes. Alternatively, if envelope cpSecY2 functions as a translocase to integrate membrane proteins, the envelope membrane might serve as an interesting integration site for translocase proteins such as Alb3, cpSecY, and cpTatC. Instead of direct integration from the stroma to thylakoid membranes, cpTatC might integrate into the envelope membrane, then move to thylakoids through membrane transport. Such an integration pathway might be particularly relevant in proplastids, which contain few thylakoid membranes.

This work makes fundamental contributions to the characterization of an important plastid Sec translocase, but much work remains. For example, no cpSecE2 has been identified. Chloroplast SecE is conserved in bacteria and exists in a complex with cpSecY at thylakoid membranes (Schuenemann et al., 1999). The bacterial homolog is involved in SecA binding to the Sec channel complex, highlighting the importance of this subunit to Sec function (Karamanou et al., 2008). In future studies, mass spectroscopic analysis might identify cpSecE2 if it is co-immunoprecipitated with cpSecY2 antibodies.

Perhaps more difficult than identifying cpSecE2, and necessary to prove that cpSec2 functions as a translocase, is the discovery of a cpSec2 substrate. Substrate-translocase relationships have been previously established by linking the chemical inhibition of substrate translocation to the energetic requirements of putative thylakoid translocases. Such was the case for translocation of OE33 and Plastocyanin (PC), which is prevented by ATPase inhibitor sodium azide (Yuan et al., 1994). The significance of azide inhibition was relevant to a plant homolog of the bacterial SecA translocase subunit, which also has an ATPase domain that is inhibited by azide (Fortin et al., 1990). The role of cpSecA in thylakoid translocation was demonstrated when purified cpSecA protein substituted for stromal extract in facilitating the

transport of cpSec substrates into isolated thylakoid membranes *in vitro* (Yuan et al., 1994). Conversely, cpSecA specific antibodies inhibited translocation of OE33 and PC into isolated thylakoids *in vitro* (Nakai et al., 1994). Similar experiments could be used to test candidate cpSec2 substrates. If azide inhibited the ATPase domain of cpSecA2, cpSec2 substrates could be identified as thylakoid proteins that are not translocated in the presence of azide or inhibitory antibodies to cpSecY2 or cpSecA2, but that are translocated in the presence of inhibitory antibodies to cpSecY or cpSecA (Nakai et al., 1994; Mori et al., 1999). Aside from testing cpSec2 substrates, specific inhibitory antibodies might also be used to test the promiscuity of cpSecY and cpSecY2 for either cpSecA.

Unfortunately, inhibitory antibodies cannot be used to test cpTatC integration via cpSecY2 because cpTatC does not integrate into isolated thylakoid membranes in an *in vitro* assay (Fincher et al., 2003). Interestingly, azide did not prevent cpTatC integration into thylakoids during an *in vitro* import reaction (Martin et al., 2009). If cpSecA2 were not involved in cpTatC integration, cpTatC might still be directly translocated by cpSecY2. Alternatively, cpTatC translocation might still involve cpSecA2 if cpSecA2 were resistant to inhibition by azide, as was observed for spinach cpSecA (Berghofer et al., 1995). In short, the inability of azide to prevent cpTatC integration into thylakoids does not rule out cpSec2 as a possible cpTatC integrase.

The embryo lethal phenotype caused by cpSecY2 knockout prevents assaying mutant tissue for membrane integrated cpTatC, as was previously done to test the necessity of cpSecY and Alb3 to cpTatC integration. Hence, inducible disruption of cpSecY2 is an attractive strategy to assess the necessity of cpSecY2 for cpTatC integration.

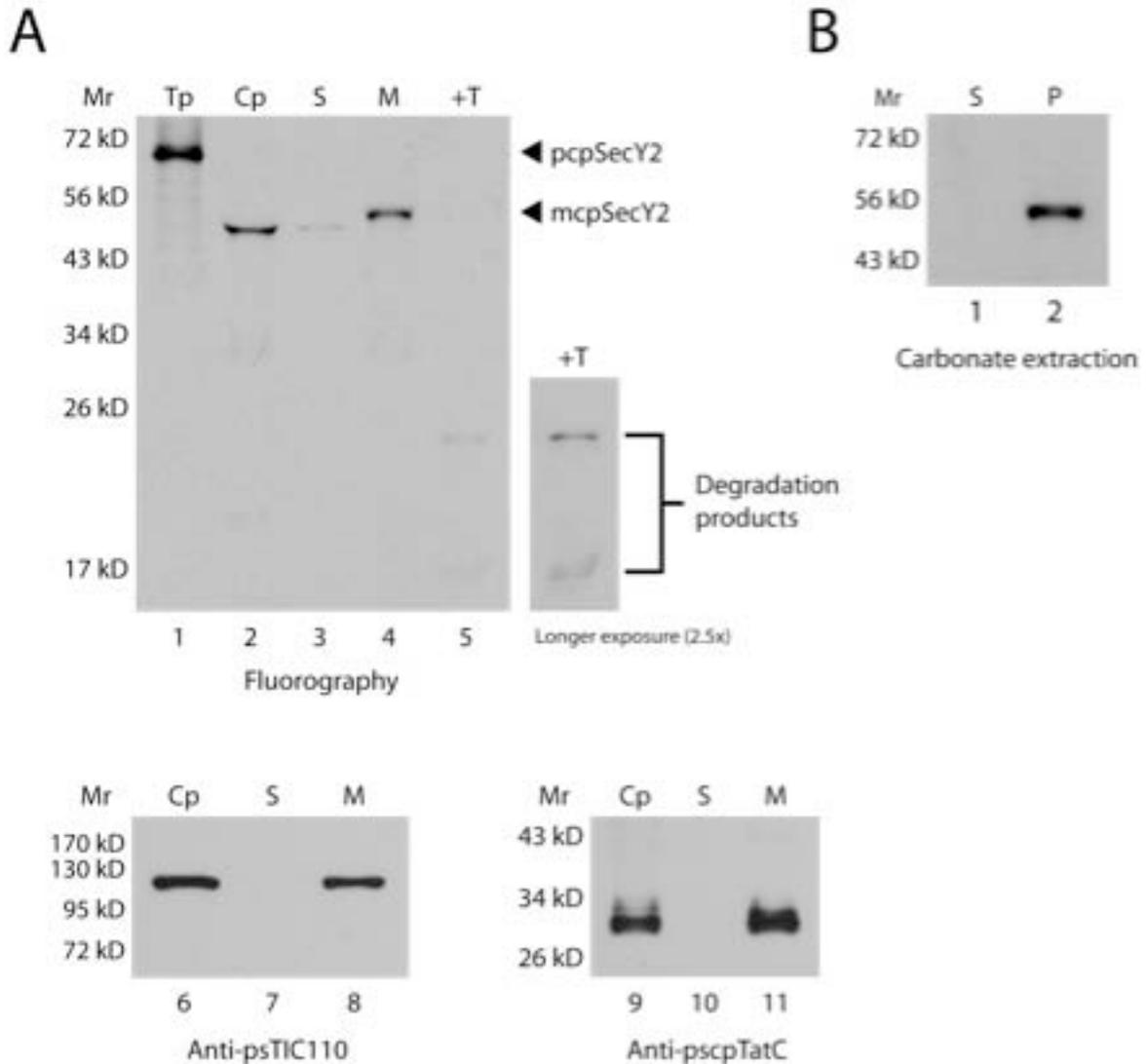


Figure 3-1. Arabidopsis SecY2 is a membrane-integrated plastid protein. A) Radiolabeled precursor to SecY2 (pcpSecY2) was incubated with isolated chloroplasts in an *in vitro* import reaction for 30 minutes (see ‘Experimental Procedures,’ Appendix). Chloroplasts were treated with 100 $\mu\text{g}/\text{mL}$ thermolysin, repurified, washed, lysed, and fractionated into stroma and membranes. Translation product (Tp) equivalent to 5% of the assay, chloroplasts (Cp), stroma (S), membranes (M), and thermolysin-treated membranes (+T) were analyzed using SDS-PAGE and fluorography. A longer exposure of thermolysin-treated membranes is shown to visualize mcpSecY2 degradation products. Chloroplasts, stroma, and membranes were also analyzed by SDS-PAGE and immunodetection with antibodies to TIC110 (applied at 1:5000) or cpTatC (applied at 1:20000). B) Membranes from the pcpSecY2 import reaction were subjected to alkaline carbonate extraction (see ‘Experimental Procedures,’ Appendix). The supernatant (S) and membrane pellet (P) were analyzed using SDS-PAGE and fluorography. The positions of molecular weight markers (Mr) are shown at left.

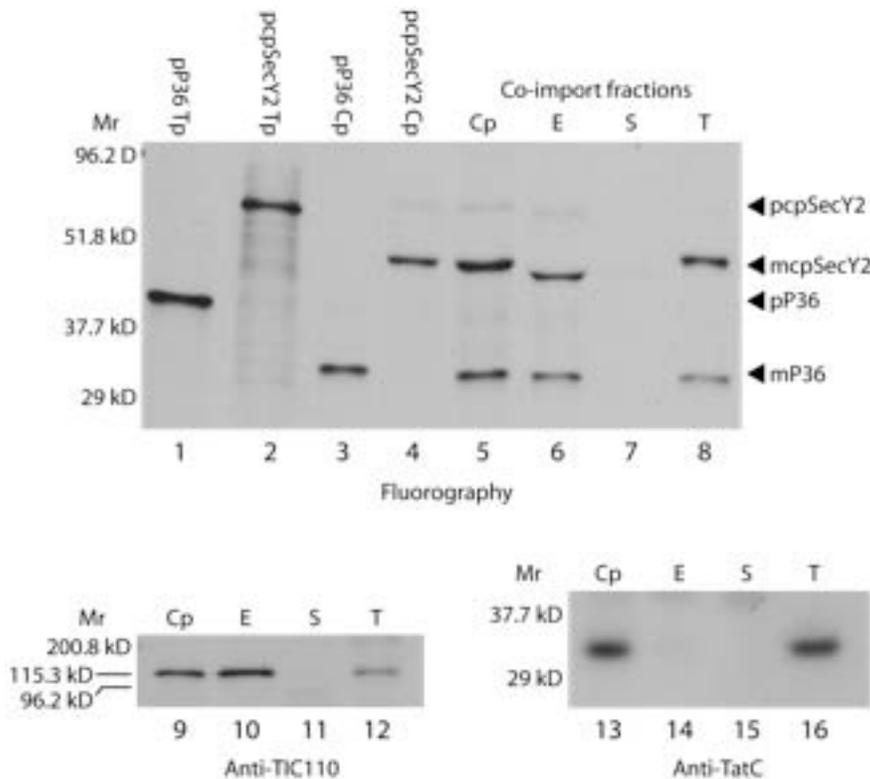


Figure 3-2. Arabidopsis plastid SecY2 localizes to the envelope membranes. Radiolabeled precursors to P36 (pP36) and cpSecY2 (pcpSecY2) were each incubated with isolated chloroplasts in *in vitro* import reactions for 15 minutes (see ‘Experimental Procedures,’ Appendix). Both precursors were also co-imported into isolated chloroplasts for 15 minutes. Chloroplasts from all three import reactions were repurified, and washed. Chloroplasts that underwent co-import were lysed and fractionated (see “Experimental Procedures,” Appendix). Translation products (Tp) equivalent to 5% of each assay, chloroplasts (Cp), envelope membranes (E), stroma (S), and thylakoid membranes (T) were analyzed by SDS-PAGE and fluorography. Chloroplasts and fractions from pcpSecY2/pP36 co-import underwent were analyzed by immunodetection with antibodies to TIC110 (applied at 1:5000) or cpTatC (applied at 1:20000). The weights of molecular markers (Mr) are shown at left.

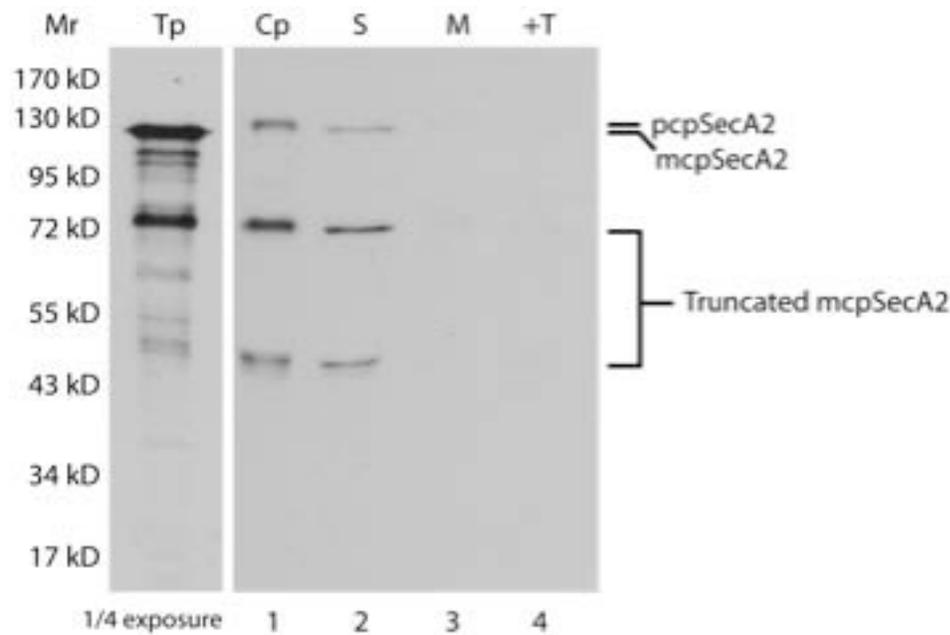


Figure 3-3. Arabidopsis SecA2 is a plastid protein. Radiolabeled precursor to SecA2 (pcpSecA2) was incubated with isolated chloroplasts in an *in vitro* import reaction for 45 minutes (see 'Experimental Procedures,' Appendix). Chloroplasts were treated with 100 $\mu\text{g}/\text{mL}$ thermolysin, repurified, washed, lysed, and fractionated into stroma and membranes. Translation product (Tp) equivalent to 0.8% of the assay, chloroplasts (Cp), stroma (S), membranes (M), and thermolysin-treated membranes (+T) were analyzed using SDS-PAGE and fluorography. Shown are translation products that were exposed for $\frac{1}{4}$ the length of time of chloroplasts and chloroplast fractions. The positions of molecular weight markers (Mr) are shown at left.

CHAPTER 4
TESTING CHROMOPHORE-ASSISTED LIGHT INACTIVATION AS A MEANS TO
DISRUPT THE FUNCTION OF CANDIDATE cpTATC INTEGRASES

Introduction

Chromophore Assisted Light Inactivation (CALI) could potentially provide a spatially and temporally selective means of disrupting protein function *in situ* in chloroplasts isolated from a normally grown plant. CALI involves a modified membrane permeable chromophore that specifically binds to a small tetra-cysteine peptide: ‘-CCPGCC-.’ The excited chromophore is exceptionally efficient at generating oxygen radicals, which chemically disrupt the target protein to which the tetra-Cys tag is genetically fused (Tour et al., 2003).

Here, CALI would be used to disrupt the candidate cpTatC integrase cpSecY2 within isolated chloroplasts *in vitro*. Although Alb4 is also a candidate cpTatC integrase, no known knockout mutants are available. A knockout in the gene that encodes the target protein is needed in order to replace the endogenous gene with a tetra-Cys tagged version. Endogenous replacement ensures that all target proteins possess the tetra-Cys tag, maximizing disruption of the target protein when CALI is applied.

To study the involvement of cpSecY2 in cpTatC integration, chloroplasts would be isolated from transgenic Arabidopsis in which the endogenous cpSecY2 was replaced by a tetra-Cys tagged cpSecY2. After incubating isolated chloroplasts with dye, light would be applied to disrupt tagged cpSecY2. The chloroplasts would then be used for *in vitro* import of radiolabeled precursor to cpTatC. Chloroplast fractions would be analyzed for cpTatC abundance. Accumulation of stromal cpTatC would constitute one line of evidence that cpTatC employs cpSecY2 for integration.

CALI holds the potential to disrupt candidate cpTatC integrases, but it is a relatively new method and is untested in chloroplasts. Furthermore, CALI has never been scaled up to prepare

chemical quantities of a reagent for a biochemical assay. As such, the application of CALI to the study of cpTatC biogenesis requires several feasibility experiments, which were conducted *in vitro* before committing the time and effort needed to replace endogenous cpSecY2 in *Arabidopsis*.

Results

Chloroplast Precursors are Translocated into Isolated Chloroplasts Despite Exposing Chloroplasts to Harsh CALI Conditions

In order to apply CALI to study what role cpSecY2 may play in cpTatC integration, chloroplasts must be able to translocate radiolabeled proteins after having undergone CALI. Under such circumstances, chloroplasts would experience intense prolonged light exposure and extended waiting periods before use in a cpTatC import reaction. Fortunately, exposing isolated chloroplasts to intense light or extended incubation on ice did not prevent thylakoid accumulation of cpSec or cpTat substrates during an *in vitro* import assay (Figure 4-1). The data show that the tested precursors are both imported into chloroplasts and transported into thylakoids. This is shown by relatively even mature protein band intensity across all time points in each treatment series. The even densities seen across the mature protein bands stand in contrast to the accumulation of intermediate sized proteins in samples that were treated with cpSec or cpTat inhibitors: Azide or Nigericin/Valinomycin.

The cpSec and cpTat substrates that each localize to the thylakoid lumen via a stromal intermediate were used in these experiments to test the effects of CALI conditions on thylakoid protein translocation in the context of a chloroplast import assay. The original intent was to use CALI to test effects on cpTatC integration in to thylakoid membranes; however, CALI feasibility experiments were conducted before demonstrating that stromal cpTatC is a direct

precursor to thylakoid-integrated cpTatC. Hence, pcpTatC was not included in the above experiments.

The Tetra-Cys Tag Does not Alter Targeting of Two Chloroplast Precursor Proteins that are to be Used for CALI Feasibility Experiments

Successful application of CALI to this study relies on an ability to bind exogenously applied dye to specific target proteins located within isolated chloroplasts. In an attempt to meet this technical requirement, tandem tetra-Cys (-2TC) tagged precursors to the soluble OE23 (pOE23-2TC) and membrane integrated cpTatC (pcpTatC-2TC) proteins were prepared as targets that could be used to test dye binding *in vitro*. CALI dyes are very expensive. Hence, before applying either protein to dye binding experiments, I tested if pOE23-2TC and pcpTatC-2TC would each target to their native localization within chloroplasts. Correct localization is necessary to demonstrate that CALI dye can cross membrane barriers to access a protein target. Although not a substitute for cpSecY2 import, I also sought data that suggested a -2TC tag would not disrupt the localization of chloroplast precursors.

Precursor to OE23-2TC was expressed in *E. coli*, purified as inclusion bodies, then imported into isolated pea chloroplasts. Antibodies that react with OE23 detected mature OE23-2TC (mOE23-2TC) in thylakoids after import (Figure 4-2A). Mature size OE23-2TC is visible as a processed band running larger than endogenous OE23. Mature size OE23-2TC does not accumulate if import is conducted in the presence nigericin and valinomycin; as expected, only stromal intermediate (iOE23-2TC) is visible. Tandemly tagged cpTatC was found assembled into the 700 kD cpTat receptor complex in thylakoid membranes that were isolated after import (Figure 4-2B). Although tetra-cys tagged cpTatC appeared to accumulate in the cpTat receptor complex at lower efficiency than did the unmodified cpTatC, results from the import of the latter

precursor stood as a particularly encouraging example of a polytopic thylakoid membrane protein whose localization and assembly will accommodate a C-terminal tetra-Cys tag.

Radiolabeled Precursors to cpTatC and Substrates of the Known Thylakoid Translocase Pathways are Translocated into and Targeted to Native Localizations Within Isolated Arabidopsis Chloroplasts In Vitro

Pea chloroplasts are useful for CALI feasibility experiments because they are easy to isolate and exhibit robust translocation activity *in vitro*. Although many CALI feasibility experiments can be conducted using pea chloroplasts, the ultimate CALI experiments require genetic replacement of endogenous cpSecY2 with a tetra-Cys tagged version. Endogenous replacement of cpSecY2 requires rescue of a heterozygous mutant, which can only be done using Arabidopsis, as no cpSecY2 mutant is available in pea.

Controls for the specific effects of CALI in Arabidopsis would involve the import of radiolabeled substrates of non-target thylakoid translocases. Assessing translocation on non-target pathways would speak to the specificity of CALI for cpSecY2. As all of our *in vitro* assays involve pea chloroplasts, I had to learn to isolate and use Arabidopsis chloroplasts in import assays. Large quantities of chloroplasts can be isolated from the leaves of soil grown Arabidopsis plants in a manner similar to that which is used to isolate pea chloroplasts (Schulz et al., 2004); however, these chloroplasts exhibit low protein translocation activity in *in vitro* import assays (data not shown). Arabidopsis chloroplasts that are isolated from young developing tissue exhibit superior translocation activity. Young leaf tissue is harvested from densely arranged populations of seedlings that are grown on sucrose media (Smith et al., 2003). Radiolabeled precursors to cpTatC, LHCP, OE33, and OE23 were each imported into Arabidopsis chloroplasts that were isolated from young leaves. Proper localization of LHCP, OE33, or OE23 was observed as mature size proteins that accumulated in chloroplasts from each import assay (Figure 4-3A, right panel). Following import and chloroplast fractionation,

radiolabeled mature cpTatC was found associated with thylakoid membranes, as expected (Figure 4-3A, left panel). Successful fractionation was evidenced by enrichment of envelope and thylakoid markers, TIC110 and cpSecY, in each respective chloroplast fraction (Figure 4-3B). It was important to demonstrate effective subfractionation of Arabidopsis chloroplasts following *in vitro* import because fractionation would be required to assess any relative changes in the proportions of stromal and thylakoid integrated cpTatC that might accumulate as a result of cpSecY2 disruption.

CALI Dye Will Cross Chloroplast Membranes and Bind a Stromal Target Protein

Precursor to OE23-2TC is a target that is better suited than pcpTatC-2TC for testing *in situ* dye binding because larger amounts of stromal OE23 can be generated within chloroplasts during an *in vitro* import assay. A stromal target is critical to dye binding because binding requires reducing conditions (Cabantous et al., 2005), which are present in the stroma. Large quantities of stromal OE23 accumulate if bacterially expressed pOE23 is applied to a chloroplast import assay (Cline et al., 1993). Stromal OE23 accumulates because expressed protein can be added in large enough quantities to saturate the thylakoid cpTat translocase. Saturation is possible because cpTat transport of intermediate OE23 occurs at a slower rate than import of pOE23 (Cline et al., 1993). The same cannot be done using pcpTatC because, to date, it has not been possible to express pcpTatC in bacteria.

In situ dye binding was tested on chloroplasts containing imported pOE23 or pOE23-2TC. Dye crossed the envelope to bind stromal iOE23-2TC, but not OE23 (Figure 4-4B). Non-specific dye binding to other chloroplast proteins, other than iOE23-2TC, was also visible (Figure 4-4A). The results of the above experiment also suggest that dye will bind a tetra-Cys tag that is fused to the stromal domain of a membrane-integrated thylakoid translocase subunit,

as would be the case for cpSecY2. However, non-specific binding of dye may preclude the application of CALI to experiments involving chloroplasts.

In Vitro CALI

CALI efficacy is typically observed as reduced target protein activity in the context of a micro-scale biochemical assay (Vitriol et al., 2007; Tour et al., 2003). It is not possible to assess cpSecY2 activity using a biochemical assay because cpSecY2 is a newly discovered translocase with no known substrate(s). Instead, I attempted to test CALI efficacy by observing any effect it had on the targeting of pcpTatC to the thylakoid membrane after being imported into isolated chloroplasts *in vitro*. In this type of assay, Precursor to tetra-Cys tagged cpTatC, that was treated with dye and light, targeted to thylakoids slightly less efficiently than precursor that had received dye but not light (Figure 4-5A, compare lanes 10 and 8). The same was not observed for pcpTatC that lacked a tetra-Cys tag (Figure 4-5A, compare lanes 10 and 8 with 5 and 3). Although CALI showed some effect on the localization of the target protein, it was unclear what caused the reduction. Possible side chain modification might be one such mechanism, which might disrupt protein function or translocation without breaking peptide bonds.

Discussion

The results of several feasibility experiments suggest CALI can be applied to *in situ* thylakoid translocase disruption within isolated chloroplasts in advance of a chloroplast protein import assay. Chloroplasts tolerate high light exposure and long periods of handling before use in an *in vitro* import reaction. Tetra-Cys tagged chloroplast precursor proteins import into isolated chloroplasts, and are targeted to their native localization within chloroplasts. CALI dye will cross the chloroplast envelope to bind stromal tetra-Cys tagged proteins. However, non-specific dye binding to non-target chloroplast proteins would complicate the application of CALI for disruption of specific thylakoid translocases (Figure 4-4A). Buffer washes reduced non-

specific dye binding, but did not remove it completely. In the context of a CALI experiment, non-specific binding could negatively affect the potency of light treatments for cpSecY2 disruption, if non-specifically labeled non-target proteins competed for photons that would otherwise disrupt tagged cpSecY2. Non-specific CALI could confuse the cause of potential cpTatC accumulation in the stroma, if it were unclear which chloroplast protein was being disrupted.

Despite the complications that could arise from non-specific dye binding, CALI did appear to have an effect on the targeting of a chloroplast precursor when the precursor was treated with dye and light in advance of import into the chloroplast. However, it is still not known if CALI will specifically disrupt a thylakoid translocase protein *in situ* in isolated chloroplasts. CALI remains an evolving method that can provide the spatiotemporal resolution needed to study the function of necessary proteins such as thylakoid translocases. Perhaps CALI will become useful to study thylakoid translocase function when the above problems are addressed.

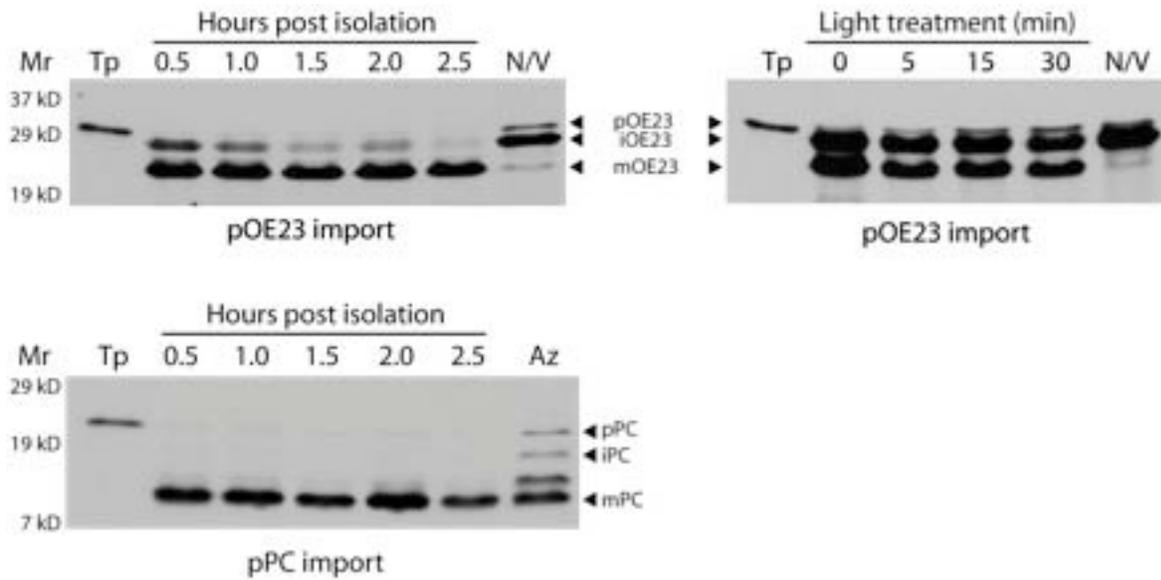


Figure 4-1. *In vitro* import of chloroplast precursor proteins proceeds after chloroplasts are exposed to CALI conditions. Samples of isolated pea chloroplasts were treated with Nigericin/Valinomycin (N/V), Azide (Az), extended exposure to 17 mW/cm² of 570nm light, or allowed to incubate on ice for increasing periods of time (see 'Experimental Procedures,' Appendix). Chloroplasts that received extended incubation on ice were incubated with radiolabeled precursors to OE23 (pOE23) or Plastocyanin (pPC) in 20 minute import reactions (see 'Experimental Procedures,' Appendix). pOE23 was incubated with light treated chloroplasts in 20 minute import reactions. After import, chloroplasts were repurified, washed, and analyzed using SDS-PAGE and fluorography. The molecular weights of markers (Mr) are shown at left.

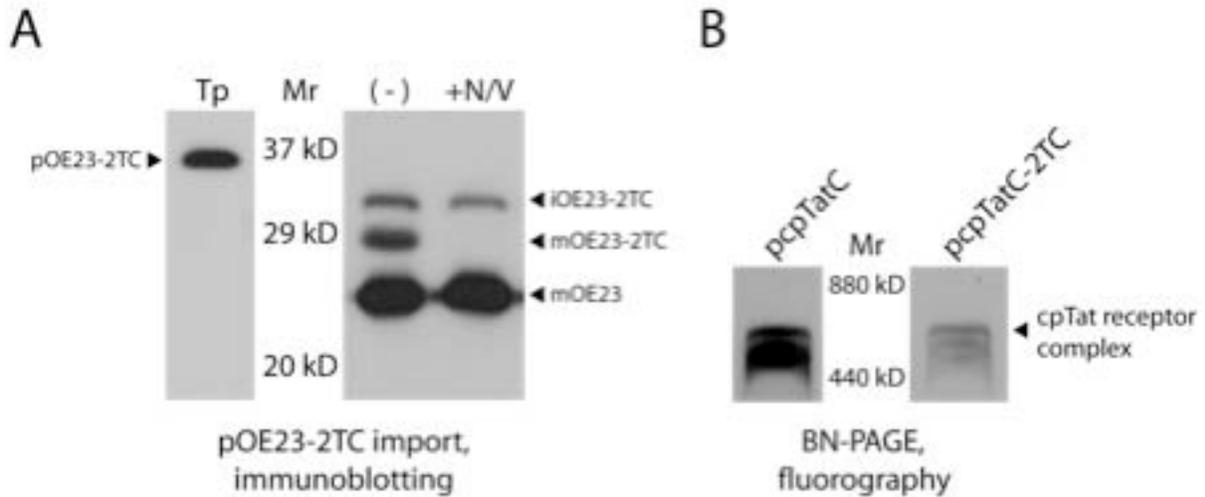


Figure 4-2. Tetra-Cys tagged precursors are targeted to native locations during import into isolated chloroplasts. A) Chloroplasts were treated with nothing or Nigericin/Valinomycin (N/V) on ice for 10 minutes. Tandem tetra-Cys tagged precursor to OE23 (pOE23-2TC) that had been expressed in *E. coli*, was purified as inclusion bodies, and solubilized to 1.5 μ M in 8 M Urea. Soluble pOE23-2TC was imported into untreated and N/V treated chloroplasts for 20 minutes (see 'Experimental Procedures,' Appendix). B) Radiolabeled precursor cpTatC (pcpTatC) or tandem tetra-Cys tagged cpTatC (pcpTatC-2TC) was imported into chloroplasts for 30 minutes. Chloroplasts from (a) and (b) were each treated with 100 μ g/mL thermolysin on ice for 30 minutes, repurified, washed, and fractionated (for pcpTatC import), or analyzed directly using SDS-PAGE (for pOE23 import). Eighty ng of pOE23-2TC (Tp) was analyzed alongside chloroplasts from the pOE23-2TC import reactions. OE23 was visualized by immunodetection using antibodies to OE23 (applied at 1:10000). The membrane fraction of chloroplasts from (b) was analyzed using BN-PAGE and fluorography. The molecular weights of markers (Mr) are shown.

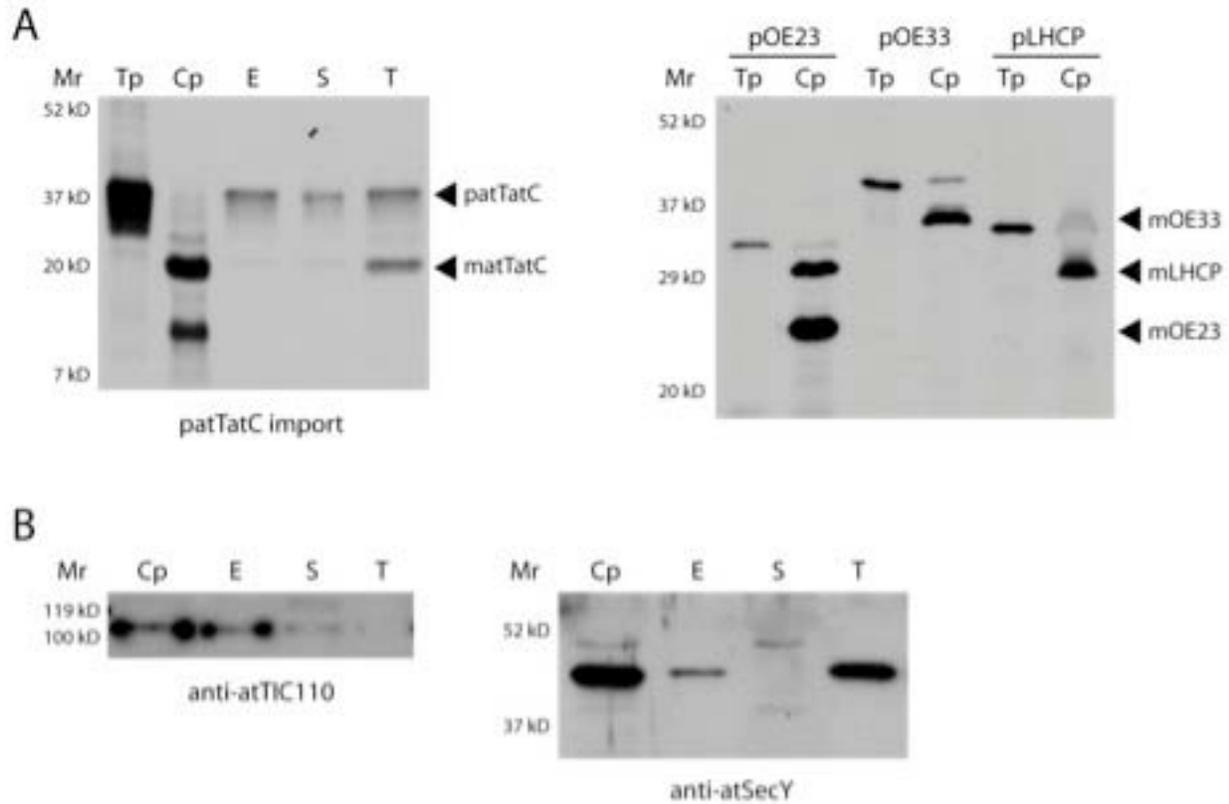


Figure 4-3. *In vitro* import of substrates to various thylakoid translocases and chloroplast fractionation are both feasible when using Arabidopsis chloroplasts. A) Radiolabeled precursors (Tp) to Arabidopsis cpTatC (patTatC) or pea OE23 (pOE23), OE33 (pOE33), or LHCP (pLHCP) were translated *in vitro* then imported into isolated Arabidopsis chloroplasts for 30 minutes (see 'Experimental Procedures,' Appendix). Samples of chloroplasts from patTatC import were fractionated into envelope membranes (E), stroma (S), and thylakoids (T) by differential centrifugation (see 'Experimental Procedures,' Appendix). Chloroplasts (Cp) from each import and chloroplast fractions from patTatC import were analyzed using SDS-PAGE and fluorography. B) Chloroplast fractions from (a) were also subjected to immunoblotting to verify the efficacy of chloroplast sub-fractionation. Gels were blotted to nitrocellulose and probed with antibodies to atTIC110 and atSecY (each applied at 1:5000). The molecular weights of markers (Mr) are shown to the left.

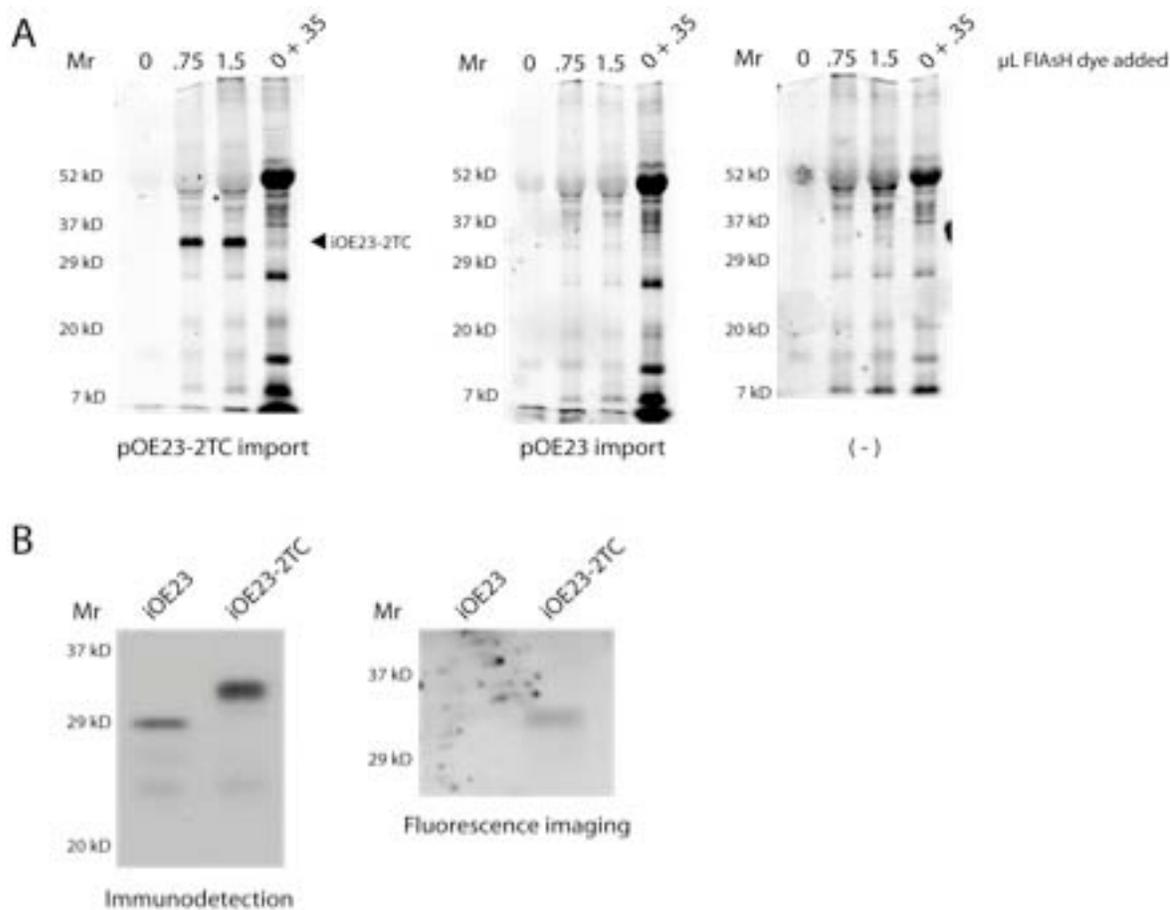


Figure 4-4. CALI dye binds a target protein within intact chloroplasts. A) Urea (-) or urea-solubilized precursors to OE23 (pOE23 import) or tandem tetra-Cys tagged OE23 (pOE23-2TC import) were imported into isolated pea chloroplasts for 20 minutes (see 'Experiment Procedures,' Appendix). Samples of chloroplasts from each import reaction were incubated with 0 μ L, 0.75 μ L, or 1.5 μ L Lumio Green dye. Treated chloroplasts were washed, lysed, and fractionated into stroma and thylakoids. A sample of stroma from the 0 μ L dye binding reaction was combined with 0.35 μ L Lumio Green dye (0 + 0.35). Samples of stroma from each dye treatment were analyzed using SDS-PAGE. Dye labeled proteins were visualized in gels by fluorescence imaging (see 'Experimental Procedures,' Appendix). B) Stromal samples from pOE23 or pOE23-2TC import reactions, that received 1.5 μ L Lumio Green dye, were immunoprecipitated using OE23 antibodies linked to Protein-A-Sepharose (see 'Experimental Procedures,' Appendix). Immunoprecipitated proteins were analyzed by replicate SDS-PAGE gels. The proteins in one gel were visualized by transfer to nitrocellulose and immunodetection using antibodies to OE23 (applied at 1:10000). The second gel was visualized using fluorescence imaging. The molecular weights of markers (Mr) are shown at left.

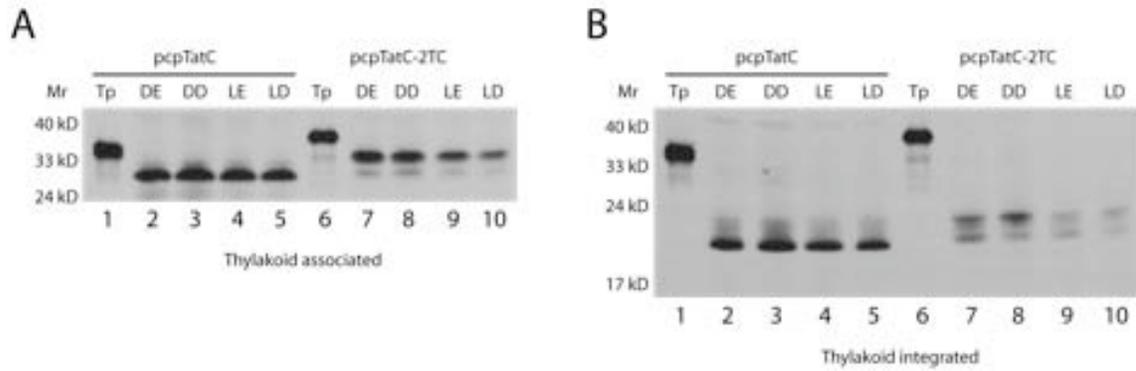


Figure 4-5. *In vitro* CALI. Radiolabeled precursors (Tp) to cpTatC (pcpTatC) and tetra-Cys tagged cpTatC (pcpTatC-2TC) were translated *in vitro* then incubated with Lumio Red dye (-D) or EDT₂ buffer (-E) for 15 minutes (see ‘Experimental Procedures,’ Appendix). Samples were treated with (L-) or without (D-) light, then imported into isolated pea chloroplasts for 20 minutes (see ‘Experimental Procedures,’ Appendix). Chloroplasts were treated with 100 µg/mL thermolysin on ice for 30 minutes, repurified, washed, and fractionated. Membranes were treated (B) or not treated (A) with thermolysin then analyzed using SDS-PAGE and fluorography. The molecular weights of markers (Mr) are shown at left.

CHAPTER 5
INDUCIBLE RNAI AS A MEANS TO KNOCKDOWN THE EXPRESSION OF CANDIDATE
CPTATC INTEGRASES

Introduction

Membranes from cpSecY or Alb3 mutants were previously probed for the presence of cpTatC to test if either translocase is required to integrate cpTatC (Martin et al., 2009). The same approach cannot be used to assess whether Alb4 or cpSecY2 are required to integrate cpTatC because Alb4 knockout mutants are not available and knocking out cpSecY2 is embryo lethal (Skalitzky, 2006; Gerdes et al., 2006). Inducible RNAi is a potential means to silence Alb4 or cpSecY2 expression during any chosen stage of plant development. As such, inducible RNAi could be used to knock down cpSecY2 expression in seedlings that germinate from embryos that have had a chance to develop normally. Alb4 could also be silenced in Arabidopsis seedlings.

Several systems have been developed to induce expression of genes of interest in Arabidopsis. These systems work with inducers such as heat, dexamethasone, alcohol, or estrogen (Zuo et al., 2000; Schena et al., 1991; Roslan et al., 2001; Masclaux et al., 2004). At the outset, heat- or steroid-inducible systems are less applicable to a study of cpTatC integration due to the concern that either inducer alone could incite a phenotype in wild type Arabidopsis seedlings (Donna Fernandez, personal communication). For this project, I would need to be confident that the phenotype arising from induction was due to RNAi of a target translocase and not to the inducer itself. The alcohol inducible system is unattractive because it is leaky in plants that are grown on sucrose media (van Hoewyk, personal communication) . Hence, leaky silencing could kill transgenic T1 seedlings during selection on sucrose media, preventing the capture of lines that silence CPSECY2 most effectively.

The estrogen-inducible expression system lacks the above problems: estrogen neither causes aberrant phenotypes when applied to wild type plants, nor does it exhibit leaky expression in plants due to culture on sterile media (Zuo et al., 2006; Zuo et al., 2000). The estrogen inducible system works as follows. In the presence of estrogen, a constitutively expressed XVE transcription factor binds a promoter to trigger expression of an open reading frame of interest (Zuo et al., 2000). The estrogen inducible system can be employed for RNAi by inserting a hairpin construct made up of sequence that is homologous to a target gene of interest (Hirano et al., 2008). The hairpin is inserted downstream of the estrogen inducible promoter.

Hairpin constructs to cpSecY, Alb4, or cpSecY2 would be prepared to silence each respective gene through estrogen inducible RNAi in Arabidopsis. Chloroplast SecY serves as a positive control for the efficacy of inducible silencing because cpSecY mutants exhibit a pale seedling lethal phenotype (Skalitzky, 2006). After Alb4, cpSecY, or cpSecY2 RNAi has been induced by estrogen, membranes could be isolated and probed for the presence of cpTatC. If either Alb4 or cpSecY2 is responsible for cpTatC integration, a reduction in membrane integrated cpTatC may be met with a corresponding increase in soluble cpTatC because soluble cpTatC is a precursor to thylakoid membrane integrated cpTatC, and soluble cpTatC is not degraded by extended incubation in stroma (Figure 2-1A). On the other hand, *in vitro* assays that previously assessed cpTatC targeting in intact chloroplasts were conducted over a period of a few hours, whereas inducible RNAi would be conducted over several days or weeks. As such, non-integrated cpTatC may be degraded instead of accumulated as a soluble protein. The latter would result in reduction in the abundance of membrane-integrated cpTatC without a corresponding increase in the abundance of soluble cpTatC.

Results

Hairpin Sequences were Constructed for Estrogen Inducible RNAi of Alb4, cpSecY, and cpSecY2 in Arabidopsis

RNAi could possibly degrade transcripts to both CPSECY2 and CPSECY if sequence that is used to construct a hairpin for CPSECY2 is highly homologous to CPSECY, and vice versa (Van Houdt et al., 2003). The same is true for ALB4; if the ALB4 hairpin sequence is nearly identical to sequence from ALB3, RNAi could potentially silence both ALB4 and ALB3. Fortunately, sizable portions of ALB4 and CPSECY2 sequence diverge from ALB3 and CPSECY, respectively, allowing large hairpins to be produced without broadening the specificity of RNAi to the transcripts of non-target gene family members. In particular, sequence within the first exon of CPSECY2 is distinct from sequence found within the CPSECY gene (Figure 5-2A and 5-2B). This region of CPSECY2 includes sequence that encodes the transit peptide. Sequence found within the third exon of CPSECY diverges from sequence found within CPSECY2 (Figure 5-2B), and sequence found within the ALB4 3' untranslated region (UTR) diverges from sequence found within ALB3 (Figure 5-2C). Further, the first, third, and last exons of CPSECY2, CPSECY and ALB4, respectively, are not homologous to other genes in Arabidopsis. Not only are the above-described sequences divergent from other Arabidopsis genes, but they are also large; each spans over 500 base pairs. Large hairpin constructs have been shown to be more potent inducers of RNAi in Arabidopsis (Bleys et al., 2006; Wesley et al., 2001).

Hairpin sequences for each target transcript were constructed using Two Fragment Multisite Gateway recombination (Invitrogen, Carlsbad, CA). Exon-intron and exon fragments were initially amplified using PCR primers that were flanked by Gateway recombination sites. Recombination fused each exon-intron with a coresponding inverted exon fragment to construct

an inverted hairpin repeat. Recombination simultaneously cloned each hairpin in the pMDC7 estrogen-inducible binary vector (Figure 5-1A) (Curtis and Grossniklaus, 2003).

Each construct was introduced into *Arabidopsis thaliana* by *Agrobacterium*-mediated floral dip transformation (Zhang et al., 2006). T1 lines carrying estrogen inducible constructs were initially identified based on resistance to hygromycin selection. PCR screening of genomic DNA from hygromycin-resistant seedlings initially involved vector primers that were designed to anneal outside the hairpin construct, but outside primers failed to amplify products (data not shown). This was likely due to complications that arise from trying to replicate a hairpin amplicon by PCR. Therefore, sets of primers designed to anneal to pMDC7 and the intron sequences of each hairpin construct (Figure 5-3A) were used. These produced fragments of expected size from Alb4-, cpSecY-, and cpSecY2-RNAi genomic DNA, which suggested that hairpin constructs for each target successfully integrated into *Arabidopsis* complete with upstream and downstream sequence from the estrogen inducible system (Figure 5-3B and 5-3C). Additionally, Sanger sequencing of each PCR product confirmed sequence identity to the estrogen inducible vector and the corresponding target gene (data not shown). Several lines contained hairpin constructs to each target: four Alb4 RNAi lines, six cpSecY lines, and nine cpSecY2 lines. Once deemed resistant to hygromycin and in possession of hairpin constructs, seedlings were transferred to soil to collect T2 seed.

Estrogen Induces Pale Phenotypes in cpSecY- and cpSecY2-RNAi Lines, but Causes no Change to Alb4-RNAi or Empty Vector Control Lines

Arabidopsis T2 lines that contained an empty estrogen inducible construct or those containing hairpin constructs to Alb4, cpSecY, or cpSecY2 were grown on selective media for two weeks, receiving exogenous 10 μ M estrogen to shoots and roots every day. Alternatively, seedlings were grown on selective media for two weeks then transferred to estrogen media and

grown for two more weeks. Neither induction regiment produced a visible phenotype in cpSecY2-RNAi or Alb4-RNAi plants that was distinct from the phenotype exhibited by control seedlings (data not shown). When germinated directly on estrogen-containing media, lines containing hairpin constructs to cpSecY and cpSecY2 produced a range of phenotypes. After growing on 10 μ M estrogen media for two weeks, cpSecY-RNAi lines bore pale cotyledons and small green true leaves, whereas cpSecY2-RNAi seedlings exhibited bleached or variegated cotyledons and green true leaves (Figure 5-4). Chloroplast SecY-RNAi line number ten (cpSecY-10) was the most responsive cpSecY-RNAi line, showing pale cotyledons when induced by estrogen. Chloroplast SecY2-RNAi line number five (cpSecY2-5) exhibited the strongest responses to inducer: bleached cotyledons with no variegation. Chloroplast SecY2-RNAi line nine (cpSecY2-9) seedlings exhibited bleached or variegated cotyledons; the remainder of cpSecY2-RNAi lines exhibited variegated cotyledons. Chloroplast SecY- and cpSecY2-RNAi seedlings were also stunted compared to the size of empty vector control seedlings (Figure 5-4). ALB4-RNAi seedlings appeared as green as the empty vector plants (data not shown). All pale, bleached, and variegated lines were green and indistinguishable from empty vector seedlings when germinated on media that lacked estrogen (Figure 5-5).

Induced Alb4-, cpSecY-, and cpSecY2-RNAi Lines Experience Transcript Knockdown

Quantitative RT-PCR (qRT-PCR) was used to compare the expression levels of target and non-target transcripts in whole seedlings of induced empty vector control and RNAi lines. Quantitative RT-PCR measured a 20% reduction in ALB4 transcript abundance in each of three lines that contained inducible hairpin constructs to ALB4 (Figure 5-6). In ALB4 RNAi lines, non-target transcripts to CPSECY and CPSECY2 were at least as abundant those in the empty vector control line, and CPTATC transcripts were generally unaffected. Measuring cpSecY

transcript abundance in cpSecY2-RNAi lines and cpSecY2 in cpSecY-RNAi lines served to assess whether RNAi was specific for targeted gene family members. Chloroplast SECY transcript abundance was reduced at least 50% in induced cpSecY-RNAi lines. ALB4 and CPSECY2 transcripts were regularly more abundant than control in cpSecY-RNAi lines, and CPTATC was comparable to that of the control. Chloroplast SECY2 fell 20% to 40% in induced cpSecY2-RNAi lines. In cpSecY2-RNAi lines, transcripts to CPSECY were at least as abundant as those measured in empty vector control, whereas CPTATC transcripts were below that of the control in cpSecY2-RNAi line five (Figure 5-6).

Weak SecY2 transcript knockdown in cpSecY2-RNAi seedlings was unexpected in plants that exhibited pale cotyledons. Apparently weak silencing was probably due to the isolation of RNA for qRT-PCR from both green and pale tissues of induced RNAi seedlings (Figure 5-4). Support for this interpretation came from qRT-PCR analysis of the green leaf tissue of induced cpSecY-10, cpSecY2-5, and cpSecY2-9 seedlings (Figure 5-7). Little or no silencing was evident in these tissues. Therefore, RNAi seedling growth was observed over two weeks to identify a stage showing the maximum phenotypic effects (Figure 5-8). At five to six days post germination, only pale or bleached cotyledons were observed in seedlings from cpSecY- or cpSecY2-RNAi lines, whereas empty vector seedlings were green (Figure 5-9). I also observed that pale true leaves emerged from induced seedlings if they were plated with space left between individual plants (Data not shown). Green true leaves were more common among seedlings that were plated together in dense clumps (Figure 5-8).

CPSECY transcript abundance was reduced to 10% of empty vector when cpSecY-10 seedlings were assayed at six days post germination (Figure 5-10A). Chloroplast SecY2-5 seedlings exhibited a 60% reduction in CPSECY2 transcript levels, effectively doubling the

degree of transcript knockdown when compared to seedlings that were assayed at 14 days post-germination (compare Figures 5-10A and 5-6). Chloroplast SECY transcripts were not reduced in cpSecY2-RNAi lines, nor was CPSECY2 transcript abundance reduced in cpSecY-RNAi seedlings (Figure 5-10A).

It was necessary to monitor the transcript abundance of non-target thylakoid translocases in order to test whether a potential reduction in cpTatC protein levels would be specific to a reduction in the thylakoid translocase that was targeted for RNAi, and not from an indirect effect of silencing a non-target translocase. Because so little is known about cpSecY2 function, it is possible that transcripts of a non-target translocase could be down regulated if the protein they encoded were integrated by cpSecY2. Reducing cpSecY2 expression could result in feedback regulation and reduced transcript abundance of a non-target translocase (Woodson and Chory, 2008). Such a result would prevent me from concluding that cpTatC is integrated by cpSecY2.

CPTATC transcript abundance was unaffected in cpSecY-RNAi seedlings, but it was reduced in both cpSecY2-5 and -9, with lower levels measured in cpSecY2-5 (Figure 5-10B). The abundances of ALB3 and ALB4 transcripts were also reduced in cpSecY2-RNAi lines, with cpSecY2-5 again experiencing a more severe knockdown. Three subunits of the plastid import apparatus were also surveyed. TOC75 transcript abundance was slightly lower than control in cpSecY-10 and cpSecY2-9 lines, whereas cpSecY2-5 experienced a 40% knockdown. Interestingly, transcripts to TIC40 and TIC110 were unaffected in all three lines in which they were at least as abundant as those levels measured in control.

Knocking Down cpSecY or cpSecY2 Transcript Abundance Results in Disruption to Thylakoid Structure

After six days on inductive media, the cotyledons of empty vector control seedlings contained plastids with large starch grains, extensive thylakoids, and numerous grana stacks

(Figure 5-11). Chloroplast SecY2-9 plastids contained fewer thylakoids than control, but not as few as were observed in cpSecY2-5 plastids (Figure 5-11). cpSecY-10 plastids mostly contained stromal vesicles and lacked thylakoid lamellae. In rare cases, cpSecY-10 plastids contained lamellae that spanned the plastid, but no grana were present (data not shown). Chloroplast SecY-10 plastids also contained inclusions that were roughly the same size and shape as starch grains, but they stained differently (Figure 5-11). Reduced numbers of thylakoids seen in cpSecY2- and cpSecY-RNAi lines is similar to what has been observed in the plastids of seedlings harboring knockout mutations in genes that encode core subunits of thylakoid protein translocases such as ALB3, SECY, and TATC (Roy and Barkan, 1998; Motohashi et al., 2001; Sundberg et al., 1997).

Knocking Down cpSecY2 Transcript Abundance Results in Reduced Levels in Plastid Envelope and Thylakoid Localized Protein Translocases

Membranes and soluble proteins that were extracted from each of the estrogen induced lines were probed for the relative abundances of Actin, Hsp70, TOC75, Alb4, cpTatC, cpSecY, TIC110, and TIC40 to assess effects that reducing cpSecY2 expression had on the abundance of target and non-target proteins. Chloroplast SecY2 could not be assessed because antibodies to cpSecY2 are not available. Actin was used as a loading control because it is a soluble non-plastid protein whose abundance might not be affected by reduced cpSecY2 expression. Hsp70 was tested as a chloroplast-specific protein that localizes to the stroma, and would not likely be affected by changes in the expression of chloroplast inner membrane translocase such as cpSecY2. The abundance of Actin, Hsp70, and TOC75 were largely unaffected in induced cpSecY2-5 knockdown seedlings (Figure 5-12). Small fluctuations in the abundance of Alb4 protein could be observed across RNAi lines. Chloroplast TatC protein was decreased in cpSecY2-5, but not in cpSecY2-9. cpSecY protein abundance is almost undetectable in

cpSecY-10 and cpSecY2-5 seedlings, and TIC40 and TIC110 abundances were specifically decreased in cpSecY2-5 RNAi seedlings.

Discussion

In this study, an estrogen-inducible expression system was employed to provide temporal control over targeting Alb4, cpSecY, and cpSecY2 for post-transcriptional gene silencing. An inducible approach was required because no Alb4 knockout mutant yet exists, and knocking out cpSecY2 causes embryo lethality (Gerdes et al., 2006; Skalitzky, 2006). Inducible RNAi was initially sought as a means to follow changes in cpTatC transcript and protein abundance that might arise in response to reductions in Alb4 or cpSecY2 transcript and protein levels. However, inducible RNAi also facilitated my following the expression of other chloroplast protein translocases, both at the transcriptional and protein levels. My attempts were fruitful in so much as I acquired Arabidopsis lines that responded to estrogen induction by knocking down Alb4, cpSecY, and cpSecY2 transcript abundance.

I started by surveying lines of estrogen-treated seedlings for pale or albino phenotypes, which are exhibited by several lines of Arabidopsis that contain knockout mutations in genes that encode thylakoid protein translocases. Alb4 transcript levels were reduced to ~80% of wild type in estrogen-treated Alb4-RNAi seedlings. Alb4-RNAi seedlings were green and grew as wild type. My results reflect those of a previous attempt to knockout Alb4, in which protein levels were reduced to 10% of wild type without producing any visible phenotype (Gerdes et al., 2006). Because I was unable to achieve a better knockdown, and that no Alb4 line appeared pale, I discontinued analysis of Alb4 RNAi lines. Chloroplast SecY knockout mutants are albino (Skalitzky, 2006), whereas inducible knockdown mutants for cpSecY were pale yellow. Although cpSecY2 knockout mutations cause an embryo lethal phenotype, the albino phenotype exhibited by induced cpSecY2-RNAi seedlings was somewhat expected because constitutive

expression of a GFP-tagged cpSecY2 previously produced white sectoring in Arabidopsis shoots (Donna Fernandez, personal communication). White sectoring was thought to arise from cpSecY2 silencing or a dominant negative effect from over-expression of a potentially deleterious cpSecY2 fusion protein (Donna Fernandez, personal communication). Furthermore pale and albino phenotypes in cpSecY- and cpSecY2-RNAi lines were inducible; if not provided with estrogen, seedlings from each RNAi line grew green as wild type.

Chloroplast SecY2-RNAi seedlings appeared to contain large quantities of anthocyanins, which were observed as purple coloration in the aerial portions of seedlings and apparently produced large dark spots in electron micrographs (Figure 5-13 and 5-8). Anthocyanins are synthesized in the cytoplasm before transport to the vacuole, where they accumulate as anthocyanic vesicle inclusions (AVIs) (Davies and K, 2003; S, 2006). At this point, it is unclear why the putative AVIs in cpSecY2-RNAi cotyledon cells are present in the cytoplasm instead of the vacuole. Anthocyanins are produced in response to biotic and abiotic stress, particularly in response to photo-oxidative stress (Chalker-Scott, 1999). Chloroplast SecY-RNAi lines were small and pale without over producing anthocyanins. From the results in this study, it would appear anthocyanin production results from a direct or indirect effect of decreased cpSecY2 expression, and not from general photo-oxidative stress that would presumably also take place in the pale tissues of cpSecY-RNAi seedlings. Aside from anthocyanin accumulation, plastids in cpSecY2-5 cotyledon cells contained large white inclusions, and mostly lacked thylakoid membranes. The plastids of cpSecY-RNAi cotyledon cells contained numerous vesicles and reduced quantities of thylakoids. A lack of thylakoid membranes and/or the accumulation of plastid vesicles are exhibited by plastids from seedlings that harbor mutations to other thylakoid

protein translocases such as ALB3, CPSECY, and TATC (Sundberg et al., 1997; Roy and Barkan, 1998; Motohashi et al., 2001).

Quantitative RT-PCR was first used to measure the abundances of transcripts to cpSecY or cpSecY2 in induced cpSecY- and cpSecY2-RNAi seedlings. I was satisfied to find that cpSecY transcripts were reduced to 10% of wild type, without non-specific silencing of cpSecY2, and that cpSecY2 transcripts could be reduced to about 40% of wild type without any reduction in cpSecY transcript levels. It was somewhat surprising that an albino phenotype could result from an arguably modest reduction of cpSecY2 transcript abundance in induced cpSecY2-RNAi seedlings. Perhaps cpSecY2 is so important that seedlings cannot tolerate more severe reductions in cpSecY2 expression. Alternatively, if cpSecY2 silencing were occurring through partial degradation of the transcript, which could disrupt cpSecY2 translation and protein stability or function, and not complete transcript degradation, my qRT-PCR primers might have amplified such transcript fragments during my qRT-PCR survey. In such a case, qRT-PCR would register truncated cpSecY2 mRNA as expressed transcripts without regard for transcript intactness, which is more relevant to protein synthesis. The latter possibility is supported by the results of maize cpSecY2 gene structure studies and RT-PCR experiments, which revealed a premature stop codon that is in frame with the translation start site (data not shown). Alternative splicing gives rise to a cpSecY2 transcript that lacks the pre-mature stop codon, which suggests that transcript truncation may be a way in which maize cpSecY2 expression is regulated. Unfortunately, I was unable to confirm protein cpSecY2 protein knockdown in RNAi lines because no antibodies are yet available. This is partly due to the fact that cpSecY2 is a newly discovered protein and is still in need of extensive characterization.

With confidence that RNAi of cpSecY or cpSecY2 was inducible and specific, I went on to survey the expression of other nuclear-encoded chloroplast genes on the transcriptional and protein levels. TOC75 or Alb4 protein levels were mostly unaffected by reductions in cpSecY or cpSecY2 transcripts, but cpTatC protein and transcript abundance were both reduced when cpSecY2 transcripts were targeted for RNAi in cpSecY2-5. The specific cause of cpTatC down regulation is unknown, but one could hypothesize that cpTatC and cpSecY2 are co-regulated, and that reducing cpSecY2 expression results in decreased cpTatC expression. Co-regulation could result from cpSecY2 being a cpTatC integrase; in such a case, reducing expression of cpSecY2 could result in feedback inhibition of cpTatC expression. Alternatively, cpTatC expression could be reduced as an indirect effect of cpSecY2 silencing. Perhaps cpSecY2 down regulation disrupts the translocation of a protein that is required for cpTatC gene expression or cpTatC protein integration or stability in the membrane. Although I cannot definitely conclude that cpSecY2 is a cpTatC integrase, the data from my inducible silencing experiments does not rule out cpSecY2 as a candidate cpTatC integrase. On the contrary, my data maintains cpSecY2 as a strong candidate cpTatC integrase.

Aside from my primary interest in cpTatC biogenesis, were interesting reductions in chloroplast proteins in cpSecY2-5 seedlings that could not be explained by reductions in transcript abundance. TIC40, TIC110, and cpSecY fit this category. Although it is interesting to interpret that cpSecY2 integrates TIC110 and TIC40, it is still possible that TIC110, TIC40, and cpSecY integration or membrane stability is indirectly regulated by cpSecY2. TIC110 plays a general role in the import of chloroplast precursor proteins (Inaba et al., 2005). TIC40 links TIC110 to the stromal, inner envelope membrane-associated chloroplast chaperone, Hsp93 (Chou et al., 2003). Although heterozygous TIC110 T-DNA insertion mutants exhibit reduced

import efficiency (Kovacheva et al., 2005), reduced TIC110 protein levels in cpSecY2-RNAi lines are apparently not sufficient to affect the import rates of stromal Hsp70 and thylakoid-integrated Alb4.

The results of *in vitro* import and localization assays place cpSecY2 at the envelope membrane, which is expected for a protein that would integrate TIC40 and TIC110. Involvement in TIC110 and TIC40 integration suggests that the cpSecY2 pore is topologically oriented toward the stroma because both TIC40 and TIC110 move through the stroma en route to the inner envelope membrane (Li and Schnell, 2006; Lubeck et al., 1997). Stroma facing topology would be consistent with other bacterially conserved chloroplast inner membrane protein translocases, including cpSecY. SecY integrates SecY in bacteria (Swidersky et al., 1992), and data here suggests that cpSecY2 is directly or indirectly involved in cpSecY integration or stability. I have observed cpSecY2 to be localized to thylakoid membranes during *in vitro* import assays, but *in vivo* localization data is still needed to examine whether cpSecY2 localizes to both thylakoid and inner envelope membranes. Perhaps after developing cpSecY2 specific antibodies, *in vivo* localization and topology studies could verify the inner envelope localization of cpSecY2 and its possible orientation toward the stroma.

The results of recent investigations into TIC40 and TIC110 biogenesis suggest that TIC40 is partly involved in both TIC40 and TIC110 integration (Chiu and Li, 2008). Evidence comes from the observed buildup of stromal intermediates to TIC110 and TIC40 when radiolabeled precursors to TIC110 and TIC40 are imported into isolated *tic40* mutant chloroplasts *in vitro*. Such experiments showed that TIC40 and TIC110 still integrate into the inner envelope membranes of *tic40* chloroplasts, but that integration is slowed. The authors explain that TIC40 might help TIC40 and TIC110 integration. It would seem that TIC40 is not required for TIC110

insertion because mutant studies have shown that TIC40 mutant seedlings are pale, slow growing, and viable, whereas TIC110 mutants do not advance beyond the globular stage of embryo development (Chou et al., 2003; Kovacheva et al., 2005). If TIC40 were necessary for TIC110 biogenesis, one could expect that TIC40 mutants would also suffer embryo lethal effects. The embryo lethal phenotype of TIC110 mutants is more congruent with the phenotype of cpSecY2 knockout mutations, which are embryo lethal, and also do not develop beyond the globular stage of embryo development (Skalitzky, 2006). In light of the previous TIC110 and TIC40 biogenesis studies and the data presented here, it is tempting to speculate that cpSecY2 is involved in TIC110 and TIC40 integration.

TIC40 integration would not seem to require cpSecA2 because TIC40 integrates without a need for ATP or stroma (Li and Schnell, 2006). TIC110 might require cpSecA2 because TIC110 integration is ATP dependent (Vojta et al., 2007). Chloroplast SecA requires ATP to help transport proteins through the cpSecY channel (Schunemann, 2007). Chloroplast SecA2 is a stromal protein that may function in an ATP-dependent manner similar to cpSecA, but future studies are required to test this hypothesis.

The results of this study are exciting because they suggest that cpSecY2 is a translocase that is involved in the biogenesis of both bacterially conserved and novel chloroplast protein translocases. Such a translocase might have facilitated the biogenesis of novel translocases within the cyanobacterial endosymbiont, which would have served to establish a key biochemical relationship as the endosymbiont developed to become a plastid. Evolution from endosymbiont to chloroplast was accompanied by relocation of genes that encode conserved thylakoid protein translocases to the nucleus. Bacterially conserved chloroplast proteins, including cpSecY2, are expressed from nuclear-encoded genes and rely on novel translocases of

the chloroplast inner and outer envelope for translocation into the chloroplast. Paradoxically, the results of this study suggest that cpSecY2 is a translocase that is both required for and depends on the biogenesis of novel subunits of the chloroplast import apparatus.

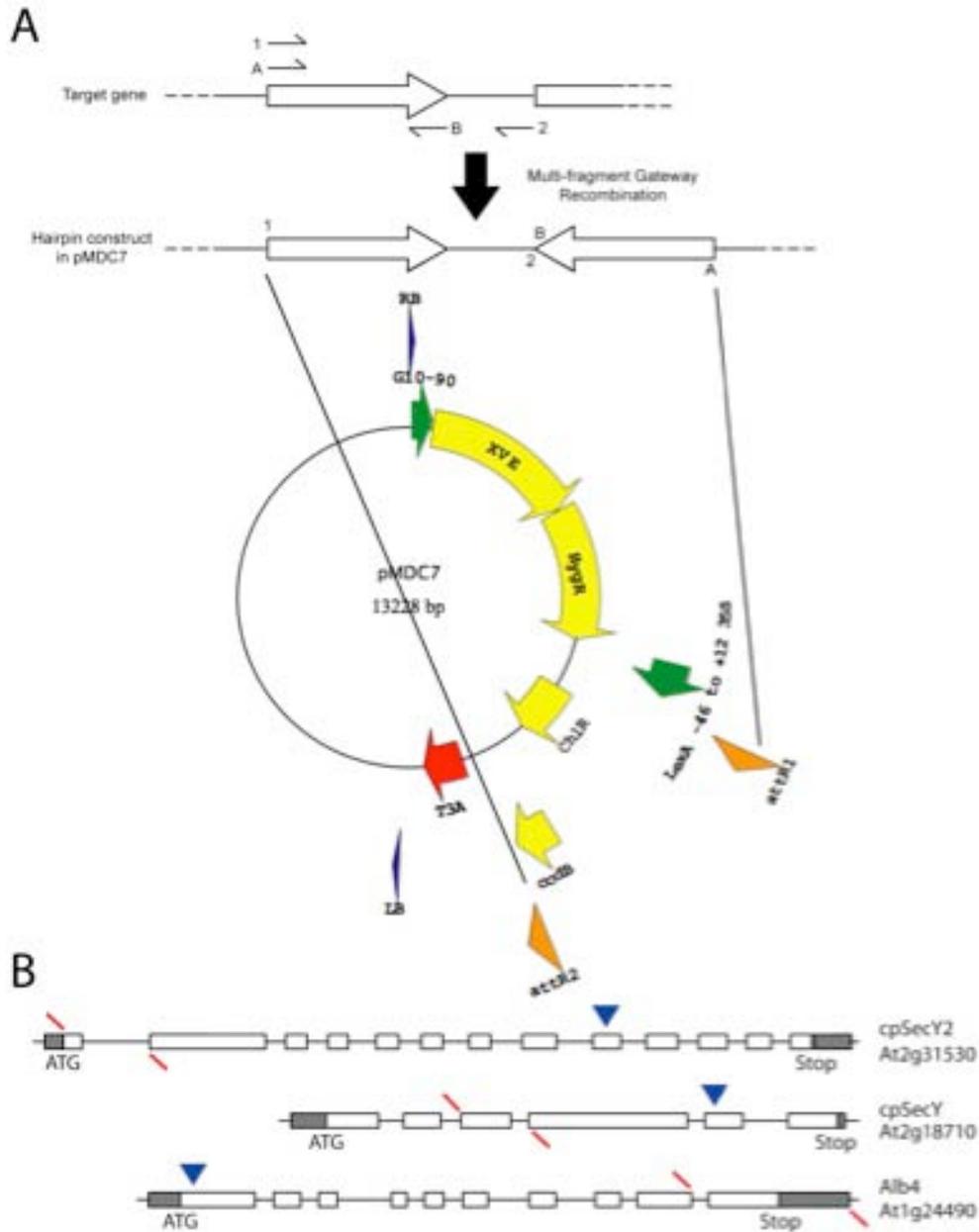


Figure 5-1. Hairpin construction scheme for inducible RNAi. A) For each target gene, primers (half arrows) flanked with Gateway recombination sites (numbers and letters) were used to PCR amplify exon-intron (large white arrow-line) and exon (large white arrow) target gene fragments from Arabidopsis genomic DNA. For each target, Multisite Gateway recombination (Invitrogen, Carlsbad, CA) oriented exon-intron and exon fragments into an inverted repeat during simultaneous insertion into the estrogen-inducible binary vector, pMDC7. B) Exons (boxes), untranslated regions (gray boxes), and introns (lines) are shown for each target gene. Regions bracketed by red lines were amplified for hairpin construction. Blue triangles point to the locations of sequences that were amplified for quantitative RT-PCR.

A

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cpSecY -----
cpSecY2 AA--ATTCTCTCAAAGTATCCAACCTTTATGAACAGAAACAATTGTTGAGGCATG--TGTT 404

cpSecY -----
cpSecY2 TTGACCTTGATAAATGTTTTTGCAGGAGACCCATTTCTGTGTTCTCA--ACCCAGAAAATG 462

cpSecY -----TCGTTGTTGTGGAGAAGGAGAAGAA--GGATAAGGATGATAACGG-T 44
cpSecY2 CTTACTACGAATTTGAATATGFCAAAGAACGAGACAAG--GGCATTCATACAAATGAAC 520
          * *      * *      * *      * *      * *      * *      * *      * *

cpSecY AAGCGA-AGTTTCTCTTACTCTTC-----TTCTTCTTCAAACCTTCGCGTCTCTGT 94
cpSecY2 AGAAGACATCTTCTTATGAAGAAGGAAG--AGTTTTTCGATAAATTATTCCGATAAGT 578
          * * * * * * * * * *      * * * *      * * * *      * * * *

cpSecY CTCGTC-TCAATC-ACAAATCATCTTCGAGATTGCGAAGCTCTTCTCTTTACAAAGGA-A 151
cpSecY2 FCAGAGATGATTCTATGAGTAG--CGAGGAGATGCATACAGATGCTTTGGATGTGGAGAT 636
          * * * * * * * * * *      * * * *      * * * *      * * * *

cpSecY GCTTCTTCTCTGTTTCGACAAAG-ACCAGGAG-----GAACACTTGTAAGCCAA-ATC 203
cpSecY2 TATTCCGCCAGATT--CACAGGACATTCGTAATFCACAGAACTCTGCTGTCTCCAACACA 694
          * * * *      * *      * *      * *      * *      * *      * *      * *

cpSecY ATGGAATCTCGGTCTTGTATCAACTCTAGAAGGTACTTCTTCTTTC-AGATTTCAGAT- 261
cpSecY2 CTACAG--GATGATAGGCCAAAGTCTTTTAGAAATAGGT-TTCTTGATTTTGTTCGGAT- 750
          * *      * *      * *      * *      * *      * *      * *      * *

cpSecY -GGGTTC--CATTTGAAATCGAAG-ATAATGATCTTGTATAG-ATATTGCGACTTAAAG 316
cpSecY2 -AAGTTCAGTACTCAATACTGCAGCAGAAAGATTCTTTAAGAG--CGAGATAA--GGAG 804
          * * * *      * *      * *      * *      * *      * *      * *      * *

cpSecY TTCGAAACTTTTCATGTGAATTTCTCTTGAATTG-TGGAGATTGA--GGGTTATTTC 372
cpSecY2 GAGGTTGTTTGAACGGCTGTTTGTCTTGTACTTAGCCGTGTTGGA--TATTTTATACCT 862
          * * * *      * *      * *      * *      * *      * *      * *      *

cpSecY TCACTAG--TTCGTAGAAATGCTGATGTTT--TGTC--CTTACTGCTGCATTG--T 421
cpSecY2 TTACCTGGATTCGACAGAAGATTAATACCTCAAGATTA--CCTTAGCTTTGTCTCGGGTT 920
          * * * *      * *      * *      * *      * *      * *      * *      *

cpSecY TTGTTGATTGGAGTTGATGTTTCAGCTCT-GAGGCTTCGGTTTTTGATCCGTTGGGGAT- 479
cpSecY2 CTGTTGGTGAGTGTCTCATGTCATTTGAG--TCTTATAGTCCCTTGTGTTGGTTGACATT 978
          * * * *      * * * *      * * * *      * * * *      * * * *      * *

cpSecY -CAATCCAGATGAAACTTCTGG--TTTAAGTAGCATTTGGGAAAGCTTTGTGAGTCTTTT 536
cpSecY2 FCTATGTATTGTAGTTAACAT--TTTATGTTTTGATAGAGGAACTGGGTGAGTTTGGGA 1036
          * * * *      * *      * *      * *      * *      * *      * *      *

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Figure 5-2. Conservation and divergence between the sequences used to construct RNAi hairpins and those of non-target gene family members. Sequences (highlighted black) that were used to construct hairpins to Alb4 (A), cpSecY (B), or cpSecY2 (C), are aligned with those belonging to each corresponding homologous translocase.

B

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cpSecY      -TTTTAGCATG-ATTGATGATGGTTGCTT-----TACTCTTTGAAAACTTCAGCTGCTA 692
cpSecY2     TTCTTTATATATTAAGATTATGATTGATGATAAAGTGCTCATGAAACACC--AGGTGCT- 1207
          * * * * *
          * * * * *

cpSecY      TTGAGGAC-AGTTCATTGATTTTGGAGATTTCCTCAAAGGGC---CATTGCCAGGGN 746
cpSecY2     TTGTCACGTTCTTCCATCCTTGGTGAAACTGCGAAAGGAAGGC--TTAGATGGTCACGAG 1265
          ***
          * * * * *

cpSecY      AGTT-TCTGAAGCTTCTTGGGTTTTAGCT-----CTCTCTCGGCTTGGAAATTAC- 796
cpSecY2     AAGA-TCAAGAGTTATATGTGTGTTTGTCTTAC--CTCTCAAGCCGTGAGATGAATT 1322
          * * * * *
          * * * * *

cpSecY      ---ATAC-CACTCGGCGGGGTTAATCGAGAAGCTTTTGTGGAAAATTGGATCAGAAT- 850
cpSecY2     CTTAACATAACAATTACTTTGTTTGTCTT--GTGACTTTTTGTCTTCATTAAA-CAAAGTT 1379
          * * * * *
          * * * * *

cpSecY      AGCATTTTGAGCACTTTGGAT--ACATTTTCTGGTGGAGGC-ATAGGTAGACT----- 900
cpSecY2     ACTAAATCGCCTTTTGGTCT--TATTTTATTAGATGGTGGCTATCGTTTTTCTTCGCTA 1437
          * * * * *
          * * * * *

cpSecY      GGGTATATGTTCT---CTCGGGATTGTTCTTTTCATCAACGCTCAGATTGTT-TCAG 955
cpSecY2     TAGTGGGAAGCATT--AGTGGTGGCTTACACTTCTCTTCAATATTAGTTTTTGCAGCCAC 1495
          * * * * *
          * * * * *

cpSecY      CTTCTGTCTCAAGTCTATCCAAAGTTGCAAGATCTTCAGAAAAAA-GAAGTGAAGCTGG 1014
cpSecY2     TGCCC--AGTAAGTTAATT--AGGATTCACGTTTCGGGGTATC--AAAATGGTGCTTT 1549
          *
          * * * * *

cpSecY      TAGAAAAAAGATTCTTCAGTATACTAGATATGCTTC-AGTTGGTTTTGCGAT--AGTAC 1070
cpSecY2     T--AAATCTATCATCCATTTTGGCCACAACCTGATGCTAAAGTAATAATGCAATG--CTTAT 1605
          * * * * *
          * * * * *

cpSecY      AAGCAATCGGACAAGTOTT-----TTACCTTCGTC--CTTACG-----TTAAT 1111
cpSecY2     TCAGGGTCAAGCATGTGATGATGACATCATCTCTGTAGTCTGTG--GTGCAATGACAAT 1663
          * * * * *
          * * * * *

cpSecY      GACTTCAGCACGGAAATGGGTTGTT---TCTTCTGTTACGTTG-TTAAACGCTTGGTTCTG 1166
cpSecY2     GACGTGGCTCTGTGATACAATATCAGAATCTGGATTT--GGTATTGATTCTCTATATCT 1721
          ***
          * * * * *

cpSecY      TTCTCACTACATATATTTGGAGAGAGAATCTCTGA-TCTGAAACTTGGAAAT---GOCAC 1221
cpSecY2     CTATCTATAAACCATTGGTAGCTTAAAG--TGATGCTGATATGGAGTAATGGTGGAGC 1779
          * * * * *
          * * * * *

cpSecY      TTCCCTAT-----TGA---TTTTCACAAGTA---TCATCT---CTTACTTGC 1259
cpSecY2     TTGCTCATAACCTGTTCTAA--GCCTTTTACAGTCAATGGCTCATCTCTGATTATTTGT 1837
          * * * * *
          * * * * *

cpSecY      CTGC-ATCATTGGGTAGAACAACAGCAGAGGCCTTACAGGAA-GGAAACTATACGGTCT 1317
cpSecY2     GTGGGAATATTGA--CGGGCTACA-CAGAGACTTTACACAAG--ATGCTCAATCAAATCT 1892
          * * * * *
          * * * * *

cpSecY      TGGAAACCATCGTTGTTTCATTTCTCCTCTTGGT-ACTCGGG--ATAGTATATGTTCAGG 1373
cpSecY2     CAGGTACC--TTTGGTTT-TTCTTACTCCACATTTCTCAAACCTTATGGTTAGTCTTTACA 1949
          * * * * *
          * * * * *

cpSecY      FACTATACCAAATTTTGTCTCTT-TTAA-TGAATCTAAGAA-GTTGG-TAT---GCT 1424
cpSecY2     T--TAGACCTGCATGTCTTCCACATTTCTGGAGGCAACTATCATTGCGCTAT--CTTCT 2005
          * * * * *
          * * * * *

cpSecY      DATATAGTCTAACAAGCAAAG----TTTTT-CAGGAAGCAGAGAGGAAAATCCGCTCA 1478
cpSecY2     CAATTGATTTATAAAAAAAGGAAGCGTTCTCTCTTTTGCAGGAA--GTTCTCGAACTG 2063
          * * * * *
          * * * * *

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Figure 5-2. Continued

C

Alb3	CTCACAAATAATGTACTTAGTACCGCCCAACAAGTATATCTGCGTAAACTAGGTGGTGCA	2169
Alb4	GTGGTGAAAAGGTGACCCCAAGTACCCCA-AACCTGGTGAAAAG STAACCAAATCTCTA	2366
	* ** * * * * * * * * * * * *	
Alb3	AAGCCAAATATGGATGAAAACGCAAGCAAATAATAAGTGCGGGACGAGCAAAGAGATCT	2229
Alb4	AAATGTGTCATCATTCGTTCTAGAACTGTTTTTTGTAATAACACGTCATGTGATCC	2426
	** * * * * * * *	
Alb3	ATTGCTCAGCCTGACGATGCTGGCGAAAGGTAAGTGAATCCCGAGTCTAGTGGAGATTAT	2289
Alb4	CAGGTTTAGGCTGCTGA-AAGAGCAAGAAGCAAAGAGACGTCGAG---AAAAGAAGAG	2481
	* * * * * * * * * * * * * * * *	
Alb3	ATCTCTCAATACGAAGCGTTTTTCTTACAAGTTTGTATTCTGATAAATGTATATTGGA	2349
Alb4	AGGCAGAAAGCTGAAGCAGCTCTATC-TAATCAAATACAGACAAGCACATGAACAAGA	2540
	* ** * * * * * *	
Alb3	TCACAACAGATTTAGACAATTAAGAGCAAGAGAAGCGCAGCAAGAAGAACAAGGCGGT	2409
Alb4	TGAAAA--ATCTGATACAGCCATTGTCGCTGAAGATGATAAAAAACAGAAGCTTT-CTGC	2597
	* * * * * * * * *	
Alb3	TGCGAAAGATACAGTTGAATTGGTAGAAGAATCTCAGTCTGAATCAGAAGAAGGGTCTGA	2469
Alb4	TGTGGATGAAACATCCGA--TGGCACAGTAGC---TGTGAATGGGAA---GCCATC	2645
	** * * * * * * * * * *	
Alb3	TGATGAGGAAGAAGAGGCTCGTGAAGGAGCGTT-AGCTTCGAGCACAACAAGCAA--GCC	2526
Alb4	CATTCAAAAGGACGAGACTACGAATGGGACATTCGGCATTGGCCATGATACAGAACAACA	2705
	* * * * * * * * * * * * * * *	
Alb3	GCTTCCTGAGGTTGGCCAACGAAGAAGCAAAAGATCGAAGCGGAAACGCACTGTATAGAA	2586
Alb4	GCATTCTCA--TGAAACAGAGAAGAGGTAAA-GACTCATGTTTTTCATGTCTTAAGGCGAA	2762
	** * * * * * * * * *	
Alb3	CCAAAGCAAACGAAATTGCAAGTAAGGAAGAGAAAAGAGAGAGATTGAAATCTGTTCCT	2646
Alb4	GCCAAACAAC-AAAGAGGAATTGAGATTTGAATAAGACGAGGAG--AAATCTTGGTACT	2819
	* * * * * * * * * * * * * * *	
Alb3	TAGTTTGTATCTTTTATATATATTTGCATACACCATGTCCGGTAATTATCTAATATTAGG	2706
Alb4	CAGG-----CCTTGTAGTCCCTCTGCA--ACAGATTCGAAGTTTATAGATCTTAAGA	2870
	** * * * * * * *	
Alb3	GCACAAAAGGTCATTCTAAAGTTAAAAGCAGTAGTTGATCAAAGTAAATTGTTTATGTG	2766
Alb4	GTGGGAAACATTAGTCTTCAGGGTTTATGATTTG--TGTACAAAGACCATAGCAGATGGG	2928
	* * * * * * * *	
Alb3	-ATTCACTTATTATAAATGTATTAACGTAATGTGTGTACTATTATGACTTTTCAACAT	2825
Alb4	TATTTGAAGCCTTCGAAGCTTGGTTTCATATTTCT-TATGTTCTTATG--TACAAGCAA	2984
	** * * * * * * *	
Alb3	GCCCGCTTCAGTTTTGTTAGCATTTCGTTGTTGTAACCTGCAATCTATTTGTTGGTGC	2885
Alb4	ACA--ATTACTTTTGTCTCGTTCACTGGTGTACTACTAATACGATAATAGTTTATG---	3038
	* * * * * * * *	

Figure 5-2. Continued

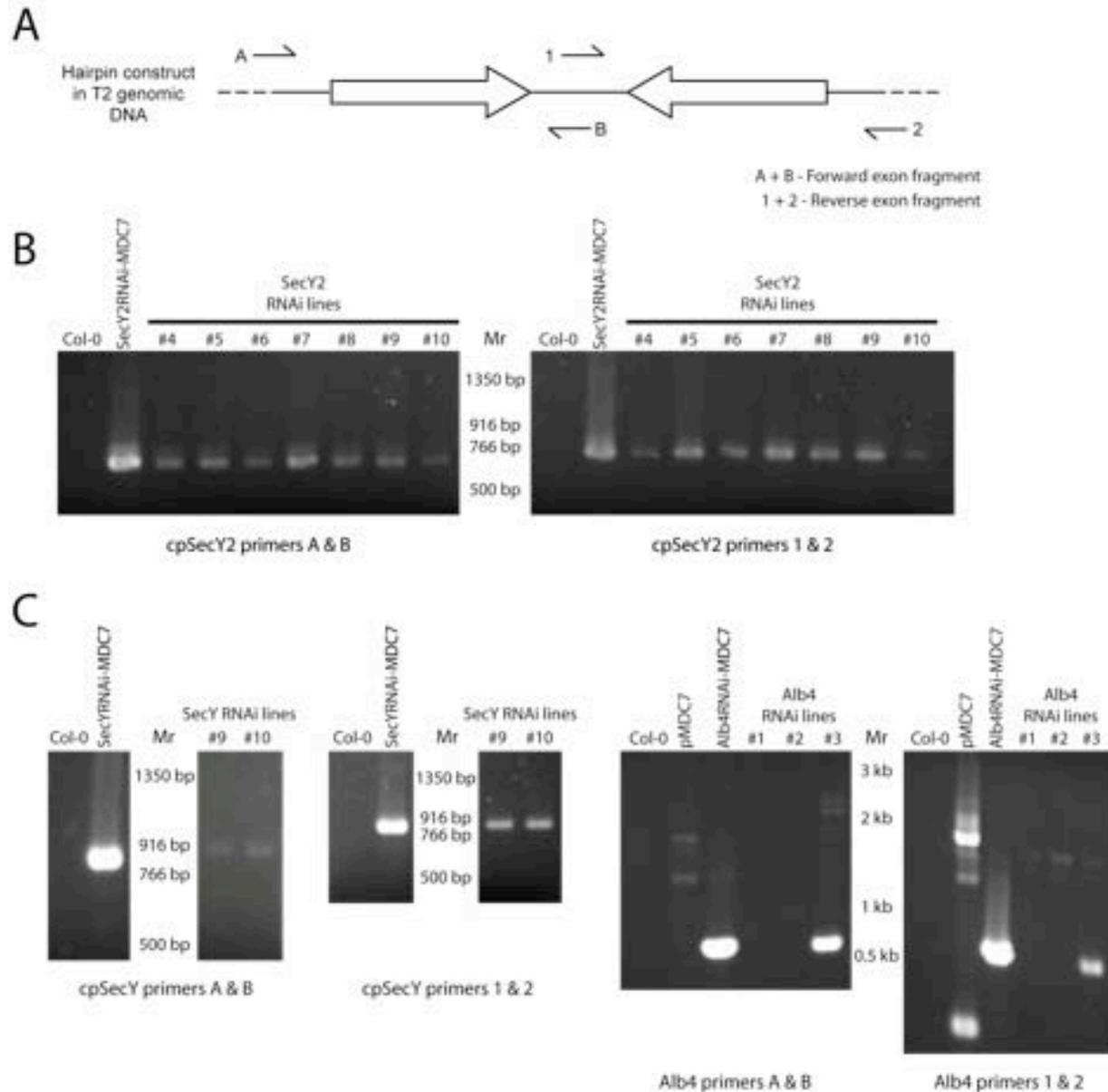


Figure 5-3. PCR screens confirm Alb4, cpSecY, and cpSecY2 hairpin constructs are present in Arabidopsis T1 lines. A) Scheme illustrating combinations of primers designed to screen T1 lines for successful transformation. Primers A and 2 anneal to pMDC7 vector sequence that flanks each hairpin construct, whereas primers B and 1 anneal to the intron sequences of each hairpin construct. B) Examples of PCR products that resulted from screening primers and genomic DNA from several T1 cpSecY2-RNAi lines. C) Examples of PCR products that resulted from screening primers and genomic DNA from several T1 cpSecY- and Alb4-RNAi lines. The sizes of DNA markers (Mr) are shown alongside each gel. Col-0, Arabidopsis wild type genomic DNA; pMDC7, empty vector plasmid.

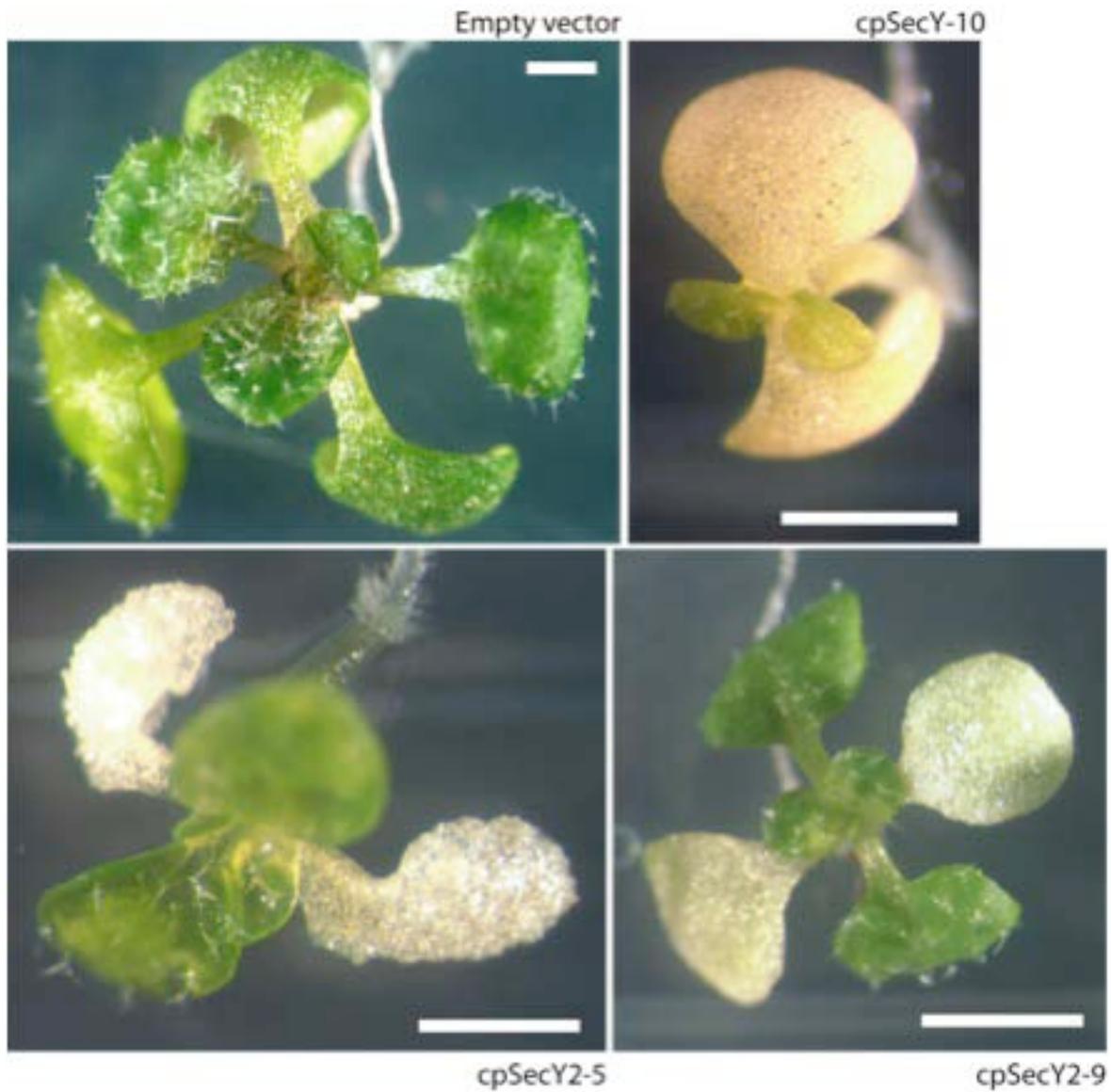


Figure 5-4. Representative phenotypes that were induced in various RNAi lines. Arabidopsis seeds from lines containing inducible hairpin constructs to empty vector, cpSecY, or cpSecY2 were grown on media containing 10 μ M estrogen for 14 days (see 'Experimental Procedures,' Appendix). Scale bars indicate 1 mm.

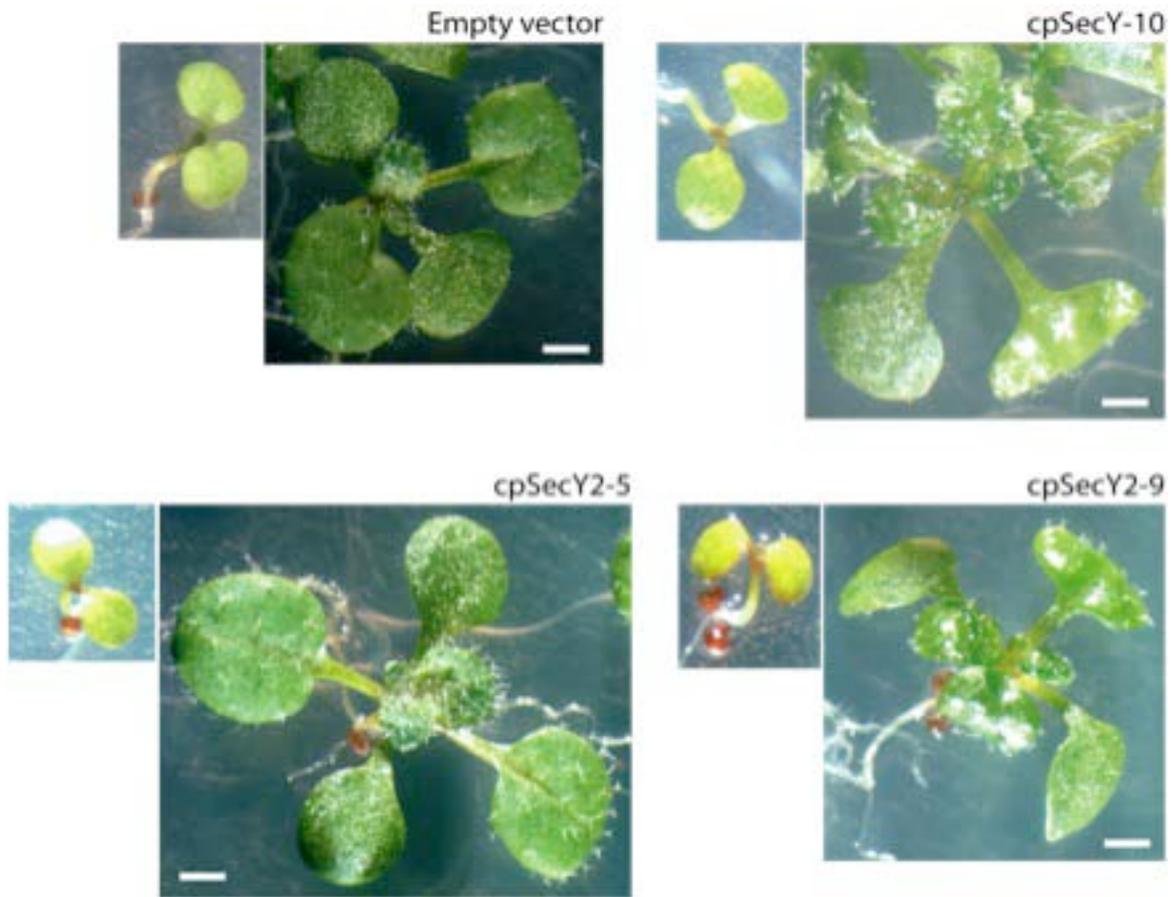
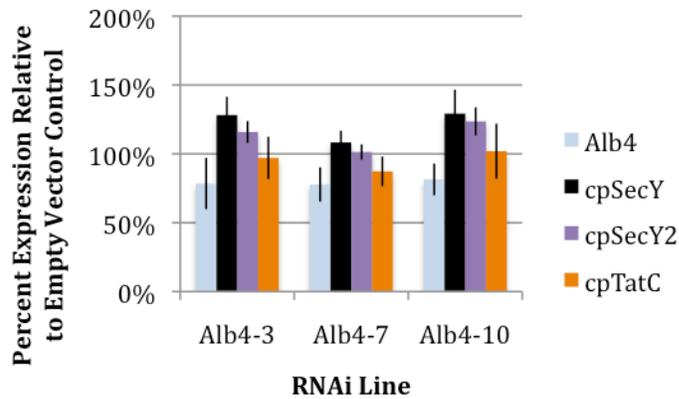
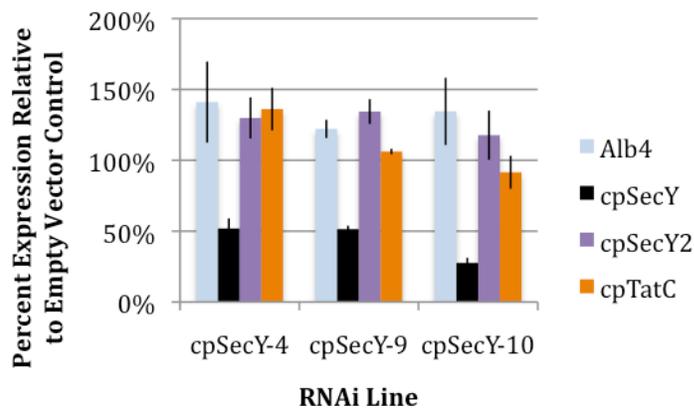


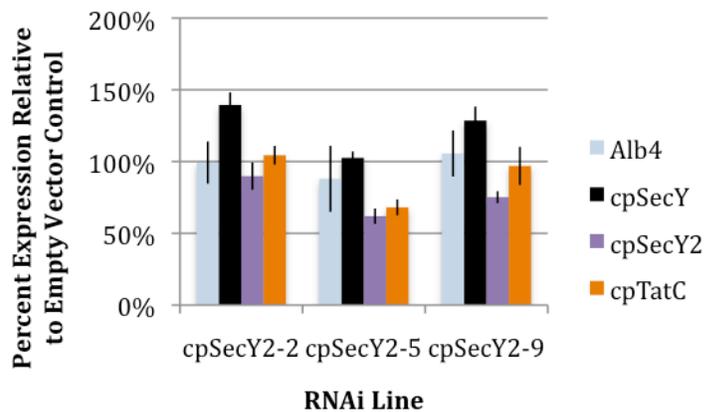
Figure 5-5. Non-induced phenotypes in various RNAi lines. Arabidopsis seeds from lines containing inducible hairpin constructs to empty vector, cpSecY, or cpSecY2 were grown on selective media lacking estrogen for two weeks (see ‘Experimental Procedures,’ Appendix). Shown are single seedlings from each line at one and two weeks post-germination. Scale bars indicate 1 mm.



Alb4-RNAi



cpSecY-RNAi



cpSecY2-RNAi

Figure 5-6. Average levels of target and non-target transcripts in induced RNAi lines. Arabidopsis plants from lines that contained empty vector, Alb4-, cpSecY-, and cpSecY2-RNAi constructs were grown on media containing 10 μ M estrogen for 14 days (see 'Experimental Procedures,' Appendix). mRNA was isolated from seedlings in each line, equal quantities of mRNA from each line were used to synthesize cDNA, and cDNA was used for qRT-PCR. The average amounts of target and non-target transcripts are plotted for several RNAi lines relative to empty vector control. Error bars depict standard error for each transcript in each line, which were calculated from two technical replicates for each of three biological replicates.

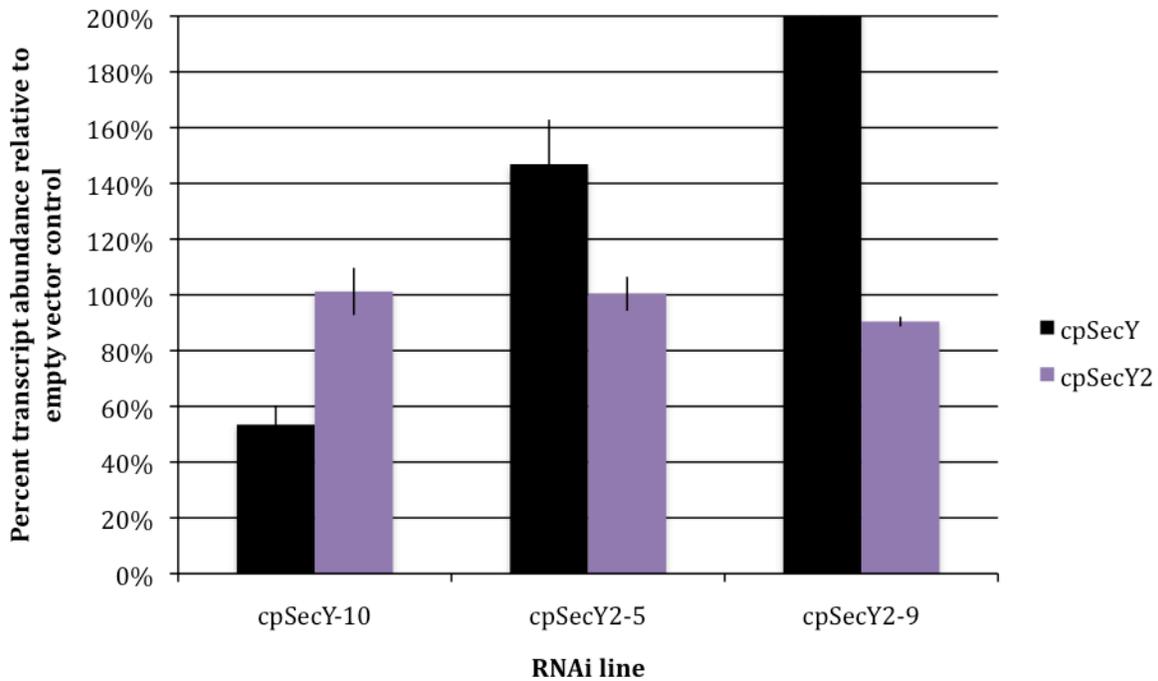


Figure 5-7. Average levels of target transcripts in green leaves of induced RNAi lines. Arabidopsis plants from lines that contained empty vector, cpSecY-, or cpSecY2-RNAi constructs were grown on media containing 10 μ M estrogen for 21 days (see 'Experimental Procedures,' Appendix). mRNA was isolated from seedlings in each line, equal quantities of mRNA from each line were used to synthesize cDNA, and cDNA was used for qRT-PCR. Plotted are the average amounts of target transcripts in several RNAi lines relative to empty vector control. Error bars depict standard error for each transcript in each line, which were calculated from three technical replicates for each of three biological replicates.

Empty vector

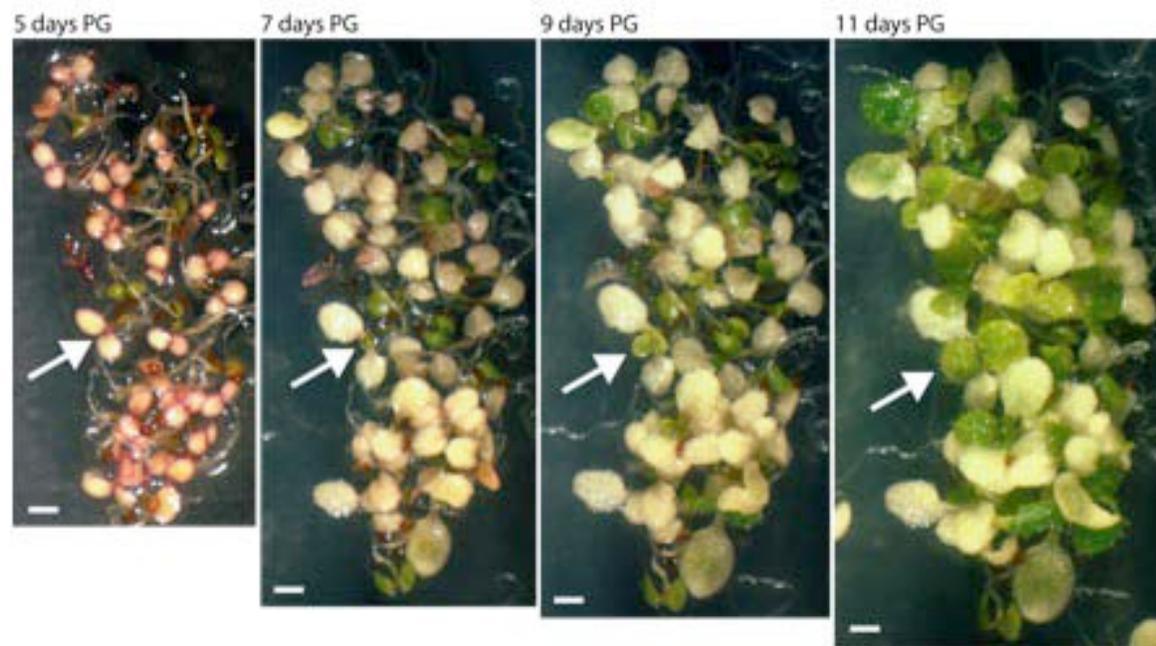


cpSecY-10



Figure 5-8. Representative phenotypes of induced RNAi seedlings over time. Arabidopsis seeds from lines containing inducible hairpin constructs to empty vector, cpSecY, or cpSecY2 were grown on selective media containing 10 μ M estrogen for two weeks (see 'Experimental Procedures,' Appendix). Shown are groups of seedlings from each line at time points beginning at five days post-germination. Scale bars indicate 1 mm. White arrows point at emerging green true leaves in seedlings that initially develop pale cotyledons.

cpSecY2-5



cpSecY2-9

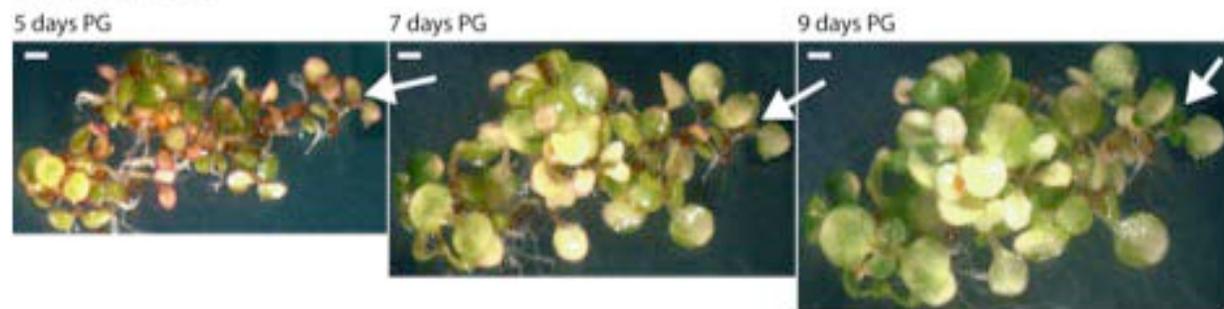


Figure 5-8. Continued.

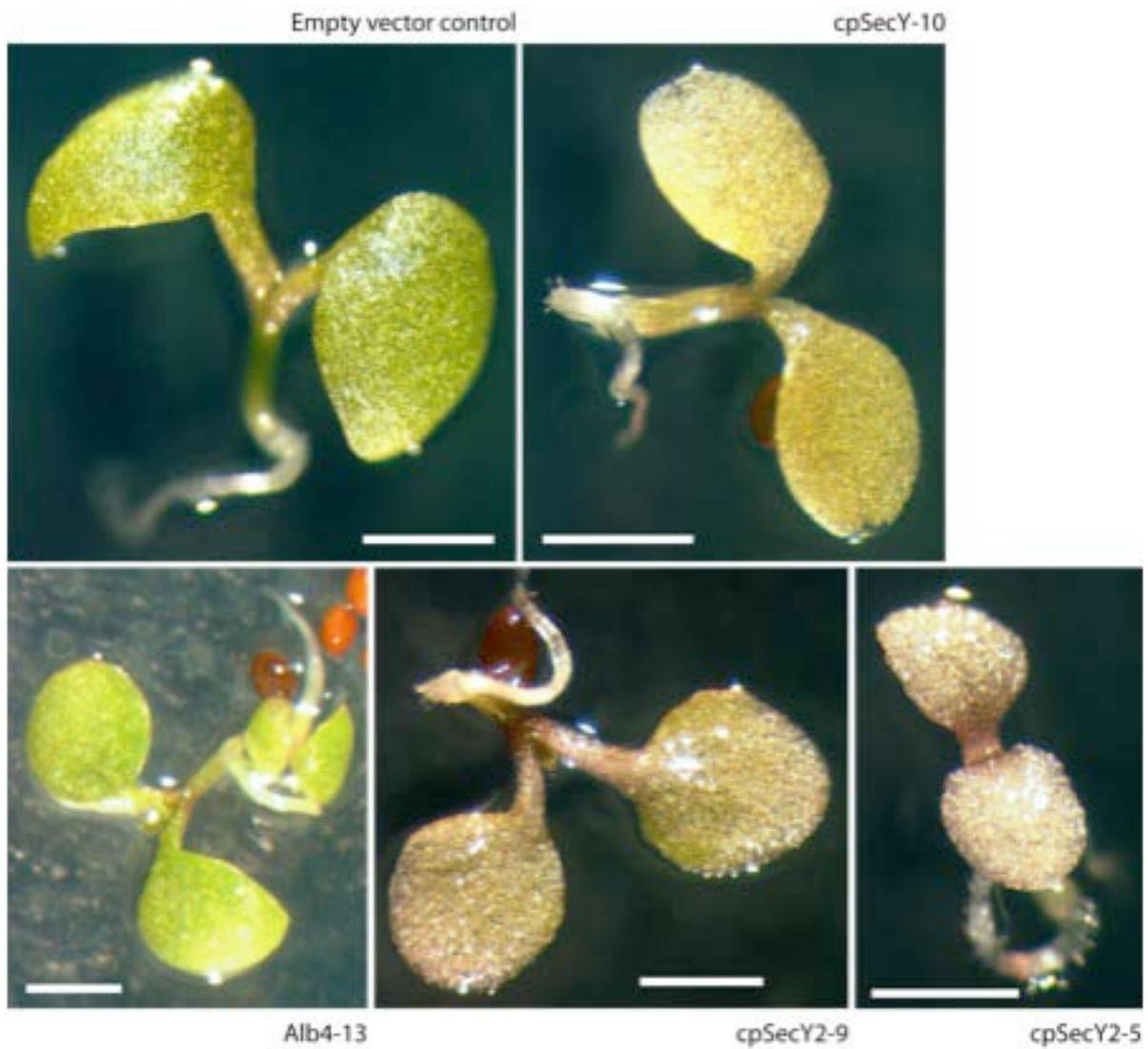


Figure 5-9. Representative early growth phenotypes that were induced in RNAi lines. Arabidopsis seeds from lines containing empty vector or inducible hairpin constructs to Alb4, cpSecY, or cpSecY2 were grown on media containing 10 μ M estrogen for six days (see 'Experimental Procedures,' Appendix). Scale bars indicate 1 mm.

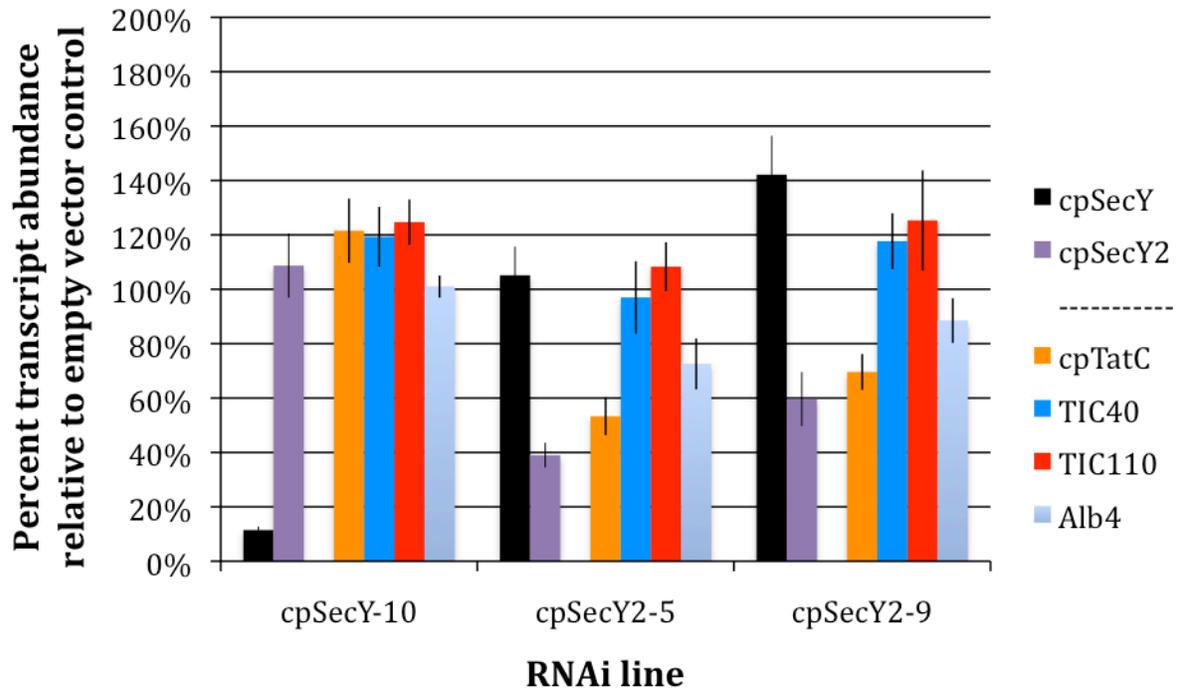


Figure 5-10. Relative abundance of various transcripts in induced RNAi lines. Arabidopsis plants from lines that contained empty vector, cpSecY-, and cpSecY2-RNAi constructs were grown on media containing 10 μ M estrogen for six days (see 'Experimental Procedures,' Appendix). mRNA was isolated from seedlings in each line, equal quantities of RNA from each line were used to synthesize cDNA, and cDNA was used for qRT-PCR. (A) The average amounts of target and non-target transcripts are plotted for several RNAi lines relative to empty vector control. Error bars depict standard error for each transcript in each line. Data was had from three technical replicates for each of three biological replicates.

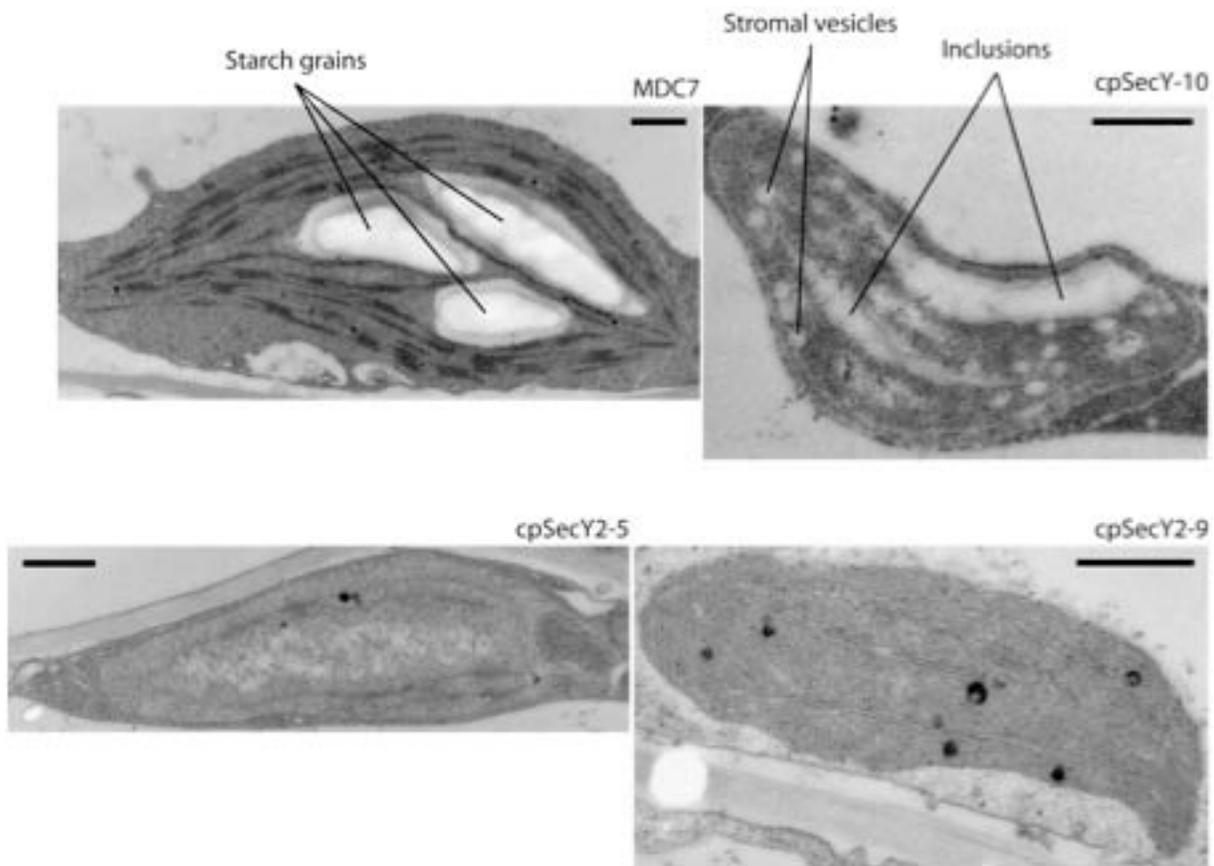


Figure 5-11. Representative TEM micrographs of plastids from induced RNAi lines. Arabidopsis seeds from lines containing empty vector or inducible hairpin constructs to Alb4, cpSecY, or cpSecY2 were grown on media containing 10 μ M estrogen for six days (see 'Experimental Procedures,' Appendix). Seedlings were harvested and plastids in the cotyledons were imaged using TEM (see 'Experimental Procedures,' Appendix). Scale bars indicate 500 nm.

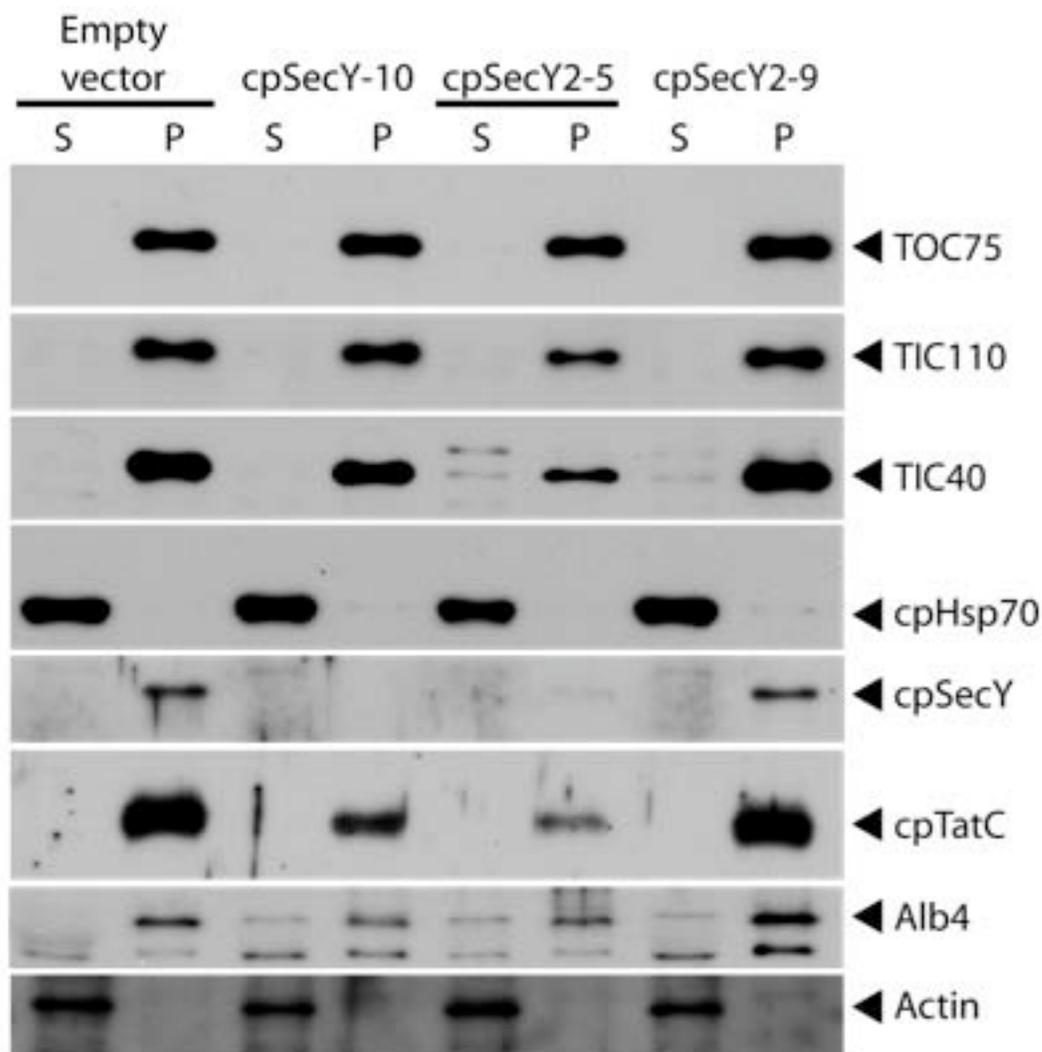
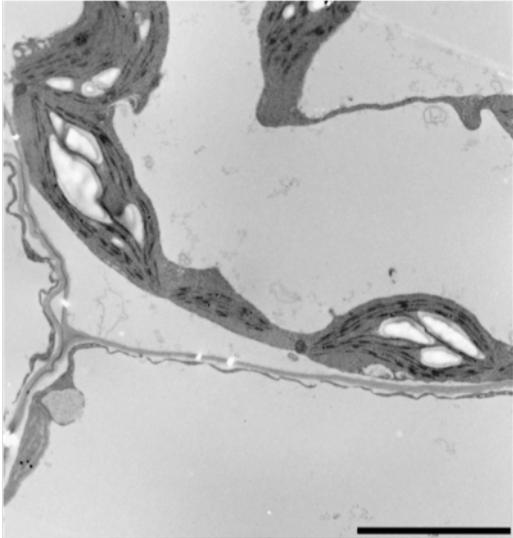


Figure 5-12. Immunoblotting membrane and soluble proteins from induced RNAi lines. Arabidopsis plants from lines that contained empty vector, cpSecY-, and cpSecY2-RNAi constructs were grown on media containing 10 μ M estrogen for six days (see 'Experimental Procedures,' Appendix). Membranes and soluble proteins were isolated from seedlings then analyzed by SDS-PAGE and immunoblotting, using antibodies to atpTatC (1:5000), atpSecY (1:5000), psOxa1p (1:5000), atTIC110 (1:5000), atTIC40 (1:5000), atTOC75 (1:2500), Hsp70 (1:5000), Actin (1:5000), or atAlb3 (1:2500). Samples were loaded as equal mass tissue.

Empty vector control



cpSecY2-5

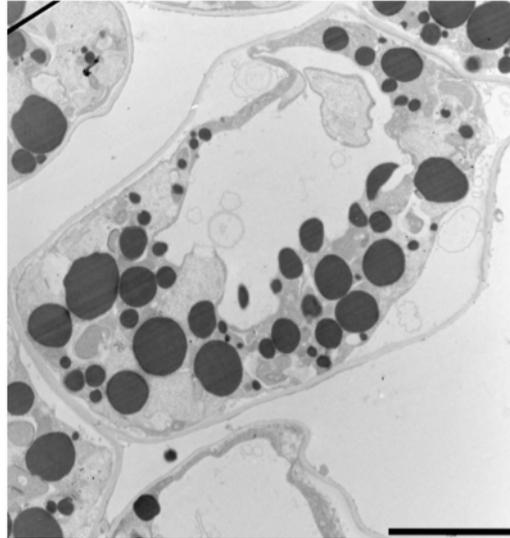


Figure 5-13. Representative TEM micrographs of cells from induced RNAi lines. Arabidopsis seeds from lines containing empty vector or an inducible hairpin construct cpSecY2 were grown on media containing 10 μ M estrogen for six days (see 'Experimental Procedures,' Appendix). Seedlings were harvested and cotyledon cells in the were imaged using TEM (see 'Experimental Procedures,' Appendix). Scale bars indicate 5 μ m.

APPENDIX EXPERIMENTAL PROCEDURES

Hairpin Construction for Inducible RNAi of Thylakoid Translocases

Two gene fragments were amplified from Arabidopsis genomic DNA to prepare hairpin constructs for CPSECY, CPSECY2, or ALB4. Exon-introns to each target were amplified using primers flanked with B1- and B5r- Gateway recombination sites (Invitrogen, Carlsbad, CA). Each exon-intron fragment was cloned into the P1-P5r Multisite Gateway 2.0 entry vector using BP Clonase II (Invitrogen). Each second fragment was made up of the exon from the first fragment, and was amplified using primers flanked with B5- and B2- Gateway recombination sites. The second fragments were cloned in the P5-P2 Multisite Gateway 2.0 entry vector using BP clonase II. Gateway LR clonase II (Invitrogen) was used to assemble fragment pairs as inverted hairpins while simultaneously sub-cloning the inverted hairpin repeat into the estrogen-inducible binary vector, pMDC7 (Curtis and Grossniklaus, 2003).

The third CPSECY exon and the following intron were used to construct the CPSECY hairpin (Figure 5-1B). Each of the two CPSECY fragments that were used to construct the hairpin were produced as nested PCR products that were amplified from a larger CPSECY gene fragment. The larger CPSECY gene fragment was synthesized by fusing two smaller CPSECY gene fragments that were amplified by from Arabidopsis genomic DNA by splicing by overlap extension (SOE) (Horton et al., 1989). The protein coding sequence in the first CPSECY2 exon and the sequence of the following intron were used to produce the cpSecY2 hairpin. The exon-intron cpSecY2 fragment was amplified directly from Arabidopsis genomic DNA. The second exon fragment was produced by fusing two half fragments of the cpSecY2 exon together by SOE. The last intron and exon were used to produce the Alb4 hairpin. The 3' Untranslated Region (3'-UTR) and intron Alb4 fragment was produced by fusing two smaller ALB4

fragments together by SOE. The 3'-UTR fragment was amplified directly from Arabidopsis genomic DNA. All primer sets that were used to synthesize the hairpin constructs are listed below (Table A-1).

Construction of Precursors

Transcription clones (Sp6 promoter) for mature size cpTatC (mcpTatC) and precursors to the light harvesting chlorophyll binding protein (pLHCP), plastocyanin (pPC), envelope phosphate translocator (pP36), cpTatC (pcpTatC), the small subunit of RuBisCO (pSSU), atAlb4, atAlb3, psAlb3 (previously called cpOxa1p), and the 23 kD (pOE23) and 33 kD (pOE33) subunits to the oxygen-evolving complex were each previously described (Anderson and Smith, 1986; Cline et al., 1989; Last and Gray, 1989; Cline et al., 1993; Moore et al., 2000; Mori et al., 2001; Gerdes et al., 2006; Schnell et al., 1990). Dr. Donna Fernandez provided SP6 transcription clones for precursors to cpSecY2 (pcpSecY2) and cpSecA2 (pcpSecA2). All novel restriction sites were inserted into constructs via Quickchange Mutagenesis (Stratagene). Reciprocal swaps between the transit peptides and mature domains of SSU and cpTatC were made as follows. *MluI* restriction sites were inserted twenty and eight amino acids after the stromal transit peptidase cleavage sites of pSSU and pcpTatC respectively, and the reciprocal chimeric precursors constructed by sub-cloning of restriction fragments (Figure 2-2B). In order to prepare cpTatC precursors lacking the non-conserved amino terminal domain, a *BssHII* restriction site was inserted into pcpTatC 47 residues after the stromal transit peptidase cleavage site. Precursors containing either the cpTatC or SSU transit peptide and cpTatC lacking the non-conserved amino terminus (Δ NCcpTatC) were prepared by an *MluI*, *BssHII* double restriction digest and subsequent ligation of fragments. The junction in SSUtp Δ cpTatC consisted of amino

acids 1-79 from pSSU and residues 198-353 from cpTatC (Figure 2-1B). The TatCtp Δ NcpTatC fusion lacked pcpTatC residues 101-197 (Figure 2-3A).

Nucleotide sequence encoding the first 126 amino acids of Arabidopsis pcpTatC was amplified from a cDNA clone by a polymerase chain reaction using primers flanked with *NdeI* and *BamHI* restriction sites. The PCR product was sub-cloned in pET-14b (Novagen) to produce an N-terminal 6x histidine-tagged fusion protein (pET14b-atcpTatC).

The tandem tetra-Cys tag (2TC) is composed of the following amino acids: - AEAAAREACCPGCCARARSAEAAAREACCPGCCARA- (Tour et al., 2003). The tag was synthesized by GeneArt. Restriction cloning was used to fuse the tag to the C-terminus of SP6 transcription clones of precursors to OE23 or cpTatC. Restriction cloning was used to place precursors to pea OE23 and OE23-2TC in the pETh3C bacterial expression vector.

Preparation of Radiolabeled Precursors

RNA transcripts were produced by transcription with SP6 polymerase (Promega) and translated with a homemade wheat germ translation system in the presence of ³[H]-leucine (Cline, 1986). Where indicated, *in vitro*-coupled transcription, translation with wheat germ TnT (Promega and NEN Life Science Products) was performed following the manufacture's guidelines. Translation products were diluted with one volume of 60 mM leucine in 2X import buffer (IB, 1X = 50 mM HEPES/KOH, pH 8.0, 0.33 M sorbitol) prior to use unless otherwise indicated in the figure legend.

Preparation of Bacterially Expressed Proteins and Antibodies

pOE23, pOE33, and tetra-cys tagged pOE23 from pea were each expressed in *E. coli*, and purified as inclusion bodies as previously described (Cline et al., 1993). Inclusion bodies were dissolved in 10 M urea, 10 mM DTT before use in *in organello* competition experiments. The amino-terminal 126 amino acids from atcpTatC were expressed in *E. coli* (strain BL21) from

the pET-14b plasmid and purified as inclusion bodies as above. Inclusion bodies were dissolved in 6 M Urea and subjected to Ni-affinity purification according to manufacturers instructions (GE Healthcare), with the modification that all buffers contained 6 M Urea. Affinity-purified atcpTatC peptide was used for the production of antibodies in rabbits (Cocalico Biologicals). Antibodies to psAlb3 have been described (Mori et al., 2001). I found that the psAlb3 antibodies react with the atAlb4 protein but not the atAlb3 protein (Figure 2-11). psAlb3 antibodies were used to detect Arabidopsis Alb4 in western blotted nitrocellulose membranes (Figure 2-10). Antibodies to atcpSecY (Schuenemann et al., 1999), atAlb3 (Moore et al., 2000), atActin monoclonal clone C4 (ICN Biochemicals, Irvine, CA), atTIC110 (Bauer et al., 2000), atTOC75 (Hiltbrunner et al., 2001), atTIC40 (Chou et al., 2003), psTOC75 (Ma et al., 1996), psTIC110 (Kessler and Blobel, 1996), psTatC (Cline and Mori, 2001), psOE23 (K. Cline, unpublished), and atAlb4 (Gerdes et al., 2006) have been described.

Plant growth conditions, preparation of Chloroplasts, Stromal Extract, and Total Cell Membranes

Peas (*Pisum sativum* L. cv. Laxton's Progress 9 Improved) used for chloroplast isolation were grown as described (Cline, 1986). Intact chloroplasts were isolated from 9- to 10-day old pea seedlings and were resuspended in IB at 1 mg/mL of chlorophyll. For preparation of stromal extract, chloroplasts were lysed hypotonically by resuspension in 10 mM HEPES/KOH, pH 8.0 and incubation on ice for 10 minutes followed by centrifugation at 150,000 x g for 30 minutes at 2°C to pellet the membranes. Chlorophyll concentrations were determined according to Arnon (Arnon, 1949). Arabidopsis seed harboring a knockout mutation in the gene encoding Alb3 were obtained from the Arabidopsis Biological Resource Center (Stock# CS16) (Rhee et al., 2003). The cpSecY knockout mutant (Ws ecotype) contains a T-DNA insertion in the third exon and was previously designated *scy1-2* (Skalitzky, 2006). Arabidopsis seeds were sterilized, plated to

media, and grown for 2 or 4 weeks (20°C, 16 hour photoperiod, 100 $\mu\text{E}/\text{m}^2/\text{s}$ of light) before total membrane or intact chloroplast isolation (Smith et al., 2003). Media used to grow *alb3* mutant seedlings contained 20 mg/L hygromycin. Seeds for Arabidopsis RNAi and empty vector lines were grown on MS media containing 25 mg/L hygromycin and 10 μM β -estradiol with a 24-hour photoperiod in 100 $\mu\text{E m}^2 \text{ sec}^{-1}$ of light, at 20°C +/- 2°C for six or 14 days.

Pale *alb3* or *secy* mutant seedlings were harvested from plates for total membrane isolation, using a method adapted from (Schaller et al., 1995). Equal masses of tissue from wild type or mutant Arabidopsis seedlings were subjected to probe homogenization (PT10-35, Kinematica GmbH) in ice-cold membrane extraction buffer (30 mM Tris pH 8, 20% glycerol, 5 mM EDTA, 5mM EGTA, 1mM PMSF) and filtration through Miracloth (Calbiochem). Filtrate underwent centrifugation at 150,000 x g, for 30 minutes, at 2°C. Membrane pellets were resuspended in IB to equivalent concentrations of tissue mass per volume.

Total proteins were extracted from six-day old estrogen-induced Arabidopsis seedlings by following the EZ extraction procedure (Martinez-Garcia et al., 1999). Sterile pestles and microfuge tubes were used to homogenize tissue in volumes of E buffer (125 mM Tris-HCl pH 8.8, 1% SDS, 10% glycerol, 50 mM $\text{Na}_2\text{S}_2\text{O}_5$) that were equal to two times the mass of tissue that was harvested. Homogenates underwent centrifugation at >13,000 x g, for 10 minutes, at room temperature. A 1/10th volume of Z buffer (125 mM Tris-HCl pH 6.8, 12% SDS, 10% glycerol, 22% β -mercaptoethanol, 0.001% bromophenol blue) was added to each sample. For membrane isolation, sterile pestles and microfuge tubes were used to homogenize tissue in volumes of buffer [30 mM Tris-HCl pH 8, 20% glycerol, 5 mM EDTA, 5 mM EGTA, 26 $\mu\text{L}/\text{mL}$ Sigma Plant Protease inhibitors (Sigma Aldrich, P9599), 1 $\mu\text{g}/\text{mL}$ chymostatin, 1 $\mu\text{g}/\text{mL}$ antipain, 1 $\mu\text{g}/\text{mL}$ aprotinin, 2 mM PMSF, 2.7 $\mu\text{L}/\text{mL}$ β -mercaptoethanol] equal to ten times the

mass of tissue that was harvested. Homogenates were filtered through equal-sized pieces of buffer-wetted Miracloth then spun at 150,000 x g, for 30 minutes, at 2°C. Membrane pellets were resuspended to volumes equal to the mass of tissues harvested using IB that contained the above protease inhibitors. Supernatants were TCA precipitated and resuspended using IB containing the above inhibitors to volumes equal to those of the membrane fractions.

Chloroplast Import and Thylakoid Protein Integration Assays

Radiolabeled precursor proteins were incubated with isolated chloroplasts (0.33 mg/mL chlorophyll), 5 mM MgATP, and IB, in 120 μ E of light in a 25°C water bath for times specified in figure legends. After import, samples were treated with 100 μ g/mL thermolysin on ice for 30 minutes. Proteolysis was stopped by addition of 0.5 M EDTA to a final concentration of 10 mM, and chloroplasts were re-isolated by centrifugation through 35% Percoll, 5 mM EDTA, in IB. Intact chloroplasts were washed, resuspended to equal concentrations of chlorophyll, then analyzed using SDS-PAGE. Alternatively, chloroplasts were lysed hypotonically by resuspension in 10 mM HEPES/KOH, pH 8.0 and incubation on ice for 10 minutes. Lysed chloroplasts were analyzed using SDS-PAGE and/or fractionated into membranes and stroma by differential centrifugation (12,000 x g, 10 minutes, 4°C). Membranes were resuspended in IB and analyzed using SDS-PAGE, and/or combined with 100 μ g/mL thermolysin on ice for 30 minutes. Proteolysis was stopped by addition of 10 mM EDTA before analysis by SDS-PAGE and fluorography.

Chloroplast fractionations that involved separation into envelope membranes, stroma, and thylakoids were conducted as follows. Chloroplasts were spun at 1500 x g, for 3 minutes, at 2°C. Chloroplasts were lysed by resuspension to 0.5 mg chlorophyll/mL in 10 mM HEPES-KOH pH 8.0 (10HK) and incubating them on ice for 10 minutes. A sample of lysate was taken aside

for analysis. Remaining chloroplast lysate was next spun at 3850 x g, for 25 seconds, in a swing bucket microfuge, at 2°C. The clear supernatant (Super1) was transferred to a separate tube. The remaining partially pelleted chloroplasts were washed in excess (1mL) 10HK then spun at 3300 x g, for 8 minutes, in a swing bucket centrifuge, at 2°C. The supernatant (Super2) was transferred to a separate tube. Both supernatants were spun at 150,000 x g, for 30 minutes, at 2°C. Super1, which represented the stroma, was transferred to a fresh tube. Super2 was discarded. The pellets, which represented the envelope membranes, were combined and resuspended in a volume of IB equal to the original lysate volume. Thylakoids were also resuspended in a volume of IB equal to the original lysate volume.

Arabidopsis chloroplasts were fractionated by hypotonic lysis and centrifugation at 2600 x g, at 2°C, for 25 seconds in a swing bucket microfuge. The thylakoid pellet was washed in 0.5 mL HK and spun at 700 x g, at 2°C, for 5 minutes. Supernatants from lysate and thylakoid centrifugation were each spun at 150,000 x g, at 2°C, for 30 minutes. Following centrifugation, the lysate supernatant was retained as the stroma fraction, while the thylakoid supernatant was discarded. The pellets, representing envelope membranes, were resuspended and combined in a volume of IB equal to that of the stroma sample. Washed thylakoids were also resuspended to stroma volume using IB.

For import time course assays, reaction samples were rapidly stopped with 3.3 mM HgCl₂ and analyzed as described (Reed et al., 1990). Chase time course assays were done similar to (Li and Schnell, 2006). Chloroplasts and MgATP were pre-incubated for 10 minutes, at 25°C, in 120 μE of light. SSUtpcpTatC precursor was then added and incubation continued for 5 minutes. Chloroplasts were diluted five-fold in ice-cold IB and treated with 200 μg/mL thermolysin on ice for 15 minutes to remove surface-bound precursor. Chloroplasts were then

washed, resuspended to the original reaction volume in IB, 5 mM MgATP and incubated in the light at 25°C. At time points, samples of chloroplasts were taken for re-isolation by centrifugation through 35% Percoll cushions. Intact chloroplasts were lysed, quantified, adjusted to equal chlorophyll concentrations, and fractionated. Chloroplasts, fractions, and thermolysin-treated membranes were analyzed by SDS-PAGE and fluorography. Radiolabelled proteins were extracted from excised gel bands and quantified by scintillation counting (Cline, 1986).

Protease accessibility assay

Radiolabeled pcpTatC was imported into chloroplasts for 10 minutes. Aliquots of the import reaction were treated with 200 µg/mL trypsin (Sigma, 6300 BAEE units/mg), 100 µg/mL thermolysin, or IB for 30 minutes on ice (Li and Schnell, 2006). Thermolysin and trypsin were inhibited by a 10 minute incubation on ice in the presence of 10 mM EDTA or trypsin inhibitors (1 mM PMSF, 0.05 mg/mL TLCK, 0.1 mg/mL soybean trypsin inhibitor, and 2 µg/mL aprotinin). Chloroplasts were re-isolated, washed, quantified, adjusted to equal chlorophyll concentration, lysed, and fractionated in the presence of all respective protease inhibitors. Stroma and membrane fractions were analyzed using SDS-PAGE and fluorography as well as by immunoblotting with antibodies to TOC75 or TIC110.

***In Organello* Competition Assay**

In organello competition for the cpTat and cpSec pathways was conducted essentially as described (Cline et al., 1993). Briefly, unlabeled inclusion bodies of pOE23 or pOE33 were dissolved in 10 M urea, 10 mM DTT for 1 hr at 37 °C. Chloroplasts, 5 mM MgATP, and 1.5 mM DTT were then incubated with the unlabeled competitors for 7 minutes in light at 25 °C in order to accumulate the stromal intermediates iOE23 and iOE33, respectively. Competitors were aliquotted from stocks, such that the final competitor concentration was either 0.5 or 0.75 µM, or

no competitor, and the urea concentration was 0.3 M in all assays. Radiolabeled precursors pcpTatC, pOE23, or pOE33 were then added (1/6 volume) and the incubation continued for an additional 15 minutes. Intact chloroplasts were recovered from assays by centrifugation through Percoll cushions. Recovered chloroplasts from the radiolabeled pOE23 and pOE33 assays were analyzed directly. Chloroplasts from cpTatC assays were lysed in 10 mM HEPES/KOH pH 8.0, 10 mM MgCl₂ buffer, the membranes recovered by centrifugation at 3,200 x g for 8 minutes and the resulting supernatant centrifuged at 100,000 x g for 20 minutes to obtain stroma. An aliquot of the membrane fraction at ~1 mg Chl per mL IB was treated with 150 µg/mL thermolysin per mL for 40 minutes at 4 °C. Proteolysis was terminated with EDTA (10 mM final concentration), the membranes recovered by centrifugation and washed with IB, containing 14 mM EDTA.

Nigericin/Valinomycin Inhibition Assay

Isolated chloroplasts were treated with IB, or 0.5 µM Nigericin and 1.0 µM Valinomycin (NigVal) for 10 minutes on ice. Radiolabeled pOE23 and pcpTatC were imported into IB- or NigVal-treated chloroplasts for 20 minutes. Un-imported precursor was removed by thermolysin. Chloroplasts were re-isolated, washed, lysed, quantified, adjusted to equal chlorophyll concentration, and fractionated. Isolated thylakoids were treated with thermolysin. Chloroplasts from pOE23 import samples and chloroplasts and fractions from cpTatC import reactions were analyzed using SDS-PAGE and fluorography. Gel band extraction and scintillation counting was used to measure the relative percentages of stromal and thylakoid membrane-associated cpTatC and OE23.

Azide Inhibition Assay

Isolated chloroplasts were treated with IB, or 5 mM or 10 mM sodium azide for 10 minutes on ice. Radiolabeled pcpTatC was imported into IB-, or azide-treated chloroplasts for 15 minutes. Chloroplasts were re-isolated, washed, and lysed. To fractionate chloroplast lysate,

stroma and envelope membranes were first separated from thylakoid membranes by differential centrifugation (3700 x g, for 30 seconds, at 4°C). Stroma and envelope membranes were next separated by high speed differential centrifugation (150,000 x g, for 30 minutes, at 4°C).

Thylakoid and envelope membranes were resuspended in a volume of IB equal to that of the isolated stroma. Isolated thylakoids were treated with thermolysin. Chloroplasts from pOE33 and pOE23 import samples and chloroplasts and fractions from cpTatC import reactions were analyzed using SDS-PAGE and fluorography. Gel band extraction and scintillation counting was used to measure the relative percentages of stromal and thylakoid membrane-associated cpTatC.

Chloroplast Exposure to Light and Prolonged Incubation

Import buffer, 10 mM Sodium Azide, or 0.5 μM Nigericin/1.0 μM Valinomycin was incubated with chloroplasts for 10 minutes on ice. Chloroplasts incubated with Azide or Nigericin/Valinomycin were directly used for *in vitro* import reactions. 50 μg aliquots of mock treated chloroplasts were incubated on ice for 0.5, 1.0, 1.5, 2.0, or 2.5 hours before being used in *in vitro* import assays. Other 50 μg aliquots of mock treated chloroplasts were exposed to 17 W/cm² of 561 nm light for 5, 15, or 30 minutes. A Shimadzu RF5301 PC spectrofluorometer produced the light, which was reflected off a front-side mirror into samples of chloroplasts that had been aliquoted into wells of a 96 well plate. After light treatment, 200 μL of IB was added to each well. Chloroplasts were transferred to microcentrifuge tubes, spun at 2200 x g, at 2°C, for 5 minutes, resuspended to 50 uL using IB, and used in *in vitro* import assays.

Transforming Agrobacterium and Arabidopsis

pMDC7 plasmids containing no insert or hairpin fragments to target transcripts were each used to transform Agrobacterium GV3101 according to (Orlic). Antibiotic resistant bacteria

were next used to transform *Arabidopsis* ecotype Columbia by the floral dip method according to (Bechtold and Pelletier).

Arabidopsis Genomic DNA Isolation and PCR Screening

3 cm² of leaf tissue was ground in 300 µL DNA extraction buffer (1% CTAB, 50 mM Tris-HCl pH 8.0, 0.7M NaCl, 10 mM EDTA, 0.1% β-mercaptoethanol) and incubated for an hour at 60°C. Samples were given equal volume of chloroform, vigorously agitated, then sedimented at >10,000 x g, for five minutes, at room temperature. The supernatant was removed and combined with 600 µL of ethanol then incubated at -20°C for 30 minutes. Five minutes of >10,000 x g centrifugation were followed by supernatant aspiration and pellet resuspension in 20 µL of sterile water. *Arabidopsis* genomic DNA from RNAi lines was PCR screened for hairpin constructs using screening primers described below (Table A-2). In PCR reactions, forward screening primers for each gene were paired with the MDC7 reverse primer, and reverse screening primers were each paired with the MDC7 forward primer.

RNA Isolation and Quantitative RT-PCR

The RNeasy Plant Mini Kit (Qiagen, Valencia, CA) was used to isolate mRNA from estrogen-induced *Arabidopsis* seedlings that contained an empty estrogen-inducible vector or estrogen-inducible hairpin constructs to ALB4, CPSECY, or CPSECY2. cDNA was synthesized from isolated mRNA by using 10 µM oligo-dT primers and the Omniscript Reverse Transcriptase kit (Qiagen) according to manufacturers instructions. A Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA) was used to measure the abundance of specific transcripts in samples of cDNA. Reactions contained 1/50 diluted cDNA, Sybr Green Power Mix (Applied Biosystems), and primers to TOC75, TIC110, TIC40, CPSECY, CPSECY2, ALB4, or CPTATC (Table A-3). The abundance of transcripts in Alb4-, cpSecY-,

and cpSecY-RNAi seedlings was calculated using the comparative $\Delta\Delta\text{CT}$ method, taking into consideration the amplification efficiencies of each primer set (Pfaffl, 2001).

Imaging and Electron Microscopy

Arabidopsis seedlings were observed growing on estrogen containing MS media at time points noted in figure legends using a Leica MZ 12.5 stereoscope (Leica Microsystems Inc., Bannockburn, IL). Images were captured by a SPOT RT Slider CCD camera and Spot Basic imaging software (Diagnostic Instruments, Sterling Heights, MI).

Membrane structures in Arabidopsis cotyledon cell plastids were observed and imaged by performing the following. Leaf samples were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.24. Fixed tissues were processed with the aid of a Pelco BioWave laboratory microwave (Ted Pella, Redding, CA, USA). The samples were washed in 0.1 M sodium cacodylate pH 7.24, post fixed with 2% OsO₄, water washed and dehydrated in a graded ethanol series 25%, 50%, 75%, 95%, 100% followed by 100% acetone. Dehydrated samples were infiltrated in graded acetone/Spurrs epoxy resin 30%, 50%, 70%, 100% and cured at 60°C. Cured resin blocks were trimmed, thin sectioned and collected on formvar copper slot grids, post-stained with 2% aqueous Uranyl acetate and Reynold's lead citrate. Sections were examined with a Hitachi H-7000 TEM (Hitachi High Technologies America, Inc. Schaumburg, IL) and digital images acquired with a Veleta 2k x 2k camera and iTEM software (Olympus Soft-Imaging Solutions Corp, Lakewood, CO).

Dye Binding to a 2TC Tagged Target Protein in Situ in Isolated Chloroplasts

Inclusion bodies of pOE23 and pOE23-2TC were each solubilized by incubation in 8 M Urea, at 37°C, for one hour. 3 μM of each precursor was incubated with isolated pea chloroplasts in an *in vitro* import assay for times specified in figure legends. Following import,

chloroplasts underwent quantification, centrifugation, and resuspension to 1 mg chlorophyll per mL in IB. Three equal volumes of chloroplasts were split into new microfuge tubes. Samples of chloroplasts were incubated with 0, 0.75, or 1.5 μ L Lumio Green dye (Invitrogen, Carlsbad, CA) for 60 minutes, at 25°C, in the dark. Following another round of centrifugation, chloroplasts were resuspended in 1 μ M ethanedithiol IB and incubated on ice for 30 minutes. Chloroplasts were hypotonically lysed and fractionated. A 5 μ L aliquot of stroma from the 0 μ L dye treatment was spiked with 0.35 μ L of Lumio Green dye. Samples of all stroma fractions were incubated in non-reducing sample buffer before analysis by SDS-PAGE. Gels were visualized by a Molecular Imager FX, set to 532 nm excitation, and Quantity One software (Biorad, Hercules, CA).

Immunoprecipitation

Stroma from Lumio dye-treated chloroplasts was combined with an equal volume of 2xTBS (1xTBS: 50 mM Tris-HCl pH 7.4, 150 mM NaCl), brought to 100 μ L using water, combined with 100 μ L 2% SDS, 1 mM EDTA, TBS, and incubated at 37°C for 5 minutes. Samples were spun at 19,000 x g for 5 minutes and supernatants were transferred to low retention tubes containing 0.5 mL IP (1% Triton X-100, 0.5% DOC, 1 mM EDTA, TBS) and 40 μ L of a 40% slurry of anti-OE23 linked protein A Sepharose beads. Antibodies were covalently linked to Sepharose beads according to manufacturers instructions (Amersham Biosciences, Pittsburg PA). Solutions were mixed end-over-end at 4°C for 1.5 hours then spun at 200 x g, for one minute, at 2°C in a swing bucket microfuge. Samples were washed three times by resuspension in IP, centrifugation, and removal of the supernatant after each centrifugation step. Beads were washed with 1 mL of 0.05% Triton X-100, TBS, then resuspended in 100 μ L TBS and transferred to Wizard Mini columns (Promega, Madison, WI) in two successive rounds.

Columns were spun at 200 x g, at 2°C, for one minute then transferred to new microfuge tubes. Columns received 25 µL of 8M Urea, 5% SDS, 125 mM Tris-HCl pH 6.8 and 1.5 hours of incubation at room temperature. Columns were spun again. Aliquots of liquid flow through were combined with equal volumes of non-reducing sample buffer. Samples were incubated at room temperature for one hour, then analyzed by SDS-PAGE. Fluorescently labeled protein was visualized by a Molecular Imager FX and Quantity One software (Biorad, Hercules, CA), as above.

In Vitro CALI

Radiolabeled precursors to cpTatC and cpTatC-2TC were translated *in vitro*. Replicate samples of each translation product were incubated with 100 nM Lumio Red dye (Invitrogen, Carlsbad, CA) or 3 µM EDT₂ for 15 minutes at room temperature. All samples were aliquoted into low retention tubes. For each translation product, one pair of dye or buffer treated samples was exposed to the dark for 15 minutes. All other samples were exposed to 17 W/cm² of 560 nm light for 15 minutes. After light or dark treatment, precursors were each imported into isolated pea chloroplasts.

Electrophoresis

cpTatC-containing samples destined for SDS-PAGE were incubated in sample buffer (0.1M Tris-HCl pH6.8, 8M urea, 5% SDS, 20% glycerol, 10% β-mercaptoethanol) for one hour at room temperature before electrophoresis, to prevent cpTatC aggregation. Samples destined for Blue Native PAGE were prepared as described (Gerard and Cline, 2007), except that 2% digitonin was included in solubilization buffers used to prepare Arabidopsis membranes. Gels were processed for fluorography or analyzed by immunoblotting (Cline, 1986; Cline and Mori, 2001). Immuno-labeled proteins were visualized by using the ECL method (Amersham

Biosciences, Pittsburg, PA). Radiolabeled proteins were extracted from dried gel slices and quantified by scintillation counting (Cline, 1986). The number of molecules of mcpTatC, iOE23, or mOE23 were calculated from the dpm of an extracted band, the specific activity of the leucine used in the translations, the number of leucine residues per molecule, and the efficiency of radiolabeled leucine incorporation during precursor synthesis *in vitro*. Leucine residues for each molecule were derived from amino acid sequence data. Gel band extraction and quantitative immunoblotting were each used to quantify precursors from *in vitro* translation reactions (Fincher et al., 2003). Leucine incorporation efficiency was determined by comparing the amount of radiolabeled precursor to the amount of total precursor produced from an *in vitro* synthesis reaction. Chloroplasts per import assay sample were calculated from the chlorophyll concentration and the number of chloroplasts per microgram of chlorophyll, which was typically about 1×10^6 .

Table A-1. Primers that were used to amplify Arabidopsis gene fragments for hairpin RNA construction. The Gateway recombination site and corresponding sequence are underlined for all relevant primers.

Primer	Sequence (5' → 3')
Outside cpSecY forward	GCATGATTGATGATGGTTGC
cpSecY SOE reverse	GCAACTTTGGATAGACTTGAGC
cpSecY SOE forward	GCTCAAGTCTATCCAAAGTTGC
Outside cpSecY reverse	GCGAGGCATAATTGAGCGG
<u>B1</u> -cpSecY forward	<u>GGGACAAGTTTGTACAAAAAAGCAGGCTCTTCAGCTGC</u> TATTGAGGACAGTTCC
<u>B5r</u> -cpSecY reverse	<u>GGGACAACCTTTTGTATACAAAGTTGCCTGAAAACTTT</u> GCTTGTTAGACTATATAAGCATACC
<u>B5</u> -cpSecY forward	<u>GGGACAACCTTTTGTATACAAAGTTGCTGAACATATACT</u> ATCCCGAGTACCAAGAGG
<u>B2</u> -cpSecY reverse	<u>GGGACCACTTTTGTACAAGAAAGCTGGGTCTTCAGCTGC</u> TATTGAGGACAGTTCC
<u>B1</u> -cpSecY2 forward	<u>GGGACAAGTTTGTACAAAAAAGCAGGCTGTGTTTTGAC</u> CTTGATAATGTTTTTGCAGGAGACCC
<u>B5r</u> -cpSecY2 reverse	<u>GGGACAACCTTTTGTATACAAAGTTGCTCACCCAGTTCC</u> TCTATCAAAAACATAAAAATGTAACTACAAATAC
<u>B5</u> -cpSecY2 forward	<u>GGGACAACCTTTTGTATACAAAGTTGATGAGAACACTC</u> ACCAACAGAACCCGAGACAAAGC
cpSecY2 SOE reverse	GTAGTGTGTTGGAGACAGC
cpSecY2 SOE forward	GCTGTCTCCAACACACTAC
<u>B2</u> -cpSecY2 reverse	<u>GGGACCACTTTTGTACAAGAAAGCTGGGTGTGTTTTGA</u> CCTTGATAATGTTTTTGCAGGAGACCC
<u>B1</u> -Alb4 reverse	<u>GGGACAAGTTTGTACAAAAAAGCAGGCTGACTAAACTAT</u> TATCGTATTAGTGTAACACCAGTGAACGAGGAC
Alb4 SOE forward	GGACGAGACTACGAATG
Alb4 SOE reverse	CATTCGTAGTCTCGTCC

Table A-1. Continued

Primer	Sequence (5' → 3')
<u>B5r</u> -Alb4 forward	<u>GGGGACAAC</u> TTTTGTATACAAAGTTGGTAACCAAATCTT CTAAAATGTGTCATCATTTCGTTCCCTAGAAGCTG
<u>B5</u> -Alb4 forward	<u>GGGGACAAC</u> TTTTGTATACAAAAGTTGGCTGCTGAAAGA GCAAGAAGCAAAGAGACGTCGAG
<u>B2</u> -Alb4 reverse	<u>GGGGACCACT</u> TTTGTACAAGAAAGCTGGGTGACTAAACTAT TATCGTATTAGTGTAACACCAGTGAACGAGGAC

Table A-2. Primers that were used to screen transformed Arabidopsis plants for hairpin constructs to cpSecY2, cpSecY, and Alb4.

Primer	Sequence (5' → 3')
MDC7 forward	CGCTGAAGCTAGTCGACTCTAGC
MDC7 reverse	CGAAAGCTGGGAGGCCTGGATCG
Alb4 hairpin screen forward	GTGTCATCATTCGTTCCCTAGAAGC
Alb4 hairpin screen reverse	GCTTCTAGGAACGAATGATGACAC
cpSecY hairpin screen forward	GGTATGCTTATATAGTCTAACAAGC
cpSecY hairpin screen reverse	GCTTGTTAGACTATATAAGCATAACC
cpSecY2 hairpin screen forward	GATAGAGGAACTGGGTGAG
cpSecY2 hairpin screen reverse	CTCACCCAGTTCCTCTATC

Table A-3. Primers used to quantify transcripts to in Arabidopsis cDNA isolated from estrogen-induced empty vector and RNAi lines.

Primer	Sequence (5' → 3')
Ubiquitin10 qRT-PCR forward	GGCCTTGTATAATCCCTGATGAATAAG
Ubiquitin10 qRT-PCR reverse	AAAGAGATAACAGGAACGGAAACATAGT
cpSecY2 qRT-PCR forward	GCTGGAATGCAACCTGTTCTC
cpSecY2 qRT-PCR reverse	AGGTGAACCCAGAATACTTGCAA
Alb4 qRT-PCR forward	TCCTCTTTCTCCACAGGCAA
Alb4 qRT-PCR reverse	CGGTGTGTGGCTGAAACG
cpTatC qRT-PCR forward	CAACGCCGGAGCAAAGG
cpTatC qRT-PCR reverse	TGGTGAATCGTCGTCATTGAG
TIC40 qRT-PCR forward	AAGAGGTAATGGATGTGTTCAACAAG
TIC40 qRT-PCR reverse	GCTTTTTCAACCCGTCATTCC
TIC110 qRT-PCR forward	CATTTCTTCTGGAGTGGATGGTT
TIC110 qRT-PCR reverse	AGACATGGCAGTCTCTCTGGATAA
TOC75-III qRT-PCR forward	ACCTCTAGCCGTAGCCTCAGTCT
TOC75-III qRT-PCR reverse	CAGAACCGACGGAAGATTCG
cpSecY qRT-PCR forward	CGGACGACGTGAGTGAACAA
cpSecY qRT-PCR reverse	CAGGTCGGACTAGAGGGATTGA

LIST OF REFERENCES

- Anderson, S. and Smith, S.M.** (1986) Synthesis of the small subunit of ribulose-bisphosphate carboxylase from genes cloned into plasmids containing the SP6 promoter. *Biochem. J.* **240**, 709–715.
- Andersson, H. and Heijne, G.v.** (1993) *Sec* dependent and *sec* independent assembly of *E. coli* inner membrane proteins: the topological rules depend on chain length. *EMBO J* **12**, 683–691.
- Andersson, M.X., Kjellberg, J.M. and Sandelius, A.S.** (2001) Chloroplast biogenesis. Regulation of lipid transport to the thylakoid in chloroplasts isolated from expanding and fully expanded leaves of pea. *Plant Physiol* **127**, 184–193.
- Andersson, M.X. and Sandelius, A.S.** (2004) A chloroplast-localized vesicular transport system: a bio-informatics approach. *BMC Genomics* **5**, 40.
- Arnon, D.I.** (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta Vulgaris*. *Plant Physiology* **24**, 1–15.
- 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.
- Aseeva, E., Ossenbuhl, F., Sippel, C. et al.** (2007) Vipp1 is required for basic thylakoid membrane formation but not for the assembly of thylakoid protein complexes. *Plant Physiol Biochem* **45**, 119–128.
- Bauer, J., Chen, K., Hiltbunner, A., Wehrli, E., Eugster, M., Schnell, D. and Kessler, F.** (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. *Nature* **403**, 203–207.
- Benning, C.** (2008) A role for lipid trafficking in chloroplast biogenesis. *Prog Lipid Res* **47**, 381–389.
- Benning, C., Xu, C. and Awai, K.** (2006) Non-vesicular and vesicular lipid trafficking involving plastids. *Current Opinion in Plant Biology* **9**, 241–247.
- Bensing, B.A., Gibson, B.W. and Sullam, P.M.** (2004) The *Streptococcus gordonii* platelet binding protein GspB undergoes glycosylation independently of export. *J Bacteriol* **186**, 638–645.

- Bensing, B.A. and Sullam, P.M.** (2002) An accessory sec locus of *Streptococcus gordonii* is required for export of the surface protein GspB and for normal levels of binding to human platelets. *Mol Microbiol* **44**, 1081–1094.
- Berghofer, J., Karnauchov, I., Herrmann, R.G. and Klosgen, R.B.** (1995) Isolation and characterization of a cDNA encoding the SecA protein from spinach chloroplasts. Evidence for azide resistance of Sec-dependent protein translocation across thylakoid membranes in spinach. *J Biol Chem* **270**, 18341–18346.
- Bleys, A., Vermeersch, L., Van Houdt, H. and Depicker, A.** (2006) The frequency and efficiency of endogene suppression by transitive silencing signals is influenced by the length of sequence homology. *Plant Physiol* **142**, 788–796.
- Block, M.A., Dorne, A.J., Joyard, J. and Douce, R.** (1983) Preparation and characterization of membrane fractions enriched in outer and inner envelope membranes from spinach chloroplasts. I. Electrophoretic and immunochemical analyses. *J Biol Chem* **258**, 13273–13280.
- Blomqvist, L.A., Ryberg, M. and Sundqvist, C.** (2008) Proteomic analysis of highly purified prolamellar bodies reveals their significance in chloroplast development. *Photosynth Res* **96**, 37–50.
- Bloois, E.v., Haan, G.J., Gier, J.-W.d., Oudega, B. and Luirink, J.** (2004) F1F0 ATP synthase subunit c is targeted by the SRP to YidC in the *E. coli* inner membrane. *FEBS Letters* **576**, 97–100.
- Gunning, B.E.S.** (2003) Plant Cell Biology on DVD.
- Brink, S., Fischer, K., Klosgen, R.-B. and Flugge, U.-I.** (1995) Sorting of Nuclear-encoded Chloroplast Membrane Proteins to the Envelope and the Thylakoid Membrane. *J Biol Chem* **270**, 20808–20815.
- Bruce, B.D.** (2001) The paradox of plastid transit peptides: conservation of function despite divergence in primary structure. *Biochimica et Biophysica Acta - Molecular Cell Research* **1541**, 2–21.
- Buchanan, B., Gruissem, W. and Jones, R.** (2000). *Biochemistry and Molecular Biology of Plants*. Rockville, MD: American Society of Plant Biologists.
- Buchanan, G., Leeuw, E.d., Stanley, N.R., Wexler, M., Berks, B.C., Sargent, F. and Palmer, T.** (2002) Functional complexity of the twin-arginine translocase TatC component revealed by site-directed mutagenesis. *Molecular Microbiology* **43**, 1457–1470.
- Cabantous, S., Terwilliger, T.C. and Waldo, G.S.** (2005) Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nat Biotechnol* **23**, 102–107.

- Carde, J.-P., Joyard, J. and Douce, R.** (1982) Electron Microscopic Studies of Envelope Membranes from Spinach Plastids. *Biology of the Cell* **44**, 315–324.
- Carrie, C., Giraud, E. and Whelan, J.** (2009) Protein transport in organelles: Dual targeting of proteins to mitochondria and chloroplasts. *FEBS J* **276**, 1187–1195.
- Cavalier-Smith, T.** (2000) Membrane heredity and early chloroplast evolution. *Trends in Plant Science* **5**, 174–182.
- Chalker-Scott, L.** (1999) Environmental Significance of Anthocyanins in Plant Stress Responses. *Phytochemistry and Photobiology* **70**, 1–9.
- Chiu, C.C. and Li, H.M.** (2008) Tic40 is important for reinsertion of proteins from the chloroplast stroma into the inner membrane. *Plant J* **56**, 793–801.
- Chou, M.L., Fitzpatrick, L.M., Tu, S.L., Budziszewski, G., Potter-Lewis, S., Akita, M., Levin, J.Z., Keegstra, K. and Li, H.M.** (2003) Tic40, a membrane-anchored co-chaperone homolog in the chloroplast protein translocon. *EMBO J* **22**, 2970–2980.
- Chua, N.H. and Schmidt, G.W.** (1978) Post-translational transport into intact chloroplasts of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase. *Proc Natl Acad Sci U S A* **75**, 6110–6114.
- Cline, K.** (1986) Import of proteins into chloroplasts. Membrane integration of a thylakoid precursor protein reconstituted in chloroplast lysates. *J Biol Chem* **261**, 14804–14810.
- Cline, K. and Dabney-Smith, C.** (2008) Plastid protein import and sorting: different paths to the same compartments. *Curr Opin Plant Biol* **11**, 585–592.
- Cline, K., Fulsom, D.R. and Viitanen, P.V.** (1989) An imported thylakoid protein accumulates in the stroma when insertion into thylakoids is inhibited. *J Biol Chem* **264**, 14225–14232.
- Cline, K. and Henry, R.** (1996) Import and routing of nucleus-encoded chloroplast proteins. *Annu Rev Cell Dev Biol* **12**, 1–26.
- Cline, K., Henry, R., Li, C. and Yuan, J.** (1993) Multiple pathways for protein transport into or across the thylakoid membrane. *EMBO J* **12**, 4105–4114.
- Cline, K. and Mori, H.** (2001) Thylakoid DeltapH-dependent precursor proteins bind to a cpTatC-Hcf106 complex before Tha4-dependent transport. *J Cell Biol* **154**, 719–729.
- Cline, K., Henry, R., Li, C.-J. and Yuan, J.** (1992) Pathways and Intermediates for the Biogenesis of Nuclear Encoded Thylakoid Proteins. *Research in Photosynthesis* **III**, 149–156.
- Cline, K. and Theg, S.M.** (2007) The Sec and Tat Protein Translocation Pathways in Chloroplasts. In *The Enzymes* (Dalbey, R., Koehler, C. and Tamanoi, F., eds). Elsevier, pp. 1-25.

- Cran, D.G. and Possingham, J.V.** (1972) Two forms of division profile in spinach chloroplasts. *Nature New Biology* **235**, 142.
- Curtis, M.D. and Grossniklaus, U.** (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol* **133**, 462–469.
- Dalbey, R.E. and Chen, M.** (2004) Sec-translocase mediated membrane protein biogenesis. *Biochim Biophys Acta* **1694**, 37–53.
- Dalbey, R.E. and Kuhn, A.** (2000) Evolutionarily Related Insertion Pathways of Bacterial, Mitochondrial, and Thylakoid Membrane Proteins. *Annual Review of Cell and Developmental Biology* **16**, 51–87.
- Davies, K. and K, S.** (2003) Transcriptional regulation of secondary metabolism. *Functional Plant Biology* **30**, 913–925.
- Dong, Y., Palmer, S.R., Hasona, A., Nagamori, S., Kaback, H.R., Dalbey, R.E. and Brady, L.J.** (2008) Functional overlap but lack of complete cross-complementation of *Streptococcus mutans* and *Escherichia coli* YidC orthologs. *J Bacteriol* **190**, 2458–2469.
- Dyall, S.D., Brown, M.T. and Johnson, P.J.** (2004) Ancient invasions: from endosymbionts to organelles. *Science* **304**, 253–257.
- Eggink, L.L., LoBrutto, R., Brune, D.C., Brusslan, J., Yamasato, A., Tanaka, A. and Hooper, J.K.** (2004) Synthesis of chlorophyll b: localization of chlorophyllide a oxygenase and discovery of a stable radical in the catalytic subunit. *BMC Plant Biol* **4**, 5.
- Emanuelsson, O., Nielsen, H., Brunak, S. and von Heijne, G.** (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* **300**, 1005–1016.
- English, A.R., Zurek, N. and Voeltz, G.K.** (2009) Peripheral ER structure and function. *Curr Opin Cell Biol* **21**, 596–602.
- Fandl, J.P., Cabelli, R., Oliver, D. and Tai, P.C.** (1988) SecA suppresses the temperature-sensitive SecY24 defect in protein translocation in *Escherichia coli* membrane vesicles. *PNAS* **85**, 8953–8957.
- Fincher, V., Dabney-Smith, C. and Cline, K.** (2003) Functional assembly of thylakoid Δ pH-dependent/Tat protein transport pathway components in vitro. *Eur J Biochem* **270**, 4930–4941.
- Flugge, U.I. and Heldt, H.W.** (1979) Phosphate translocator in chloroplasts: Identification of the functional protein and characterization of its binding site. In *Function and Molecular Aspects of Biomembrane Transport* (al., I.Q.e., ed). Amsterdam: Elsevier, pp. 373.
- Fortin, Y., Phoenix, P. and Drapeau, G.R.** (1990) Mutations conferring resistance to azide in *Escherichia coli* occur primarily in the secA gene. *J Bacteriol* **172**, 6607–6610.

- Fuhrmann, E., Gathmann, S., Rupprecht, E., Golecki, J. and Schneider, D.** (2009) Thylakoid membrane reduction affects the photosystem stoichiometry in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Physiol* **149**, 735–744.
- Fulgosi, H., Gerdes, L., Westphal, S., Glockmann, C. and Soll, J.** (2002) Cell and chloroplast division requires ARTEMIS. *Proc Natl Acad Sci U S A* **99**, 11501–11506.
- Funes, S., Hasona, A., Bauerschmitt, H., Grubbauer, C., Kauff, F., Collins, R., Crowley, P.J., Palmer, S.R., Brady, L.J. and Herrmann, J.M.** (2009) Independent gene duplications of the YidC/Oxa/Alb3 family enabled a specialized cotranslational function. *Proc Natl Acad Sci U S A* **106**, 6656–6661.
- Funes, S., Nargang, F.E., Neupert, W. and Herrmann, J.M.** (2004) The Oxa2 protein of *Neurospora crassa* plays a critical role in the biogenesis of cytochrome oxidase and defines a ubiquitous subbranch of the Oxa1/YidC/Alb3 protein family. *Mol Biol Cell* **15**, 1853–1861.
- Gao, H. and Xu, X.** (2009) Depletion of Vipp1 in *Synechocystis* sp. PCC 6803 affects photosynthetic activity before the loss of thylakoid membranes. *FEMS Microbiol Lett* **292**, 63–70.
- Garab, G. and Mannella, C.A.** (2008) Reply: On Three-Dimensional Models of Higher-Plant Thylakoid Networks: Elements of Consensus, Controversies, and Future Experiments. *The Plant Cell Online* **20**, 2549.
- Gerard, F. and Cline, K.** (2007) The thylakoid proton gradient promotes an advanced stage of signal peptide binding deep within the Tat pathway receptor complex. *J Biol Chem* **282**, 5263–5272.
- Gerdes, L., Bals, T., Klostermann, E., Karl, M., Philippar, K., Hünken, M., Soll, J. and Schünemann, D.** (2006) A Second Thylakoid Membrane-associated Alb3/Oxa1/YidC Homologue Is Involved in Proper Chloroplast Biogenesis in *Arabidopsis thaliana*. *J Biol Chem* **281**, 16632–16642.
- Gillingham, A.K. and Munro, S.** (2007) The small G proteins of the Arf family and their regulators. *Annu Rev Cell Dev Biol* **23**, 579–611.
- Gohre, V., Ossenbuhl, F., Crevecoeur, M., Eichacker, L.A. and Rochaix, J.D.** (2006) One of two alb3 proteins is essential for the assembly of the photosystems and for cell survival in *Chlamydomonas*. *Plant Cell* **18**, 1454–1466.
- Hasona, A., Crowley, P.J., Levesque, C.M., Mair, R.W., Cvitkovitch, D.G., Bleiweis, A.S. and Brady, L.J.** (2005) Streptococcal viability and diminished stress tolerance in mutants lacking the signal recognition particle pathway or YidC2. *Proc Natl Acad Sci U S A* **102**, 17466–17471.
- Haucke, V.** (2003) Vesicle budding: a coat for the COPs. *Trends Cell Biol* **13**, 59–60.

- Hiltbrunner, A., Bauer, J., Vidi, P.A., Infanger, S., Weibel, P., Hohwy, M. and Kessler, F.** (2001) Targeting of an abundant cytosolic form of the protein import receptor at Toc159 to the outer chloroplast membrane. *J Cell Biol* **154**, 309–316.
- Hirano, H., Harashima, H., Shinmyo, A. and Sekine, M.** (2008) Arabidopsis RETINOBLASTOMA-RELATED PROTEIN 1 is involved in G1 phase cell cycle arrest caused by sucrose starvation. *Plant Mol Biol* **66**, 259–275.
- Hofmann, K. and Stoffel, W.** (1993) TMbase - A database of membrane spanning proteins segments. *Biol. Chem. Hoppe-Seyley* **374**, 166.
- Hooper, J.K., Bednarik, D., Keller, B.J. and Marks, D.B.** (1982) Regulatory aspects of thylakoid membrane formation in *Chlamydomonas reinhardtii* y-1. *Prog Clin Biol Res* **102 Pt B**, 127–137.
- Hooper, J.K., Boyd, C.O. and Paavola, L.G.** (1991) Origin of Thylakoid Membranes in *Chlamydomonas reinhardtii* y-1 at 38 degrees C. *Plant Physiol* **96**, 1321–1328.
- Hooper, J.K. and Stegeman, W.J.** (1976) Kinetics and regulation of synthesis of the major polypeptides of thylakoid membranes in *Chlamydomonas reinhardtii* y-1 at elevated temperatures. *J Cell Biol* **70**, 326–337.
- Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R.** (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61–68.
- Hruz, T., Laule, O., Szabo, G., F, W., S, B., L, O., P, W., W, G. and P, Z.** (2008) Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. *Advances in Bioinformatics* 420747.
- Hutin, C., Havaux, M., Carde, J.P., Kloppstech, K., Meierhoff, K., Hoffman, N. and Nussaume, L.** (2002) Double mutation cpSRP43--/cpSRP54-- is necessary to abolish the cpSRP pathway required for thylakoid targeting of the light-harvesting chlorophyll proteins. *Plant J* **29**, 531–543.
- Inaba, T., Alvarez-Huerta, M., Li, M., Bauer, J., Ewers, C., Kessler, F. and Schnell, D.J.** (2005) Arabidopsis tic110 is essential for the assembly and function of the protein import machinery of plastids. *Plant Cell* **17**, 1482–1496.
- Inaba, T. and Schnell, D.J.** (2008) Protein trafficking to plastids: one theme, many variations. *Biochem J* **413**, 15–28.
- Inoue, K., Baldwin, A.J., Shipman, R.L., Matsui, K., Theg, S.M. and Ohme-Takagi, M.** (2005) Complete maturation of the plastid protein translocation channel requires a type I signal peptidase. *J Cell Biol* **171**, 425–430.

- Inoue, K. and Keegstra, K.** (2003) A polyglycine stretch is necessary for proper targeting of the protein translocation channel precursor to the outer envelope membrane of chloroplasts. *The Plant Journal* **34**, 661–669.
- Orlic, I.J.D.** Introduction of plasmid into *Agrobacterium* (Method II).
- Jackson, D.T., Froehlich, J.E. and Keegstra, K.** (1998) The Hydrophilic Domain of Tic110, an Inner Envelope Membrane Component of the Chloroplastic Protein Translocation Apparatus, Faces the Stromal Compartment. *Journal of Biological Chemistry* **273**, 16583–16588.
- Karamanou, S., Bariami, V., Papanikou, E., Kalodimos, C.G. and Economou, A.** (2008) Assembly of the translocase motor onto the preprotein-conducting channel. *Mol Microbiol* **70**, 311–322.
- Kessler, F. and Blobel, G.** (1996) Interaction of the protein import and folding machineries of the chloroplast. *Proc Natl Acad Sci U S A* **93**, 7684–7689.
- Kieselbach, T. and Schroder, W.P.** (2003) The proteome of the chloroplast lumen of higher plants. *Photosynth Res* **78**, 249–264.
- Kim, S.J., Jansson, S., Hoffman, N.E., Robinson, C. and Mant, A.** (1999) Distinct "assisted" and "spontaneous" mechanisms for the insertion of polytopic chlorophyll-binding proteins into the thylakoid membrane. *J Biol Chem* **274**, 4715–4721.
- Kim, S.J., Robinson, C. and Mant, A.** (1998) Sec/SRP-independent insertion of two thylakoid membrane proteins bearing cleavable signal peptides. *FEBS Lett* **424**, 105–108.
- Kim, S.J., Robinson, D. and Robinson, C.** (1996) An *Arabidopsis thaliana* cDNA encoding PS II-X, a 4.1 kDa component of photosystem II: a bipartite presequence mediates SecA/delta pH-independent targeting into thylakoids. *FEBS Lett* **390**, 175–178.
- S, K.** (2006) Transport of Flavonoids. In *The Science of Flavonoids* (Grotewold, E., ed). New York: Springer, pp. 123-146.
- Knight, J.S. and Gray, J.C.** (1995) The N-Terminal Hydrophobic Region of the Mature Phosphate Translocator Is Sufficient for Targeting to the Chloroplast Inner Envelope Membrane. *The Plant Cell* **7**, 1421–1432.
- Kobayashi, K., Kondo, M., Fukuda, H., Nishimura, M. and Ohta, H.** (2007) Galactolipid synthesis in chloroplast inner envelope is essential for proper thylakoid biogenesis, photosynthesis, and embryogenesis. *PNAS* **104**, 17216–17221.
- Koch, H.-G., Moser, M., Schimz, K.-L. and Mueller, M.** (2002) The Integration of YidC into the Cytoplasmic Membrane of *Escherichia coli* Requires the Signal Recognition Particle, SecA and SecYEG. *Journal of Biological Chemistry* **277**, 5715–5718.

- Kovacheva, S., Bedard, J., Patel, R., Dudley, P., Twell, D., Rios, G., Koncz, C. and Jarvis, P.** (2005) In vivo studies on the roles of Tic110, Tic40 and Hsp93 during chloroplast protein import. *Plant J* **41**, 412–428.
- Kroll, D., Meierhoff, K., Bechtold, N., Kinoshita, M., Westphal, S., Vothknecht, U.C., Soll, J. and Westhoff, P.** (2001) VIPP1, a nuclear gene of *Arabidopsis thaliana* essential for thylakoid membrane formation. *Proc Natl Acad Sci U S A* **98**, 4238–4242.
- 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.
- Last, D.I. and Gray, J.C.** (1989) Plastocyanin is encoded by a single-copy gene in the pea haploid genome. *Plant Mol Biol* **12**, 655–666.
- Leech, R.M., Rumsby, M.G. and Thomson, W.W.** (1973) Plastid Differentiation, Acyl Lipid, and Fatty Acid Changes in Developing Green Maize Leaves. *Plant Physiol* **52**, 240–245.
- Li, M. and Schnell, D.J.** (2006) Reconstitution of protein targeting to the inner envelope membrane of chloroplasts. *J Cell Biol* **175**, 249–259.
- Li, X., Henry, R., Yuan, J., Cline, K. and Hoffman, N.E.** (1995) A chloroplast homologue of the signal recognition particle subunit SRP54 is involved in the posttranslational integration of a protein into thylakoid membranes. *Proc Natl Acad Sci U S A* **92**, 3789–3793.
- Lorkovic, Z.J., Schroder, W.P., Pakrasi, H.B., Irrgang, K.D., Herrmann, R.G. and Oelmüller, R.** (1995) Molecular characterization of PsbW, a nuclear-encoded component of the photosystem II reaction center complex in spinach. *Proc Natl Acad Sci U S A* **92**, 8930–8934.
- Lubeck, J., Heins, L. and Soll, J.** (1997) A nuclear-coded chloroplastic inner envelope membrane protein uses a soluble sorting intermediate upon import into the organelle. *J Cell Biol* **137**, 1279–1286.
- Luirink, J., Samuelsson, T. and de Gier, J.W.** (2001) YidC/Oxa1p/Alb3: evolutionarily conserved mediators of membrane protein assembly. *FEBS Lett* **501**, 1–5.
- Ma, Y., Kouranov, A., LaSala, S.E. and Schnell, D.J.** (1996) Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope. *J Cell Biol* **134**, 315–327.
- Maloney, M.A., Hooper, J.K. and Marks, D.B.** (1989) Kinetics of Chlorophyll Accumulation and Formation of Chlorophyll-Protein Complexes during Greening of *Chlamydomonas reinhardtii* y-1 at 38 degrees C. *Plant Physiol* **91**, 1100–1106.
- Mannella, C.A.** (2006) Structure and dynamics of the mitochondrial inner membrane cristae. *Biochim Biophys Acta* **1763**, 542–548.

- Mant, A., Woolhead, C.A., Moore, M., Henry, R. and Robinson, C.** (2001) Insertion of PsaK into the thylakoid membrane in a "Horseshoe" conformation occurs in the absence of signal recognition particle, nucleoside triphosphates, or functional albino3. *J Biol Chem* **276**, 36200–36206.
- Martin, J.R., Harwood, J.H., McCaffery, M., Fernandez, D.E. and Cline, K.** (2009) Localization and integration of thylakoid protein translocase subunit cpTatC. *Plant J*
- Martinez-Garcia, J.F., Monte, E. and Quail, P.H.** (1999) A simple, rapid and quantitative method for preparing Arabidopsis protein extracts for immunoblot analysis. *Plant J* **20**, 251–257.
- Masclaux, F., Charpentreau, M., Takahashi, T., Pont-Lezica, R. and Galaud, J.P.** (2004) Gene silencing using a heat-inducible RNAi system in Arabidopsis. *Biochem Biophys Res Commun* **321**, 364–369.
- Mayer, A.** (1999) Intracellular membrane fusion: SNAREs only? *Curr Opin Cell Biol* **11**, 447–452.
- Mayer, A., Wickner, W. and Haas, A.** (1996) Sec18p (NSF)-driven release of Sec17p (alpha-SNAP) can precede docking and fusion of yeast vacuoles. *Cell* **85**, 83–94.
- Michl, D., Robinson, C., Shackleton, J.B., Herrmann, R.G. and Klosgen, R.B.** (1994) Targeting of proteins to the thylakoids by bipartite presequences: CFoII is imported by a novel, third pathway. *EMBO J* **13**, 1310–1317.
- Mistou, M.Y., Dramsi, S., Brega, S., Poyart, C. and Trieu-Cuot, P.** (2009) Molecular dissection of the secA2 locus of group B Streptococcus reveals that glycosylation of the Srr1 LPXTG protein is required for full virulence. *J Bacteriol* **191**, 4195–4206.
- Moore, M., Harrison, M.S., Peterson, E.C. and Henry, R.** (2000) Chloroplast Oxa1p homolog albino3 is required for post-translational integration of the light harvesting chlorophyll-binding protein into thylakoid membranes. *J Biol Chem* **275**, 1529–1532.
- Mori, H. and Cline, K.** (2001) Post-translational protein translocation into thylakoids by the Sec and DeltapH-dependent pathways. *Biochim Biophys Acta* **1541**, 80–90.
- Mori, H. and Ito, K.** (2001) The Sec protein-translocation pathway. *Trends Microbiol* **9**, 494–500.
- Mori, H., Summer, E.J. and Cline, K.** (2001) Chloroplast TatC plays a direct role in thylakoid (Delta)pH-dependent protein transport. *FEBS Lett* **501**, 65–68.
- Mori, H., Summer, E.J., Ma, X. and Cline, K.** (1999) Component specificity for the thylakoidal Sec and Delta pH-dependent protein transport pathways. *J Cell Biol* **146**, 45–56.

- Morre, D.J., Minnifield, N. and Paulik, M.** (1989) Identification of the 16 degrees C compartment of the endoplasmic reticulum in rat liver and cultured hamster kidney cells. *Biol Cell* **67**, 51–60.
- Morre, D.J., Sellden, G., Sundqvist, C. and Sandelius, A.S.** (1991) Stromal Low Temperature Compartment Derived from the Inner Membrane of the Chloroplast Envelope. *Plant Physiol* **97**, 1558–1564.
- Motohashi, R., Nagata, N., Ito, T., Takahashi, S., Hobo, T., Yoshida, S. and Shinozaki, K.** (2001) An essential role of a TatC homologue of a Delta pH- dependent protein transporter in thylakoid membrane formation during chloroplast development in *Arabidopsis thaliana*. *PNAS* **98**, 10499–10504.
- Mustardy, L., Buttle, K., Steinbach, G. and Garab, G.** (2008) The three-dimensional network of the thylakoid membranes in plants: quasihelical model of the granum-stroma assembly. *Plant Cell* **20**, 2552–2557.
- Nakai, M., Goto, A., Nohara, T., Sugita, D. and Endo, T.** (1994) Identification of the SecA protein homolog in pea chloroplasts and its possible involvement in thylakoidal protein transport. *J Biol Chem* **269**, 31338–31341.
- Nakai, M., Sugita, D., Omata, T. and Endo, T.** (1993) Sec-Y protein is localized in both the cytoplasmic and thylakoid membranes in the cyanobacterium *Synechococcus* PCC7942. *Biochem Biophys Res Commun* **193**, 228–234.
- Bechtold, N. and Pelletier, G.** *In-planta Agrobacterium* mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration and floral dip. *EMBO Course: Practical Course on Genetic and Molecular Analysis of Arabidopsis* **5**,
- 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.
- Nohara, T., Asai, T., Nakai, M., Sugiura, M. and Endo, T.** (1996) Cytochrome f encoded by the chloroplast genome is imported into thylakoids via the SecA-dependent pathway. *Biochem Biophys Res Commun* **224**, 474–478.
- Pavlov, P.F., Rudhe, C., Bhushan, S. and Glaser, E.** (2007) In vitro and in vivo protein import into plant mitochondria. *Methods Mol Biol* **372**, 297–314.
- Payan, L.A. and Cline, K.** (1991) A stromal protein factor maintains the solubility and insertion competence of an imported thylakoid membrane protein. *J Cell Biol* **112**, 603–613.
- Peltier, J.B., Emanuelsson, O., Kalume, D.E. et al.** (2002) Central functions of the luminal and peripheral thylakoid proteome of *Arabidopsis* determined by experimentation and genome-wide prediction. *Plant Cell* **14**, 211–236.

- Pfaffl, M.W.** (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45.
- Preuss, M., Ott, M., Funes, S., Luirink, J. and Herrmann, J.M.** (2005) Evolution of mitochondrial oxa proteins from bacterial YidC. Inherited and acquired functions of a conserved protein insertion machinery. *J Biol Chem* **280**, 13004–13011.
- Race, H.L., Herrmann, R.G. and Martin, W.** (1999) Why have organelles retained genomes? *Trends Genet* **15**, 364–370.
- Rawyler, A., Meylan-Bettex, M. and Siegenthaler, P.A.** (1995) (Galacto) lipid export from envelope to thylakoid membranes in intact chloroplasts. II. A general process with a key role for the envelope in the establishment of lipid asymmetry in thylakoid membranes. *Biochim Biophys Acta* **1233**, 123–133.
- Rawyler, A., Meylan, M. and Siegenthaler, P.A.** (1992) Galactolipid export from envelope to thylakoid membranes in intact chloroplasts. I. Characterization and involvement in thylakoid lipid asymmetry. *Biochim Biophys Acta* **1104**, 331–341.
- Reed, J.E., Cline, K., Stephens, L.C., Bacot, K.O. and Viitanen, P.V.** (1990) Early events in the import/assembly pathway of an integral thylakoid protein. *Eur J Biochem* **194**, 33–42.
- Reinbothe, C., Bartsch, S., Eggink, L.L., Hooper, J.K., Brusslan, J., Andrade-Paz, R., Monnet, J. and Reinbothe, S.** (2006) A role for chlorophyllide a oxygenase in the regulated import and stabilization of light-harvesting chlorophyll a/b proteins. *Proc Natl Acad Sci U S A* **103**, 4777–4782.
- Rhee, S.Y., Beavis, W., Berardini, T.Z. et al.** (2003) The *Arabidopsis* Information Resource (TAIR): a model organism database providing a centralized, curated gateway to *Arabidopsis* biology, research materials and community. *Nucleic Acids Res* **31**, 224–228.
- Richter, S. and Lamppa, G.K.** (1999) Stromal Processing Peptidase Binds Transit Peptides and Initiates Their ATP-dependent Turnover in Chloroplasts. *Journal of Cell Biology* **147**, 33–44.
- Robinson, D.R., Karnauchov, I., Herrmann, R.G., Klosgen, R.B. and Robinson, C.** (1996) Protease-sensitive thylakoidal import machinery for the Sec-, DeltapH- and signal recognition particle-dependent protein targeting pathways, but not for CF0II integration. *Plant Journal* **10**, 149–155.
- Rohl, T. and van Wijk, K.J.** (2001) In vitro reconstitution of insertion and processing of cytochrome f in a homologous chloroplast translation system. *J Biol Chem* **276**, 35465–35472.
- Roslan, H.A., Salter, M.G., Wood, C.D. et al.** (2001) Characterization of the ethanol-inducible alc gene-expression system in *Arabidopsis thaliana*. *Plant J* **28**, 225–235.

- 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.
- Schaller, G.E., Ladd, A.N., Lanahan, M.B., Spanbauer, J.M. and Bleeker, A.B.** (1995) The Ethylene Response Mediator ETR1 from Arabidopsis Form a Disulfide-linked Dimer. *J Biol Chem* **270**, 12526–12530.
- Schena, M., Lloyd, A.M. and Davis, R.W.** (1991) A steroid-inducible gene expression system for plant cells. *Proc Natl Acad Sci U S A* **88**, 10421–10425.
- Schnell, D.J., Blobel, G. and Pain, D.** (1990) The chloroplast import receptor is an integral membrane protein of chloroplast envelope contact sites. *J Cell Biol* **111**, 1825–1838.
- 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.
- Schulz, A., Knoetzel, J., Scheller, H.V. and Mant, A.** (2004) Uptake of a fluorescent dye as a swift and simple indicator of organelle intactness: import-competent chloroplasts from soil-grown Arabidopsis. *J Histochem Cytochem* **52**, 701–704.
- Schunemann, D.** (2007) Mechanisms of protein import into thylakoids of chloroplasts. *Biol Chem* **388**, 907–915.
- Scotti, P.A., Valent, Q.A., Manting, E.H., Urbanus, M.L., Driessen, A.J.M., Oudega, B. and Luirink, J.** (1999) SecA Is Not Required for Signal Recognition Particle-mediated Targeting and Initial Membrane Insertion of a Nascent Inner Membrane Protein. *J Biol Chem* **274**, 29883–29888.
- Shimoni, E., Rav-Hon, O., Ohad, I., Brumfeld, V. and Reich, Z.** (2005) Three-Dimensional Organization of Higher-Plant Chloroplast Thylakoid Membranes Revealed by Electron Tomography. *The Plant Cell* **17**, 2580–2586.
- Shipman-Roston, R.L., Ruppel, N.J., Damoc, C., Phinney, B.S. and Inoue, K.** (2010) The Significance of Protein Maturation by Plastidic Type I Signal Peptidase 1 for Thylakoid Development in Arabidopsis thaliana Chloroplasts. *Plant Physiol*
- Shipman, R.L. and Inoue, K.** (2009) Suborganellar localization of plastidic type I signal peptidase 1 depends on chloroplast development. *FEBS Lett* **583**, 938–942.
- Siboo, I.R., Chaffin, D.O., Rubens, C.E. and Sullam, P.M.** (2008) Characterization of the accessory Sec system of Staphylococcus aureus. *J Bacteriol* **190**, 6188–6196.
- Sirrenberg, C., Bauer, M.F., Guiard, B., Neupert, W. and Brunner, M.** (1996) Import of carrier proteins into the mitochondrial inner membrane mediated by Tim22. *Nature* **384**, 582–585.

- Skalitzky, C.A.** (2006) Genetic Analysis of SCY1 and SCY2 Function in *Arabidopsis thaliana*. *Botany Master of Science*, 62.
- Smith, M.D., Fitzpatrick, L., Keegstra, K. and Schnell, D.J.** (2003) In Vitro Analysis of Chloroplast Protein Import. In *Current Protocols in Cell Biology* (eds). pp. 16.1-16.21.
- Spence, E., Sarcina, M., Ray, N., Moller, S.G., Mullineaux, C.W. and Robinson, C.** (2003) Membrane-specific targeting of green fluorescent protein by the Tat pathway in the cyanobacterium *Synechocystis* PCC6803. *Mol Microbiol* **48**, 1481–1489.
- Staehelein, L.A.** (2003) Chloroplast structure: from chlorophyll granules to supra-molecular architecture of thylakoid membranes. *Photosynth Res* **76**, 185–196.
- Steiner, J.M., Kocher, T., Nagy, C. and Löffelhardt, W.** (2002) Chloroplast SecE: evidence for spontaneous insertion into the thylakoid membrane. *Biochem Biophys Res Commun* **293**, 747–752.
- Sundberg, E., Slagter, J.G., Fridborg, I., Cleary, S.P., Robinson, C. and Coupland, G.** (1997) ALBINO3, an *Arabidopsis* nuclear gene essential for chloroplast differentiation, encodes a chloroplast protein that shows homology to proteins present in bacterial membranes and yeast mitochondria. *Plant Cell* **9**, 717–730.
- Swidersky, U.E., Rienhofer-Schweer, A., Werner, P.K., Ernst, F., Benson, S.A., Hoffschulte, H.K. and Mueller, M.** (1992) Biochemical analysis of the biogenesis and function of the *Escherichia coli* export factor SecY. *European Journal of Biochemistry* **207**, 803–811.
- Tour, O., Meijer, R.M., Zacharias, D.A., Adams, S.R. and Tsien, R.Y.** (2003) Genetically targeted chromophore-assisted light inactivation. *Nat Biotechnol* **21**, 1505–1508.
- Valent, Q.A., Scotti, P.A., High, S., Gier, J.-W.L.d., Heijne, G.v., Lentzen, G., Wintermeyer, W., Oudega, B. and Luirink, J.** (1998) The *Escherichia coli* SRP and SecB targeting pathways converge at the translocon. *EMBO J* **17**, 2504–2512.
- van de Meene, A.M., Hohmann-Marriott, M.F., Vermaas, W.F. and Roberson, R.W.** (2006) The three-dimensional structure of the cyanobacterium *Synechocystis* sp. PCC 6803. *Arch Microbiol* **184**, 259–270.
- Van Houdt, H., Bleys, A. and Depicker, A.** (2003) RNA target sequences promote spreading of RNA silencing. *Plant Physiol* **131**, 245–253.
- van Wijk, K.J., Knott, T.G. and Robinson, C.** (1995) Evidence for SecA- and delta pH-independent insertion of D1 into thylakoids. *FEBS Lett* **368**, 263–266.
- Vitriol, E.A., Utrecht, A.C., Shen, F., Jacobson, K. and Bear, J.E.** (2007) Enhanced EGFP-chromophore-assisted laser inactivation using deficient cells rescued with functional EGFP-fusion proteins. *Proc Natl Acad Sci U S A* **104**, 6702–6707.

- 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.
- Vojta, L., Soll, J. and Bolter, B.** (2007) Requirements for a conservative protein translocation pathway in chloroplasts. *FEBS Lett* **581**, 2621–2624.
- Vothknecht, U.C. and Soll, J.** (2005) Chloroplast membrane transport: interplay of prokaryotic and eukaryotic traits. *Gene* **354**, 99–109.
- Wesley, S.V., Helliwell, C.A., Smith, N.A. et al.** (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* **27**, 581–590.
- Westphal, S., Heins, L., Soll, J. and Vothknecht, U.C.** (2001a) *Vipp1* deletion mutant of *Synechocystis*: a connection between bacterial phage shock and thylakoid biogenesis? *Proc Natl Acad Sci U S A* **98**, 4243–4248.
- Westphal, S., Soll, J. and Vothknecht, U.C.** (2001b) A vesicle transport system inside chloroplasts. *FEBS Lett* **506**, 257–261.
- Westphal, S., Soll, J. and Vothknecht, U.C.** (2003) Evolution of chloroplast vesicle transport. *Plant Cell Physiol* **44**, 217–222.
- White, R.A. and Hooper, J.K.** (1994) Biogenesis of Thylakoid Membranes in *Chlamydomonas reinhardtii* y1 (A Kinetic Study of Initial Greening). *Plant Physiol* **106**, 583–590.
- Woodson, J.D. and Chory, J.** (2008) Coordination of gene expression between organellar and nuclear genomes. *Nat Rev Genet* **9**, 383–395.
- Woolhead, C.A., Thompson, S.J., Moore, M., Tissier, C., Mant, A., Rodger, A., Henry, R. and Robinson, C.** (2001) Distinct Albino3-dependent and -independent pathways for thylakoid membrane protein insertion. *J Biol Chem* **276**, 40841–40846.
- Wu, H., Bu, S., Newell, P., Chen, Q. and Fives-Taylor, P.** (2007) Two gene determinants are differentially involved in the biogenesis of Fap1 precursors in *Streptococcus parasanguis*. *J Bacteriol* **189**, 1390–1398.
- Yi, L., Jiang, F., Chen, M., Cain, B., Bolhuis, A. and Dalbey, R.E.** (2003) YidC Is Strictly Required for Membrane Insertion of Subunits a and c of the F1F0ATP Synthase and SecE of the SecYEG Translocase. *Biochemistry* **42**, 10537–10544.
- Yuan, J., Henry, R. and Cline, K.** (1993) Stromal factor plays an essential role in protein integration into thylakoids that cannot be replaced by unfolding or by heat shock protein Hsp70. *Proc Natl Acad Sci U S A* **90**, 8552–8556.
- Yuan, J., Henry, R., McCaffery, M. and Cline, K.** (1994) SecA homolog in protein transport within chloroplasts: evidence for endosymbiont-derived sorting. *Science* **266**, 796–798.

- Yusa, F., Steiner, J.M. and Loffelhardt, W.** (2008) Evolutionary conservation of dual Sec translocases in the cyanelles of *Cyanophora paradoxa*. *BMC Evol Biol* **8**, 304.
- Zerges, W. and Rochaix, J.D.** (1998) Low density membranes are associated with RNA-binding proteins and thylakoids in the chloroplast of *Chlamydomonas reinhardtii*. *J Cell Biol* **140**, 101–110.
- 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.
- Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W. and Chua, N.H.** (2006) Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat Protoc* **1**, 641–646.
- Zuo, J., Hare, P.D. and Chua, N.H.** (2006) Applications of chemical-inducible expression systems in functional genomics and biotechnology. In *Methods in molecular biology* (eds). Clifton: Humana Press, pp. 329–342.
- Zuo, J., Niu, Q.W. and Chua, N.H.** (2000) Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J* **24**, 265–273.

BIOGRAPHICAL SKETCH

Jonathan Martin took an early interest in biology. His brother and he had much exposure to biology while hunting, fishing, and backpacking with their father, who worked as a wildlife biologist with the State of California. After he entered UC Davis to pursue a B.S. in Biology, Jon quickly felt that his major was too broad. In his early course work, Jon was most impressed by discoveries made in molecular scale plant biology. Jon focused his major on Plant Biotechnology, which provided more exposure to plant biochemistry, and molecular and cellular biology.

Jon initially worked with Dr. John Duniway on developing a means to biologically control *Verticillium Wilt* in Strawberry. Working in Dr. Duniway's lab was a good experience: Jon made friends and experienced what lab science was like, but Dr. Duniway did not study biology on a molecular scale. Some persistence earned Jon a job at the National Science Foundation Center for Engineered Plant Resistance Against Pathogens, working with Drs. Lincoln and Gilchrist. Jon managed a few small projects, and made friends with Jagger Harvey, a PhD candidate in the genetics program, who encouraged Jon to pursue a PhD in plant molecular biology.

Jon enjoyed his interview for a graduate research assistantship in the Program for Plant Molecular and Cellular Biology at the University of Florida. After rotating through three labs, Jon accepted Dr. Ken Cline's invitation to study thylakoid protein translocation. Jon's experiences as a graduate student have been trying and rewarding. Along with help from Dr. Cline, his committee members, and lab colleagues, Jon is glad to have attained PhD candidacy, published his work in a prestigious journal of plant biology, and to have had an opportunity to present his work at a prestigious scientific meeting.